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Chromatography of 99 amino acids and other ninhydrin-reactive compounds in the Pickering lithium gradient system

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ABSTRACT

High-performance liquid chromatographic systems can be adapted to the high-resolution determination of free amino acids with hardware and reagents from Pickering Labs. The lithium-based eluent gradients used allow good separations to be achieved isothermally in 2 h. Although the overall elution pattern correlates strongly with those of established automated methods, the differences can be large, and are numerous enough that one type of system cannot serve as a predictor for the other. Relative retention times in the Pickering system were determined for 99 ninhydrin-positive compounds: imino acids, ureides, amino sugars, amino acids and derivatives, with emphasis on those occurring in plants.

INTRODUCTION

Automated analyses of the amino acids derived from proteins, based on cation-exchange chromatography with post-colum ninhydrin derivatization [1], found widespread application with a variety of instruments dedicated to that purpose. Many commercial amino acid analyzers have been produced worldwide, including, in the USA, those from Technicon (Tarrytown, NY, USA), Beckman Instrument (Fullerton, CA, USA), Durrum Instruments (Palo Alto, CA, USA) and Phoenix Precision Instruments (Philadelphia, PA, USA). Run times were usually several hours, and for the much more difficult separations of so-called physiological amino acids (the highly complex mixtures of free amino acids and related compounds found in both animal and plant materials) could range up to 25 h. These systems used automated changes of eluent buffers, stepwise or gradient, and automated changes in column temperature, to achieve critical separations [2]. The advent of higher performance resins and lithium-based eluents facilitated such separations in much shorter run times [3].

Further developments included greater sensitivity from postcolumn reagents allowing fluorescence detection, the rapid derivatizations at ambient temperature encouraging the use of non-dedicated high-performance liquid chromatographic (HPLC) equipment [4]. In addition, the introduction of several precolumn reagents yielding relatively hydrophobic derivatives allowed the entirely different chromatographic principles of reversed-phase HPLC to be exploited [4]. Rapid high-resolution separations thus became attainable, although not without drawbacks. Some reagents yield unstable derivatives; others may produce artefactual peaks and/or multiple peaks from single amino acids; most do not react with secondary amines; and the complex matrices of physiological mixtures can interfere with smooth derivatization [5,6]. More importantly, matrix variation can lead to considerable uncertainty in elution times [7].

In contrast, ion exchange is relatively insensitive to matrix components other than easily removed proteins. The ion-exchange columns and postcolumn hardware developed by Pickering Labs. permit the adaptation of suitable (*i.e.*, rapid-pulse or essen-

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tially pulseless) HPLC systems to the high-resolution determination of free, non-derivatized amino acids. For physiological amino acids (lithium system) the eluent gradients employed permit good separations to be achieved isothermally in a 2 h run to arginine. We adopted this system for the dual purpose of determining the basic amino acid S-methylmethionine in corn [8] and the free amino acids in plant roots. Ninhydrin was the reagent of choice because it reacts with a wide range of compounds, including secondary amines, and can provide valuable structural information when absorbance is measured at more than one wavelength.

With the Pickering system, despite the overall similarities, there are many substantial differences in elution order compared with automated sodium-based eluent methods, stepwise or gradient [9–11]. The points of difference with a lithium gradient technique [12] are fewer, but still significant. We present here the results of a chromatographic survey of 99 amino acids and related compounds, most chosen because they occur in plants or are known metabolic inhibitors [13–15].

EXPERIMENTAL

Biochemicals

Forty-four compounds are present in the solution (Amino Acid Calibration Standard) provided by Pickering Labs. (Mountain View, CA, USA) for their lithium Physiologic Fluid columns. The remainder were purchased from Sigma (St. Louis, MO, USA), except for the two dimethylated arginines (N^{G1}Me,N^{G2}Me; N^{G1}diMe), which were gifts from Pickering.

Chromatography

The compounds were separated by the lithium system for physiological fluids, the Pickering 150 × 3 mm I.D., 5-μm column ("high efficiency", formerly "fast run") being used. This system was combined with an HP1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA), modified by dead-heading the pressure transducer to prevent its corrosion by the lithium citrate–chloride eluents [8]. The detector was an Altex/Hitachi Model 100-10 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) and the integrator/recorder was a Shimadzu (Columbia, MD, USA) Model CR3A. Li280 and Li750 eluents (eluents A and B), RG003 regenerant (lithium hydroxide–chloride, eluent C), diluent and ninhydrin reagent were all from Pickering.

The chromatographic conditions were as follows. The column temperature was 42°C and the gradient program was as shown in Table I. This is the standard Pickering program modified slightly to improve some separations, plus an extension to remove homoarginine and to clean the column with 100% (or 99.6%) regenerant between runs. The latter, plus a more essential *ca.* 10-min regenerant wash prior to the first run of the day, was found to be necessary to eliminate ghost peaks. The 99.6% regenerant was used rather than 100% solely because the controlling HP 85B computer cannot handle gradients between eluents B and C alone, *i.e.*, with 0% A. Therefore, 0.4% A was the minimum used. Each regenerant wash was followed by equilibration with eluent A for 25–30 min prior to injection. Injected mixtures were in pH 2.2 diluent and the loop size was 10 μl. The eluent flow-rate was 0.30 ml/min, ninhydrin flow-rate *ca.* 0.3 ml/min and post column reaction temperature 130°C. The monitored wavelength was 570 nm throughout. (Multi-wavelength monitoring with the HP 1040A diode-array detector proved impossible. The system's software prevents the use of this instrument, at any wavelength, when the liquid phase absorbs very strongly in the ultraviolet region, as does the ninhydrin reagent.)

TABLE I

GRADIENT PROGRAM

Time (min)	Eluent A (Li280) (%)	Eluent B (Li750) (%)	Eluent C (RG003) (%)
0.0	100.0	0.0	0.0
9.0	100.0	0.0	0.0
21.0	90.5	9.5	0.0
33.0	80.0	20.0	0.0
49.0	65.0	35.0	0.0
88.0	0.4	99.6	0.0
94.6	0.4	99.6	0.0
120.0	0.4	93.6	6.0
120.5	0.4	93.6	6.0
126.5	0.4	0.0	99.6
134.5	0.4	0.0	99.6
135.0	100.0	0.0	0.0

RESULTS AND DISCUSSION

The results are given in Table II. The twenty protein amino acids are designated by their standard single-letter symbols, the remaining compounds by number in order of elution. The precise retention times may vary slightly from one run to another, one influence being length of preinjection equilibration, and some differences would be expected with other HPLC configurations. However, these retention times will be good indicators of *relative* positions under the stated conditions. In this regard, however, it should be noted that this project was begun with one kind of column and finished with another, packed with resin from a newly acquired source. This resin's properties were sufficiently different that the composition of eluent A, previously designated Li275, was changed to compensate. The new designation became Li280, although both eluents A were actually of pH 2.75 [16]. Differences in relative retention times between the two columns were minor in most instances, one such resulting in a poorer proline–glycine separation. However, all three amino sugars eluted from the new column 3 min earlier in relation to adjacent amino acids (suggesting a higher degree of cross-linking in the new resin [10]). The retention times and profiles presented here were all obtained with the latter column and Li280.

Of the 99 compounds surveyed, 62 elute in the first one-third of the profile. Therefore, to illustrate peak positions and shapes, the compounds were split between three different runs, shown in Figs. 1–3. As markers for profile 2, the following from profile 1 were added: glutamic acid (E), valine (V), β-alanine (67), δ-hydroxylysines (77) and canavanine (91). For profile 3, the markers were aspartic acid (D), proline (P), valine and β-alanine. Included in Table II but not in these profiles were homoglutamine (31), oxidized glutathione (33) and selenomethionine (58). In profile 1 the amount of each compound injected was *ca.* 2 nmol, except for creatinine (80), 20 nmol, and dimethylarginine (95), unknown. In profile 2 each was at 2 nmol except for N^{G1}-methyl, N^{G2}-methylarginine (96), unknown (the two dimethylarginines were provided by Pickering as solutions of unstated concentration). Some oxidation of homocysteine (51) to homocystine (69) was apparent. In profile 3 the following components, which react weakly with ninhydrin and/or yield derivatives that absorb sub-optimally at 570 nm, were at 20 nmol: allantoic acid (4), allantoin (7), hydroxyproline (16), aminooxyacetic acid (20), 5-hydroxypipecolic acid (30), proline, pipecolic acid (49) and 1-aminocyclopropane-1-carboxylic acid (55). The remainder were at 2 nmol. The urea (11) in profile 3 was derived from the breakdown of allantoic acid, which is markedly unstable under these conditions. Carbamyl phosphate (1) and argininosuccinic acid (61) are also unstable at pH 2.2, the latter giving rise to an additional unidentified peak (X) at 57.8 min.

N-Acetylglutamic acid and N-acetylglutamine, eluting at 2.80 and 2.88 min, respectively, react with ninhydrin at only *ca.* $1 \cdot 10^{-4}$ and $3 \cdot 10^{-4}$ times the rates of the corresponding non-acetylated compounds, and are not included in Table II or the profiles. Similarly, N-acetylglucosamine and adenosine-5′-monophosphate also were chromatographed but did not react significantly with ninhydrin under these conditions, as monitored at 570 nm; nor did cytidine-5′-monophosphate, even though the oxidative deamination of cytosine is known to occur in DNA as a long-established pathway of spontaneous mutation [17]. The chromatography of octopine [N-(D-1-carboxyethyl)arginine], widely distributed in plants, yielded a detectable peak only in the position of free arginine.

With regard to the lack of matrix influence on the behavior of amino acids in ion exchange chromatography, our work confirms that such influences appear to be very minor. The only noticeable matrix effects so far have been on aspartic acid. From extracts of both corn kernels [8] and pumpkin roots (unpublished work) made with 3.5% sulfosalicylic acid, ultrafiltered through a membrane with a 5000 molecular weight cut-off, adjusted to pH 2.2 and diluted 1:1 with diluent prior to injection, aspartic acid eluted 1 min *later* than usual. In addition, as a comparison of Figs. 1 and 3 shows, the sharpness, though not the retention time, of the aspartic acid peak can be affected by other amino acids in the mixture. In Fig. 1 the aspartic acid and valine peaks are of similar height, but in the mixture in Fig. 3 the aspartic acid peak (now off-scale) is reproducibly about 60% taller.

The resolving power, speed, matrix indifference and cost of the Pickering system appear to present a

TABLE II

ORDER OF ELUTION

Retention time (min)	Symbol/No.	Compound	Described in plants (P)
2.25	1	Carbamyl phosphate	P
2.28	2	L-Cysteic acid	
2.42	3	O-Phospho-DL-serine	P
2.95	4	Allantoic acid	P
3.2	5	D-Glucosamine-6-phosphate	
3.4	6	Taurine (2-aminoethanesulfonic acid)	P
3.6	7	Allantoin	P
3.7	8	DL-*threo*-β-Hydroxyaspartic acid	
3.9	9	O-Phosphoethanolamine	P
4.5	10	Mannopine [N^{α}-(1-D-mannityl)-L-glutamine]	
5.1	11	Urea	P
6.3	12	β-Cyano-L-alanine	P
6.5	13	N-Methyl-L-aspartic acid	
8.8	14	Glutathione (reduced form)	P
9.4	D	L-Aspartic acid	P
10.5	16	L-Hydroxyproline	P
10.7	17	O-Acetyl-L-serine	P
12.5,13.0	18	L-Methionine sulfoxides	
13.0	T	L-Threonine	P
13.3	20	Aminooxyacetic acid	
13.7	S	L-Serine	P
14.9	N	L-Asparagine	P
15.8	E	L-Glutamic acid	P
16.1	24	L-Azetidine-2-carboxylic acid	P
16.9	Q	L-Glutamine	P
17.4	26	DL-β-Hydroxynorvaline	
17.5	27	L-Homoserine	P
17.5+	28	L-Albizziine	P
19.4	29	Sarcosine	P
19.5	30	5-Hydroxy-L-pipecolic acid	P
19.6	31	L-Homoglutamine	
19.9	32	γ-Methylene-DL-glutamic acid	P
20.5B	33	Glutathione (oxidized)	P
21.2	34	DL-α-Aminoadipic acid	P
21.6	C	L-Cysteine	P
22.5	36	S-Methyl-L-cysteine	P
25.2	P	L-Proline	P
25.8	G	Glycine	P
25.9	39	D-Glucosamine	P
27.3	A	L-Alanine	P
27.4B	41	D-Mannosamine	P
27.9	42	L-Citrulline	P
28.8B	43	D-Galactosamine	P
29.1	44	N^{ε}-Acetyl-L-lysine	P
29.5	45	L-α-Aminobutyric acid	P
31.1	V	L-Valine	P
32.8	47	L-Cystine	P
33.3	48	L-Saccharopine [N^{ε}-(L-glutar-2-yl)-L-lysine]	P
33.7	49	L-Pipecolic acid	P
34.7	50	α-Methyl-DL-methionine	
34.8	51	DL-Homocysteine	P

TABLE II (*continued*)

Retention time (min)	Symbol/No.	Compound	Described in plants (P)
35.8	M	L-Methionine	P
36.6	53	Glycylglycine	
37.4	54	L-Cystathionine	P
38.3	55	1-Aminocyclopropane-1-carboxylic acid	P
38.7	56	N^{α}-Acetyl-L-ornithine	
38.8	I	L-Isoleucine	P
40.3	58	L-Selenomethionine	P
40.4	L	L-Leucine	P
40.6	60	DL-α,ε-Diaminopimelic acid	P
40.9	61	Argininosuccinic acid[b]	P
42.3	62	L-Norleucine	
44.2	Y	L-Tyrosine	P
44.2	64	L-Methionine sulfoximine	
47.1	F	L-Phenylalanine	P
47.2	66	L-Glutamic acid amide (isoglutamine)	
51.1	67	β-Alanine	P
53.9	68	DL-β-Aminoisobutyric acid	P
55.8	69	DL-Homocystine	P
56.9	70	δ-Aminolevulinic acid	P
61.4B	71	5-Hydroxy-L-tryptophan	P
62.9	72	γ-Aminobutyric acid	P
64.1	73	DL-Kynurenine	P
64.3	74	3-Hydroxy-DL-kynurenine	
72.5B	W	L-Tryptophan	P
76.5	76	Ethanolamine	P
80.0,81.3	77	δ-Hydroxylysines (DL- and DL-*allo*-)	P
82.4	78	Ammonia	P
84.4	79	ε-Amino-*n*-caproic acid	
85.8	80	Creatinine	P
90.1	81	L-Ornithine	P
90.2	82	L-α,γ-Diaminobutyric acid	P
93.3	K	L-Lysine	P
96.3	H	L-Histidine	P
98.2	85	3-Methyl-L-histidine (τ-methyl-L-histidine)	P
99.3	86	N^{ε}-Methyl-L-lysine	P
100.1	87	1-Methyl-L-histidine (π-methyl-L-histidine)	
102.4	88	L-Homocarnosine (γ-aminobutyryl-L-histidine)	
102.7	89	L-Carnosine (β-alanyl-L-histidine)	
104.3	90	L-Anserine (β-alanyl-1-methyl-L-histidine)	
107.1	91	L-Canavanine	P
108.0	92	S-Methyl-DL-methionine	P
108.5	93	L-α-Amino-β-guanidinopropionic acid	
109.4	94	L-Leucinamide	
116.8	95	N^{G1}-Dimethyl-L-arginine	
117.3	96	N^{G1}-Methyl,N^{G2}-methyl-L-arginine	
120.4	97	N^{G}-Methyl-L-arginine	
120.6	R	L-Arginine	P
128.1	99	L-Homoarginine	P

[a] B denotes broad peak (ratio of height to width at half-height $\leqslant 10$, with the chromatography and chart parameters used here).
[b] Isomeric constitution unknown.

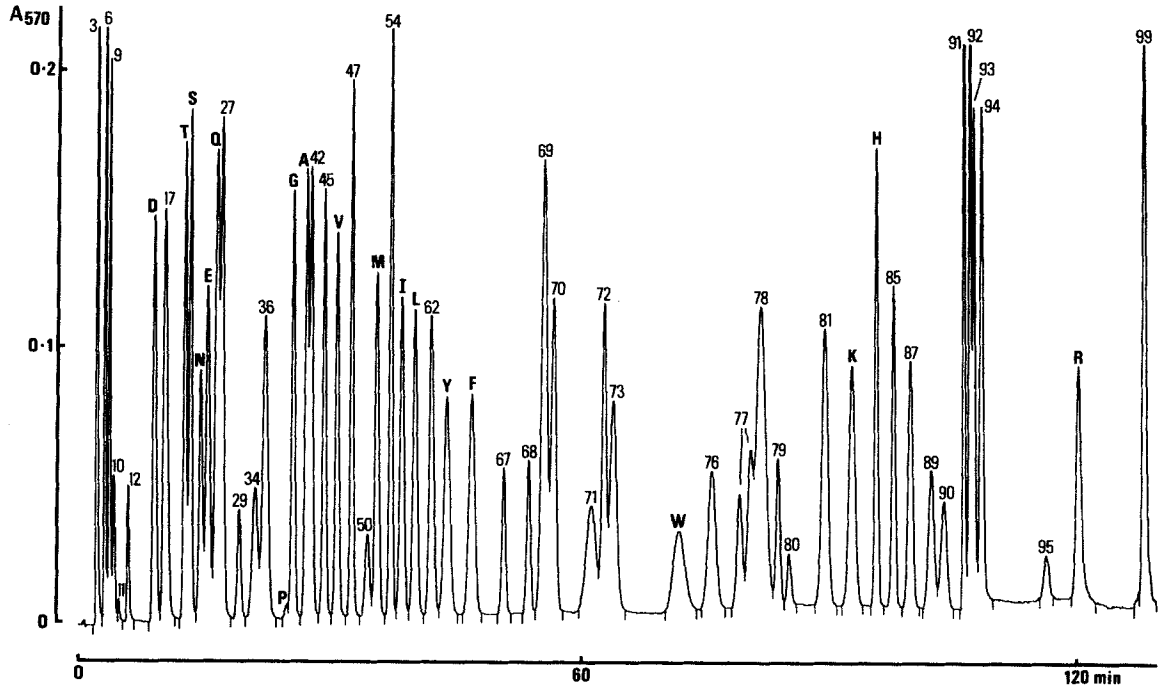

Fig. 1. Chromatographic profile of 59 amino acids and related compounds. Gradient program as in Table I. Injected amounts *ca.* 2 nmol except for 80 (20 nmol) and 95 (not determined).

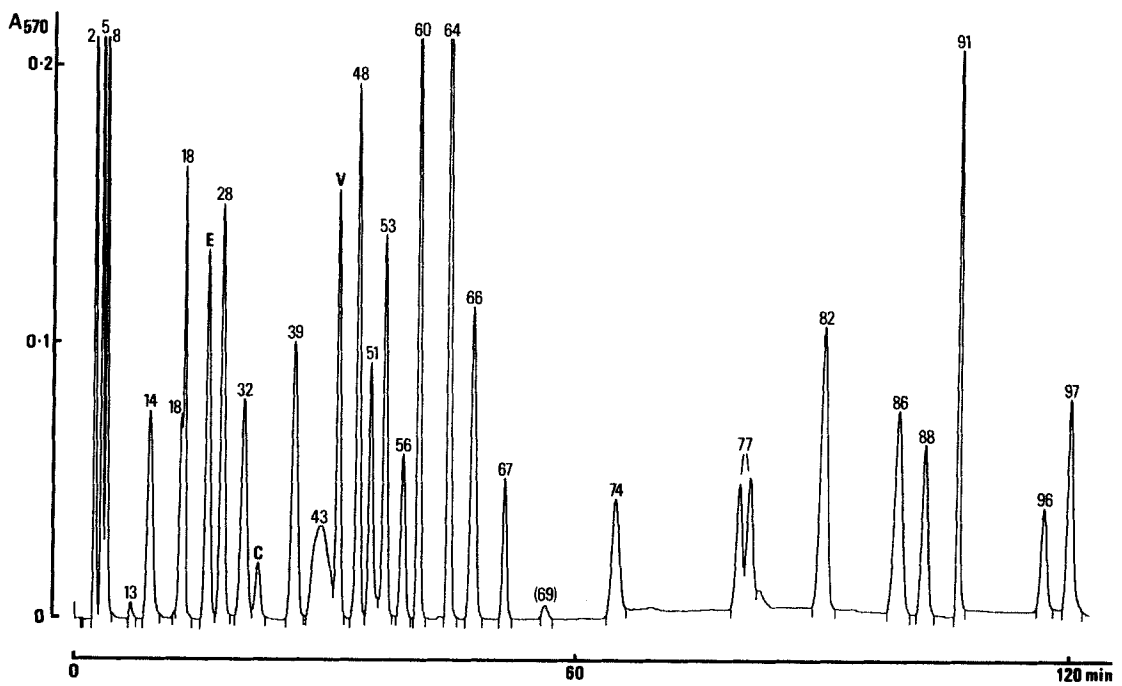

Fig. 2. As for Fig. 1, for 29 compounds. All at 2 nmol except for 96 (not determined). Peak 69: oxidation product of 51.

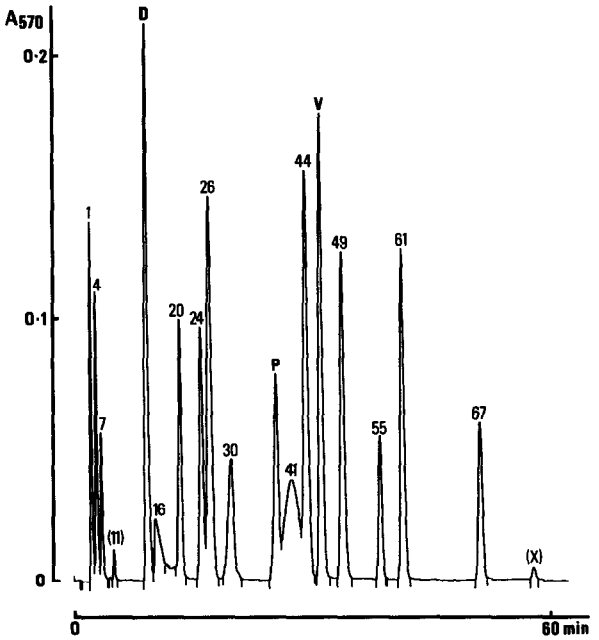

Fig. 3. As for Fig. 1, for 17 compounds. Peaks D, V, 1, 24, 26, 41, 44, 61 and 67 injected at 2 nmol, the remainder at 20 nmol. Peak 11: breakdown product of 4. Peak X: unidentified breakdown product of 61.

relatively favorable combination. Also, because of the first two factors, almost all amino acids elute within small volumes of eluent, 0.2–0.6 ml for the nanomolar amounts employed in this study. This suggests that the most challenging separation problems, those likely to be encountered in the often crowded first 40 min of a profile, especially when concentrations of neighboring components differ widely, could profitably be attacked in the following way. Rather than stretch the profile by using shallower gradients, a more promising approach would be first to apply this system, minus ninhydrin, in order to collect selected narrow bands of the eluate, then, after suitable derivatization in this now-simple matrix, to take advantage of the different separation principles of reversed-phase HPLC. Conceivably, the same piece of HPLC equipment could be utilized for both modes.

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