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Note

Optimized method for the automated analysis of ninhydrin-positive substances in plant extracts

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Plant extracts, which are an exclusive source of most natural amino acids[1], frequently also contain other types of ninhydrin-positive compounds[2–4], as well as contaminants, which affect the automated ion-exchange chromatography of the amino acids. The methods used for the automated analysis of free amino acids[5–11] are therefore unsatisfactory for the analysis of plant extracts because of resolution problems. Moreover, these methods do not meet the requirement of our laboratory for a routine analytical procedure as they are either time consuming or require the use of special ion exchangers and/or advanced high-pressure analysers.

In amino acid analysis, there are generally three ways of improving the resolution: the enhancement of column efficiency and/or selectivity by using special ion exchangers, the reduction of the extra-column peak broadening with modified detection systems, and the optimization of the eluent composition.

Unfortunately, the optimal mobile phase cannot be selected on the basis of published experimental data[5,6,12,13], which are not directly comparable and lack any theoretical interpretation. In this situation, we have found that recently published equations[14] based on an earlier theoretical work[15] can be employed to predict the effects of the pH and counter-ion concentration of the mobile phase on the retention of most ninhydrin-positive compounds. The number of test runs required for the selection of the optimal conditions was thus minimized.

We attempted to devise a flexible concept of plant extract analysis, based on an accelerated two-column method capable of resolving all common ninhydrin-positive constituents of plant extracts at low pressure without requiring temperature programming. The applicability of this method can be extended to materials that contain less common compounds by using complementary column chromatographic techniques for the preliminary fractionation of the sample. This extra step also substantially improves the preliminary identification of peaks.

EXPERIMENTAL

A Model 4020 amino acid analyser (Development Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia) was used. This instrument was modified by inserting a six-port sample application valve (Mikrotechna, Prague,

Czechoslovakia), with an internal loop volume of 30 μl, immediately below the outlet of the chromatographic column. This arrangement was used for the determination of the extra-column contributions to the peak broadening.

Ostion high-resolution cation-exchange resins were obtained from the United Chemical and Metallurgical Works (Ústí nad Labem, Czechoslovakia).

The optimal analytical conditions for particular tasks were as follows. Acidic and neutral amino acids were analysed using a 63 × 0.95 cm resin column, Ostion LG KS 0803 (Li^+) particle size 17.1 ± 1.9 μm, eluted at a flow-rate of 60 ml/h with a column temperature of 37.2°. Elution was started with buffer A (for composition, see Table I), changing to buffer B when the maximal absorbance of the aspartic acid peak was recorded and to buffer C after citrulline had been eluted. Basic amino acids and co-eluted neutral compounds (see Fig. 2) were separated using the same flow-rate and column temperature, a 20,4 × 0.96 cm resin column, Ostion LG KS (Na^+) particle size 11.0 ± 1.3 μm being eluted with buffer E.

TABLE I

COMPOSITION OF BUFFERS

Parameter	Buffer				
	A	B	C	D	E
Resin counter-ion concentration (M)	0.25	0.30	0.30	1.50	0.60
Citrate concentration (M)	0.10	0.05	0.18	0.50	0.10
pH (at 25°)	2.82	3.03	4.40	2.20*	4.34
LiOH·H_2O (g)	21.0	25.2	25.2	126.0	—
NaCl (g)	—	—	—	—	49.6
Sodium citrate·$2H_2O$ (g)	—	—	—	—	34.6
Citric acid·H_2O (g)	42.0	21.0	75.6	210.0	17.4
Concentrated HCl (ml)	33	45	**	260	**
EDTA (g)	—	0.4	0.4	—	—
Thiodiglycol (ml)	5.0	5.0	5.0	200	10.0
Isopropanol (ml)	—	10.0	—	—	70.0
Caprylic acid (ml)	0.2	0.2	0.2	2.0	0.2
Final volume (l)	2	2	2	2	2

* This pH value is reached after 5-fold dilution of the buffer with distilled water.
** Buffers C and E usually do not require any pH adjustment with HCl.

Samples that did not require any preliminary separation were prepared for analysis as follows. An aliquot of a vacuum-concentrated ethanolic extract (1–2 ml) was mixed with 0.5 ml of an internal standard (various concentrations of norleucine in 10% isopropanol), 1 ml of buffer D was added, the final volume was made to 5 ml with distilled water and the solution was centrifuged if necessary. The clear supernatant was immediately applied to the column in order to prevent the hydrolysis of glutamine.

The most advantageous method of the preliminary fractionation of extracts was based on the use of Dowex 1-X8 (100–200 mesh) in the acetate form. An aliquot of the vacuum-concentrated extract representing 2–12 g of the fresh plant material was introduced to a column containing 5 ml of the settled resin. Basic and neutral amino acids were eluted together with some other compounds that give a positive
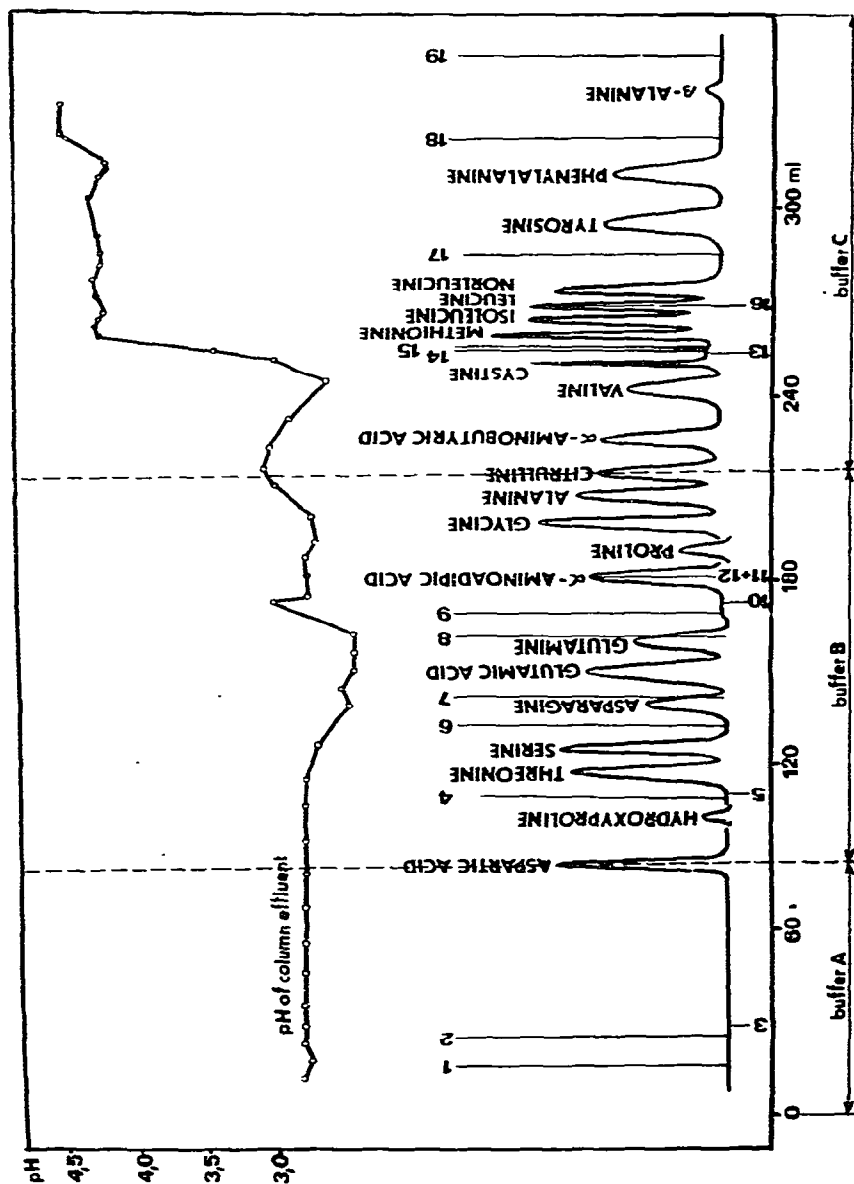
Fig. 1. Separation of acidic and neutral amino acids. Column pressure, 9.5–12.4 bar. Elution volumes indicated: 1 = cysteic acid; 2 = acetic acid; 3 = levulinic acid; 4 = methionine sulfone; 5 = methionine sulfoxide; 6 = β-D-gluconuramic acid; 7 = azetidinecarboxylic acid; 8 = homoserine; 9 = sarcosine; 10 = γ-methylenglutamic acid; 11 = S-methylcysteine; 12 = γ-methylenglutamine; 13 = pipecolic acid; 14 = glucosamine; 15 = cystathionine; 16 = 3,4-dihydroxyphenylalanine (DOPA); 17 = galactosamine; 18 = homocystine; 19 = β-aminoisobutyric acid. Elution volumes are corrected for the internal volume of extra-column parts of the analyser. Changes in pH exhibited by buffer sequence are shown in the upper part of the chromatogram.

response with the present detection system (sugars and pigments) with 25 ml of distilled water. Acidic ninhydrin-positive compounds and the other strongly adsorbed neutral aromatic compounds (pigments and tryptophan) were eluted with 25 ml of 25% acetic acid solution. The fractions were separately vacuum-dried and prepated for analysis as described above. Other sample pre-separation methods, including the separation of amino acids from peptides in the form of Cu^{2+} complexes (which does not require the elimination of copper prior to the automated analysis) and adsorption chromatography of aromatic compounds on Sephadex columns, have been described elsewhere[14].

RESULTS AND DISCUSSION

The equations[14] mentioned in the Introduction were found to be particularly useful for the preduction of the effects of eluent composition on the retention of basic amino acids and co-eluted compounds, as the separation of these substances does not require buffer changes. For example, in an experiment involving simultaneous changes of the pH and the resin counter-ion concentration of the eluent, exhibited by additions of sodium chloride, the average difference between experimentally determined capacity factors of γ-aminobutyric acid, ornithine, lysine, ammonia, tryptophan and arginine and the values calculated on the basis of independent experimental data was only 2.8%.

The methods that gave the results presented in Fig. 1 and 2 can be combined to form a complete fully automated procedure, involving partial co-elutions of both columns. This method has been successfully employed for the routine analyses of different plant extracts (sugar beet, hops, wheat, onion, winter rape, cabbage, etc.) and also for occasional analyses of samples of human, animal and microbiological origin.

The chromatographic efficiency of the proposed procedure was compared with the commonly used combination of methods developed by Benson and co-workers for the analysis of basic[5] and neutral plus acidic[6] amino acids, which served as a reference method. The proposed method, performed with identical column and flow-rate conditions, requires only about 65% of the time required for the completion of the reference method. The resolution of most peaks was improved, so that R values ≥ 1.3 were ensured for all common plant extract constituents shown in Fig. 1 and 2 (for the definition of R, see ref. 16).

This improvement in resolution is believed to be caused exclusively by favourable changes in the capacity factors of the eluted compounds, as the differences in measurable plate heights between the two methods were statistically insignificant for all compounds except arginine. Moreover, the proposed method, which produces narrower peaks of most amino acids, loses a significant part of its column efficiency owing to the extra-column peak broadening effects. These effects result in a decrease in resolution of the lysine–ornithine pair (Fig. 2) of about 18%.

Ostion cation exchangers proved to be highly efficient column packings. Plate heights calculated on the basis of peak variances, corrected for extra-column contributions[17], were in the range from $48.2 \cdot 10^{-6}$ m (serine) to $81.6 \cdot 10^{-6}$ m (glutamic acid) for the results shown in Fig. 1, whereas on the column packed with a finer resin in the sodium form (Fig. 2) plate heights between $28 \cdot 10^{-6}$ m (ornithine, histidine) and
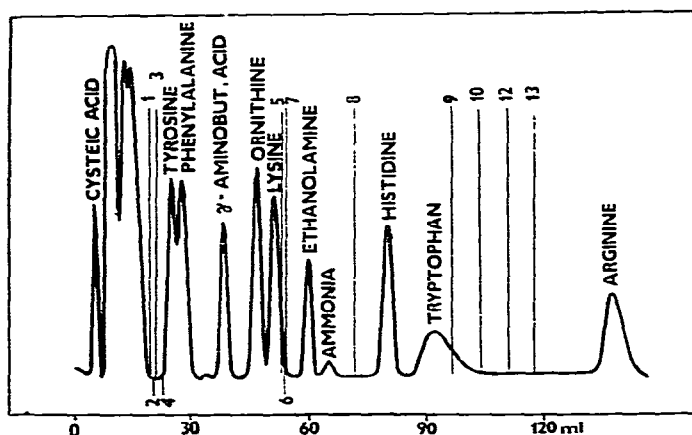
Fig. 2. Separation of basic ninhydrin-positive compounds and of other neutral amino acids. Column pressure, 10.6–12.0 bar. Elution volumes indicated: 1 = homocystine; 2 = β-alanine; 3 = 3,4-DOPA; 4 = β-aminoisobutyric acid; 5 = α,γ-diaminobutyric acid; 6 = N-methyllysine; 7 = S-methylmethionium iodide; 8 = α, β-diaminopropionic acid; 9 = homoserine lactone; 10 = canavanine; 12 = α-amino-β-guanidinopropionic acid; 13 = ε-aminocaproic acid. Elution volumes are corrected for the extra-column volume of the analyser.

$39.1 \cdot 10^{-6}$ m (arginine) were attained for basic amino acids and an exceptionally high value ($227 \cdot 10^{-6}$ m) was observed for tryptophan.

One of the main reasons why we have been disappointed with the use of previously published methods employing lithium citrate buffers was the occurrence of broad, asymmetrical peaks with shifted retention times in two areas of the chromatograms (between aspartic acid and glutamine and between cystine and norleucine), which made the analysis of some crude acidified plant extracts (e.g., of sugar beet) impossible. In the proposed procedure, this problem was eliminated by increasing the citrate content of buffers A and C. The baseline drop before cystine, observed on the chromatograms of samples containing Cu^{2+}, was suppressed by the addition of EDTA to buffers B and C. When increasing the buffering capacity of buffer A, care was taken not to affect the pH of the ninhydrin reaction and not to affect adversely the resolution of threonine from serine and the separation of proline from glycine. It was also noted that an increase in the citrate content of buffer A requires a simultaneous decrease in the pH of this eluent in order to maintain an acceptable resolution of the asparagine–glutamic acid –glutamine triplet and causes statistically significant improvements in the separation of the methionine sulphoxide–threonine, glycine–alanine and citrulline–α-aminobutyric acid pairs.

The reproducibility of the separation of basic amino acids was not affected by the sample composition, which permitted the resolution in the area of the chromatogram between the lysine and histidine peaks (for a comparison, see ref. 18) to be improved by maintaining a low citrate content of the mobile phase. An improvement in the separation of lysine from the S-methylmethionium cation can be obtained by increasing the pH of eluent to 4.50.

The advantages of a proper choice of the sample pre-separation method for the enhancement of the analytical capacilities of the basic procedure are illustrated in Fig. 3. The onion is known to be a very complex source of ninhydrin-positive
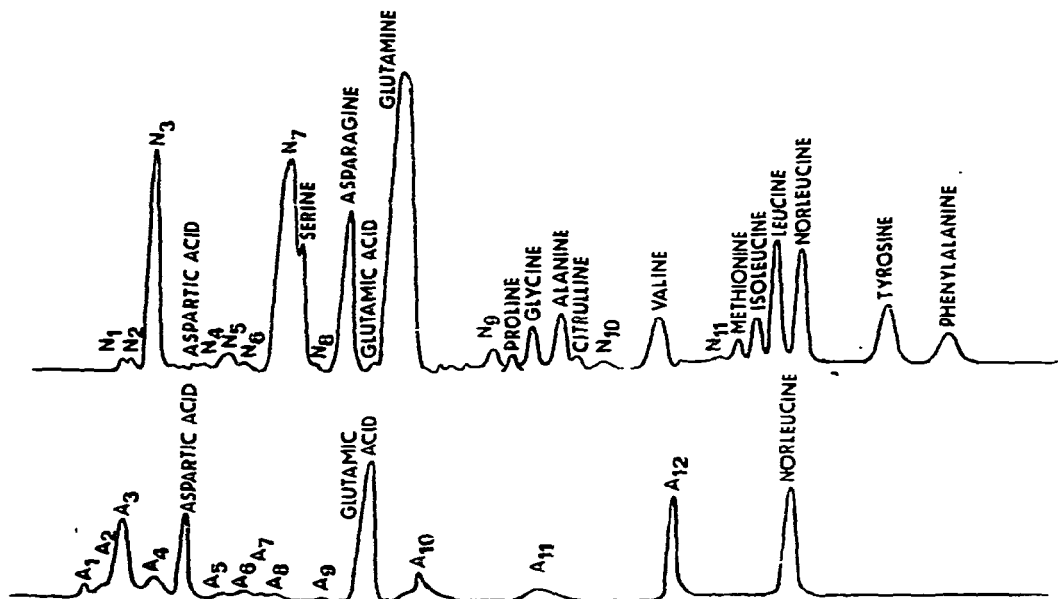
Fig. 3. Analysis of fractions obtained by a preliminary separation of an onion extract on the acetate form of Dowex 1. Pre-separation conditions as described under Experimental. Analytical conditions: an increased Li^+ concentration (0.3 M) of buffer A was used. Upper trace, neutral fraction; lower trace, acidic fraction.

compounds[19], containing several γ-glutamyl peptides (peaks A_1–A_9 and A_{11}–A_{12} on the chromatogram of the acidic fraction in Fig. 3) as well as some uncommon sulphur amino acids and their sulphoxides eluted in the neutral fraction. In the analysis of a complete extract, many acidic and neutral compounds are co-eluted and even clearly separated peaks may be incorrectly identified [for example, γ-glutamylphenylalanine (Fig. 3' peak A_{12}) may be identified as cystine].

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