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NEW LIQUID CHROMATOGRAPHIC APPROACHES FOR FREE AMINO ACID ANALYSIS IN PLANTS AND INSECTS

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SUMMARY

A simple and rapid method of analysis for free amino acids in plants and insects is presented. The amino acids were determined as their 5-dimethylaminonaphthalene-1-sulfonyl (Dns) derivatives. Two-dimensional thin-layer chromatography on polyamide sheets and high-performance liquid chromatography on μBondapak C_{18} columns were used to resolve the Dns derivatives. R_F values of 45 Dns derivatives are reported.

INTRODUCTION

Researchers in the field of agriculture believe that information on the free amino acid contents of plant tissues and exudates could be relevant to the expression of plant disease and the feeding preference of certain insects. Differences in the pattern of free amino acid contents of disease-resistant plants from those of non-resistant plants, if correlated to the specific needs of the insect vector or the microorganism involved, could be a way of determining what plants or plant varieties can be grown in infested areas. Amino acid patterns could also be a clue to the nutrients needed for the artificial rearing of insects and/or culture of microorganisms in question.

Since the pioneering works of Hartley and Gray[1–3] in the early sixties, a number of reports[4–8] have appeared in the literature using 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) as a labelling reagent in protein chemistry. However to our knowledge, there are no published reports on the use of free amino acid Dns derivatization as a possible routine method of analysis for free amino acids in plants and plant disease carrier insects.

We report here a simple method of extraction, separation, derivatization and resolution of free amino acids applicable to plants as well as insect specimens.

EXPERIMENTAL

Extraction and separation of the free amino acids

Leaves. After weighing, 10 g of leaves were cut into small pieces and placed in a

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blender (spiking with standard amino acids to determine recovery was done at this point). The leaves were blended for 2 min with 50 ml of a solution of methanol–water–12 *M* hydrochloric acid (90:5:5). The supernatant liquid was vacuum filtered. This procedure was repeated using the remaining leaf pulp. The combined total filtrate was *ca.* 90 ml.

The resulting leaf extract was divided into two aliquots, for two separate analyses. In each, *ca.* 40 ml of the leaf extract were diluted with 15 ml of deionized water and extracted with 15 ml of chloroform. The chloroform extract removes most of the non-polar organic components of the leaf extract, leaving the free amino acids in the acidic aqueous layer. The aqueous layer was passed through an ion-exchange column (7 × 1 cm; Amberlite IR-120 H, medium porosity). The column was washed with deionized water until neutral to pH paper. The amino acids were eluted using 10 ml of 4 *M* NH_4OH followed by 5 ml of deionized water. The eluate was evaporated to dryness in a rotary evaporator. The resulting residue was then dansylated.

Phloem sap. Phloem exudate (15–20 ml) was mixed with an equal volume of the above methanol–water–hydrochloric acid mixture and treated in the same way as the leaf extract.

Insects. Approximately 0.1 g of the insect in question was carefully ground to a thick paste using a mini agate mortar and pestle. The resulting paste was dissolved in *ca.* 1.5 ml of methanol–water–hydrocholoric acid (90:5:5). The mixture was filtered through a Pasteur pipet plugged with glass wool. The filtered solution was diluted with an equal volume of deionized water and then extracted with chloroform. The rest of the procedure is the same as that followed for the leaf extract.

Preparation of the Dns derivatives

Standard amino acids and/or amines. Some 10–20 mg of a standard amino acid and/or amine (Sigma, St. Louis, MO, U.S.A.) were dissolved in 1.5 ml of $NaHCO_3$ buffer (pH 10.5, 0.1 *M*). Next, 0.5 ml of Dns-Cl (Sigma) solution (15 mg/ml acetone) was added, mixed thoroughly, incubated at 40°C for 2 h, and evaporated to dryness under reduced pressure. The residue was taken up in methanol (HPLC grade; J. T. Baker, Phillipsburgh, NJ, U.S.A.), and filtered through a Pasteur pipet plugged with glass wool. The resulting clear solution was used directly for spotting with no further purification.

Amino acids from plant and insect extracts. The procedure followed for the Dns derivatization of the free amino acids in the plant and insect extracts was basically the same as above, except for an occasional need for a clean-up step after Dns derivatization. For the clean-up step, the acetone in the reaction mixture was removed by passing nitrogen gas. Excess unhydrolyzed Dns-Cl was then removed by extracting the reaction mixture two to three times with toluene. Alternatively, the Dns-amino acids were extracted into ethyl acetate leaving the bulk of Dns-OH and Dns-NH_2 in the aqueous layer.

Chromatographic separation of Dns derivatives

High-performance liquid chromatography (HPLC). HPLC was done using Waters Associates solvent delivery system Models 6000-A and M-45; solvent programmer Model 660; injector Model U6K; and μBondapak C_{18} column. The detector was a Tracor 970A variable wavelength, and the recorder was a Houston Instrument Omni Scribe recorder.

TABLE I

THE 45 AMINO ACIDS AND RELATED COMPOUNDS STUDIED

	Abbreviation		*Abbreviation*
1 Alanine	Ala	24 Lysine	Lys
2 Asparagine	AsN	25 ε-lysine	ε-Lys
3 Aspartic acid	Asp	26 Methionine	Met
4 Arginine	Arg	27 Methionine sulfone*	Mes
5 Aziridine	Azi	28 Methionine sulfoxide*	Meo
6 α-butyric acid	AABA	29 Norleucine	Nle
7 γ-butyric acid	GABA	30 Norvaline	Nval
8 Cadaverine	Cad	31 Ornithine*	Orth
9 Carboxymethyl cysteine*	Cmc	32 Phenylalanine	Phe
10 Cysteic acid	Cya	33 Pimelic acid*	Pim
11 Cystine	Cys	34 Proline	Pro
12 Dansylic acid	Dns-OH	35 Sarcosine	Sar
13 Dansylsulfonamide	Dns-NH_2	36 Serine	Ser
14 Diaminobutyric acid*	di-ABA	37 Speridine*	Spd
15 Diaminopropionic acid	di-APA	38 Spermine*	Spm
16 Glutamic acid	Glu	39 Taurine*	Tau
17 Glutamine	GlN	40 Threonine	Thr
18 Glycine	Gly	41 Tryptamine	TrN
19 Histidine*	His	42 Tryptophan	Trp
20 Hydrazine	Hydz	43 Tyrosine	Tyr
21 Hydroxyproline	HO-Pro	44 bis-Tyrosine	bis-Tyr
22 Isoleucine	Ile	45 Valine	Val
23 Leucine	Leu		

* Dns derivative prepared as outlined in the Experimental section.

Thin-layer chromatography (TLC). Spotting was done on 5 × 5 cm sheets (Schleicher & Schüll, Keene, NH, U.S.A.) using a 10-μl Hamilton syringe. Development of the plates was carried out in a filter-paper-lined 250-ml beaker with two pieces of glass rod cut to fit diagonally at the bottom of the beaker to hold the plates upright and covered by an inverted 600-ml beaker. Solvent I (1.5% formic acid) and solvent II (benzene–acetic acid, 4.5:1) were run perpendicular to each other. The chromatograms were viewed under a UV chromatogram viewer equipped with both long-wave (366 nm) and short-wave (254 nm) UV light source.

DISCUSSION

To investigate the feasibility of using Dns derivatization coupled with HPLC[9–13] and/or two-dimensional TLC on polyamide sheets[14,15] as a routine method of analysis for free amino acids in plants and insects, we experimented first on the standard Dns derivatives of the twenty common amino acids. After successfully resolving the Dns derivatives of the twenty common amino acids, we experimented on the Dns derivatives of 25 other very closely related compounds to determine if they would interfere in the resolution of the twenty common amino acids of interest. Of the 45 Dns derivatives (Table I), 34 were obtained commercially and II were prepared in our laboratory as outlined in the Experimental section.

Our first attempt to resolve the twenty amino acids was made using HPLC with

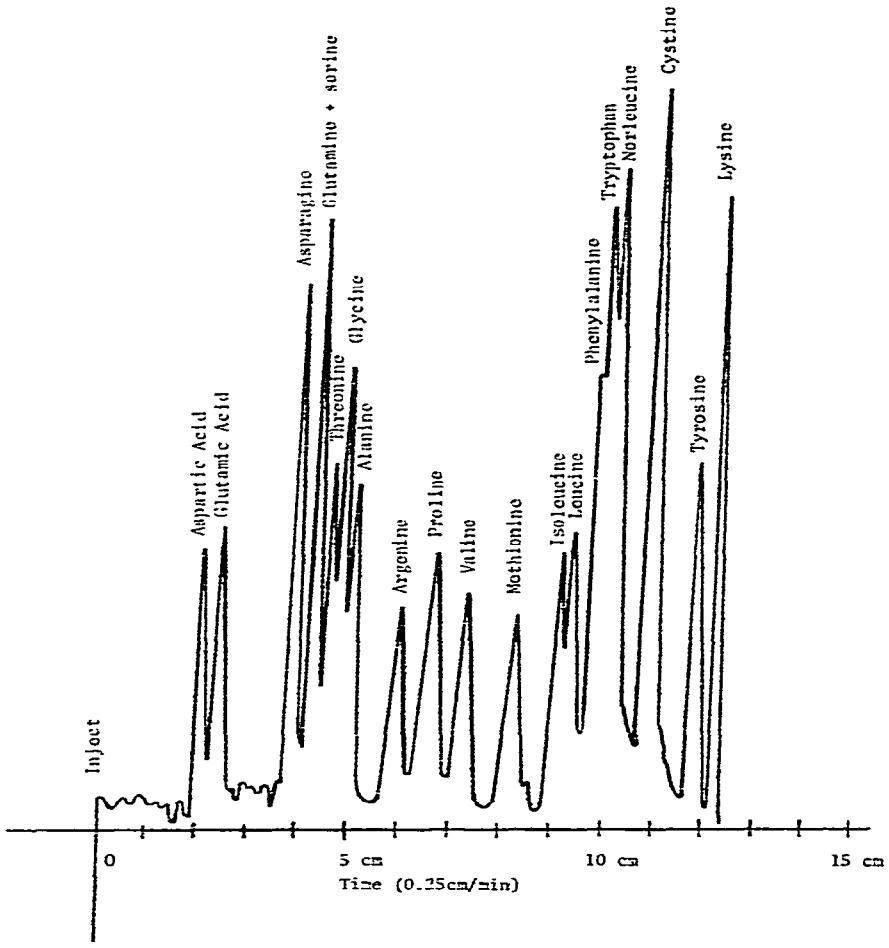

Fig. 1. Separation of a mixture of twenty standard amino acids as their Dns derivatives. Packing, μBondapak C_{18}; column, 30 cm × 4 mm; solvent, (A) 0.01 *M* K_2HPO_4 pH 6.7, (B) acetonitrile, isocratic for 10 min at 10% B, then curve No. 8: 15% B → 45% B in 35 min; flow-rate, 1.4 ml/min; detector, UV at 254 nm; chart speed, 0.25 cm/min.

a gradient solvent system (acetonitrile–phosphate buffer) on a μBondapak C_{18} column. The parameters varied in the attempt to resolve the common amino acids were: the ion concentration and pH of the phosphate buffer; the solvent strength or amount of acetonitrile; the time; and the flow-rate. Our best results gave eighteen peaks (Fig. 1) for a mixture of twenty Dns amino acids. Each run required *ca.* 1 h, not including re-equilibration time.

We then tried the two-dimensional TLC on a 5 × 5 cm polyamide sheet. As a first step we tried resolving a mixture containing the twenty common amino acids using different solvent systems. We tested solvent systems suggested by other workers[16] as well as solvent systems that we thought could be of some use. The solvent system suggested by Lee and Safille[15] gave us the best results. This method completely resolved the twenty common amino acids (Fig. 2a). Each run from the initial spotting to the final air-drying takes only 15–20 min.

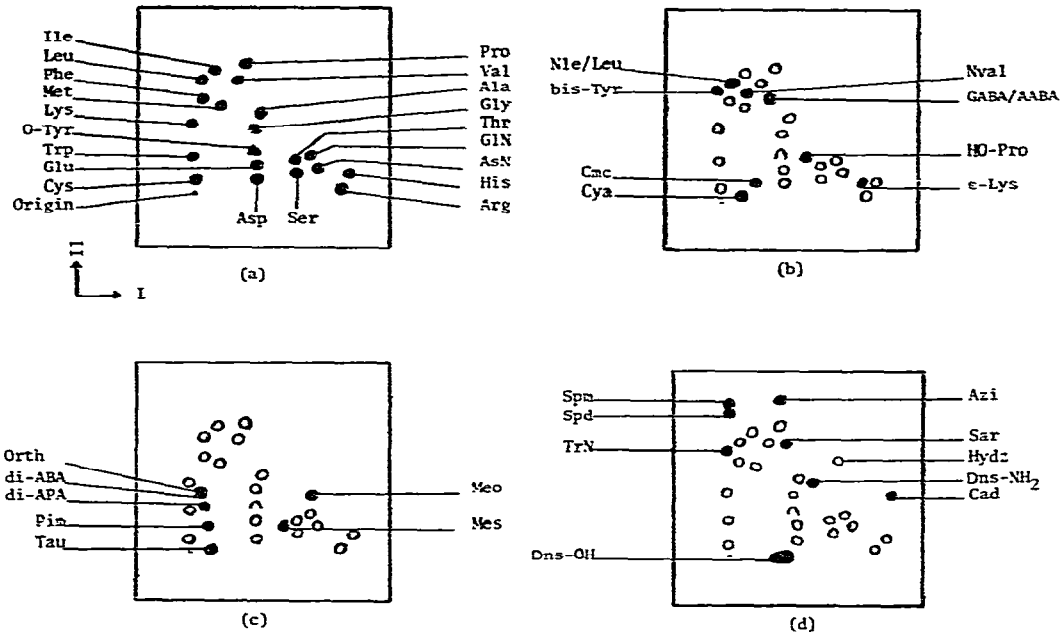

Fig. 2. (a) Chromatogram of a mixture of the twenty common amino acids. (b)–(d) Chromatograms showing the position of 25 other compounds [●] relative to those of the twenty common amino acids [○].

RESULTS AND EVALUATION

HPLC

Our attempts to use HPLC to separate the Dns-amino acids did not result in the complete resolution of the twenty amino acids in a single run, but there was a reasonably good resolution (Fig. 1). The fact that the pairs glutamine/serine and phenylalanine/tryptophan completely overlapped did not pose serious problems in the identification of the amino acids. It was possible to zero in on these pairs and resolve them by altering the conditions of the chromatogram after the other amino acids had been identified. However, after evaluating our results, we thought that for a routine analysis, merely for identification and not necessarily for a detailed or quantifying analysis, the use of HPLC may not be warranted. Another unfavorable factor was the time element, since each HPLC run required approximately three times longer than the two-dimensional TLC, which completely resolves the twenty most common amino acids.

TLC

The use of polyamide TLC in protein chemistry has been popular for some time now. The literature reports the sensitivity of detection of Dns derivatives to be in the nanomole range[5,17] and in some cases in the picomole range[18]. Therefore we started experimenting on the Dns derivatives of standard amino acids. From our results we found that the modified solvent system suggested by Lee and Safille[15] completely resolved the Dns derivatives of the twenty common amino acids. Fig. 2a is a tracing of the chromatogram of a mixture containing all twenty common amino

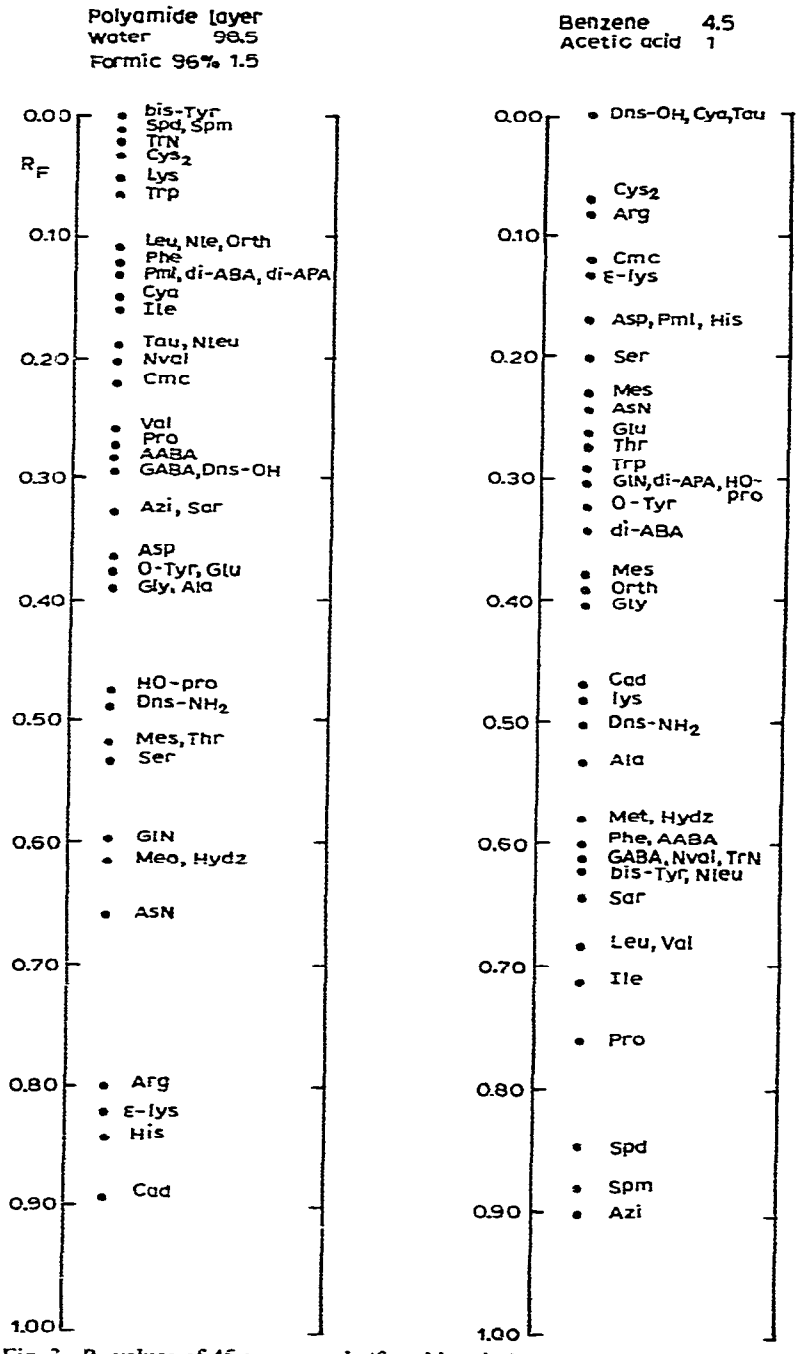

Fig. 3. R_F values of 45 compounds (for abbreviations see Table I).

acids, after it had been developed using solvent systems I and II (see Experimental section) at right angles to each other.

While our primary interest was to separate and identify the twenty common amino acids, it was necessary to determine the relative positions or R_F values of

compounds that are often associated with the amino acids found in living organisms, such as taurine, ornithine, tryptamine, etc. Figs. 2b–2d are plates showing the positions of 25 such compounds relative to those of the twenty common amino acids. As can be observed, with the exception of the pair norleucine/leucine, none of the 25 other compounds studied overlaps with any of the common amino acids. Mono-Dns-lysine, if present in large amounts, may fuse with arginine and histidine. The presence of large amounts of mono-Dns-lysine, however, is not likely under the conditions we use in the preparation of the Dns derivatives. Under saturating concentrations of Dns-Cl, most of the lysine will be present in the di-Dns form, which has an R_F value quite distinct from any of the other compounds.

In Fig. 3, we give the R_F values of all 45 compounds in solvent systems I and II separately.

Application

Once we had determined the R_F values of the Dns derivatives of appropriate compounds, we turned our attention to application of the method in the identification of free amino acids in plants and insect specimens. We devised a general scheme (see Experimental section) for the extraction, separation, and derivatization of the free amino acids found in the above specimens.

TABLE II

AMINO ACID ANALYSIS

− = not observed; + = observed; ++ = bright; ± = faint.

Amino acids	*Sample specimens*		
	Phloem sap Veitchia merrilli (palm)	*Leaves Syzigium jambos (rose-apple)*	*Insect Myzus persicae (green pea aphids)*
Alanine	++	++	++
Asparagine	++	+	+
Aspartic acid	+	+	+
Arginine	+	±	−
α-butyric acid/γ-butyric acid	++	+	+
Carboxymethyl cysteine	+	−	−
Cysteic acid	−	+	+
Cystine	−	−	−
Glutamic acid	++	+	+
Glutamine	+	+	+
Glycine	++	+	+
Histidine	−	−	+
Hydroxyproline	+	−	−
Isoleucine	+	+	+
Leucine/Norleucine	+	+	++
Lysine	+	+	++
Methione	+	−	−
Phenylalanine	+	+	+
Proline	++	++	++
Serine	++	+	+
Threonine	+	+	+
Tryptophan	+	++	+
O-Tyrosine	−	−	+
bis-Tyrosine	±	+	++
Valine	−	++	+

To determine if the amino acids would survive the proposed procedure of extraction, separation and derivatization, we chose eight different amino acid standards at random (alanine, isoleucine, lysine, methionine, proline, threonine, tyrosine and valine) and used them to spike eight different leaf samples. Approximately 0.1 g of standard amino acid was added to a 10-g sample of leaves. The chromatograms of these test runs gave us bright spots for the free amino acids found in leaves and very intense spots for the added standard amino acids. The intensities of the added standard amino acids were such that the readings of adjacent spots were rendered difficult. This indicates that 0.1 g amino acid in 10 g of leaves was a concentration that was out of proportion relative to the concentration of free amino acids found in 10 g of leaves. The spiking was done to test the effectiveness of the work-up procedure. The amount used for spiking was arbitrary and was chosen for convenience. Evidently the sensitivity of the method is good. At this stage, no effort had been made to quantify the sensitivity limits of the method. Our present interest is geared more to identification and only to a relative or rough quantification.

In Table II we report the results of sample analysis obtained following the scheme outlined in the Experimental section; an analysis of phloem sap, leaves, and insects. Besides the twenty common amino acids, we have also included in Table II a number of other amino acids that were observed in the specimens cited. It is interesting to note that while cystine was not observed in any of the three sample analyses, cysteic acid was observed in both the rose-apple leaves and the green pea aphids, and carboxymethylcysteic acid in the palm phloem sap. Tyrosine, like lysine, seemed to be present mostly in the di-Dns form, except in the green pea aphids where tyrosine was observed in both forms, with the di-Dns-tyrosine in relatively large concentration.

Identification of the amino acids was done by spiking with marker mixtures of standard Dns-amino acids, as well as by comparison with identification maps.

We feel that this method of analysis for free amino acids, using the scheme for extraction, separation, derivatization and resolution in two-dimensional TLC as described in this report, can be of value because the method is simple, does not require sophisticated instrumentation, is reliable and is easily adaptable to a variety of specimens.

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