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QUALITATIVE AND QUANTITATIVE ANALYSIS OF COMMON AND UNCOMMON AMINO ACIDS IN PLANT EXTRACTS

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SUMMARY

A method is described for the automatic qualitative and quantitative analysis of free amino acids, common and uncommon, present in plant extracts. An automatic amino acid analyser has been modified to allow the absorption of ninhydrin colours to be measured at various wavelengths and at different stages of colour development, permitting the identification of amino acids by ninhydrin colour as well as by elution time. Data are processed using a programmable calculator, and the complete spectrum of the amino acids from each plant species is stored in a coded form on magnetic tape. Comparisons of different spectra can be made rapidly either by simultaneous visual display on an oscilloscope or by direct computer correlation.

INTRODUCTION

The twenty protein amino acids that occur universally in living systems are of limited taxonomic use, whereas the three hundred or so "uncommon" amino acids, by reason of their more restricted distributions, are potentially valuable as taxonomic characters[1]. The techniques of high-voltage paper electrophoresis and two-dimensional paper chromatography have been widely used to survey the distribution of uncommon amino acids in plant species, and the results of these surveys have been used to establish or confirm phylogenetic relationships between different taxa[2]. The use of commercially available automatic amino acid analysers for this type of work has, however, been limited since the basic design of the apparatus is appropriate to the detection and estimation of common amino acids but not to the uncommon ones.

With the exception of proline, the twenty protein amino acids react with ninhydrin to give purple colours which show maximum absorbance at approximately 570 nm. Other naturally occurring amino acids give a wide variety of colours with ninhydrin, however, ranging from the bright red of lathyrine through the brown of γ-methyleneglutamic acid, the grey-purple of *p*-aminophenylalanine to the green of 4-hydroxypipecolic acid (Table I). Such differences in colour are a valuable aid to the

TABLE I

WAVELENGTHS OF ABSORPTION MAXIMA* AND ABSORBANCE RATIOS** OF NINHYDRIN REACTION PRODUCTS OF VARIOUS AMINO ACIDS

Amino acid	*Wavelengths of maxima (nm)*	*Absorbance ratio*				
		A_{405} (3 min) / A_{570} (3 min)	A_{416} (3 min) / A_{570} (3 min)	A_{434} (3 min) / A_{570} (3 min)	A_{475} (3 min) / A_{570} (3 min)	A_{570} (3 min) / A_{570} (15 min)
Alanine	405, 570	1.05	0.79	0.21	0.13	0.11
Tryptophan	405, 570	1.00	0.76	0.50	0.35	0.45
Proline	415	7.21	7.12	6.48	4.54	0.69
4-Hydroxyproline	393, 447	8.43	7.86	7.07	4.71	0.65
Pipecolic acid	405, 432, 570	0.94	0.77	0.34	0.26	0.51
4-Hydroxypipecolic acid	438	6.08	12.17	17.00	11.83	0.58
4-Methylglutamic acid	405, 570	1.35	1.03	0.15	0.05	0.39
4-Methyleneglutamic acid	408, 470, 570	2.89	2.78	2.89	3.22	0.06
4-Ethylideneglutamic acid	405, 462, 570	1.10	0.90	0.31	0.31	0.51
Homoarginine	405, 570	1.05	0.75	0.12	0.06	0.56
3,4-Dihydroxyphenylalanine	405, 570	1.11	0.82	0.14	0.07	0.86
2,4-Diaminobutyric acid	405, 570	1.13	0.88	0.15	0.10	0.47
2-Amino-3-methylaminopropionic acid	405, 570	1.08	0.89	0.39	0.39	0.50
N-Methyltyrosine	405, 570	1.00	0.75	0.13	0.13	0.44
Lathyrine	415, 470	6.33	6.56	6.67	6.56	1.00
Canavanine	405, 570	1.18	0.85	0.18	0.09	0.85
Albizzine	405, 570	1.05	0.81	0.13	0.06	0.50
3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	398, 570	2.20	1.60	0.50	0.01	0.30
Dichrostachinic acid	405, 570	1.11	0.79	0.14	0.08	0.57
Djenkolic acid	405, 456, 570	1.15	0.90	0.26	0.18	0.78
Azetidine-2-carboxylic acid	417, 570	1.67	1.33	0.52	0.38	0.78
Mimosine	405, 570	1.09	0.78	0.12	0.07	0.88

* The wavelengths of absorption maxima of ninhydrin reaction products were measured under the conditions described in the text.

** The ratios of the absorbances of a ninhydrin reaction product at different wavelengths after a 3-min reaction time give an indication of the colour of the product mixture: the ratio of absorbances at 570 nm after 3-min and 15-min development is a measure of the rate of production of the colour.

identification of uncommon amino acids after electrophoresis or paper chromatography, and are frequently most pronounced during the early stages of colour development. Using a standard amino acid analyser measuring absorption at 440 and 570 nm, not only is much of this valuable qualitative information lost, but the sensitivity of the method for the detection and quantitative determination of those amino acids giving ninhydrin colours with λ_{max} values other than at the normal wavelengths is greatly reduced. In the system described we have modified a standard automatic analyser to allow for and take advantage of the various colours given by different amino acids with ninhydrin. The method also takes into account the fact that the wavelength or wavelengths of maximum absorption of a ninhydrin colour may change during the period of development, and that the time taken for maximum colour development at constant temperature varies from amino acid to amino acid.

The survey of the free amino acids in seeds of the Leguminosae which we are presently undertaking involves the analysis of thousands of extracts. The processing of data on this scale is not practicable using the normal chart recorder, hence we have devised a data-handling system, based on a cheap programmable calculator, that enables a record of the complete amino acid profile to be stored in a form that is immediately accessible for computer analysis and visual or computer comparison with profiles obtained from other species.

METHOD

Apparatus

An LKB (South Croydon, Great Britain) Model 4101 automatic amino acid analyser equipped with a 6 mm diameter column was used. The analyser was modified to employ six colorimeters each with a flow cell of a path length of 10 mm. The first and the last colorimeter monitor absorption at 570 nm, the remaining four at 475, 434, 416, and 407 nm, respectively. In the original analyser the effluent from the ion-exchange column was mixed with ninhydrin reagent and passed into a reaction coil, 55 m long, held in a boiling water-bath, to allow colour development. At the recommended flow-rates of buffer and ninhydrin reagent this coil length corresponded to a reaction time of 15 min. As most of the unusual ninhydrin colours given by uncommon amino acids are converted by prolonged heating to the more familiar purple colour, absorbances at the five wavelengths indicated are measured in the modified analyser after only 3 min of reaction time (corresponding to passage through 11 m of the reaction coil). After passing through the first five colorimeter cells the reaction mixture is returned to the boiling water bath and the 570 nm absorbance remeasured using the sixth cell after a total of 15 min reaction time.

The output signals (range 0–*ca.* 20 mV) from the six-channel colorimeter are fed into the signal conditioner (eight-channel signal conditioning unit model Digitronix Dataforce 100; Digitronix, Milton Keynes, Great Britain) and amplified to the range 0–1 V to give a suitable input for Digitronix Model AL8 eight-channel data logger (Digitronix). All six channels are monitored throughout each analysis, points being recorded at 20-sec intervals: Under these conditions a C60 magnetic tape cassette is sufficient for 63 h continuous use of the analyser, a C90 cassette increases this to 95 h.

TABLE II

BUFFER SYSTEM FOR THE ANALYSIS OF AMINO ACIDS IN PLANT EXTRACTS

Ninhydrin reagent: ninhydrin buffer, 750 ml; 2-methoxyethanol, 2250 ml; ninhydrin, 30.0 g; stannous chloride, 1.00 g.
Programme: Buffer 1, 1.7 h; buffer 2, 3.0 h; buffer 3, 4.0 h; regeneration (0.3 *M* LiOH), 25 min; flow-rate, 0.50 ml/min for buffer, 0.25 ml/min for the ninhydrin reagent; temperature, 37° for 36 min and 70° for the remainder of the analysis.

	pH	*Lithium conc.*	*Citrate conc.*	*LiCl (anhyd.) (g/l)*	*LiOH (monohyd.) (g/l)*	*Citric acid (monohyd.) (g/l)*	*Glacial acetic acid (ml/l)*	*Phenol (g/l)*	*Thiodiglycol (25% aq.) (ml/l)*
Sample buffer	1.60	0.2000 *N*	0.050 *N*	8.48	–	10.5	–	–	–
Buffer 1	2.20	0.2114 *N*	0.050 *N*	8.48	0.48	10.5	–	1.0	10.0
Buffer 2	2.94	0.600 *N*	0.055 *N*	22.30	3.11	11.56	–	1.0	10.0
Buffer 3	3.53	1.700 *N*	0.100 *N*	65.03	6.97	21.02	–	0.5	5.0
Ninhydrin buffer	5.20	–	–	–	168.0	–	293.0	–	–

Analysis

Preparation of samples. Seed material was ground to a fine powder and a sample (200 mg) was extracted with ethanol–water (75:25, 1.00 ml) for 24 h with constant agitation. An aliquot (0.50 ml) of the supernatant was passed into a small column containing Dowex 50-X8 (H^+) and the column was washed with water (10 ml). Amino acids were eluted from the column with 2 *N* ammonia solution (5 ml) and the alkaline effluent was evaporated to dryness, redissolved in water (5 ml), and again evaporated. This process was repeated three times, after which the residue was taken up in the sample buffer (1.00 ml; Table II).

Buffer system. The programme used for ion-exchange analysis was an adaptation of an LKB procedure based on A6 resin with stepwise elution by three buffers of increasing pH and increasing Li^+ concentration; details of this system are given in Table II.

Storage of results

Data from all six channels are recorded on the data logger tape (with the inclusion of a time count) for the period of elution with buffers 1 to 3. A remote control facility allows the recording of data during regeneration and equilibration to be suppressed. In order that only the essential data of the amino acid spectrum are stored permanently, the recorded data are processed using a Hewlett-Packard (Wokingham, Great Britain) Model 9830A programmable calculator which, when fitted with an Extended I/O ROM (Model 11272B) and a BCD Input Interface Card (Model 11203A), accepts recorded data directly from a Digitronix replay unit (RU-HP).

Data are accepted and sorted into the correct memory file in the calculator initially in a block of 60 readings per channel, and updated in blocks of 35 readings per channel throughout the program. The 3- and 15-min reaction time 570-nm channels are scanned for peak maxima and shoulders, and for each detected event the readings from the other four channels corresponding to the event are recalled from memory and assembled in a 60 × 6 array. Finally all of the data from the 15-min reaction time 570-nm channel are set up in a 53 × 30 array, the arrays are combined and, together with identification and protocol codes, are transferred to magnetic tape memory in a 65 × 30 matrix. Seventeen such coded spectra can be stored on each C60 tape cassette.

Display, calculation and correlation of data

The direct display of data is carried out using the information stored in the matrix form. Information is transferred from the tape cassette to the computer memory; the spectral information for each peak is displayed in printout whilst the data from the 15-min reaction time 570-nm channel are displayed on a digital oscilloscope. The oscilloscope used (Nicolet Model 1020A with interface Model 191) has the capacity to accept four separate spectra and to display them simultaneously, hence visual correlation between spectra is possible. Expansion of the spectra (by up to a factor of 64×) with respect to both time and absorbance allows detailed study of trace components and peak separation. Spectra can be drawn out from the oscilloscope trace using an X:Y plotter.

Calculation of data is also carried out using the information stored in the matrix. The detected events already coded for in the array are used as markers and

the approximate width of each peak or peak complex established. The points within this band width are fitted to a fourth-order polynomial and the first- and second-order derivatives of the curve at each point are followed. Peak detection and area assignment are then carried out using an adaptation of the method of Bartoli *et al.*[3]. The printout of the amino acid spectrum for a sample includes retention data and degree of resolution for each peak, area and percentage of the total area and, where a positive correlation based on retention and spectral data between the unknown component and amino acid standard already stored in the memory can be made, the name of each component and amount present in the sample. The complete report for each amino acid spectrum is assembled into a 60 × 6 matrix, at least eighty of which, together with identification and protocol codes, can be stored on a C60 tape cassette. Once stored the spectral data can be sorted into any order required, *viz.*, based on uncommon amino acid content or on taxonomic relationship of the plant source, for computer correlation. Thus a newly acquired spectrum from an unknown plant species can be compared with all previously recorded results for best match, or all species found to contain a specific set of uncommon amino acids at specific concentrations can be recalled.

RESULTS

Fig. 1 shows the spectra of the free amino acids extracted from the seeds of various species of *Indigofera*. It is clear that the quantitative and qualitative patterns of the common and uncommon amino acids are similar in all the spectra depicted (*e.g.*, peaks corresponding to Asp, Hypro, Thr, Ser, and Glu). This result has been confirmed[4] for 30 species of *Indigofera* with respect to both computer correlation and to the time/wavelength ratios. Results from this study have suggested that the reaction time/wavelength ratios for the common amino acids may be of use in peak correlation and identification by computer, and we hope to report on this aspect at a future date.

The two major uncommon amino acids that occur in the *Indigofera*, canavanine and indospicine, are positively identified by the computer method described: The unknown basic component has been previously observed in *I. schimperi*[5], but its identity has not yet been established.

DISCUSSION

The system described in the present paper allows rapid identification and quantitative estimation of free amino acids, common and uncommon, in biological fluids or extracts, or in protein hydrolysates. The measurement of the absorption values of ninhydrin colours given by different amino acids at five different wavelengths and at two stages of colour development allows greater use to be made of ninhydrin colour differences as criteria of amino acid identity than is possible with a single 570/440 nm ratio measured after an arbitrary period of ninhydrin-amino acid interaction.

The use of magnetic computer tape rather than chart paper for the recording of analytical data provides many major advantages. Tapes are cheaper than chart paper and require less storage space; data can be retrieved easily and with speed; known amino acid peaks are identified and their areas integrated automatically; unknown peaks are recorded in terms of their retention time, area, and absorption characteristics. A computer can also be programmed to correct for the slight variations

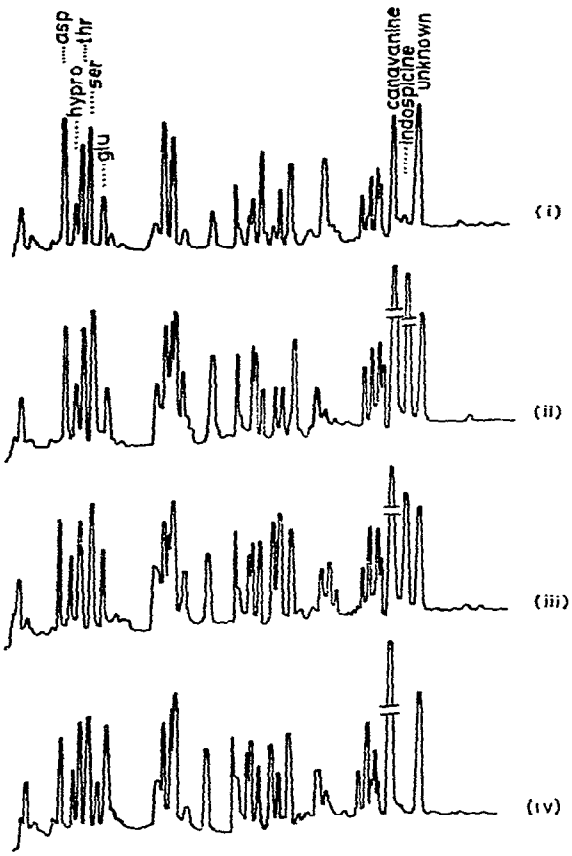

Fig. 1. Analyser traces of the free amino acids present in extracts of seeds of various species of *Indigofera*. i = *I. astragalina*; ii = *I. spicata*; iii = *I. mucronata*; iv = *I. teysmanii*.

in retention times of amino acids which occur owing to slight variation in operating conditions of the analyser; such corrections ensure that all analytical traces are directly comparable. Up to four amino acid profiles may be displayed visually on the oscilloscope screen, allowing direct comparison between the analytical data from different extracts. Different regions of the amino acid profile may be expanded to allow the closer study of individual peaks and the recognition of asymmetry in a peak which indicates the presence of two separate compounds.

The primary value of storing data in a computer bank is the ease with which these data can be recalled. In studying the free amino acid content of Legume seeds it has proved possible, for example, to determine similarities and differences between species with varying degrees of evolutionary relationship. It is also possible to scan the records rapidly for information on the distribution of a single amino acid. The same system is of course equally applicable to the storage and handling of data obtained by the analysis of protein hydrolysates and could, for example, be usefully employed to provide immediate information on the distribution of lysine, methionine, and cystine in seed protein of plant species from various taxa.

The programs referred to in this paper are written in BASIC and further information concerning them is available from the authors. The system described is very flexible and can easily be adapted to make use of a computer facility that is already available.

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