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Note

Method for the separation of glutamine and homoserine by ion-exchange chromatography

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Moore and Stein[1] were first to develop an amino acid analyser and used an ion-exchange resin for the separation of amino acids. Spackman *et al.*[2,3] made further improvements by introducing devices to make it fully automatic, and also added a recording device to the instrument. Since then a large number of commercially manufactured analysers became available, and the separation of ninhydrin positive compounds in physiological fluids, animal and plant tissues and many other liquids has become almost a routine matter. A large number of methods and techniques are described[4,5] for the separation of different amino acids; however, to the best of our knowledge there is no suitable method available for the separation of glutamine and homoserine, which elute together. The separation of these two compounds was important for our work on aphids, especially in the light of previous reports[6–8] where a high concentration of homoserine was shown in the pea plant (*Pisum sativum* L.) extract, and also in the hemolymph and honeydew of the pea aphid, *Acyrthosiphon pisum* (Harris). A method was therefore developed to separate glutamine and homoserine by ion-exchange chromatography.

EXPERIMENTAL

The amino acid analyser used in the present study was a Beckman Model 121M with a system AA computing integrator. A computerized program describing the series of events for the single column automated ion-exchange analysis is given in Table I, and the technical details of the procedure are as follows.

Operating parameters

Model, 121M; column, 2.8 × 690 mm; resin, AA20 (supplied with the analyser); resin bed size, 0.28 × 37.0 cm; buffer, lithium citrate pH 2.85 ± 0.02 (0.24 *N*); buffer flow-rate, 9.9 ml/h; ninhydrin reagent, prepared by dissolving 0.7 g of hydrindantin (anhydrous) in 900 ml of dimethyl sulphoxide (DMSO); ninhydrin flow-rate, 4.9 ml/h; temperature, 63°; optical density, 0.2.

TABLE I
INSTRUMENTAL ANALYSIS SEQUENCE FOR THE ANALYTICAL RUN

Step	*Step time (min)*	*Events*
1	25.0	Instrument on standby. Integrator on standby. Pumps 2 and 4 on. Buffer to column 2. Column 2 effluent to reaction coil.
2	0.1	Stop program recycle. Transfer sample to metering loop 2.
3	2.0	Complete transfer to metering loop 2.
4	0.1	Inject sample to column 2.
5	54.0	Complete injection. Start computing integrator for column 2 data acquisition. Elution of amino acids from column 2.
6	15.0	Start regeneration of column 2 with lithium hydroxide. Start pump 1. Column 1 effluent to drain.
7	25.0	Equilibrate column 2 with buffer. Column 1 to reaction coil. Column 2 to drain.
8	15.0	Complete equilibration of column 2. Stop pump 1. Column 2 effluent to reaction coil. Column 1 effluent to drain. Data acquisition system to standby.
9	0.1	Start program recycling.
10	20.0	Stop ninhydrin pump (pump 4). Ninhydrin diverter valve to drain. Flush reaction coil with buffer.
11	0.0	Program shut down.

System AA

Peak width, 2.0; slope sensitivity, 5000; T_1, 6.0; minimum area, 0.50; window, 2.0, scaling factor, 99.99.

Standard solution

A stock solution containing L-glutamine, L-glutamic acid and DL-homoserine, each at 2.5 μmoles/ml, was prepared in deionized water. A 0.5-ml volume of this stock solution was diluted to 25 ml with lithium citrate buffer pH 2.2 (0.15 *N*). The diluted solution was used as a standard and contained 2.5 nmoles of each of these three amino compounds in a 50-μl sample, the amount injected on the column for analysis. This solution was freshly used since glutamine hydrolyses in acid medium. Similar solutions at the same concentration were also prepared with each of the individual amino compounds mentioned above and stored frozen till used.

Amino acid calibration standard type P-AN and P-B (purchased from Hamilton, Reno, Nev., U.S.A.) containing 40 amino compounds were diluted with lithium citrate buffer pH 2.2. To this were added known quantities of L-glutamine and DL-homoserine so that the final concentration of most of the compounds was 2.5 nmoles in a 50-μl sample. This standard in the text will be referred to as Hamilton standard.

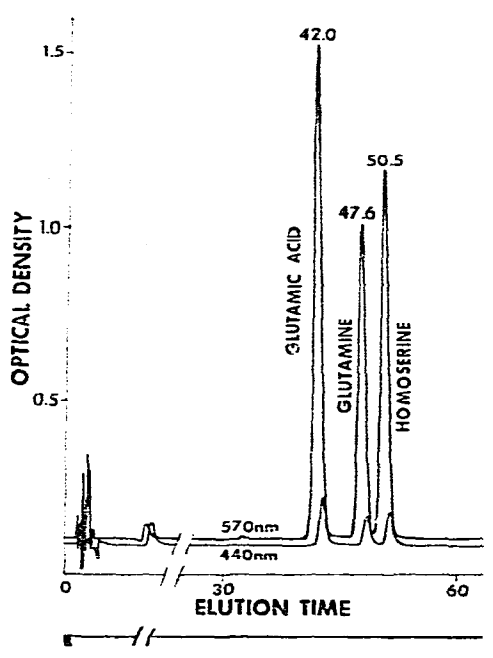
Method for collecting insect hemolymph

Pea aphid adults reared on broad bean (*Vicia faba* L., variety Windsor) plants as described earlier[9] were taken and immersed in hot deionized water at 62° for 2 min, and dried out on a clean filter paper. Under a dissecting stereomicroscope the tip of the antenna of each aphid was cut and the oozing hemolymph sucked in a calibrated micropipette; 5 μl of blood was collected in this way by bleeding 10–12 adults. The collected hemolymph was blown into a micro-centrifuge tube containing 400 μl of 95% ethanol, the pipette rinsed twice with 10 μl of deionized water, and once with

fresh ethanol (10 μl). The tube was centrifuged for 4 min at *ca.* 9500 *g* and the supernatant collected in a small round-bottom glass container, the precipitate was dispersed twice, each time in 200 μl of ethanol and centrifuged, the supernatant mixed with the previous collection and the precipitate finally discarded. The collected sample was evaporated to dryness in a vacuum desiccator. The dried sample was redissolved in 1 ml of lithium citrate buffer pH 2.20 $\pm$ 0.02 (0.15 *N*) and filtered through a Millipore filter (0.22 μm), a few sample storage loops were filled for analysis, and the rest stored frozen.

RESULTS AND DISCUSSION

Our results indicated that glutamine and homoserine which coeluted at lower temperatures separated out at 63°. The average elution time (from 15 analyses) for glutamic acid, glutamine and homoserine whether run singly, or together, was 41.9 (S.D. $\pm$ 0.6), 47.7 (S.D. $\pm$ 0.5) and 50.8 (S.D. $\pm$ 0.7) min respectively (Fig. 1). Other amino compounds did not interfere, or coelute with these two (*i.e.* glutamine and homoserine), since even in the Hamilton standard, which in the present study contained more than 40 ninhydrin positive compounds, glutamine and homoserine separated out clearly (Fig. 2). Pea aphid hemolymph showed the presence of glutamine and homoserine (Fig. 3), as previously reported[8]. Clear separation of these two compounds in hemolymph confirms that the method can be reliably used for biological



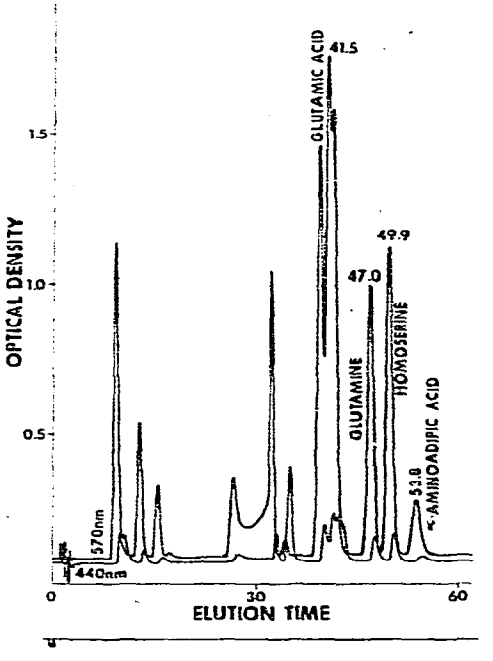


Fig. 1. Chromatogram showing the elution time in minutes of the three amino compounds, L-glutamic acid, L-glutamine and DL-homoserine.

Fig. 2. Chromatogram of Hamilton standard. Note the clear separation of L-glutamine and DL-homoserine. Elution time is given in minutes.

fluids. However, this method is limited to the separation of glutamine and homoserine since many other amino acids at this temperature form a lump and elute together (Fig. 2); therefore for a complete separation, a second sample has to be analysed.

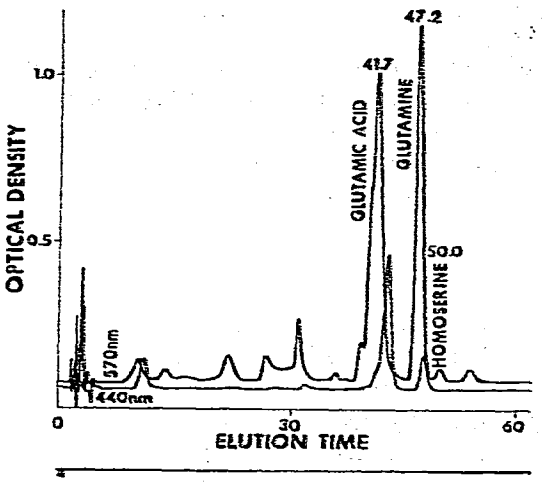


Fig. 3. Chromatogram of deproteinized pea aphid (*A. pisum*) hemolymph showing the separation of L-glutamine and DL-homoserine. Elution time is given in minutes.

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