

Sequential Injection Chromatography: Automated Sample Preparation, Derivatization and Separation of Amino Acids.

Ilkka Lähdesmäki, Andrea D. Carroll, Jonathan Wood, Garth E. Klein, Louis D. Scampavia

SICrom Systems Inc., 14450 NE 29th Place, Bellevue, WA, 98007 USA

Summary

This paper describes a novel instrument, designed to suit the needs of Sequential Injection Chromatography (SIC). The system includes a medium-pressure pump (up to 500 psig) capable of precision control of forward, stopped and reversed flow at microliter levels. Such capabilities allow flow programming, which is essential for performing SIC protocols. The features of the instrument and advantages of SIC methods are presented in form of an automated amino acid assay that includes on-line protein removal, as well as pre-column derivatization. The method highlights the ability of SIC to automate complex sample processing procedures and combine them with liquid chromatographic separation.

Introduction

HPLC is a widely used laboratory technique but remains largely a bench-top instrument due to its overall large size, high power consumption and need for manual sample pretreatment/preparation prior to analysis. Sequential Injection Chromatography (SIC) is a novel approach that integrates several technologies that allow for fully automated sample preparation/analysis using Sequential Injection and RP-LC techniques. A significant feature to the SIC approach is the capability of performing RP-LC analysis at very low pressures (<500 psig), which allows the instrument design to be compact and portable. It is envisioned that the versatility and robustness of a SIC instrument will find wide and diverse applications in many fields where a remote and autonomous analyzer is desired, including online process control, batch fermentation or in-situ environmental monitoring.

The concept of Sequential Injection Chromatography was proposed and developed by Šatínský *et al.*, shortly after monolithic C₁₈ columns became commercially available.¹ The group of Šatínský and Solich has demonstrated the applicability of SIC in several publications, which can be found summarized in a recent review.² Briefly, in contrast to other chromatographic techniques, SIC uses flow programming to select injected sample volumes and to produce concentration gradients for chromatographic separations. Other investigators have also used monolithic columns connected to low pressure pumps, however without flow programming, which is the essential part of SIC.³ So far, the SIC applications have focused on sample injection without pretreatment. This work aims to expand the scope of SIC applications by: 1) demonstrating versatility of sample preparation, derivatization and separation enabled by flow programming, 2) introducing a

novel instrument design, which allows the SIC system to operate at higher pressures compared to previous designs.

In order to illustrate the versatility and novel features of SIC, we have applied the technique to amino acid determination. Amino acids are routinely assayed in foodstuffs, proteins and peptides, and frequently monitored as essential nutrients in fermentors and bioreactors. The most common methods employed for this purpose are ion-exchange chromatography (IEX), capillary electrophoresis (CE), gas chromatography (GC) and reverse-phase liquid chromatography (RP-LC).⁴ Naturally, each method has some advantages and presents certain disadvantages. For RP-LC, the biggest advantage is that most analytical laboratories have strong expertise in this field, making method development, operation and troubleshooting easier for the analysts. The chief downside is the necessity for pre-column chemical derivatization, required to render amino acids retentive on reverse-phase columns and to allow their detection by means of absorbance or fluorescence.

Derivatization adds labor and complexity to the analytical procedure. Manufacturers offer instrument configurations that allow for pre-column derivatization of amino acids in an automated fashion. Typically, an autosampler is required to aspirate aliquots of the sample and the derivatization agent from separate vials and to inject the derivatized sample into the RP-LC instrument.⁵ This is an adequate solution for samples like protein/peptide digests that do not contain appreciable levels of intact proteins. However, for protein-containing samples, such as serum or bioreactor media, further sample processing is necessary to remove the protein background. This is required because proteins are known to foul reverse-phase columns⁶, as well as to interfere with the amino acid derivatization reactions.⁷ Even though automated protein removal methods do exist for RP-LC⁸, these have not been applied to amino acid analysis. There may be several reasons for this: 1) an automated protein removal method would need to be compatible with automated pre-column derivatization, 2) since proteins and amino acids share the same functional groups, many protein removal methods may result in significant losses of some amino acids, 3) non-standard hardware configuration (auxiliary pumps and valves) would be required to implement some of the removal methods.

The complex sample preparation requirements for protein-containing samples also have another corollary: they prevent or at least significantly complicate interfacing an RP-LC instrument to reactors for true on-line amino acid monitoring. That puts RP-LC at a disadvantage compared to ion exchange chromatography, which has been interfaced directly to a bioreactor for this purpose.⁹

Presented here is an instrument design that eliminates the above obstacles by performing automated on-line protein removal, pre-column derivatization and RP-LC separation of amino acids in a fully automated fashion. The experimental protocol we propose comprises the following sections: 1) on-line derivatization integrated with separation, enabled by programmable flow, 2) sample preparation to remove interfering proteins.

Experimental

Instrumentation

The experiments were carried out with a SICrom instrument for Sequential Injection Chromatography (FIALab Instruments, Bellevue, WA, USA). It features a medium pressure syringe pump (volume 4 mL) capable of operating at up to 500 psig back pressure, as well as a multi-position valve with a holding coil (volume 3500 μ L) for sample/reagent introduction and gradient generation. In this particular case, the system was augmented by an auxiliary module to allow automated protein removal. This module consisted of an on-line microdialyzer (compartment volume 100 μ L, FIALab Instruments), a SciQ 400 peristaltic pump (Watson-Marlow, Wilmington, MA, USA), as well as a VICI EMH 8-position valve (Valco Instruments, Houston, TX, USA). A schematic view of the setup is shown in Fig. 1. The dialysis membrane inside the microdialyzer was a Type C Cuprophane membrane (Pulse Instrumentation, Saskatoon, SK, Canada). The entire setup was controlled by FIALab for Windows software ver 5.0 (FIALab Instruments).

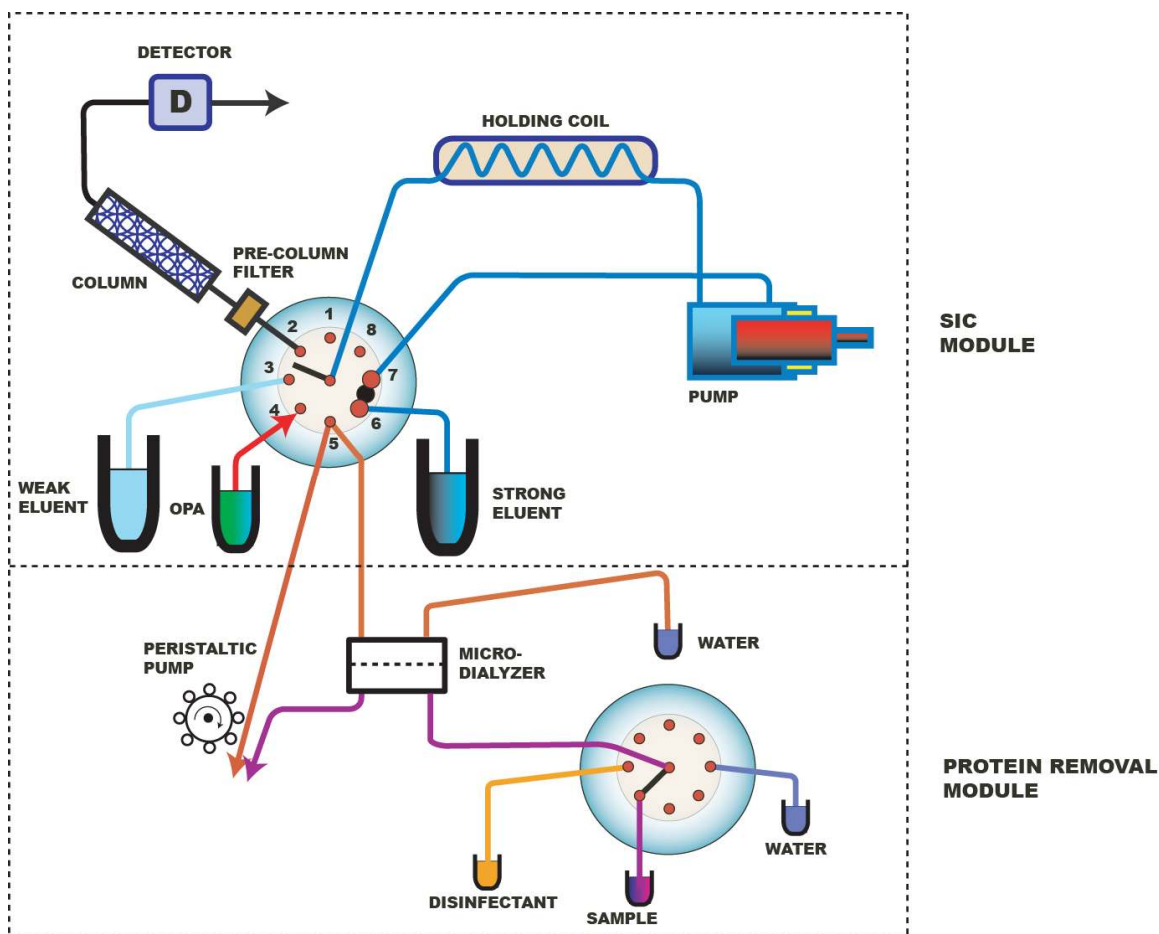


Fig. 1 A schematic diagram of the instrument setup.

The core of the SICrom instrument is an eight position valve, on top of which is mounted a transparent “lab-on-valve” manifold. Three of the valve ports (Fig. 1 and 2) have been modified in the following fashion. Port 5 has been made into a flow through port that allows automatic sample introduction into the system from the dialyzer unit. Inlets to ports 6 and 7 have been enlarged in such a way that a circular divot, machined onto the valve rotor, connects the ports when the rotor is in the “fill” position.. By attaching the mobile phase container to one of the enlarged ports and the pump inlet to the other, the pump can be filled by piston reversal (Fig. 2).

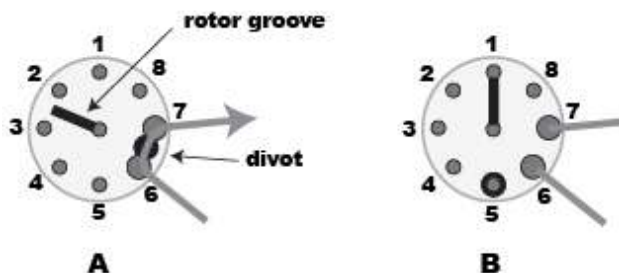


Fig. 2 Pump fill operation. (A) With the rotor in the “fill” position, the divot connects the pump to the mobile phase container, allowing the pump to fill. (B) In any other rotor position, the divot will not connect adjacent channels.

The instrument was equipped with a fluorescence detector, built around a SMA fluorescence flowcell (FIALab Instruments). A 340 nm UVTop LED (Sensor Electronic Technology, Columbia, SC, USA) was directly attached to the flowcell to provide the excitation light. The LED was powered by the 24 V outlet on the SICrom unit. A 350/50 nm bandpass excitation filter (Chroma, Rockingham, VT, USA) was mounted between the LED and the flow channel. Fluorescence emission was collected by a 1000 μm UV-VIS optical fiber (Ocean Optics, Dunedin, FL, USA) and relayed to a photomultiplier tube (Electron Tubes, Ruislip, United Kingdom). A 450/40 nm emission filter (Edmund Optics, Barrington, NJ, USA) was mounted between the optical fiber and the photomultiplier tube.

For dialysis verification and gradient visualization, the instrument was modified to use absorbance detection. The separation column was removed and a Z-type absorbance flowcell (FIALab Instruments) was directly attached to the multi-position valve. The light source used for the absorbance measurements was a Mikropack DH-2000 deuterium lamp and the absorbance detector was a USB 4000 miniature spectrometer (both from Ocean Optics). The light source and the spectrometer were connected to the flowcell by two 600 μm UV/VIS optical fibers (FIALab Instruments).

Reagents

Bovine serum was from HyClone (Logan, UT, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA). These chemicals included PBS pH 7.4 powder, bovine serum albumin, L-tryptophan, L-glutamine, Hank's buffered salt solution powder, folic acid, choline chloride, nicotinamide, pyridoxal HCl, thiamine HCl, DMEM cell culture solution powder, benzalkonium chloride (50 % solution), amino acid standard solution, OPA reagent solution, mineral oil, potassium phosphate (monobasic), potassium phosphate (dibasic), methanol and water (HPLC grade).

Column

Amino acid separations were performed with an Onyx C₁₈ 4.6×25 mm monolithic column (Phenomenex, Torrance, CA, USA). A pre-column filter of 2 µm porosity (Upchurch, Oak Harbor, WA, USA) was used in front of the separation column to prevent clogging of the latter.

Solution preparation

PBS pH 7.4. Phosphate buffered saline pH 7.4 (PBS pH 7.4) was prepared by dissolving a bag of PBS pH 7.4 powder in water.

Amino acid standard solutions (- matrix). Glutamine stock solution (2.5 mM) was prepared by dissolving L-glutamine in PBS pH 7.4. Amino acid standard solutions (10, 50, 100, 500, 1000 µM) were prepared by diluting a commercial amino acid standard (2.5 mM) with PBS pH 7.4. Since the standard did not contain glutamine, appropriate aliquots of glutamine stock solution were added before diluting to the full volume.

HBSS pH 7.4. A vial of Hank's buffered salt solution powder was dissolved in water. pH was adjusted to 7.4 with 1 M NaOH and glucose (1 g/L) was added before diluting to the full volume.

Vitamin stock solutions. A stock solution of folic acid (1000 mg/L) was prepared by dissolving folic acid in 0.1 M NaHCO₃. Stock solutions of choline chloride (1000 mg/L), nicotinamide (1000 mg/L), pyridoxal HCl (1000 mg/L) and thiamine HCl (1000 mg/L) were prepared by dissolving in water.

Matrix solution. Bovine serum albumin (BSA) was dissolved in HBSS pH 7.4. Aliquots of vitamin stock solutions were added and the solution was diluted to the final volume with HBSS pH 7.4. The final concentrations in the solutions were 4 mg/L BSA, 4 mg/L folic acid, 14 mg/L choline chloride, 4 mg/L nicotinamide, 4 mg/L pyridoxal HCl and 4 mg/L thiamine. The components in the matrix solution were selected to represent the protein- and vitamin-rich environment present in many cell culture media.

Amino acid standard solutions (+ matrix). Amino acid standard solutions (10, 50, 100, 500, 1000 μ M) were prepared by diluting a commercial amino acid standard (2.5 mM) with the matrix solution. Appropriate aliquots of glutamine stock solution were added before diluting to the full volume.

o-phthalaldehyde (OPA) derivatization reagent. An aliquot of a commercial OPA reagent solution (Sigma), was transferred to a glass vial. The solution was overlaid with mineral oil to prevent oxidation. The vial was closed with a cap and a septum. The closed vial was connected to the reagent inlet port by piercing the septum and pushing the inlet tube through.

Mobile phases. A 20 mM phosphate buffer solution pH 7.0 was prepared by dissolving the appropriate amounts of KH_2PO_4 and K_2HPO_4 in water. Mobile phase A (weak phase) was prepared by mixing 20 mM phosphate buffer pH 7.0 and methanol in proportions 74:26 (v:v). Mobile phase B (strong phase) was prepared by mixing 20 mM phosphate buffer pH 7.0 and methanol in proportions 44:57 (v:v). Mobile phases were filtered through grade 202 filter paper (Reeve Angel, Clifton, NJ, USA) prior to use.

Culture medium. DMEM cell culture solution was prepared by dissolving a vial of DMEM powder in water. L-glutamine (4 mM) and HEPES (10 mM) were added. pH was adjusted to 7.4 with 1 M NaOH before diluting to the full volume. Finally, serum-supplemented medium was prepared by combining nine parts of the above solution with one part of bovine serum.

Solutions for dialysis verification. A solution of 0.06 mM (4 mg/mL) bovine serum albumin (BSA) was prepared by dissolving BSA in PBS pH 7.4. A solution of 0.4 mM tryptophan was prepared by dissolving L-tryptophan in PBS pH 7.4.

Instrument operation

The instrument performs on-line amino acid derivatization by injecting aliquots of the sample solution and the OPA derivatization reagent into the holding coil. In the process of injection, the solutions mix and the derivatization reaction starts to take place. Sufficient time (1 min) is allowed for the reaction to go to completion, after which the mixture is transferred by a flow reversal onto the column for separation.

OPA-derivatized amino acids exhibit a wide range of hydrophobicity, requiring gradient elution for their separation. A gradient was generated by filling the holding coil initially with the strong mobile phase and then aspirating weak mobile phase into it. As the weak mobile phase advances up the holding coil, it mixes with the strong phase and creates a solvent gradient along the coil. In order to achieve reasonable gradient duration, it is necessary to use a large volume holding coil. This approach is a simplified version of the generic pulse modulation method for gradient generation developed by Herbelin *et al.*¹⁰

Automated protein removal was carried out by attaching the sample inlet port to an auxiliary sample processing module, comprised of a microvolume dialyzer, a peristaltic

pump and a multiposition valve (Fig. 1). The sample solution is stopped in the donor compartment of the dialyzer for a period of time, after which protein-free sample can be drawn into the SICrom instrument from the acceptor compartment. By including a multiposition valve in the sample processing module, standard solutions can be subjected to the exact same processing steps that the sample undergoes. The multiposition valve also makes it possible to introduce washing solutions to periodically disinfect and clean the dialyzer. All these steps can be performed in an automated manner.

The experiments were conducted by having the instrument programmed for continuous operation, thereby mimicking on-line measurements. One measurement cycle consisted of protein removal by dialysis, injection of the protein-free sample and the OPA derivatization reagent into the holding coil, derivatization period (in the holding coil, under stopped flow), separation and detection. The measurements were carried out in such a way that while one sample was undergoing separation and detection, the next sample was in the dialyzer, undergoing protein removal. One measurement cycle lasted 14 min.

Duration	Processing of sample (N)	Processing of sample (N+1)
30 s	Fill pump with strong eluent.	
70 s	Form gradient in holding coil by aspirating weak eluent.	
5 s	Aspirate OPA (20 µL) into holding coil.	
2 s	Aspirate <i>sample N</i> (2 µL) from acceptor compartment of dialyzer into holding coil.	
5 s	Aspirate OPA (20 µL) into holding coil.	
60 s	Wait for derivatization reaction to go to completion.	Wash dialyzer. Bring <i>sample N+1</i> into donor compartment of dialyzer.
230 s	Send derivatized <i>sample N</i> to column, followed by gradient in holding coil.	
70 s	Fill pump with strong eluent.	
350 s	Send strong eluent to column to elute remaining amino acids.	
2 s	Save data file for <i>sample N</i> .	

Table 1 Instrument operation cycle. Note that samples *N* and *N+1* are partially processed in parallel.

Experimental protocols

Gradient generation and visualization. The gradient was visualized by using water in place of the weak mobile phase and 0.1 % acetone in water in place of the strong mobile phase. Recording the absorbance signal at 265 nm generated a tracer curve for the gradient profile.

Linearity. Amino acid standard solutions (- matrix) of 10, 50, 100, 500 and 1000 µM were measured in linearity experiments. Four replicate injections were made per

concentration level (n=4). The peak heights were determined and the linearity of each amino acid signal was evaluated by a least squares linear regression fit to the equation

$$\text{Peak height}_i = \text{Intercept}_i + \text{Slope}_i \times C_i$$

where Peak height is given in terms of fluorescence intensity values (PMT counts)
C is the amino acid concentration (μM)

Accuracy. Accuracy was evaluated by comparing the signals from “amino acid standard solutions (- matrix)” to those obtained from “amino acid standard solutions (+ matrix)”. Such experiments were conducted at 100 μM and 500 μM amino acid levels. The number of replicate injections was four (n=4). The peak heights were determined and an accuracy % was calculated as

$$\text{Accuracy \%} = \frac{\text{Average peak ht (+ matrix)}_{\text{amino acid } i \text{ conc level } j}}{\text{Average peak ht (- matrix)}_{\text{amino acid } i \text{ conc level } j}} \times 100\%$$

Precision. The precision experiments consisted of repeated injections of amino acid standard solutions (- matrix). The experiments were conducted at 100 μM and 500 μM amino acid levels. The number of replicate injections was six (n=6). Precision was evaluated by determining the peak heights and calculating their relative standard deviations.

Limit of detection. The limit of detection for amino acid “i” (LOD_i) was calculated from

$$\text{LOD}_i = 3 \times \frac{\text{SD}_{\text{baseline}}}{\text{Slope}_i}$$

where SD_{baseline} = standard deviation of the baseline signal
Slope_i = slope from the calibration curve for amino acid “i”

Application to monitoring. The application of the method to monitoring a real cell culture medium sample was demonstrated as follows. The instrument was connected to a stirred vessel containing culture medium supplemented with 10 % serum (no cells were present in the medium). It was programmed to draw a sample every 1h 16 min, process it and analyze it for amino acids. The program included steps for washing and disinfecting the dialyzer by passing 0.15 % solution of benzalkonium chloride through it. In addition, amino acid standard solution (- matrix) was injected periodically to verify the system response.

Dialysis verification. These experiments were performed in order to verify that the dialyzer functions as intended. A solution of 0.06 mM BSA or a solution of 0.4 mM tryptophan was brought into the donor compartment of the dialyzer. The dialysis was allowed to proceed for varying periods of time (1, 2, 5 or 10 min), a 30 μ L sample was injected from the acceptor compartment and its absorbance at 280 nm was measured.

Tryptophan was selected as a marker amino acid due to its strong native absorbance at 280 nm. The concentrations of the BSA and tryptophan solutions were selected in such a way that they had roughly equal absorbance at 280 nm. The equal absorbance, as well as the linearity of the BSA and tryptophan signals was verified by removing the dialyzer and injecting serial dilutions of both solutions directly into the instrument (data not shown).

Data processing

Peak heights were determined using the peak detection and measurement capabilities in the FIALab for Windows software (FIALab Instruments). An exception was the lysine and glutamine peak heights that were determined manually.

Results and discussion

Amino acid separation

A chromatogram of a 1000 μ M amino acid standard solution is depicted in Fig. 3A. It shows that some of the amino acids (Gly+Arg, Tyr+Ala, Trp+Met) co-elute as one peak. In addition, there is little resolution between Gln and His. The remaining amino acids are separated from one another. One reason for the incomplete resolution is the limited length of the gradient and the resulting duration of the elution period. An extended, less steep gradient would be required for complete resolution. Generating such a gradient with this instrument configuration is not possible, since it would require a holding coil with a volume considerably larger than the volume of the syringe pump. Another reason for co-elution lies in the limitation of the separation column: some of the amino acids remained unresolved even when the column was connected to a conventional HPLC instrument and run with a long gradient attainable with conventional instrumentation.¹¹

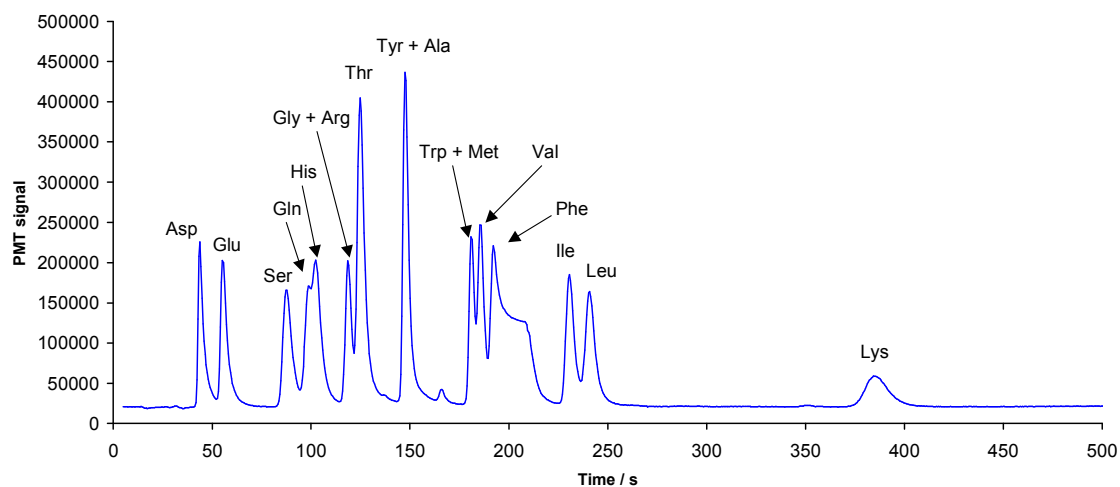
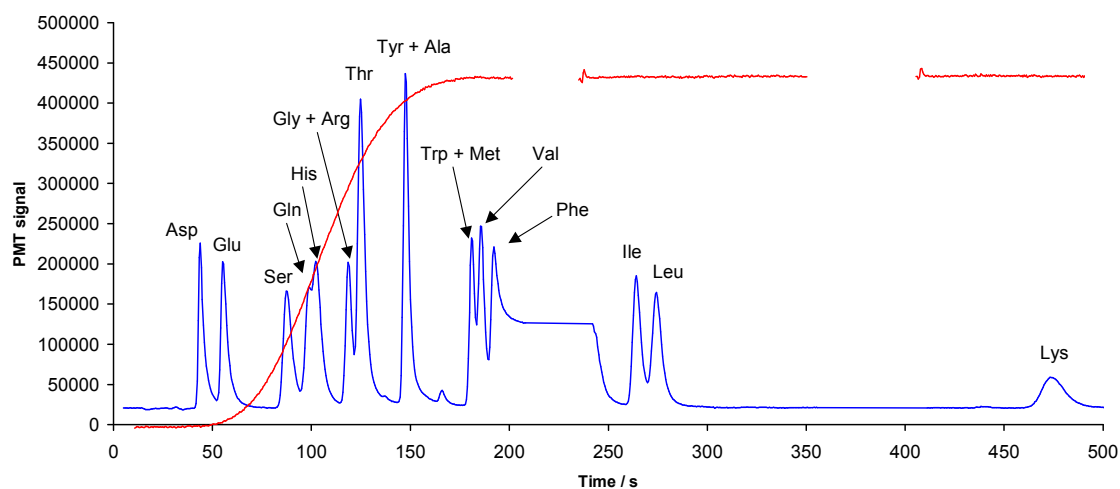


Fig. 3 (A) A chromatogram of the amino acid standard solution (-matrix) at 1000 µM level. The plateau between Phe and Ile is caused by a stopped flow period due to pump fill. The red line shows the gradient profile, representing the ratio [20 mM phosphate buffer pH 7.0 : methanol] going from 74:26 to 44:57. (B) A parsed version of the same chromatogram after removing the pump fill periods.

It should be noted that cysteine and proline do not appear in the chromatogram despite being present in the standard solution. This is because there was only a very weak spectral response for the former and no response for the latter.

The gradient profile (visible in Fig. 3A) consists of a linear portion and an isocratic portion, connected by a short non-linear segment. Backpressure measurements (carried out with a Sper Scientific handheld pressure meter (Cole Parmer, Vernon Hills, IL, USA)) showed that the maximum pressure during the measurement cycle was ca. 300 psig. It should be noted that such values would be out of reach for conventional Sequential Injection pumps whose upper pressure limit is 60-100 psig.

The necessity to fill the syringe pump periodically may create plateaus in the chromatogram, as can be seen in Fig. 3A. Since the plateau regions are simply lines connecting two data points, they can be removed without losing any information content. A chromatogram “parsed” in such a way is shown in Fig. 3B.

Linearity

The results of the linearity experiments are summarized in Table 2. All the responses show acceptable linearity.

Peak	Intercept	Slope	r ²
Asp	4901	193	0.9983
Glu	1839	180	0.9999
Ser	1689	140	1.0000
Gln	2854	186	0.9918
His	2477	200	0.9962
Gly+Arg	1463	177	1.0000
Thr	7152	360	0.9978
Tyr+Ala	2	438	0.9999
Trp+Met	2883	220	0.9984
Val	4655	227	0.9984
Phe	4139	188	0.9994
Ile	2519	158	1.0000
Leu	2089	138	0.9999
Lys	1367	33	0.9988

Table 2 Method linearity: regression fit parameters. The data was fitted to an equation $P = Intercept + Slope \times C$, where P = peak height and C = the amino acid concentration. The number of replicate injections was four (n=4).

Accuracy

The results of the accuracy experiments are summarized in Table 3. A partial loss of analyte is seen for isoleucine and leucine in the presence of the matrix background. This may be caused by isoleucine and leucine binding to BSA and being partly retained in the donor compartment of the dialyzer. The other amino acids do not show signs of being affected by the matrix solution.

Peak	Accuracy %, 100 µM level	Accuracy %, 500 µM level
Asp	101.2	96.9
Glu	99.5	98.5
Ser	101.0	98.3
Gln	98.2	99.8
His	98.0	99.5
Gly+Arg	96.1	100.5
Thr	98.5	99.3
Tyr+Ala	104.4	99.2
Trp+Met	98.7	98.4
Val	99.4	97.9
Phe	99.9	98.3
Ile	101.4	89.1
Leu	101.7	87.2
Lys	95.7	99.9

Table 3 Method accuracy results. The number of replicate injections was four (n=4).

Precision

The results of the precision experiments are summarized in Table 4. They show that the precision lies within 2-5 % for the 500 µM level, and within 4-10 % for the 100 µM level.

Peak	RSD, 100 µM level (%)	RSD, 500 µM level (%)
Asp	5.8	2.2
Glu	6.2	2.0
Ser	7.1	3.0
Gln	5.5	3.2
His	6.1	2.9
Gly+Arg	7.0	3.3
Thr	5.7	3.0
Tyr+Ala	6.7	3.3
Trp+Met	5.0	2.1
Val	4.4	2.4
Phe	4.7	2.1
Ile	6.5	4.6
Leu	7.1	5.0

Lys 9.6 3.0

Table 4 Method precision results. The number of replicate injections was six (n=6).

Limit of detection

The limits of detection ranged from 2 to 5 μM , with the exception of lysine that has a considerably larger LOD (21 μM). The reason for this behavior is the late elution of lysine: since it elutes late in the isocratic portion of the chromatogram, the peak becomes broader and shorter compared to the other peaks. The individual LOD values are presented in Table 5.

Amino acid	LOD (μM)
Asp	4
Glu	4
Ser	5
Gln	4
His	4
Gly+Arg	4
Thr	2
Tyr+Ala	2
Trp+Met	3
Val	3
Phe	4
Ile	5
Leu	4
Lys	21

Table 5 Limits of detection (LOD).

Application to monitoring

The results of culture medium monitoring are shown in Fig. 4. Except for some minor shifts in peak positions due to column equilibration, the initial and final chromatograms are identical. The results show no signs of column fouling or response degradation.

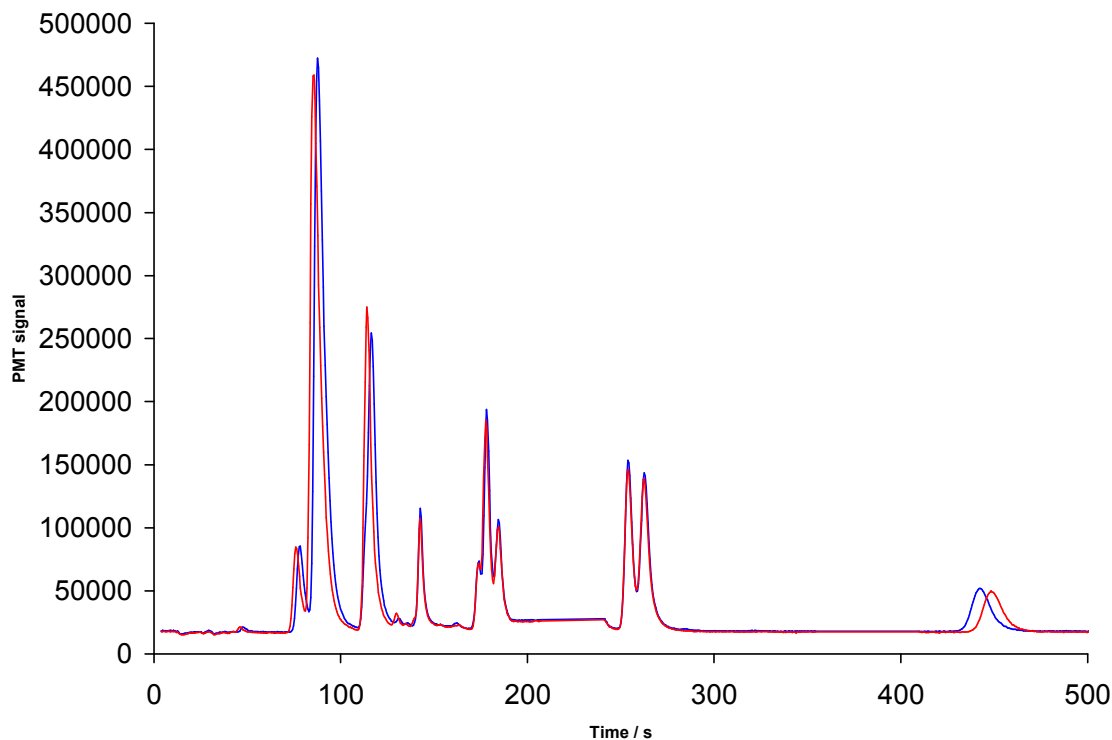


Fig. 4 Application to monitoring cell culture medium. The two chromatograms represent the initial results (blue line), as well as results obtained 30 h (and 27 injections) later (red line).

Dialysis verification

The signals from BSA- and tryptophan-dialysates are shown in Fig. 5. The results show that the dialysis membrane blocks BSA while allowing tryptophan to pass.

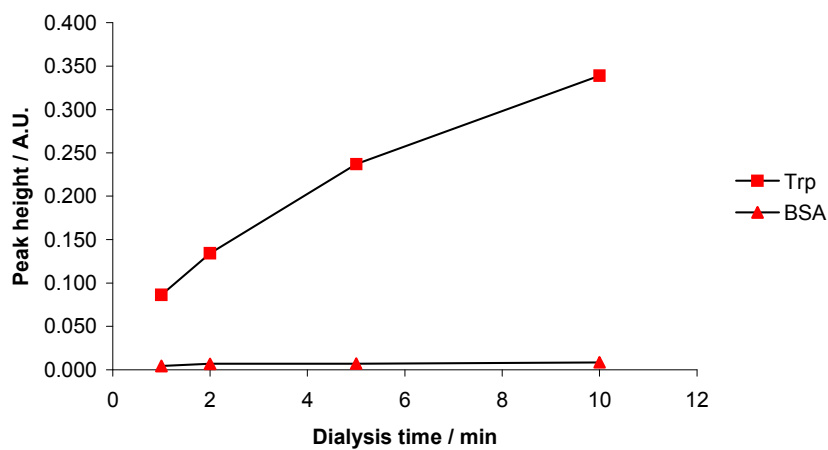


Fig. 5 The absorbance signal from BSA-dialysates (▲) and tryptophan-dialysates (■).

Conclusions

The instrument described in this paper is the first system specifically designed for SIC. Due to its ability to operate at backpressures up to 500 psig, it makes possible for SIC to utilize the full range of monolithic column sizes, conventional LC flow rates and viscous mobile phases. The amino acid analysis application demonstrates the high degree of automatic sample processing that can be achieved by SIC, and demonstrates the advantages that Sequential Injection can lend to liquid phase separation methods. Due to flow programming and the “stop and go” type of operation that is inherent for Sequential Injection, SIC has the advantage of saving fluids as compared to continuous flow methods like Flow Injection and RP-LC. The overall compact size, visual transparency of the flow path, as well as the unique reagent/solvent conservation methodology are especially useful for on-line monitoring applications where intermittent operation is the norm.

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