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AMINO ACID ANALYSIS USING STANDARD HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY EQUIPMENT

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SUMMARY

The components normally employed for high-performance liquid chromatography, with the addition of an interface, have been used to construct an amino acid analyser. Detection was effected with either ninhydrin or *o*-phthalaldehyde. The separation of amino acids normally found in protein hydrolysates was performed within 45 min. Reproducibility of the time for peak elution enabled peak-height or -area measurements to be used for quantitation in the range 10 pmol to 25 nmol. The compositions of the buffers that achieve these fast analysis times and permit quantitation at the low pmol level using *o*-phthalaldehyde detection are given.

INTRODUCTION

In any study of the primary structure of proteins the most frequently required analysis is that of amino acids. The successful application of high-performance liquid chromatography (HPLC) techniques to this analysis has been demonstrated by the performance of modern amino acid analysers such as the Durrum D-500. To date, HPLC of amino acids or derivatives thereof has been performed on either sulphonated polystyrene or silica-based supports[1,2].

Commercially available instruments basically utilize the method of analysis originally described by Spackman *et al.*[3]. Essentially, amino acids are separated by cation exchange on cross-linked sulphonated polystyrene using citrate buffers, and detection is effected by post-column derivatization utilising ninhydrin[4] or the more recently introduced fluorogenic reagent *o*-phthalaldehyde (OPA)[5]. The use of a "micro-bead" resin with a uniform diameter of about 10 μm or less, improved buffer composition and high-pressure chromatographic techniques have led to a decrease in the analysis time from the original 24 h to approximately 1 h. Moreover, a 100-fold increase in sensitivity makes it possible to analyse samples containing less than 1 nmol of each amino acid, utilising ninhydrin. This is a consequence of the use of microbore

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columns and improved spectrophotometer design. Analysis at the level of a few picomoles is possible when OPA is used[6].

Silica-based chromatographic supports are less compressible than polystyrene and therefore flow-rates can be increased, allowing, in some instances, a shorter analysis time. Numerous reports on the HPLC of a variety of amino acid derivatives have appeared, *e.g.*, PTH-[7,8], DABTH-[9] and dansyl-[10,11]. The derivatization step, however, is tedious if numerous samples have to be processed and may introduce errors. Separation of all the free amino acids found in normal protein hydrolysates within 30 min has been achieved by normal-phase chromatography on NH_2-silica[1]. Detection is effected by absorption at 200 nm. Interference with the analysis by contaminants should always be considered, especially at high sensitivity. Reversed-phase ion-pair chromatography of amino acids, utilizing OPA post-column derivatization, has been reported[2]; however, Glu and Gly are not separated and Thr is poorly resolved from the latter. The speed of analysis (30 min) combined with the sensitivity of OPA detection make this a promising alternative to analysis on polystyrene supports.

We describe here how an HPLC apparatus normally employed for peptide or protein separation[12–15] can be used for amino acid analysis. The reproducibility, speed of analysis and sensitivity are equal to or better than those of most commercially purpose-built analysers.

EXPERIMENTAL

Buffers and reagents

Unless stated otherwise, the chemicals used were of the best available grade from either Merck (Darmstadt, G.F.R.) or Fluka (Buchs, Switzerland). The cation-exchange resins used were DC-4A, DC-6A (Durrum, Sunnyvale, CA, U.S.A.) and Aminex A-9 (BioRad Labs, Richmond, CA, U.S.A.). Stainless-steel columns (250 × 4.6 or 3.2 mm I.D.) were slurry packed (reservoir volume, 10 ml) in buffer C, at 70°C, with a flow-rate of 2 ml/min (4.6 min I.D. column) or 1 ml/min (3.2 mm I.D. column).

The compositions (per litre) of the buffers used for detection with OPA were as follows:

Buffer A 0.2 *N* Na^+; pH 3.25: 3 g of NaOH, 8.9 g of Na_2SO_4, *ca.* 8.2 ml of formic acid, 10 ml of 1-propanol.

Buffer B (0.2 *N* Na^+; pH 4.25); 8 g of NaOH, *ca.* 10 ml of formic acid.

Buffer C (1.1 *N* Na^+; pH 7.9) 28 g NaOH, 40 g of trisodium citrate, *ca.* 26.4 ml of formic acid.

Sample application buffer: 14.2 g of Na_2SO_4, 20 ml of formic acid.

Detection buffer (pH 10.3): 43 g of KOH, 62 g of boric acid, 1 ml of 2-mercaptoethanol, 2 ml of 30% (w/w) Brij (Pierce, Rockford, IL, U.S.A.), 200 mg of OPA (in 2 ml of methanol).

Formic acid was distilled from ninhydrin. Ammonia contamination in buffers A and B can be reduced by filtration through DC-3A resin (Na^+ form) (Durrum, Palo Alto, CA, U.S.A.). The detection buffer was stored in amber-glass bottles under nitrogen.

When ninhydrin detection was utilized, the buffers for column elution were Pico buffer systems II A, B and C (Pierce, Rotterdam, The Netherlands) and in later work Hi-Phi eluents Na-A, B and C (Dionex, Sunnyvale, CA, U.S.A.). The solution

or ninhydrin detection was prepared as follows: 20 g of ninhydrin and 2 g of hy-
lrindantin (Pierce) were dissolved in 750 ml of dimethylsulphoxide (Merck), 250 ml
of 4 *M* sodium acetate pHix-buffer (Pierce) was added and the solution was kept
under nitrogen.

For both detection systems, sodium hydroxide (0.2 *N*) was used for column
egeneration.

Apparatus

The system used for OPA detection is shown in Fig. 1. Buffers A, B and C and
).2 *N* sodium hydroxide solution were connected to a Model 110A buffer pump
Altex, Berkeley, CA, U.S.A.) via an eight-port motorized valve (Kontron, Zürich,
Switzerland). For analysis of peptide hydrolysates, buffers A, B and C and regener-
ation were connected to positions 1, 2, 3 and 4 respectively. Sample application was
effected via a Kontron Model 100 automatic sample injector fitted with a 100-μl loop.
The column was fixed in a stainless-steel water-jacket and the temperature was con-
trolled via a Haake Proportional water-bath (Haake, Karlsruhe, G.F.R.). A pressure
gauge (0–4000 p.s.i.) was connected via a Swagelok $\frac{1}{16}$-in. T-piece between the sample
injector and the buffer pump. Detection solution was pumped (Altex Model 110A)
and mixed with the eluate from the column via a Swagelok $\frac{1}{16}$-in. T-piece. For OPA

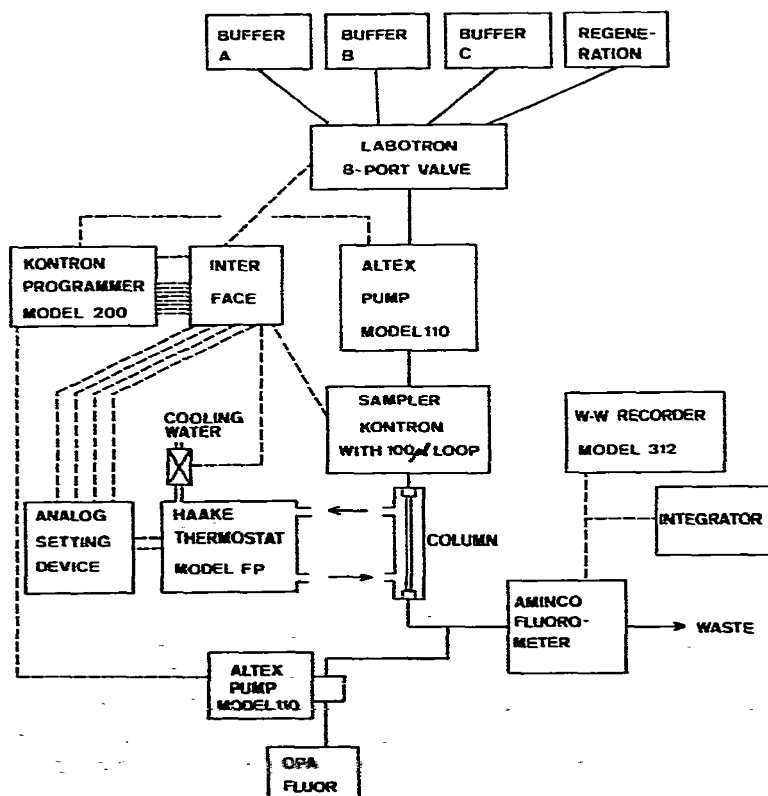

Fig. 1. Apparatus for amino acid analysis.

the reactants were mixed in 3 m of PTFE tubing (0.3 mm I.D.) and detection was carried out with an Aminco Fluoro Monitor (American Instrument Co., Silverspring, MD, U.S.A.) set at the least sensitive range, and equipped with a 70-μl flow cell; filters were as supplied for fluram detection. For detection with ninhydrin, the solutions were mixed through 10 m of PTFE tubing (0.3 mm I.D.) maintained at 120°C by submersion in a Haake Model SK58 oil-bath, and absorption at 440 and 570 nm was measured with a Kontron spectrophotometer.

Located after the derivatization pump and connected in series were a stainless-steel column (250 × 4.6 mm I.D.) containing a 10-cm high bed of DC-6A (to reduce pulsation noise), 2 m of PTFE tubing (0.3 mm I.D.) and a pressure gauge (0-200 p.s.i.). PTFE back-pressure coils 3 m × 0.3 mm I.D.) were connected to the outlet of each detector. Chromatograms were plotted by a two-channel recorder (W + W Model 312, Kontron) and integrated by a Supergrator 3 (Columbia Scientific Industries, Austin, TX, U.S.A.).

A microprocessor (Altex Model 420, modified for eight external flags, or a Kontron Model 200, normally employed to control the flow-rate of two pumps for grandient elution) was used to control all functions of the analyser. The buffer pump was controlled as A and the derivatizing pump as B. Flow-rates were as follws: 3.2 mm I.D. column, column eluant and OPA solution 0.4 ml/min, ninhydrin 0.2 ml/min; 4.6 mm column, column eluent and OPA solution 0.62 ml/min, ninhydrin 0.32 ml/min. The recorder chart speed could be controlled by the microprocessor. Flag 7 controls the buffer selection; one contact closure (duration 0.01 min) caused the valve to rotate by one port. Flag 8 (duration 0.05 min), activated after column regeneration, returned the buffer valve to the start position (buffer A). The sample injector and integrator were started by a contact closure, duration 10 sec, of flag 5. Five flags were used for temperature control of the column. Flags 1–4, by switchin a 10-turn potentiometer in series with the programming input of the Haake thermostat, enabled up to four temperatures to be used. Rapid cooling was achieved by circulation, via a magnetic valve (controlled by flag 6), of mains water through a coil in the Haake bath. Details of the interface between microprocessor and rotary valve are given in Fig. 2.

RESULTS AND DISCUSSION

HPLC is useful for the analysis and preparative isolation of peptides and proteins[12–17]. An apparatus of this kind, which can be used for both isolation and amino acid analysis, has the advantages of lower cost than analysers built for exclusive use in amino acid analysis and of greater versatility. This, in turn, allows more readily the most effective use of equipment.

The equipment commonly employed for HPLC (Altex or Kontron) consists of a microprocessor which controls the flow-rates of two pumps; gradient elution from two solvents is achieved by adjustment of these rates. Detection of peptides is carried out either by post-column derivatization, via a sampling valve, with the fluorogenic reagents fluorescamine[12,13,18] or OPA[13,19], or directly by absorption in the UV region[14,15,20]. We have used these elements, with a small number of additions, to construct an amino acid analyser.

In Fig. 3A the elution profile of standard mixture of amino acids using ninhyd-

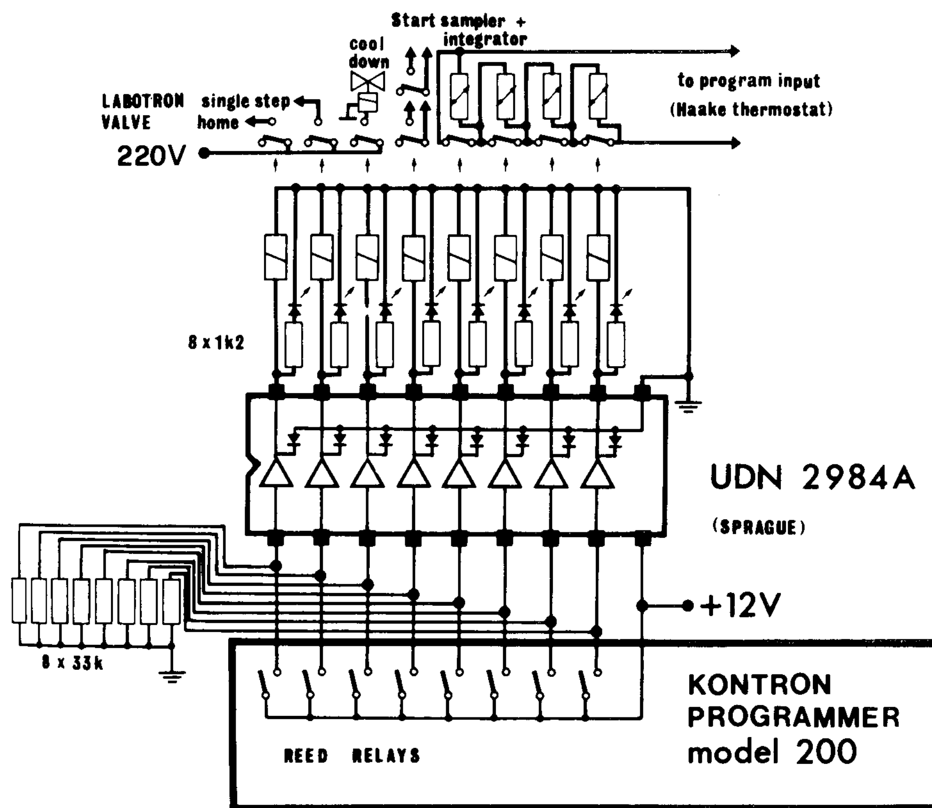

Fig. 2. Circuit diagram for the interface between the microprocessor and the rotary valve. The cost of the components was approximately half that of a standard analytical HPLC column, and the time for construction was 12 h. k = kΩ.

rin is shown. DC-4A resin packed in a stainless-steel column (250 × 4.6 mm I.D.) was eluted with the Pico system II buffers. Stainless-steel columns permit the use of higher pressure than the more commonly used glass columns and thus higher flow-rates are possible. The analysis time, including regeneration and equilibration, for a standard hydrolysate (not including tryptophan) is 60 min, the buffer is pumped at a flow-rate of 0.62 ml/min and the back-pressure is 1500–2500 p.s.i. Similar chromatograms were obtained using DC-6A resin, but, Thr and Ser were not as well resolved. Aminex A9 resin gave unsatisfactory resolution under these conditions. Commercially available buffers were used for elution because of convenience and their constant chemical composition. Initial work was carried out with Pico system II buffers, and although the elution times remained fairly constant, recent stocks of buffer A produced an increase of the ammonia plateau, equivalent to approximately 5 nmol of amino acid. The contamination may be largely removed by filtration through Dowex 50 (Na^+). Hi-Phi eluent buffers give equivalent resolution under the same conditions, with no appreciable interference with the baseline, but the analysis time is 6 min longer than with Pico buffers.

Reproducibility of the chromatography is evident from a comparison of stan-

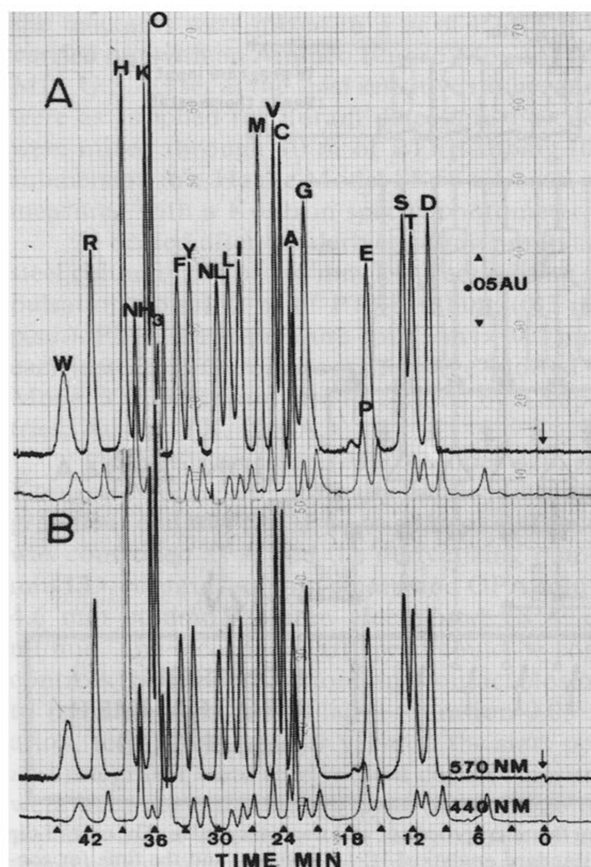

Fig. 3. Chromatograms of a standard mixture of amino acids (10 nmol) using ninhydrin for detection. B was obtained 50 analyses after A. The column (250 × 4.6 mm I.D.) of DC-4A was eluted with Pico system II buffers A, B and C for 17, 15 and 14 min, respectively. Buffers A and C contain 1 and 5% of propan-1-ol, respectively. The equilibration temperature was 40°C and changes to 60, 69 and 75°C were at 6, 28 and 32 min, respectively. Regeneration and re-equilibration were for 6 and 18 min, respectively. Mains water cooling for 6 min was initiated immediately after regeneration. The column back-pressure was 2200 p.s.i. at 40°C and 1500 p.s.i. at 75°C. Abbreviations are standard one-letter codes with the exceptions of NL = norleucine and O = ornithine.

dard runs, 50 analyses apart, shown in Fig. 3A and B. The time of elution for each amino acid is constant to within 20 sec and the peak width is constant. To achieve this reproducible separation, ammonia filtration columns and other sources of large dead volumes must be excluded. A gradual accumulation of air from the buffers in such devices causes alteration of the dead volume, which consequently produces a difference in eluent composition at any buffer change. A constant time of peak elution has two advantages: (1) interpretation of an analysis in which only one or few amino acids are present is easier; (2) peak heights can be used for quantitation.

The use of ninhydrin for detection limits the sensitivity to the high picomole level. When a higher sensitivity is required, fluorescence detection using OPA is available. Below the 1 nmol level, impurities present in commercial buffers make the

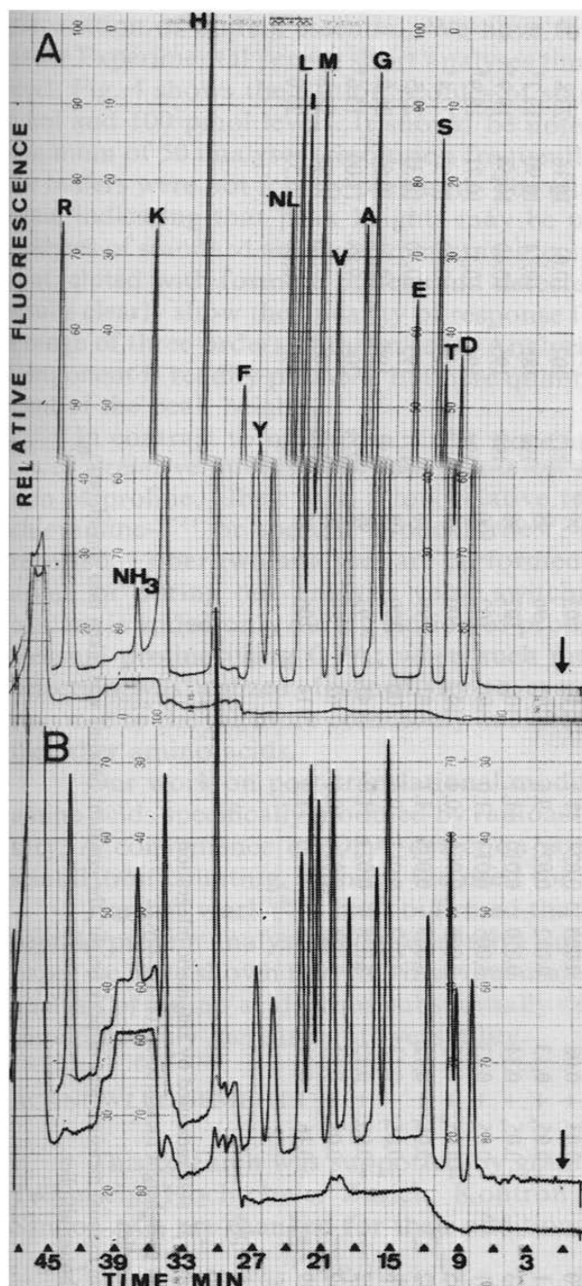

Fig. 4. Elution profile of a standard mixture of amino acids using OPA for detection. A, 1 nmol per component; B, 100 pmol per component. The column (250 × 3.2 mm I.D.) of DC-4A was eluted with formate buffers (see Experimental). Elution with buffers A, B and C for 13, 11 and 16 min, respectively. The equilibration temperature was 38°C and changes to 59 and 75°C were at 8 and 26 min, respectively. Regeneration and re-equilibration were for 4 and 14 min, respectively. The column back-pressure was 2500 p.s.i. at 38°C and 1600 p.s.i. at 75°C. Injection to injection time was 60 min.

TABLE I

ANALYSIS OF STANDARD AMINO ACID MIXTURES WITH CALIBRATION AT 1.0 nmol USING OPA FOR DETECTION

Amino acid	*Elution time (min)**		*Standard deviation (nmol) for 1.0 nmol***		*10 pmol*‡‡	*100 pmol*‡‡			*200 pmol*‡‡		*10 nmol*‡‡		*25 nmol*‡‡
	Mean	*Maximum deviation*	*a****	*b*‡	*c*‡‡‡	*a****	*b*‡	*c*‡‡‡	*a****	*b*‡	*a****	*b*‡	*a****
Asp	7.98	± 0.03	0.013	0.015	11.0	99	97	98	206	202	10.02	10.00	25.9
Thr	9.35	± 0.01	0.012	0.013	10.8	99	101	95	193	195	10.02	9.95	26.5
Ser	9.92	± 0.02	0.010	0.008	12.5	101	103	100	197	200	10.31	10.53	20.5†
Glu	11.90	± 0.03	0.017	0.014	9.5	110	105	102	217	204	9.84	10.30	24.6
Gly	15.37	± 0.02	0.011	0.011	11.8	101	99	101	195	193	10.56	10.63	18.7†
Ala	16.32	± 0.02	0.008	0.010	11.5	102	99	100	198	197	10.21	10.24	23.0†
Val	18.70	−0.03, + 0.08	0.020	0.024	11.1	116	103	99	212	202	10.01	10.16	23.8
Met	20.11	−0.05, + 0.13	0.012	0.023	9.6	103	96	95	194	185	10.74	10.90	19.2†
Ile	21.33	−0.05, + 0.14	0.010	0.010	11.1	102	102	98	198	195	10.40	10.54	19.9†
Leu	22.01	−0.05, + 0.13	0.005	0.013	11.0	100	102	100	194	196	10.51	10.71	18.9†
NorLeu	22.85	−0.06, + 0.12	0.008	0.005	10.4	102	100	98	198	195	10.40	10.50	22.4†
Tyr	25.32	−0.06, + 0.12	0.009	0.005	11.1	101	99	98	191	193	10.30	10.33	25.5
Phe	26.78	−0.05, + 0.13	0.012	0.013	10.6	100	100	98	191	194	10.34	10.50	26.0
His	30.22	−0.06, + 0.13	0.028	0.023	11.0	141	93	98	223	204	9.88	11.00	15.6†
Lys	34.69	−0.08, + 0.11	0.024	0.026	10.6	62	89	100	228	185	9.33	10.18	18.8†
Arg	42.99	−0.16, + 0.21	0.013	0.020	11.2	114	106	99	197	196	10.27	10.53	23.0

* Average for 30 samples.
** 7 standard runs.
*** Determined from peak area measured by automatic integration.
‡ Determined from peak height measured automatically.
‡‡ Average for 2 samples.
‡‡‡ Determined from manual measurement of peak height; the constant baseline was taken into consideration.
† Saturation of photomultiplier.

preparation of buffers essential. We have found that the formate buffers described under Experimental permit short analyses times and quantitation at the low picomole level. Fig. 4 shows the elution profiles of standard mixtures of amino acids at the 1 nmol and 100 pmol levels. It should be noted that the column had been through a minimum of 50 analyses (resolution frequently diminishes with column age) and that the buffers were not freshly prepared. The elution times and peak widths are similar, again indicating that peak heights may be used for quantitation. Results from the analyses of standard amino acid mixtures, containing 10 pmol to 25 nmol per component, eluted with formate buffers and detected with OPA, are given in Table I. The results clearly show the linearity of response for both peak area and peak height over a range of three orders of magnitude. Analysis on as little as 10 pmol per amino acid component is readily possible, but here quantitation is restricted to manual measurement of the peak height.

In contrast to ninhydrin, OPA does not react with proline and cysteine produces a relative fluorescence that is too low for practical use. Methods for the detection of proline, albeit with a low relative response, by post-column oxidation with chloramine-T[21] or sodium hypochlorite[22], prior to reaction with OPA, have been reported. Either two analyses are performed, one with and one without an oxidizing agent, or during one analysis valve switching is arranged in such a way that the oxidant is added only during elution of proline. In this work no attempt was made to measure proline using OPA; when such measurements were obligatory, ninhydrin detection was utilized. Insensitivity to cysteine has not presented problems as the response to the far more commonly analysed alkylated derivatives is equal to that of the other amino acids.

Our work on post-translational modification of proteins made the analysis of amino acids specifically modified by radioactively labelled ^{14}C and ^{3}H reagents necessary. A convenience of OPA detection is that fractions may be used directly for scintillation counting, without the need for split-stream valves.

Further work[13–15] has confirmed that the application of HPLC technology to peptide/protein analysis and preparative isolation has become a powerful tool. In this paper we have shown that this instrumentation can be used with equal success for the analysis of amino acids, thus substantially decreasing the cost of the equipment while simultaneously increasing its versatility.

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