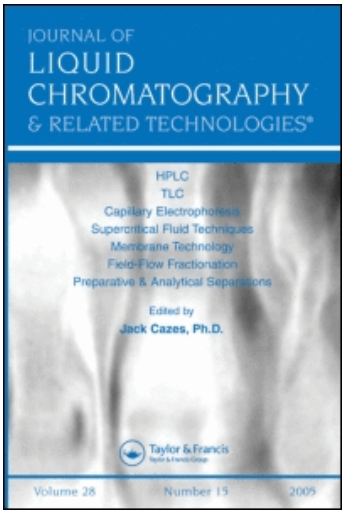

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Inexpensive, Computer-Automated HPLC for Ion Exchange Separation and Quantification of Amino Acids in Physiological Fluids

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INEXPENSIVE, COMPUTER-AUTOMATED HPLC FOR ION EXCHANGE SEPARATION AND QUANTIFICATION OF AMINO ACIDS IN PHYSIOLOGICAL FLUIDS

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ABSTRACT

An inexpensive, computer-automated HPLC for separation and quantification of amino acids in physiological fluids is described. The system offers fully automated equipment control, data collection, processing and storage capabilities. The component nature of the system and the software flexibility permit extensive system modification, accomodating a wide variety of different separatory procedures which are not possible with many dedicated amino acid analysers. The system uses a lithium-based ion exchange column with post-column o-phthalaldehyde derivatization. A time of 128 minutes, including column regeneration, is required for separation of all amino acids through to arginine. The advantages of post-column derivatization over pre-column derivatization methods for post-separatory amino acid techniques are discussed. Accurate quantification of radiolabel in amino acids is demonstrated.

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INTRODUCTION

Quantification of amino acids in physiological fluids has been achieved using a wide variety of methods [1, 2, 3, 4, 5, 6, 7]. The traditional liquid chromatographic approach involved separation by ion exchange and subsequent spectrophotometric detection following post-column derivatization with ninhydrin. In recent years, techniques have been developed for the separation of amino acids by reverse phase HPLC using silica-based columns. While rapid separation of amino acids has been achieved, pre-column derivatization is required. A number of derivatizing reagents have been used including danysl chloride (Dns) [1], o-phthalaldehyde (OPA) [2, 5], phenylthiohydatoin (PTH) [4], and phenylisothiocyanate (PITC) [6, 7]. However, problems associated with pre-column derivatization techniques include lack of column-to-column reproducibility, derivative instability, reagent interference, lack of reactivity and lengthy derivative preparation times [5, 8].

Recently, ion exchange columns have been developed which can withstand the high pressures characteristic of HPLC systems and therefore are able to provide rapid and efficient separations [8]. When combined with post-column derivatization using OPA, this approach offers a number of advantages for analysis of amino acids, including non-destructive amino acid separations, program flexibility for separation of unusual amino acids, detection of all amino acids (including imino acids by hypochlorite

oxidation), high sensitivity and good column-to-column reproducibility. Both ion exchange and reverse phase HPLC also provide access to a variety of separation techniques such as column switching, trace enrichment, multicolumn chromatography and recycle chromatography [10] that permit complete analysis of extracts containing a large number of unknown amino acids.

Instrumentation for amino acid analysis of physiological fluids must be able to accomodate unknown samples from a variety of sources. In addition, use of the instrument for routine analysis requires that the procedure be simple, flexible and high speed [5]. Lithium based ion exchange chromatography has proved to be among the best of methods available for these purposes. Currently available dedicated amino acid analyzers meet these requirements and thus are convenient for routine analysis of physiological fluids and offer good data handling capabilities but are cost-prohibitive for many laboratories [9].

A number of microcomputer-based systems have been developed to control chromatographic equipment and to aid in the data analysis [11, 12, 13, 14]. For HPLC applications, requirements include the capability for universal equipment control, rapid data acquisition, extensive data processing and ease of programming. Modern A/D (analog to digital) converters provide digital control outputs for external equipment control and analog inputs for data collection and equipment monitoring. Quality of A/D conversion rests mainly upon the number of incoming and

outgoing channels that can be monitored, the bit resolution (precision with which two measurements can be resolved in discrete digital quantities), adjustibility and capacity of conversion rate (sampling rate of analog signal) and software quality [15, 16]. Several software/hardware packages for processing chromatographic data are currently marketed.

We report here on the development of an inexpensive computer-automated system for the routine amino acid analysis of physiological fluids. Despite the low cost, this system gives high-quality separations similar to those reported for other, dedicated lithium-based ion exchange systems. In addition, the data handling capacities and elution program flexibility afforded by this system are comparable to those of a dedicated software/hardware analyzer.

MATERIALS AND METHODS

HPLC Apparatus

A full schematic of the system is given in Figure 1. Buffers A through E and regenerant solution were pumped from acid-washed 2 L bottles through an Altex 6 way valve (Altex Scientific Operations, Berkeley, CA.). Samples were injected into the buffer stream via a Beckman 340 Organizer using a 1000 μL loop. Since the system used step changes in buffer rather than a continuous gradient, the static mixer was bypassed. A lithium-based ion exchange column (Model AA503, Interaction

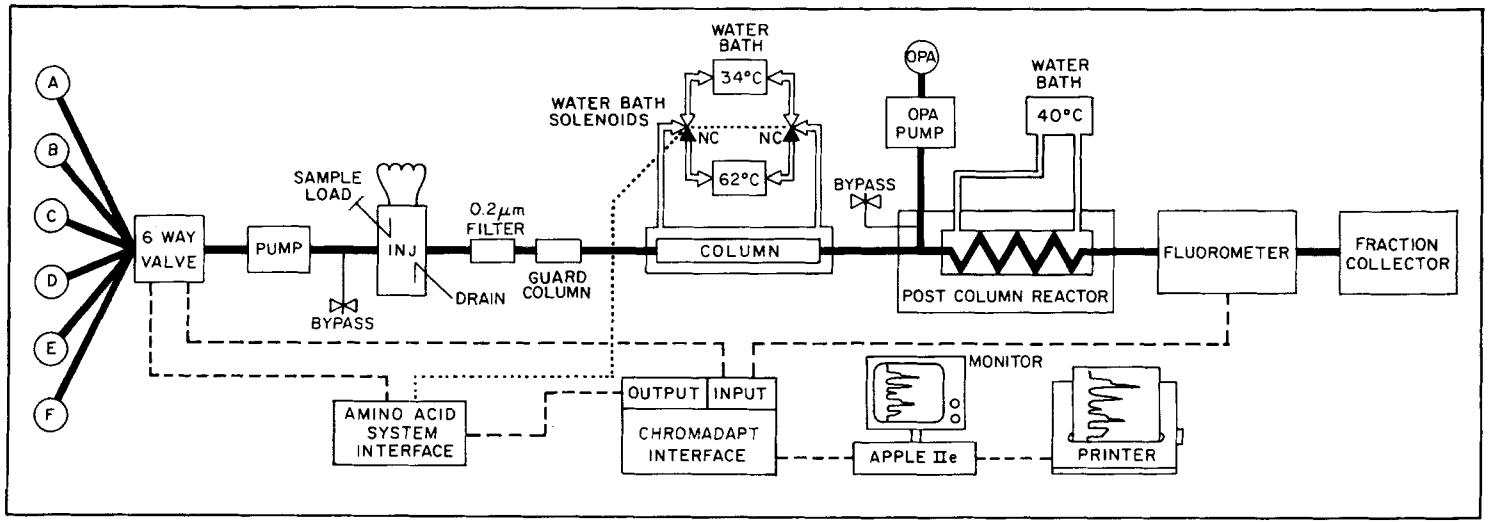

FIGURE 1: Schematic of components for computer-automated amino acid analysis by ion exchange HPLC. Heavy lines indicate solvent flow (A, B, C, D, and E are buffers; F is the regenerant). Dashed lines indicate incoming or outgoing 5V logic signal to or from the Chromadapt interface module (Interactive Microware Inc., State College, PA.) and also indicate incoming fluorometer signal (0-10 mV FS) to the Chromadapt interface. Dotted lines indicate outgoing 115V AC supplied to the solenoid valves. The power (115V AC) for all equipment, except the computer, was supplied through the amino acid system interface as shown in Figure 2.

Chemicals Inc., Mountain View, CA.) was used for the separation. The column was water-jacketed (Alltech Associates, Deerfield, IL.) and temperature regulation achieved by solenoid control of two heating water bath circulators (Haake D1, Berlin, W. Germany). The OPA mixture was delivered by a low pressure mini-pump (Milton Roy Company, St. Petersburg, FLA.) and amino acids in the column effluent were derivatized with OPA in a Beckman 230 Post-Column Reactor. The mixing volume for derivatization was enclosed in a water jacket and maintained at 40°C (Haake D1 water circulator). A Gilson Spectra/Glo fluorometer with 15 μL flow cell (Beckman Instruments, Berkeley, CA.) was used for detection.

Hardware/Software

An Apple IIe microcomputer was equipped with the following components: an Apple 80 column extender card, a Pkaso/u graphics card (Interactive Structures Inc., Bala Cynwyd, PA.), a Legend 64K RAM card (Legend Industries, Pontiac, MI.), an Adalab analog-to-digital data acquisition control card and a Chromadapt interface module (Interactive Microware Inc., State College, PA.). The software package Chromatochart v1.07 (Interactive Microware Inc., State College, PA.) and associated Chromadapt interface module were used with the computer to: (a) control valve position, column temperature and equipment power supply; (b) record and integrate the incoming fluorometer signal; and (c) store the data to disk.

Interface Electronics Hardware

Outgoing 5V logic signals were available from the Apple IIe through the Chromadapt interface for equipment control. A second interface box was constructed (Biomedical Engineering, Queen's University, Ont.) to convert 5V logic to 115V AC. The wiring diagram is given in Figure 2. Valve switching was actuated from logic output terminal 0 on the Chromadapt interface. The available 5V logic signal was used to drive an optically coupled contact closure which, when actuated, engaged the valve drive stepper motor. Outgoing voltages from the valve pin-outs (D, E, F) were used to monitor the position of the valve through Chromadapt interface logic input terminals 0, 1 and 2 (Figure 2). Column temperature was regulated using two heating water circulators connected to two three way solenoid valves (ITT General Controls Division, Glendale, CA.). The solenoids were activated from the interface box with a 115V AC relay driven by the 5V logic at Chromadapt interface logic output terminal 1 (not activated: Temp 1=34.0^{o}C; activated: Temp 2=62.0^{o}C). Power to all HPLC equipment, except the computer, was provided through a relay driven by the 5V logic signal at the Chromadapt interface logic output terminal 2 through the second interface box. Current for the three heating water baths was drawn from three independent 15 Amp circuits (Figure 2).

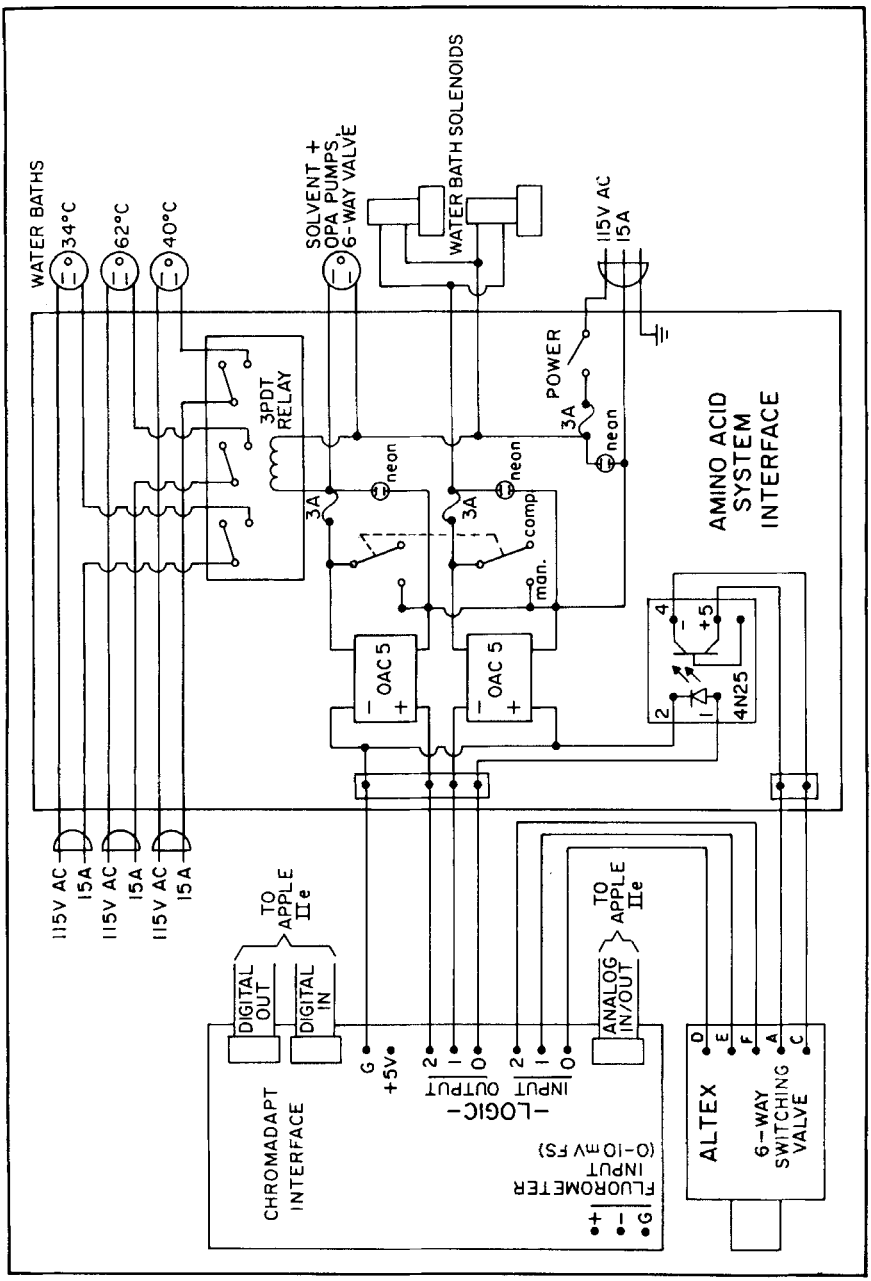

FIGURE 2: Wiring diagram of the amino acid system interface. The diagram shows the connections between the computer, the Chromadapt interface, the amino acid system interface and the system components. Components description: OAC5=5V DC input/115V AC output photo-isolated module relay; 4N25=5V DC optically-coupled contact closure; 3PDT relay=115V AC three-pole double throw relay. All components were obtained from Electro Sonic Inc. (Willowdale, Ont.).

Software Control

The bit configuration for the three valve pin-outs was determined by directly reading the voltage from each pin for each valve position (Table 1). This configuration was then used to construct a series of logic conditions that returned the valve to a desired position. The program called START shown in Table 2 returned the valve to position 1. The START program was used to initialize the system before a running a series of amino acid samples. The function of the program was to ensure that the Chromadapt interface output terminals were inactive and that the valve was in position 1 before an amino acid sample was run. A second program (AMINOACID) was then loaded with the appropriate event control schedule for amino acid separations.

TABLE 1

Bit Configuration of the Altex 6 Way Valve Pin-Outs, Determined from Output Voltages, Used to Monitor Valve Position.

Valve Position	PIN D	E	F
1	0 [a]	1	1
2	1	0	1
3	0	0	1
4	1	1	0
5	0	0	0
6	1	0	0

[a] Value of 0 indicates off, value of 1 indicates on. D, E and F represent pin designations.

TABLE 2

START Program to Monitor Valve Position and Actuate Valve Switching until Desired Valve Position is Reached.

EVENT	SECONDS	COMMAND/ BIT [a]	ON/ OFF	GOTO	COMMENTS
1	0	STATUS 1			Adalab A/D channel 0 on; system active
2	1	SWITCH 0	OFF		Chromadapt output terminal 0 off (valve switch)
3	2	SWITCH 1	OFF		Chromadapt output terminal 1 off (solenoid power)
4	3	SWITCH 2	ON		Chromadapt output terminal 2 on (system power)
5	5	IFBITS 1	OFF	8	
6	10	SWITCH 0	ON		advances 6-way valve one position
7	11	SWITCH 0	OFF		logic sequence to test valve position [b]
8	15	IFBITS 1	ON	6	
9	20	IFBITS 2	OFF	6	
10	22	SWITCH 0	OFF		
11	25	STATUS 0			Adalab A/D channel 0 off; system inactive

[a] The logic input and output connections were as shown in Figure 2. STATUS events control activation of A/D channels, clock, gradient and sampling. SWITCH events will activate or inactivate output terminals while IFBITS events are used to monitor input terminals on the Chromadapt interface module. The Chromadapt interface module operates such that the BIT value specified for the SWITCH command refers to output terminal "n," whereas the BIT value specified for the IFBITS command refers to the binary value (2^n) associated with terminal "n." For example, SWITCH 2 ON activates Chromadapt interface output terminal 2, whereas IFBITS 2 ON causes the computer to check if Chromadapt interface input terminal 1 is active.

[b] This logic sequence returns the 6-way valve to position 1 as indicated by pins E and F being on and pin D being off (see Table 1).

Sample Preparation

Soybeans (Glycine max) were inoculated at planting with Rhizobium japonicum (USDA 16) and grown as previously described [17]. The root systems of 12 plants were placed in a sealed vessel and exposed to $^{14}CO_2$ for 2 min. Plants were harvested immediately and the root nodules were ground in 50 mL of 80% aqueous EtOH acidified with formic acid. Insoluble material was removed by centrifugation, the pellet rinsed several times and the resulting supernatant evaporated to dryness. The crude extract was resuspended in 5.0 mL distilled H_2O. Lipids, proteins and hydrophobic species were removed by a 1:1 chloroform : water separation. The aqueous phase was applied to a Dowex AG50W-X8 column (1 cm^3) and rinsed through with 20 mL H_2O to remove acidic and neutral species. The basic (amino acid) fraction was eluted with 35 mL 2N HCl. The eluant was evaporated to dryness and resuspended in 5.0 mL H_2O. Subsamples were adjusted to the pH and ionic strength of the loading buffer by two-fold dilution in Buffer A before being loaded onto the column. All samples were filtered through 0.45 μm luer-lock filters before injection.

HPLC

Separation was achieved using a series of five lithium citrate buffers (Pierce Pico-Buffer system, Pierce Chemical Co., Rockford, IL.) as the mobile phase. The pH and ionic strength of

each buffer is given in Figure 3. LiOH 0.3 M (pH 12.3) was used to regenerate the column. These solutions were prepared by dilution of the stock solutions using double de-ionized, glass distilled water. All solvents and samples were passed through 0.45 m in-line filters (Rheodyne) before reaching the column. The OPA mixture used was supplemented with Brij 35, as described elsewhere [3].

Chromatography

Samples were injected into a continuous stream of buffer, eliminating the pressure peak created by stop-flow injectors. Both solvent flow rate and OPA flow rates were 0.5 mL/min. Sample volumes for injection ranged from 10 to 500 μL. The conditions for separation were similar to those reported for lithium-based ion exchange columns [18] and are shown in Figure 3. Column back-pressure was approximately 1500 psi. Total run time for detection of all amino acids was 128 min, which included a 5 min column regeneration in 0.3 M LiOH and a further 15 min to reequilibrate the column in Buffer A following each run.

Quantification of ^{14}C Incorporation into Amino Acids

Column effluent was collected after fluorescence detection using a Gilson FC-100 microfractionator. Fractions of 350 μL were collected for the first 25 min, of 500 μL from min 25 to 85 and then of 1000 μL for the remainder of each run. ScintiVerse I

(Fisher Scientific Co.) was added to each vial and radiolabel detected and quantified by liquid scintillation using the channels ratio method.

RESULTS AND DISCUSSION

Figure 3 shows the printout of a standard separation of an amino acid mixture generated by the computer-automated amino acid analyzer described here. The elution program used is given at the top of Figure 3. This type of separation is comparable to that reported for many other ion exchange systems [3, 8, 9, 19]. The software package (Chromatochart v1.07) used with this system recognizes the elution program or method file in four parts: (1) file identification; (2) event control schedule; (3) data collection; and (4) data processing. In the first part of the method file the gradient, standards and data filenames for each run are specified. The event control schedule is used to control 5V logic signals available from the Chromadapt interface and to initiate and/or terminate sampling. Data collection options permit one to adjust chart speed, sample rate and X and Y scale compression. These options control the way in which data is acquired and displayed once stored in RAM. Data processing options affect the processing of the analog signal after it has been digitized. Most of the parameters in this part of the method file control the baseline calculation, peak recognition and integration of peak areas. A convenient feature of this

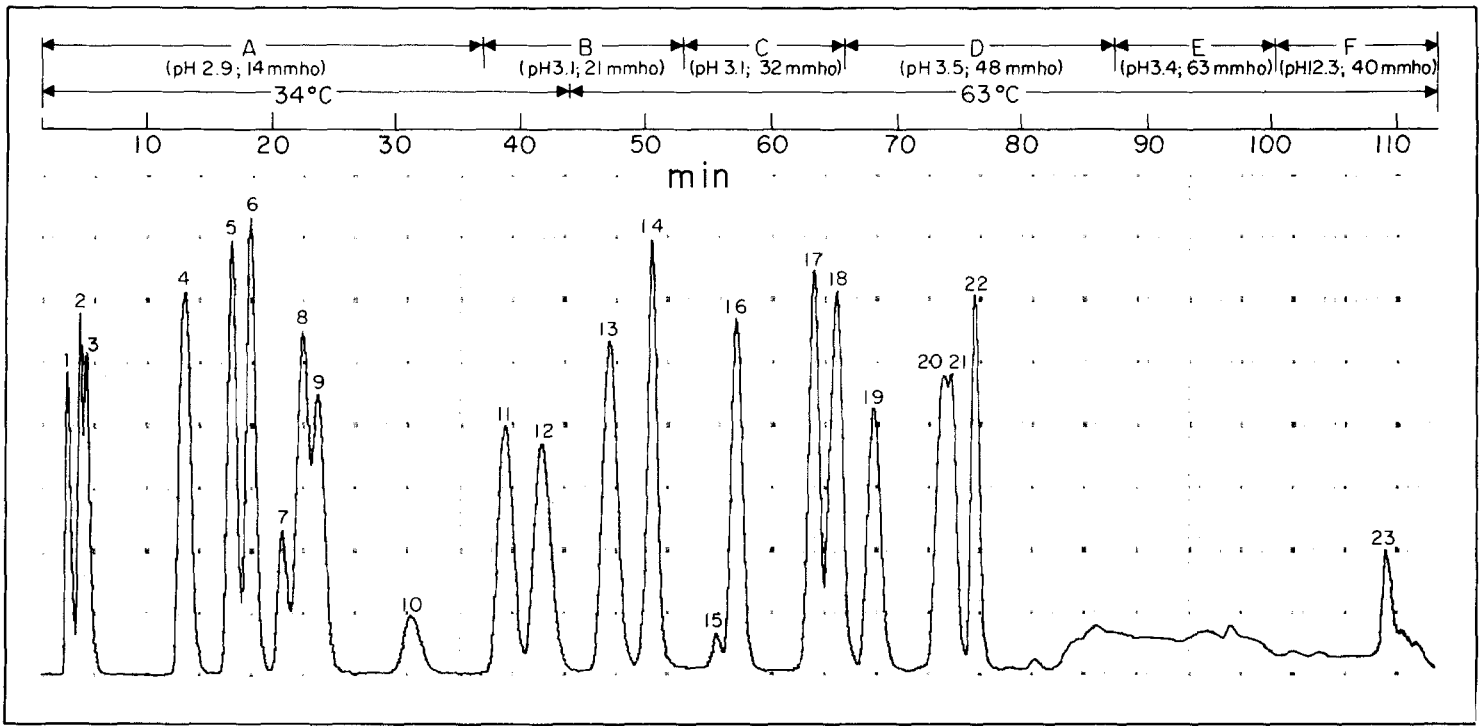

FIGURE 3: Standard separation of an amino acid mixture as displayed using Chromatochart v1.07. The elution program and buffer composition are shown above the peaks. Peaks: 1=Pser; 2=Tau; 3=Peta; 4=Asp; 5=Thr; 6=Ser; 7=Asn; 8=Glu; 9=Gln; 10=α-Aaa; 11=Gly; 12=L-Ala; 13=Cit; 14=Val; 15=Cys; 16=Met; 17=Ile; 18=Leu; 19=Tyr; 20=β-Ala; 21=Phe; 22=Aiba; 23=Arg.

system is that the raw data is stored to disk and can be recalled and re-integrated if desired.

Ion exchange with step-changes in buffer concentration was used in this system for reasons discussed above and in Benson and Woo [8]. However, it should be noted that this type of computer automation is entirely suitable for gradient control.

An important requirement of amino acid HPLC is program flexibility. Using this system, separation and resolution of amino acid peaks of particular interest may be markedly improved with minor modifications to the program. Figure 4A shows the effect of temperature on the separation of Asn, Glu and Gln. These amino acids are especially important in studies of plant N metabolism and consequently good separations are required. Better separations of Glu and Gln can be achieved at higher temperatures (36°C) at the expense of the Asn peak separation. For routine samples where all three amino acids were to be quantified, 34°C was used. Adjustments in the ionic strength of the mobile phase provided a more satisfactory method for effecting differential separation of amino acids. Figure 4B shows the effect of decreasing ionic strength on the separation of Asn, Glu and Gln at 34°C. Dilutions of 1/10, 1/15 and 1/20 of Buffer A were made to obtain the ionic strengths shown in Figure 4B. With this system any number of buffer and temperature modifications could be used to enhance separations of particular amino acids.

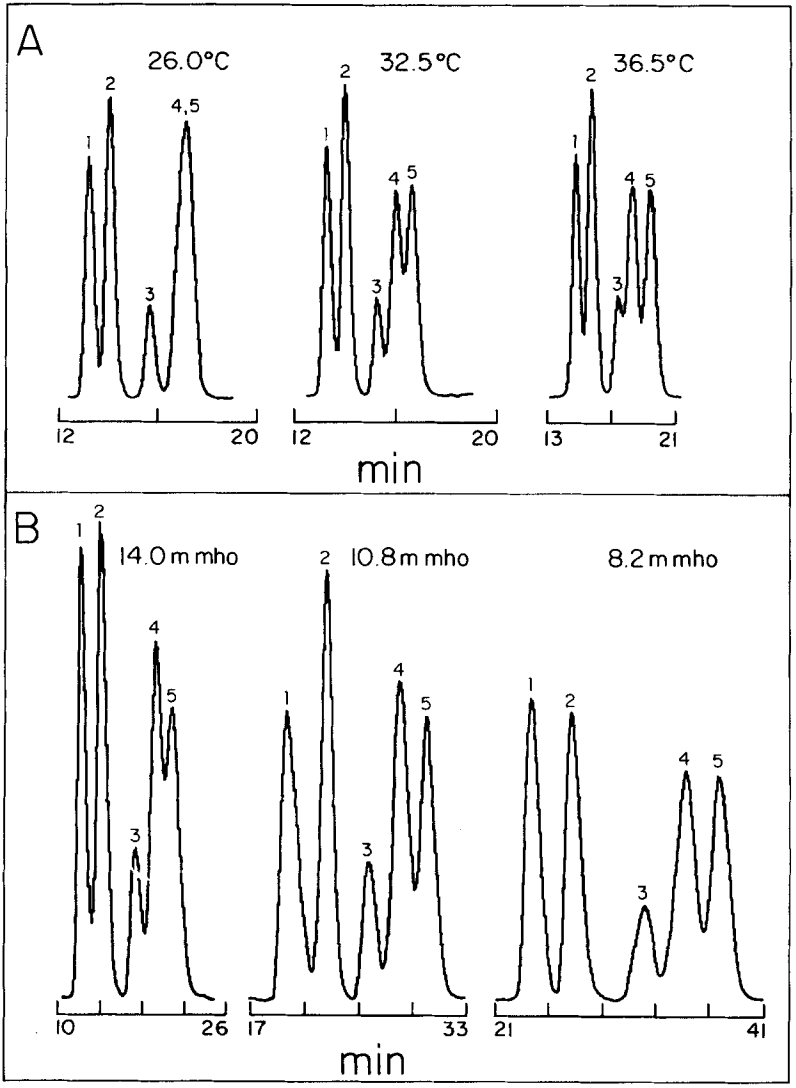

FIGURE 4: Effects of column temperature and ionic strength on the separation of Asn, Glu and Gln. Panel A shows the effect of increasing temperature at an ionic strength of 14.0 mmho. Panel B shows the effect of decreasing ionic strength at 34°C. Peaks: 1=Thr; 2=Ser; 3=Asn; 4=Glu; 5=Gln.

Linearity of fluorometer response was tested over a wide range of amino acid concentrations. The detection limits for OPA derivatives of amino acids have been well established [3, 8, 20] to be in the low pmol range. Frequently in biological samples it is important to detect radiolabel in the amino acid fraction in order to quantify incorporation. This often requires loading high concentrations of amino acid extracts in order to detect low levels of label. The high capacity of ion exchange columns renders them particularly suitable for these types of separations. To this end, linearity was tested at high amino acid concentrations. Response was linear for all amino acids over the range from 0.625 to 45.0 nmol/inj. Results for several amino acids are shown in Figure 5. Separations of most amino acids were still satisfactory at 62.5 nmol/inj. However, Thr and Asp showed significant peak broadening and tailing at this concentration indicating partial column saturation under these separation conditions.

Two main advantages of ion exchange with post-column derivatization for amino acid detection are that: (1) non-destructive separations are possible; and (2) breakdown products are unimportant considerations in the quantitation of radiolabel incorporation into amino acids. Current physiological methods require that a variety of post-separatory analytical procedures be available for complete sample analysis. Non-destructive separations are particularly important when

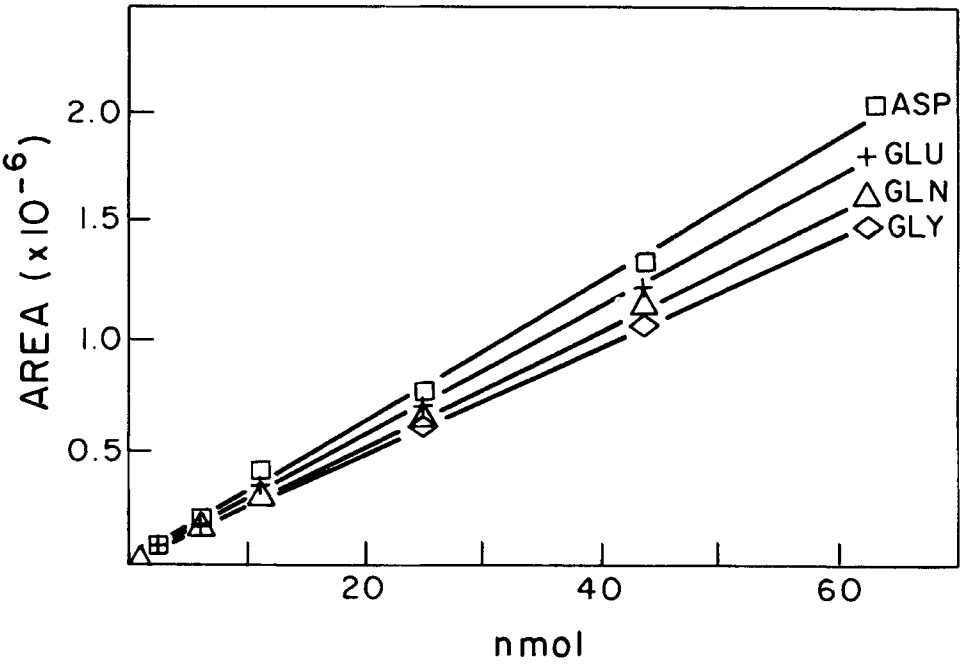

FIGURE 5: Peak areas versus nmol per injection are plotted for Asp, Glu, Gln and Gly to demonstrate linearity of peak area over a range of amino acid concentrations.

purification of a labelled amino acid or synthesis and recovery of unusual amino acids in their native form is desired. Post-column systems allow access to these techniques by simply turning off the OPA pump and monitoring the known retention time of the amino acid of interest. Breakdown products caused by the OPA reaction [see 21] pose a major problem for quantification of radiolabel in amino acid samples that are separated by pre-column reversed-phase systems. This is because the breakdown products

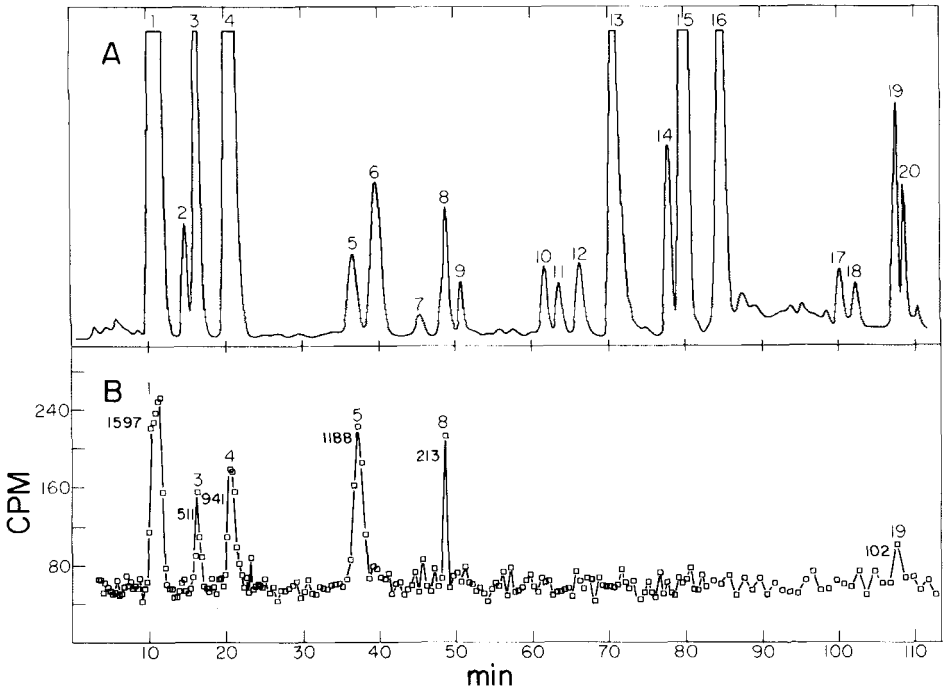

FIGURE 6: Separation of amino acids from a soybean root nodule extract and quantification of ^{14}C incorporation into the amino acid fraction. Panel A shows the distribution and quantification of amino nitrogen in the extract. Panel B shows the quantification of $^{14}CO_2$ incorporation into specific amino acids. The numbers beside each peak represent the total CPM in that peak. Peaks: 1=Asp; 2=Thr; 3=Ser; 4=Gln; 5=Gly; 6=L-Ala; 7=Cit; 8=Val; 9=unknown; 10=Ile; 11=Leu; 12=Tyr; 13=unknown; 14= β-Ala/Phe; 15=Aiba; 16= γ-Aba; 17=Orn; 18=Lys; 19=Arg; 20=unknown.

do not elute at the same time as the native amino acid. With ion exchange and post-column derivatization, this problem is eliminated. Any breakdown that occurs does so after separation; consequently fractions containing the amino acid of interest also contain the breakdown products of that particular amino acid. Thus quantification of all the label in that amino acid is possible.

Figure 6 illustrates one post-separatory procedure that is possible with this system. Labelled amino acids from soybean root nodules were extracted as outlined in the methods. Figure 6A shows the amino acid composition of the sample. In Figure 6B the radiolabel in the fractions associated with each peak is plotted. Clearly the incorporation of $^{14}CO_2$ is greater into some amino acids than into others. Figure 6 underscores the necessity for amino acid analysis techniques that allow further sample treatment following separation. Quantification of nitrogen incorporation into amino acids is also possible with this system in samples pretreated with ^{15}N. Column effluent can then be collected as described here and split, one aliquot for ^{14}C analysis, the other for mass or emission spectrophotometer detection of ^{15}N.

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