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AMINO ACID ANALYSES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

AN EVALUATION OF THE USEFULNESS OF PRE-COLUMN Dns DERIVATIZATION

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SUMMARY

The utility of pre-column amino acid Dns derivatization, liquid chromatographic separation and subsequent quantitation has been evaluated. Relative to the more conventional method, *e.g.*, the chromatography of free amino acids on ion-exchange resins and post-column detection, the techniques are equally suitable when beginning with nanomole amounts of material. At the lower picomole level, an area where few if any analyzers can perform routinely, the results are significantly better. Not only is the amino acid composition expected from an investment of 100–300 pmol of peptide but also the identification of the amino-terminal residue. The Dns modifications have been performed under conditions very similar to those described by Tapuhi *et al.* (*J. Chromatogr.*, 205 (1981) 325–337 and *Anal. Biochem.*, 115 (1981) 123–129) which ensure complete modification. Reversed-phase high-performance liquid chromatography was carried out on a Brownlee Aquapore RP-300 column, employing a gradient of methyl ethyl ketone–2-propanol at 55°C, under conditions which allow for the separation of all Dns derivatives expected from a "normal" peptide/protein hydrolysate.

INTRODUCTION

The quantitation of amino acid concentrations from such widely differing sources as grains, physiological fluids and hydrolyzed peptides/proteins has for years relied on the method developed by Spackman *et al.*[1]. Here the amino acids are chromatographically separated on a cation-exchange resin, mixed with ninhydrin as they are eluted, and heated; the absorbance of the Ruhemann's purple complex is then measured photometrically. Concentrations are calculated by comparing the

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TABLE I

RECENT METHODOLOGY FOR AMINO ACID ANALYSIS UTILIZING HPLC EQUIPMENT

	Chromatographic conditions		*Detection*	*Sensitivity limits (pmol)*	*Analysis times (min)*	*Remarks*	*Ref.*
	Column support	*Buffers**					
I	Free amino acids						
	1 NH_2-bonded phase	KH_2PO_4–AN gradient	200 nm (UV)	Not given	30	N, Q and G poorly separated	4
	2 RP-18	Ion-pairing-AN–MeOH	Post-column (OPA)	< 40	30	E, G and T poorly separated	5
	3 Cation exchange	Sodium formate, step gradient	Post-column (OPA)	10	45	All amino acids separated	3
II	Dns-amino acid derivatives						
	1 RP-8	Sodium acetate–phosphate–AN gradient	Fluorescence	>1000	58	Adequate separation; low *M* value	6
	2 RP-18	MeOH–THF–AcOH–TEA gradient	Fluorescence	Not given	40	Good separation of all amino acids; comment that can be used for pico- and femtomole ranges	7
III	OPA-amino acid derivatives						
	1 RP-18	Na_2HPO_2–AN gradient	Fluorescence	5	45	T and G not separated	8
	2 Several RP-supports	TEA–AcOH–AN gradients	Fluorescence	25	50	Chromatographic conditions with several different supports tested	9

* Abbreviations: AcOH = acetic acid; AN = acetonitrile; MeOH = methanol; TEA = triethylamine; THF = tetrahydrofuran.

peak areas in the sample with those for known concentrations of the same amino acids in a standard. Instruments utilizing this basic method have been improved over the years from the standpoints of both sensitivity and shorter analysis times. Changes in resin synthesis and sizing methods, reduction of the column dimensions, advances in photometer design and higher chemical purity have all been significant contributing factors toward improving the technique. The introduction of fluorescence detection methods have, in turn, provided another 5–10 fold increase in sensitivity (see Benson[2] for a review of some of these points). Most commercially available instruments are currently capable of performing routine analyses at the *ca.* 500 pmol level within 60–90 min.

Recent advances in high-performance liquid chromatography (HPLC) technology have also provided instrumentation suitable for utilization in amino acid analysis (see Table I). In a previous report[3] the conversion of a dual-pump, automated HPLC instrument into a highly sensitive (> 10 pmol), quick (60 min) analyzer was described. The chromatography was carried out on a Durrum DC-4A resin, and *o*-phthalaldehyde (OPA) was used as the detection reagent. A variable-temperature water-bath, buffer selector valve, fluorometer and microprocessor interfacing unit were the additional items required. We have subsequently improved upon these results through the substitution of Kontron AS-70 (7 μm) resin and the utilization of either isocratic or gradient programmes for amino acid elution[10].

Other approaches have been to use various silica derivatives as the chromatographic support material (Table I). Free amino acids have been separated using a gradient on NH_2 silica employing UV detection[4], and on reversed-phase (RP) supports with post-column OPA derivatization[5]. Pre-column derivatizations with Dns chloride (Dns-Cl)[6,7,11–14] followed by RP-HPLC have also been investigated. Although yielding fluorescent amino acid derivatives which reportedly can be detected in the low femtomole range by chemiluminescence[15], there appear to be a number of serious drawbacks inherent to using a pre-column reaction with either Dns-Cl or OPA[8,9]: (1) no reaction, incomplete reaction, and/or formation of secondary peaks from the same amino acid; (2) lack of stability of the derivatives with respect to time, solvent or exposure to light; (3) widely differing detector responses for equal amounts of derivative; and (4) introduction of contamination in the form of amino acids or reaction byproducts during the derivatization process. None of these needs serious consideration when a post-column reaction is used.

The purpose of this communication is to compare the suitability of (a) pre-column Dns derivatization and subsequent RP-HPLC separations of the derivatives with (b) post-column OPA or ninhydrin detection of amino acids separated by ion-exchange chromatography. As will be shown, each method has its own advantages, and their utilities depend on the specific application.

EXPERIMENTAL

Chemicals and buffers

Dns-Cl, Li_2CO_3 and methylamine–HCl were purchased from Fluka (Buchs, Switzerland), methyl ethyl ketone "LiChrosolv" from Merck (Darmstadt, G.F.R.) and HPLC-quality 2-propanol from J. T. Baker (Gross-Gerau, G.F.R.). Both free amino acids and their Dns derivatives were from Sigma (St. Louis, MO, U.S.A.).

Additionally, a type H amino acid standard solution from Hamilton (Bonaduz, Switzerland) was used. Water was quartz bi-distilled.

The following peptides were obtained from Bachem (Dubendorf, Switzerland): (1) D-leu^6-renin inhibitor (His-Pro-Phe-His-Leu-Leu-Val-Tyr), (2) angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), (3) lys^8-vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-amide), (4) iodo-phe^1-experimental allergic encephalitogenic peptide (iodo-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg), (5) eledoisin (Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-amide), (6) physalaemine (Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-amide), (7) γ-MSH (Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly), (8) D-arg^6-dynorphin-(1–13) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys), (9) somatostatin (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys) and (10) des-tyr-γ-endorphin (Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu). The homogeneity of each peptide was checked by RP-HPLC in a $NaClO_4$–acetonitrile buffer system[16] prior to acid hydrolysis.

The stock solution used in buffer preparation consisted of 37.5 ml of 1 *N* NaOH, 2.5 ml of formic acid and 3.75 ml acetic acid per litre of quartz bi-distilled water. For the buffers used in Dns-amino acid separations the stock solution was made 10% (v/v) in methyl ethyl ketone for *A* buffer and 3% (v/v) methyl ethyl ketone–35% 2-propanol (v/v) for *B* buffer.

Instrument

The HPLC unit consisted of two Altex Model 110 pumps (Beckman, Berkeley, CA, U.S.A.), a Kontron Model 200 microprocessor (Kontron, Zürich, Switzerland), and a WI-SP sampler (Waters, Milford, MA, U.S.A.). Effluent monitoring was with a Fluoro-Monitor from Aminco (Silver Spring, MD, U.S.A.), which was equipped with a 70-μl flow-through cell and filters for fluram detection, and was operated with the damping switch in the ON position. Similarly, an Uvikon 725 Spectrophotometer (Kontron) with an 8-μl flow-through cell was employed for UV-visible detection. The output of either detector was plotted by a two-channel recorder (Model 600, W + W Electronic, Basel, Switzerland) and peak areas determined using a 3390 A integrator from Hewlett-Packard (Avondale, PA, U.S.A.). A stainless-steel pressure coil (*ca.* 200 cm × 0.25 mm I.D.) was connected to the detector outlet in order to prevent degassing and bubble formation in the flow-through cell.

Separations were carried out at 0.8 ml/min on columns thermostated at 55°C in a stainless steel jacket using a Haake type FJ water-bath (Karlsruhe, B.R.D.). The columns (250 × 4.6 mm I.D.) tested were: RP-8 (10 μm), RP-18 (5 μm), RP-18 (10 μm) and RP-300 (10 μm) from Brownlee (Santa Clara, CA, U.S.A.); Partisil-10 ODS-2 and ODS-3 from Whatman (Clifton, NJ, U.S.A.); Zorbax ODS from DuPont (Wilmington, DE, U.S.A.); and a 150 × 4.6 mm I.D. column of Ultrasphere-ODS (5 μm) from Altex.

Methods

Dns derivatization was carried out under conditions similar to those used by Tapuhi *et al.*[7], using Dns-Cl in acetonitrile (1.5 mg/ml, 5.56 m*M*) and Li_2CO_3 (40 m*M*) as the buffer. The ratio of Dns-Cl to buffer was 1:2 and the volume used dependent on the *total* concentration of amino groups present in the sample to be dansylated. For example, standards were reacted at 37°C for 60 min in the following

volumes: 20 μl of standard solution (1 nmol per amino acid/μl), 800 μl of buffer and 400 μl of Dns-Cl. The reaction was terminated by the addition of 40 μl of 2% methylamine and a further 5 min incubation at 37°C; the total volume was 1260 μl. Reaction volumes were decreased progressively by a factor of two for each halving of the standard concentrations, *e.g.*, 630 μl for 10 nmol, 315 μl for 5 nmol, 165 μl for 1 nmol and 85 μl for 500 pmol (in the latter two the standard concentration was diluted 10-fold to 0.1 nmol/μl). For samples with total concentrations of 10 nmol amino groups or less a final volume of 85 μl was employed, the minimal amount which we could conveniently manipulate. The derivatized samples were diluted into a sufficient amount of buffer *A* to lower the pH to *ca.* 4.0 and injected automatically. Peptides and proteins were hydrolyzed in distilled 6 *N* hydrochloric acid for 22 h at 110°C under vacuum. A Durrum D-500 analyzer (Dionex, Sunnyvale, CA, U.S.A.) was used for determining amino acid compositions/concentrations by "standard" methods. All glassware that came in contact with the reagents for Dns derivatization was carefully washed; tubes (60 × 5 mm I.D.) for hydrolyses were heated at 500°C for *ca.* 4 h to pyrolyze any organic material present. Solutions were transferred with automatic pipettes using polypropylene tips, and Dns derivatization reactions were carried out in micro sample tubes with caps (250 μl or 1.5 ml total volume) made of the same material. No attempts were made to clean either the tips or sample tubes.

RESULTS AND DISCUSSION

When the first report by Tapuhi *et al.*[7] on the utilization of Dns derivatization and subsequent separation of the Dns-amino acids by HPLC appeared, we thought that this might well be a technique suitable for determining the amino acid compositions of peptides which were being isolated at below the nanomole level using RP-HPLC techniques[16–19]. Their use of Dns-Cl in acetonitrile, a Li_2CO_3 reaction buffer and methylamine (for terminating the reaction) were directly applied. We were not, however, able to utilize the large volumes (3 ml total) or the apparent high amino acid concentrations (exact values not given) which they were. This was based on the necessity to develop a method which would allow one both to identify the amino terminus of a peptide and to determine its amino acid composition by using less than 200 pmol of starting material. Numerous experiments led to the final reaction conditions (see Experimental), which maintain a ratio range of [Dns-Cl]:[amino acid] of between 6:1 and 8:1, for *total* amino acid concentrations from *ca.* 20 to 400 nmol. For peptide hydrolysates or for unhydrolyzed peptides, where the *total* primary amino group concentrations are usually much less (0.1–5 nmol), the ratio increases to values far in excess of 8:1. It is interesting to note that a similar ratio range (5:1 to 10:1) was reported by Tapuhi *et al.* in a later publication[14].

Dns-amino acids were separated by Tapuhi *et al.*[7] on a 5-μm C_{18} Hypersil column (150 × 4.6 mm I.D.) employing a methanol gradient at pH 4.0. Since such a column was not available we checked a number of other C_8 and C_{18} packings from both the same and different suppliers (see Experimental). The only one found which gave acceptable results, *i.e.* good peak shape and separation of *all* Dns derivatives found in hydrolysates of "normal" proteins, was the Brownlee RP-300 resin. This support material has also recently been shown to be useful for both peptide and protein HPLC[20].

It was during the search for another suitable HPLC resin that the methyl ethyl

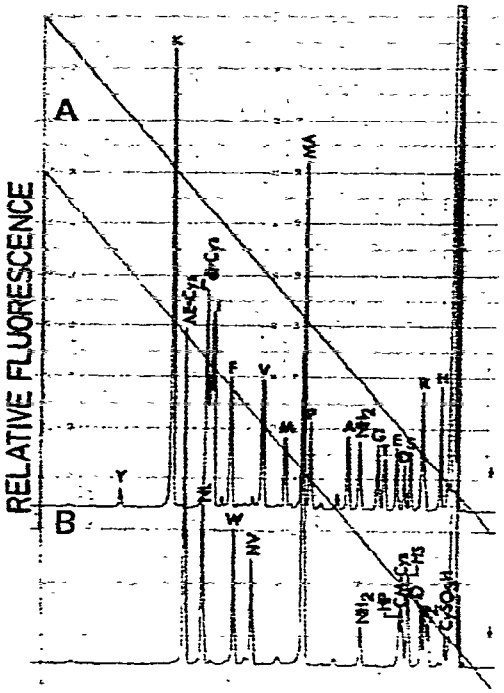

Fig. 1. Reversed-phase HPLC of Dns-amino acids. A, Dns derivatives of the amino acids found in protein hydrolysates; B, Dns derivatives of amino acids often resulting from chemical modifications, cleavages at specific side-chains or used as internal standards. Amount per derivative, *ca.* 200 pmol; photomultiplier setting, 30; 20-mV recorder range. The one-letter code for the amino acids is used except for the following abbreviations: NH_2 = amide; MA = methylamine; di-Cys = cystine; $CySO_3H$ = cysteic acid; HS = homoserine; CM-Cys = carboxymethylcysteine; HP = hydroxyproline; NV = norvaline; NL = norleucine; AE-Cys = aminoethylcysteine.

ketone–2-propanol-based gradient system was developed (Fig. 1). The gradient is started at 0% buffer *B* in order to achieve maximum separation between Dns-His, as well as Dns-$CySO_3H$, and the polar reaction by-products which elute with the void volume of the column. Elution can be commenced at higher values (12–15% buffer B), in order to shorten run times, but Dns-His and often Dns-Arg are lost when the concentration ratio of by-products to amino groups is too high. The ammonia originating from the acid deamination of Asn and Gln, as well as that contaminating the HCl used for hydrolysis, yields the Dns-NH_2 derivative during modification. Since we were unable to separate this derivative from that of Glu using the methanol gradient of Tapuhi *et al.*[7] on the RP-300 resin, it was necessary to develop the indicated system. Separations of Dns-Pro and Dns-MA are sometimes problematic and will be discussed later. The elution of Dns-di-Cys between Dns-Ile and Dns-Leu is not thought detrimental since most samples are either alkylated or oxidized prior to hydrolysis, thereby converting Cys and/or di-Cys residues into acid-stable derivatives.

In comparison with the other amino acid derivatives (see Fig. 2A), the amount of Dns-Tyr observed is low and represents one of the potential problems of employing pre-column Dns derivatization for quantitative analysis. The sample chromato-

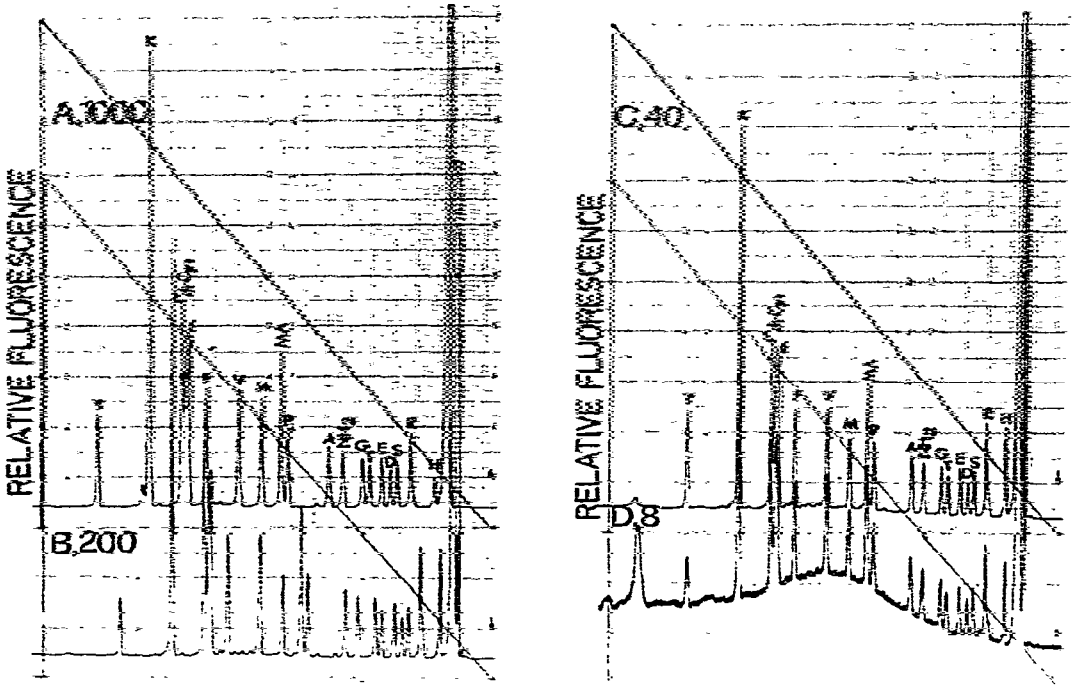

Fig. 2. Chromatography of various concentrations of Dns-amino acids. The amounts (picomoles of each derivative) are indicated on the chromatograms. Photomultiplier setting was held constant at 30 while recorder sensitivity was progressively increased, *i.e.*, 100 mV (A), 20 mV (B), 5 mV (C) and 1 mV (D). Sample volumes injected from a stock solution (5 pmol per derivative per μl) were 200 μl (A), 40 μl (B), 8 μl (C) and 8 μl from a five-fold dilution of the original solution (D).

graphed here (Fig. 1) was allowed to remain for a few (4–5) days at room temperature (diluted into the *A* buffer for HPLC) and exposed to the light in the laboratory, both natural and from overhead irridescent lamps. This loss is not observable when the samples are analyzed within a reasonable time (1 day) after Dns derivatization while remaining somewhat protected from direct light, *i.e.*, as in the micro-injection vials for many automatic sampling systems: However, for greatest accuracy, it is recommended that injections be carried out as soon as possible following sample preparation. A second problem concerning Dns-Tyr stability was observed while testing the chromatographic properties of the various C_8/C_{18} columns and utilizing the tetrahydrofuran (THF) containing buffer system of Tapuhi *et al.*[7]. Decreased amounts, or even total lack, of this derivative were often noted and attributed to the presence of oxidants in the reagent. Thus, instead of attempting to purify the reagent, our gradient system was developed employing methyl ethyl ketone as a substitute for the THF.

Fig. 1B illustrates the elution positions of the Dns derivatives of Norleu and Norval, analogues often used as internal/external standards, and of those amino acids which are normally destroyed during HCl hydrolysis, *e.g.*, Asn and Gln (for reasons discussed above) and Trp. Similarly, the positions of three Cys derivatives, namely cysteic acid, carboxymethyl- and aminoethylcysteines, are indicated. Dns-homoserine, a product arising from the CNBr cleavage at Met residues, co-chromato-

TABLE II

AMINO ACID COMPOSITIONS OF PEPTIDES DETERMINED BY (1) PRE-COLUMN Dns DERIVATIZATION AND RP-HPLC SEPARATION WITH (2) THOSE DETERMINED BY CATION-EXCHANGE SEPARATION AND NINHYDRIN DETECTION*

Italicized values indicate which amino acid was used for calculations of composition.

Peptide	*No. residues*	*Analysis method*	*Amino acid*																		*Amount (pmol/μl)*
			D	*T*	*S*	*E*	*P*	*G*	*A*	*C*	*V*	*M*	*I*	*L*	*Y*	*F*	*H*	*K*	*R*	*W*	
1	8	1					1.0				0.8			2.0	0.6	*1.0*	1.5				188
		2					1.0				0.6			2.1	0.8	*1.0*	1.9				184
2	8	1	1.2				*1.0*				1.2		0.6		0.6	1.0	1.1		1.1		520
		2	0.9				*1.0*				0.8		0.8		0.8	0.9	0.8		0.9		508
3	9	1	1.1			1.0	1.0	1.1		ND					0.6	*1.0*		1.0			392
		2	0.9			1.0	1.0	0.9		ND					0.7	*1.0*		0.8			430
4	9	1			*1.0*	2.1		1.9	0.9							0			1.0	0	267
		2			*1.0*	2.2		2.1	1.1							0			1.0	0	224
5	11	1	1.4		1.2	1.1	1.1	1.1	1.2			1.0	0.6	1.0		*1.0*		0.9			572
		2	1.1		0.9	1.1	1.1	1.0	1.0			0.9	0.9	1.0		*1.0*		0.9			587
6	11	1	2.1			1.2	1.2	1.1	1.2			1.1		1.0	0.9	1.1		*1.0*			390
		2	1.7			1.0	1.1	1.0	0.8			1.0		1.0	0.9	0.8		*1.0*			421
7	12	1	1.3					2.1			1.2	1.1			1.0	*2.0*	0.6		2.1	0	660
		2	0.8					1.9			1.0	1.1			1.0	*2.0*	0.8		1.7	0	601
8	13	1					1.1	2.1					0.7	2.0	0.6	*1.0*		2.1	3.1		336
		2					1.1	2.0					1.0	2.0	0.9	*1.0*		2.2	2.9		356
9	14	1	*1.0*	1.7	1.2			1.2	1.3	ND						3.0		1.9		0	334
		2	*1.0*	2.0	0.9			1.1	0.9	ND						3.2		1.9		0	283
10	16	1		2.6	2.2	2.3	1.3	2.1			1.0	0.8		2.1		*1.0*		1.0			204
		2		3.0	1.9	2.0	1.1	1.8			1.0	1.0		2.1		*1.0*		0.8			213

* Peptides hydrolyzed were: 1 = renin inhibitor; 2 = angiotensin II; 3 = Lys^8-vasopresin; 4 = iodo-Phe^1-experimental allergic encephalitogenic peptide; 5 = eledoisin; 6 = physalaemine; 7 = γ-MSH; 8 = D-Arg^6-dynorphin (1–13); 9 = somatostatin; 10 = des-Tyr-γ-endorphin. See Experimental for their respective sequences. The amounts or concentrations of the peptides are given in the last column and have been determined by the respective methods.

graphs with the Asp derivative, and 4-hydroxyproline, present in high amounts in collagen, with Dns-Glu.

The concentrations of Dns derivatives which can be easily chromatographed with the present system range from *ca.* 25 nmol down into the low picomole levels. Fig. 2 shows a concentration series of 1000, 200, 40 and 8 pmol of each Dns-amino acid. The extended elution time for the 1000 pmol sample (Fig. 2A), as compared with the others (Fig. 2B–D), was due to a slightly lower flow-rate being delivered from both HPLC pumps. At the 8-pmol level the signal-to-noise ratio ranges from *ca.* 70:1 for Dns-Lys down to 7.5:1 for Dns-Asp. For this reason, as well as that of amino acid contamination of specially distilled 6 *N* hydrochloric acid and cleaned glassware, attempts are seldom made to carry out analyses at much below the 50-pmol level. Note that at the 8 pmol level the Dns-Tyr peak is significantly lower than would be expected. This appears not to be due to irradiation, since the samples were injected consecutively, but rather to an apparent loss or destruction during chromatography. For a series of 200-pmol injections the elution times of the Dns derivatives were found to be extremely reproducible (maximum deviations between 0.01 and 0.04 min, depending on the particular derivative). Similarly, the standard deviations for the Dns-amino acids range between 2 and 5 pmol.

In addition to fluorescence detection, the absorption properties of the methyl ethyl ketone–2-propanol buffers are such that the elution of the Dns derivatives can be followed using the ultraviolet range between 320 and 340 nm. The variations in peak-area ratios for the different Dns-amino acids, a factor of *ca.* 13 when comparing the fluorescence yields of Dns-Lys and Dns-Ser, are much less pronounced. Although its use is between 10 and 100 times less sensitive, depending on the Dns derivative, nanomole concentrations can be conveniently detected and should be considered as an alternative in those laboratories where either a fluorometer is not available or analyses in the picomole range are not required.

The utility of the Dns derivatization reaction and subsequent RP-HPLC of the products is indicated in Table II. Here, various amounts of the peptides (2–12 nmol) were hydrolyzed in distilled 6 *N* hydrochloric acid and dried by rotary evaporation, and the resultant amino acids were dissolved in a known volume of water. Aliquots containing *ca.* 1 nmol of hydrolyzed peptide were required for determining the indicated compositions by cation-exchange-based amino acid analysis (denoted as 2 under *Analysis method* in Table II). To have carried out the analyses on either of our Durrum instruments at significantly lower concentrations would have required a formidable effort, *i.e.* running the instrument at elevated detection levels, readjustment or replacement of photometer lamp(s), etc. In a similar manner, aliquots of 200–500 pmol of hydrolysate were derivatized and analyzed at the 100–300 pmol range (see Figs. 3A and B for representative chromatograms). Although the compositions determined by the two methods are seen to agree rather well (compare 1 and 2 under *Analysis method* in Table II), there are exceptions: Asp values in peptides 5 and 7, due to poor integration; Pro value in peptide 6, poor peak integration due to large amount of Dns-MA (see later discussion); His value in peptide 7, reduced due to excessive amounts of polar by-product material eluting just prior to the amino acid derivative. The peptide concentrations as determined by both methods (last column, Table II) also agree reasonably well. This result contrasts with the latest report of Tapuhi *et al.*[14], where significant differences between the two types of analysis were

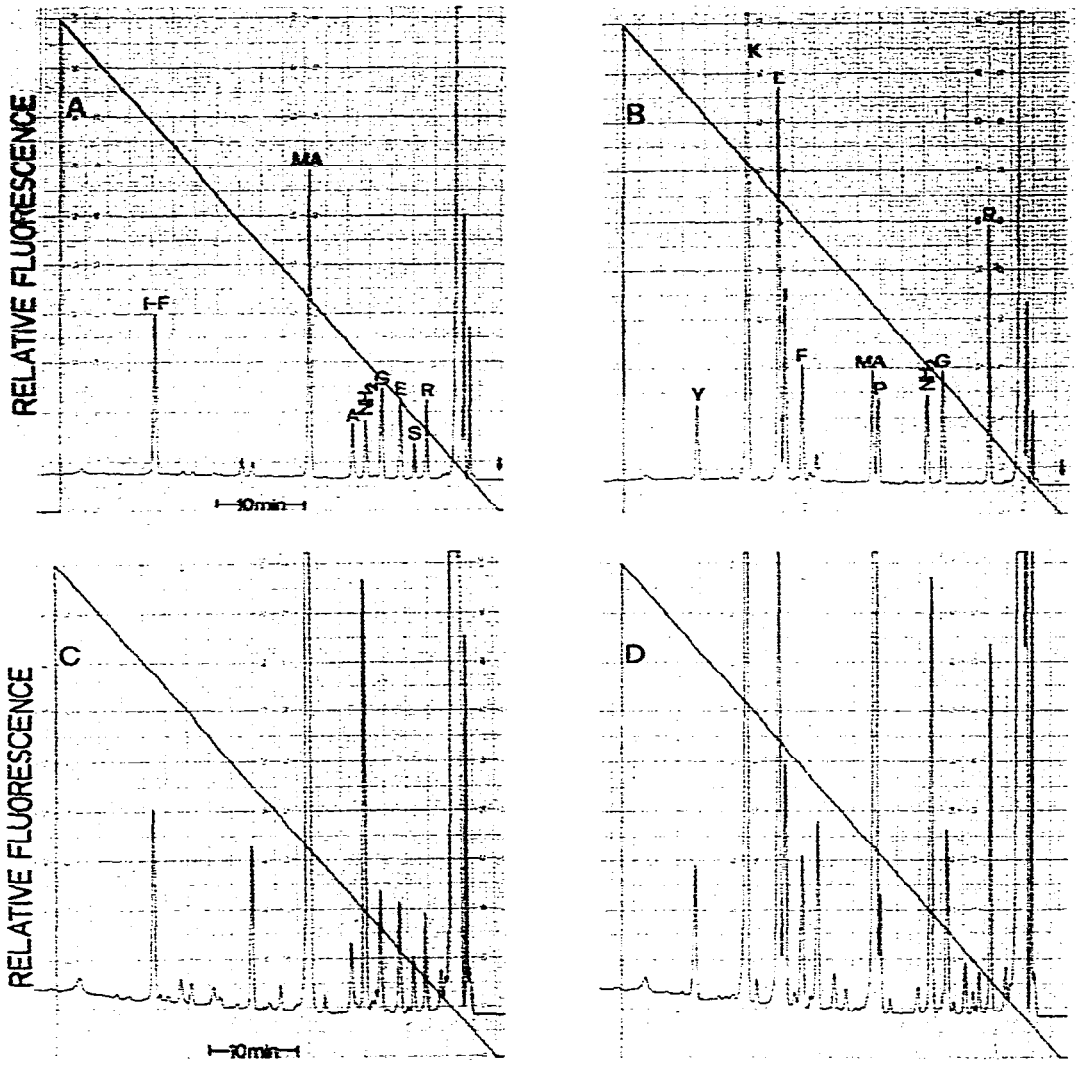

Fig. 3. Representative chromatograms of Dns-amino acid analyses. A, 1 μl (224 pmol) of hydrolyzed peptide 4 was Dns derivatized and 50% (112 pmol) chromatographed. B, 1 μl (514 pmol) of hydrolyzed peptide 8 was Dns derivatized and 50% (257 pmol) chromatographed. For both A and B the photomultiplier setting was 30 and the recorder sensitivity was 20 mV. C, 298.6 pmol of peptide 4 were Dns derivatized and two-thirds of the sample hydrolyzed for 18 h. Dns derivatization was repeated and 25% (*i.e.* one-sixth of original sample or 49.8 pmol) chromatographed. D, 457 pmol of peptide 8 were processed in the same manner as for C (above), 76.2 pmol being chromatographed. Fluorometer photomultiplier and recorder settings were 10 and 20 mV, respectively.

observed. However, as they pointed out, this might well have been due to the sample undergoing analysis (fermentation broth) and/or manipulations required prior to the analysis itself.

As stated in the introduction, one prime objective was to find conditions which would allow the simultaneous determination of amino acid compositions and identifi-

cation of amino-terminal residues on as small an amount of material as possible. To this end it was necessary to determine the rate at which Dns derivatives are destroyed under the conditions employed for peptide/protein hydrolysis. Table III indicates the percentages of Dns-amino acids remaining after exposure to hydrolysis conditions (6 *N* hydrochloric acid at 110°C under vacuum) for various times. The results clearly indicate why a short hydrolysis time is necessary when Pro is the amino-terminal residue. The decrease in the values for Dns-Ile represents the destruction of Dns-di-Cys (since they co-elute it was not possible to give separate values); the reason for the low Dns-Met percentages are presently not yet clear. Thus, during the hydrolysis of a Dns-peptide not only are the labeled residues (N-terminal and ε-Lys) being released through peptide-bond cleavage, but destruction of the Dns derivatives also occurs. For these reasons a Dns-peptide is usually hydrolyzed for both 4 h and 18 h and the chromatographic results compared (see below).

TABLE III
PERCENTAGE OF Dns-AMINO ACID REMAINING AFTER ACID HYDROLYSIS
Duplicate samples of Dns-amino acids (737 pmol each) were dried following Dns derivatization and hydrolyzed in 150 μl of distilled 6 *N* hydrochloric acid under vacuum for the indicated time. Following evaporation the hydrolysates were dissolved in buffer *A* and a portion (207 pmol of each) analyzed. The averaged peak areas for the duplicates of each time period were then compared with those of an unhydrolyzed sample of identical concentration and expressed as percentage of Dns derivative remaining.

Amino acid	*Hydrolysis time (h)*				
	1	*2*	*4*	*8*	*18*
Asp	97	101	105	117	121
Thr	100	96	90	79	58
Ser	96	92	75	58	29
Glu	100	101	94	92	79
Pro	85	58	18	0	0
Gly	96	89	74	57	31
Ala	97	100	91	80	57
Val	100	92	89	84	68
Met	35	27	43	51	38
Ile	130	103	100	94	76
Leu	101	91	91	85	72
Tyr	85	–	109	–	94
Phe	100	91	92	92	79
His	100	97	90	85	77
Lys	97	75	94	92	58
Arg	100	89	76	72	61

The chromatograms illustrated in Fig. 3C and D are examples of where a relatively small amount of starting material (300 and 457 pmol of peptides 4 and 8, see Table II) was used for both compositional and amino-terminal analyses. Following Dns derivatization, a portion of each (one third) was hydrolyzed for 4 h and the remaining (two-thirds) for 18 h. Each 18-h hydrolysate was divided equally; on one half derivatization was repeated and aliquots (49.8 and 76.2 pmol, respectively) were injected. A comparison of Fig. 3A with 3C and 3B with 3D thus represents the difference between the derivatization of the amino acids in a peptide hydrolysate and those

Fig. 4.

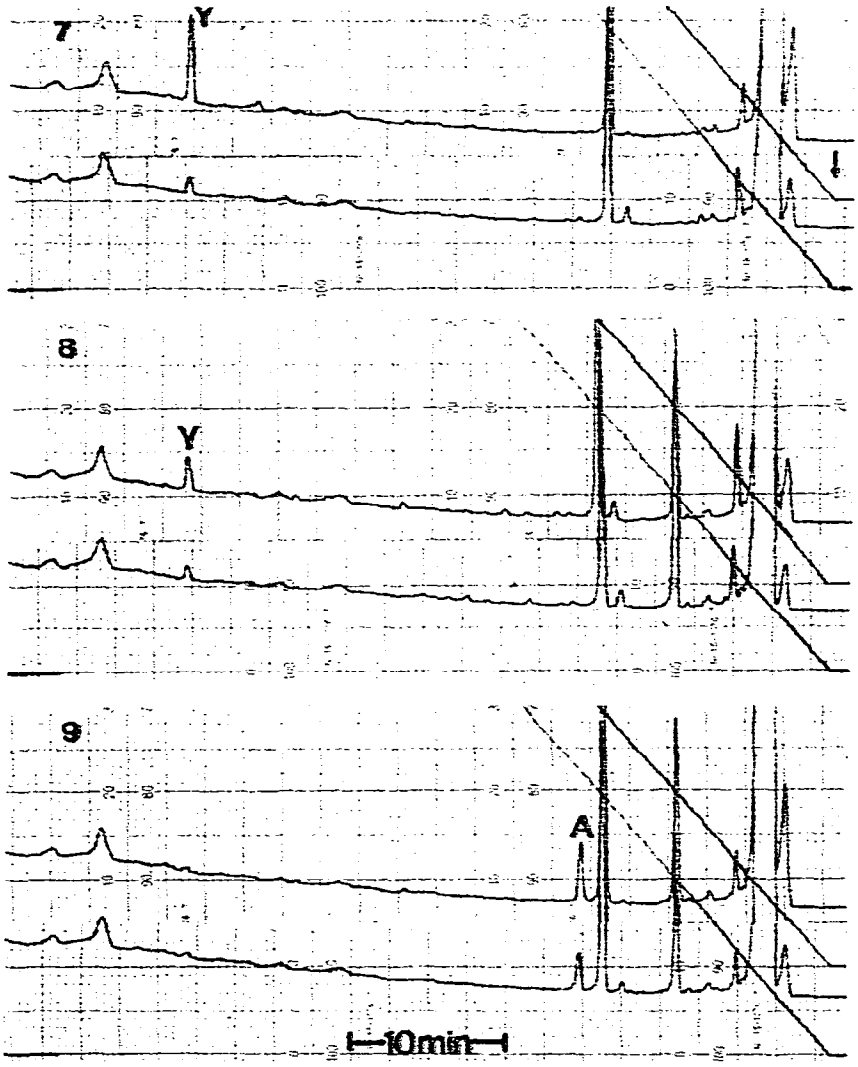

Fig. 4. Amino-terminal analyses of various peptides. Aliquots of known amounts of peptides 1–9 (see Table II) were Dns derivatized, one-third was hydrolyzed for 4 h and 50% of the hydrolysate (*i.e.* one-sixth of original) was chromatographed (upper chromatogram for each peptide). Hydrolysis for 18 h was carried out on the remaining two-thirds; the sample was divided equally and 25% (again one-sixth of original) chromatographed (lower chromatogram). The remaining sample was Dns derivatized, and the results were compared with those given in Table II (results not given). The amounts (pmol) of Dns-peptide chromatographed following both 4 h and 18 h of hydrolysis were: 1, 81.8; 2, 56.4; 3, 63.7; 4, 49.8; 5, 58.7; 6, 56.1; 7, 133.6; 8, 76.2; and 9, 58.4. Fluorometer photomultiplier and recorder settings were 10 and 20 mV, respectively. Additional abbreviations used; ε-NH_2 = Dns-ε-Lys; i-F = Dns-iodo-Phe.

arising from the hydrolysate of a peptide which had been previously dansylated at the available primary amino groups. From the latter it is apparent that more peaks are visible (perhaps from the hydrolyzed by-products of the first derivatization or from the second derivatizaton of degradation products thereof) and that the relative ratios between the Dns-amino acids and Dns-MA, as well as the reaction by-products

eluting at the beginning of each chromatogram, are quite low. However, the ratios between each of the peaks agree well with those noted when only a single derivatization of the hydrolyzed peptide was performed. In fact the amino acid compositions of the peptides agreed extremely well with the values given in Table II, *i.e.*, for peptide 4: *1.0 Ser*, 1.94 Glu, 2.05 Gly, 0.80 Ala and 0.79 Arg; and Peptide 8: 1.90 Gly, 0.65 Ile, 2.08 Leu, 0.84 Tyr, *1.0 Phe*, 1.84 Lys and 2.62 Arg. No value for Pro is given for peptide 8 and, as can be seen from the chromatogram (Fig. 3D), is a consequence of the peak not being detected for integration, *e.g.*, due to the large Dns-MA peak partially obscuring the Dns-Pro derivative. By comparing the relative peak heights, however, it is clear that the peak area approximates the value expected.

All of the other peptides given in Table II were similarly Dns derivatized, hydrolyzed, re-Dns derivatized and chromatographed as outlined above (results not given). Although integration problems were noted with each peptide containing a prolyl residue, the amino acid compositions were in good agreement with the given compositions (Table II), exceptions being the Met values which were constantly below average (40–50%). In those cystine-containing peptides (numbers 3 and 9) no Dns derivatives of the amino acid or degradation products thereof were observed. Values for those residues which were capable of Dns derivatization, *e.g.*, primary amino groups at the amino-termini and the ε-amino groups of lysyl side-chains, were not, as might have been expected, low. This suggests that although destruction of the Dns derivatives occurs during acid hydrolysis (Table III) the resulting free amino acid is subsequently capable of being re-Dns derivatized.

The chromatograms from those portions of peptides 1–9 which were derivatized and subsequently hydrolyzed for either 4 h or 18 h are shown in Fig. 4. For those peptides not blocked at the amino terminus (5 and 6) or involved in disulphide formation (3), a single discrete peak was observed which correctly identified the first residue. In those peptides containing internal lysyl residues an additional peak was observed, with a retention time slightly less than that of Dns-Asp, which has been identified as the Dns-ε-Lys derivative. Since even on new columns these two derivatives are poorly separated, it can be expected that, as the column ages, identification will become more and more difficult. We have found that washing (regeneration) with a few column volumes of a 1:1 mixture of chloroform and methanol often improves subsequent performance. However, if this fails then either the elution gradient must be changed or a "newer" column used for the reanalysis. A comparison of those peptides containing amino acids which are deaminated during acid hydrolysis (*e.g.*, Asn, Gln and/or carboxy-terminal amides) with those lacking such residues, indicates little correlation between the relative sizes of the Dns-NH_2 peaks and the amount of peptide hydrolyzed. Thus, the contaminating NH_3 must be coming from other sources, such as the buffer used for originally dissolving the peptides, the bi-distilled water used for diluting them and/or the distilled hydrochloric acid employed for hydrolysis.

In summary the above examples demonstrated that the Dns derivatization reaction, and subsequent separation of the derivatives via RP-HPLC, is well suited to quantitative peptide amino acid analysis. The lower limits for which the methods can be utilized are principally defined by the amount of contamination present in/on all solutions/surfaces which come in contact with the sample during handling. Practical amounts, or those with which it is convenient to work, have been shown to be in the 100–300-pmol range. Such amounts provide sufficient material for both determi-

nation of the amino acid composition and identification of the amino-terminal residue. However, it should be strongly emphasized that the usefulness depends on assuring near-quantitative derivatization and the availability of an instrument equipped for fluorescence detection and, preferably, automatic injection as well as peak integration. Such an instrument is then useable for not only amino acid analysis as shown but also for analyses based on either isocratic[3,10] or gradient[10] elutions of cation-exchange resins. In these cases either a motorized selector valve for the buffers/regeneration solutions or a third pump for post-column OPA transfer are additional requirements. Thus, for specialized protein laboratories which are interested in determining amino acid compositions and/or amino terminal residues on low picomole amounts of material, or for those laboratories requiring only a few analyses per given time period, and not wishing to buy an extra pump or valve, then this method must be considered essential.

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