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CAPILLARY GAS CHROMATOGRAPHY MICRO-ASSAY FOR PYROGLUTAMIC, GLUTAMIC AND ASPARTIC ACIDS, AND GLUTAMINE AND ASPARAGINE*

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SUMMARY

Glutamine (GLN) and asparagine (ASN) are acid labile and thus are degraded by acid-catalysed procedures for preparing volatile derivatives for analysis by gas–liquid chromatography (GLC). Evidence is presented that GLN and ASN form chromatographically stable N(O)-dimethyl-*tert.*-butylsilyl derivatives, without cyclisation or conversion to the corresponding acids via loss of the amide moiety, on reacting with N-methyl-N-(*tert.*-butyldimethylsilyl)-trifluoroacetamide in pyridine at 75°C for 30 min. The structures of the derivatives have been confirmed by chemical ionisation mass spectrometry. Pyroglutamic (pGLU), glutamic (GLU) and aspartic (ASP) acids react similarly and, on a 15 m × 0.25 mm I.D. SPB-1 fused-silica capillary column, all five derivatives are resolved from each other and from the derivatives of the other proteic amino acids. The above reaction constitutes the basis for a direct GLC assay for pGLU, GLU, ASP, GLN and ASN.

INTRODUCTION

In a previous report[1], we described a simple and fast assay for glutamine (GLN) and asparagine (ASN) in the presence of the corresponding acids and other proteic amino acids. Its application to biological fluids was also demonstrated. This report addressed two separate, but interrelated, problems in the analysis of GLN and ASN by gas–liquid chromatography (GLC). First, the procedures most commonly used for assaying amino acids by GLC are based on the acid catalysed formation of alkyl esters followed by the formation of perfluoro acyl derivatives. These result in the conversion of GLN and ASN to the corresponding acids. Secondly, the procedures consequently developed for assaying GLN and ASN are not appropriate for the other proteic amino acids. In summary, apart from our earlier report, there is no direct, single-derivatisation, GLC-based procedure for assaying GLN and ASN in the presence of, and resolved from, *all* the other proteic amino acids.

There appears to be no method available for assaying pyroglutamic acid (pGLU), glutamic acid and glutamine in a single GLC procedure. Similarly, there is no simple method based on high-performance liquid chromatography (HPLC). Recently, Shih[2] showed that pretreatment of pGLU with hypochlorite does not open the pyrrolidone ring. Consequently, the o-phthalaldehyde derivative was not obtained and Shih used an independent method for evaluation of pGLU.

In addition to the papers cited in our earlier report[1], several other papers have described alternative procedures for assaying ASN and GLN by GLC.

Husek and Felt[3] condensed the amino acids and their amides with 1,3-dichlorotetrafluoroacetone (DCTFA) in a weakly basic medium. This reaction was followed by an acylation, an extraction and a second acylation reaction. Phenylalanine was used as an internal standard (I.S.) but, otherwise, resolution of the compounds of interest from the other proteic amino acids was not demonstrated, nor was the assay applied to a real sample.

Later, Husek[4] demonstrated the resolution of ASN and GLN from the other proteic amino acids using packed columns. However, although most of the amino acids were resolved on a slightly polar column (OV-17), a second analysis on a non-polar column was necessary to resolve histidine, tryptophan and cystine. The need for a second column renders the procedure rather cumbersome for application to routine amino acid analysis. Husek et al.[5] also studied the use of capillary columns. The best results were obtained using a 12 m × 0.5 mm I.D. wall-coated open-tubular glass (OV-17) column but, although all the other proteic amino acids shown were well resolved, both the ASN and GLN peaks clearly contained at least two components. GLN was resolved using a 25 m × 0.31 mm I.D. fused-silica open-tubular (OV-101 cross linked) column but ASN was not adequately resolved from isoleucine.

Frank et al.[6] developed a procedure based on the conversion of GLN to pGLU. The conversion is highly dependent on reaction conditions but, by using the D-amino acid as an internal standard, quantitation of GLN was simply a matter of determining the ratio of the D- and L-isomers of pGLU after resolving them on a Chirasil-Val column operated isothermally at 160°C. This elegant procedure was applied to the determination of GLN in cerebrospinal fluid, but the problem of assaying the other amino acids, including ASN, in the sample in the same analysis was not addressed.

The procedure we described earlier[1] was based on the transformation of ASN and GLN into their N(O)-dimethyl-tert.-butylsilyl derivatives by reaction with N-methyl-N-(tert.-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) followed by quantitation by capillary gas chromatography. Asparagine was derivatised without degradation of the amide moiety. It was concluded, on the basis of mass spectrometric evidence, that the derivative obtained for GLN was, in fact, a pGLU derivative and that GLN had been quantitatively converted to pGLU during the derivatisation reaction.

However, the standard GLN solution, which was prepared in 0.01 M hydrochloric acid was not stored at a sufficiently low temperature. Glutamine is converted to ammonia and pGLU by heating in the presence of dilute acid or alkali[7,8], but the reaction proceeds slowly even at refrigerator temperatures. It appears, therefore, that the conversion to pGLU occurred before the derivatization reaction.

We now present evidence that GLN can be derivatised with MTBSTFA, with-

out loss of the amide moiety and without cyclisation to produce a chromatograph-ically stable compound. The GLN derivative is separated from the derivatives of pGLU, GLU, ASP and ASN and of the other proteic amino acids. No significant conversion to pGLU occurs during the derivatisation reaction. This is the basis of a direct assay for pGLU, GLN, GLU, ASP and ASN, in the presence of the other proteic amino acids, using a single derivatisation reaction and a single chromato-graphic analysis. Resolution of the derivatives of these compounds from the other proteic amino acids is demonstrated using a standard amino acid calibration mixture and physiological samples.

EXPERIMENTAL

Reagents
        MTBSTFA was obtained from Pierce (Rockford, IL, U.S.A.) and redistilled before use to minimise interfering reagent peaks. The fraction distilling in the range 168–170°C was collected. Molar quantities of the reagent were calculated on the basis of a density of 1.121 g/ml[9]. All other reagents and solvents were of analytical grade.

Sample preparation
        Physiological samples were "cleaned-up" by ion-exchange chromatography, the procedure being based on that of Adams[10]. A Dowex 50 × 8 column (6 × 1 cm I.D.) was first regenerated with 25 ml of 2 *M* hydrochloric acid and washed to pH 6–7 with distilled water. A volume of 100 μl of crude xylem or phloem sap was acidified with 0.1 *M* hydrochloric acid to pH 2–3, transferred to the resin column using a pasteur pipet and allowed to pass through the column at about one drop every two seconds using very little air pressure. The column was first washed with 25 ml of water and the amino acids fraction was eluted with 25 ml of 2 *M* ammonium hydroxide. This fraction was evaporated *in vacuo* on a Buchi rotavapor at 45°C and the residue was dissolved using two times 1 ml of distilled water. A volume of 500 μl (equivalent to 25 μl of xylem sap) was analysed.

Derivatization
        A TECAM, Model DB-3H, Driblock heater was used to heat samples during derivative preparation.
        The mixture of amino acids (10 μl of a solution containing 25 μmol/ml; pre-pared daily) was dispensed into a 1 ml Reactival (Pierce) and excess solvent was evaporated at 35°C using a stream of dry nitrogen (100–200 ml/min). A molar equiv-alent of octadecane was added and the solvent evaporated. After the addition of MTBSTFA (15 μl), pyridine (15 μl) and triethylamine (1 μl), the vial was heated at temperatures and for times indicated in the text. After cooling, an aliquot of the reaction mixture was injected directly in the gas chromatograph.

Chromatography
        All chromatography was performed using a Varian Vista 6000 gas chromato-graph equipped with a capillary injection system and dual flame ionization detectors. The column, a 15 m × 0.25 mm I.D. SPB-1 fused-silica capillary column (Supelco, Oakville, Canada), was operated in the split mode (1:20). The carrier gas (helium)

flow was 60 cm/s. Data were acquired and processed using a Varian DS-601 data system.

Mass spectrometry

Mass spectra of the derivatives of GLN, pGLU, GLU, ASN and ASP were obtained using a Finnigan Model 3300 mass spectrometer operated in the positive chemical ionization (CI) mode using methane as the reagent gas.

RESULTS AND DISCUSSION

Mass spectral interpretation

The positive CI mass spectrum of the dimethyl-*tert.*-butyl silyl (DMBS) derivative of GLN is shown in Fig. 1 and the structures of specific ions are presented in Table I. In the table and the text which follows, charge designations have been omitted for the sake of clarity. For the same reason, the fragments are indicated as derived from the molecular ion rather than the protonated molecular ion. A molecular mass of 488 is indicated by the adduct ions at m/z 489 [M + 1] and m/z 517 [M + C_2H_5]. The even molecular mass further indicates that the molecule contains an even number of nitrogen atoms.

The presence of a silylated carboxylic acid group is indicated by the ions at m/z 357 [M − ODMBS] and 329 [M − COODMBS]. The formation of a neutral species at m/z 356 [M − ODMBS − H] is indicated by the adduct ions at m/z 385 (356 + C_2H_5) and 397 (C_3H_5).

The presence of three silylated groups is indicated by the ions at m/z 357, 243
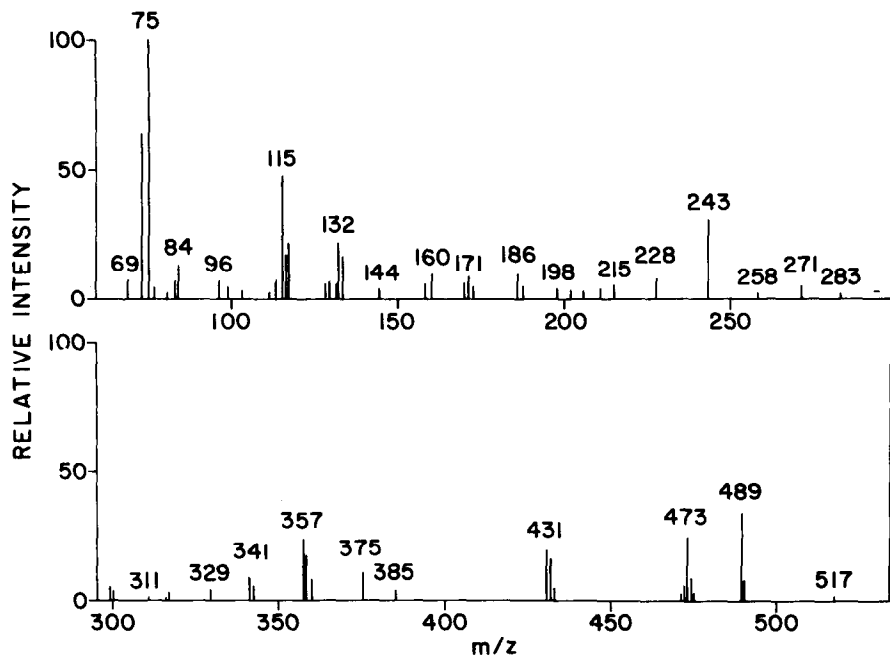

Fig. 1. Methane CI spectrum of the dimethyl-*tert.*-butylsilyl derivative of glutamine.

TABLE I

CI MASS SPECTRUM OF N(O)-DIMETHYL-*tert.*-BUTYLSILYL GLUTAMINE

DMBS denotes the dimethyl-*tert.*-butylsilyl moiety.

Ion	m/z	Ion	m/z
$[M + C_3H_5]$	—	$[M - DMBS + 2H]$	375
$[M + C_2H_5]$	517	$[M - ODMBS]$	357
$[M + H]$	489	$[M - COODMBS]$	329
$[M]$	488	$[M - ODMBS - C_4H_9]$	300
$[M - CH_3]$	473	$[M - DMBS - NHDMBS]$	243
$[M - C_4H_9]$	431	$[M - 2(DMBSNH) - DMBS + 2H]$	115
$[M - ODMBS - H + C_2H_5]$	385	$[COODMBS]$	159
$[M - C_4H_9 - C_4H_8]$	375	$[DMBS]$	115

and 115 the structures of which are rationalised in Table I. Isobaric structures have not been presented in all cases. Overall, the spectrum is consistent with the structure of a tri-DMBS derivative of glutamine.

TABLE II

EFFECT OF TIME, AND REACTION TEMPERATURE ON RELATIVE MOLAR RESPONSE OF pGLU, ASP, GLU, ASN AND GLN DIMETHYL-*tert.*-BUTYLSILYL DERIVATIVES

Abbreviations: RMR = relative molar response; C.V. = coefficient of variation ($n = 3$). Values are relative to octadecane = 1.

| Time (min)	Temperature (°C)	pGLU		ASP		GLU		ASN		GLN	
		RMR	C.V.	RMR	C.V.	RMR	C.V.	RMR	C.V.	RMR	C.V.
30	25	0.622	5.6	0.939	2.6	0.772	2.5	0.678	4.5	0.732	2.5
10	50	0.768	10.9	0.938	5.1	0.760	5.2	0.841	16.9	0.820	6.0
20	50	0.827	1.7	1.006	1.5	0.830	1.4	0.928	7.6	0.846	0.6
30	50	0.879	1.5	1.041	1.6	0.872	1.2	1.054	0.9	0.859	0.6
45	50	0.857	0.6	1.046	0.7	0.884	1.8	1.067	1.5	0.821	1.6
60	50	0.872	1.1	1.032	1.2	0.868	1.1	1.052	1.4	0.883	1.1
10	75	0.859	1.7	1.048	1.2	0.888	1.2	1.053	1.2	0.903	0.9
20	75	0.908	0.7	1.121	0.7	0.947	0.6	1.130	0.6	0.945	0.6
30	75	0.892	0.5	1.146	0.3	0.983	0.3	1.165	0.4	1.015	1.3
45	75	0.892	0.6	1.088	0.8	0.928	0.3	1.094	0.7	0.833	1.2
60	75	0.925	0.7	1.071	0.5	0.892	0.2	1.056	0.5	0.849	0.7
10	100	0.860	0.9	1.043	1.2	0.886	1.3	1.034	0.9	0.883	0.7
20	100	0.895	1.1	1.062	0.7	0.885	0.8	1.012	0.5	0.867	0.6
30	100	0.868	0.3	1.075	0.5	0.904	1.0	1.020	0.2	0.941	0.3
45	100	0.880	1.7	1.099	2.6	0.926	3.8	1.009	3.6	0.960	3.6
60	100	0.934	0.8	1.087	0.9	0.915	0.7	1.027	0.5	0.853	0.9
30	150	0.997	0.6	1.021	0.1	0.816	2.4	0.559	1.6	0.735	2.4
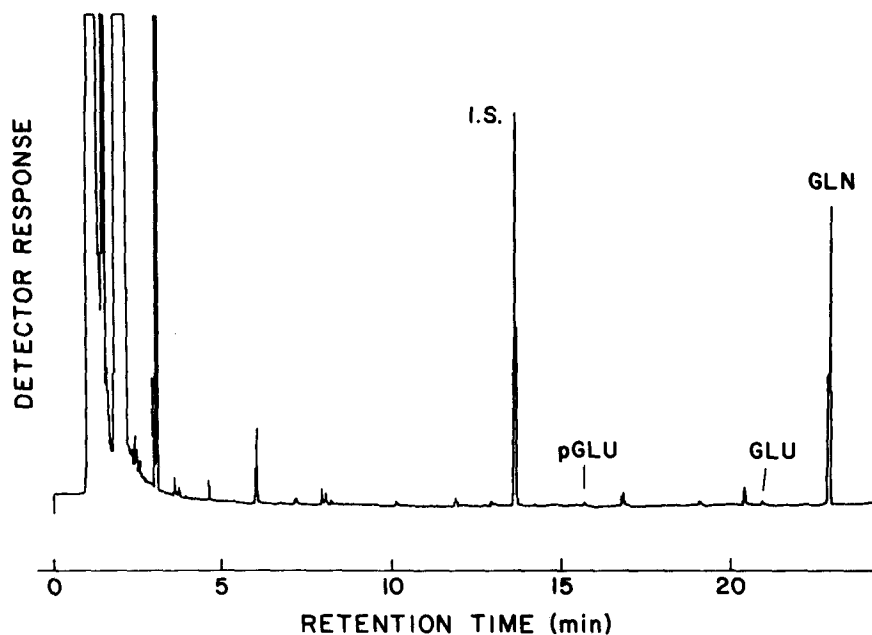
Fig. 2. Chromatogram illustrating chromatography of the dimethyl-*tert.*-butylsilyl derivative of glutamine.

Derivatisation

The effect of varying reaction time and temperature on the formation of the DMBS derivatives of the five amino acids of primary interest is indicated in Table II. The relative molar response (RMR) (octadecane = 1) directly reflects the amount of each derivative formed. As found previously[1], heating at 75°C for 30 min produced optimal overall results while avoiding excessive reaction time and temperature. Under these conditions, the conversion of GLN to pGLU and GLU is insignificant (<2%) (Fig. 2).

TABLE III

EFFECT OF REAGENT EXCESS ON FORMATION OF MTBSTFA DERIVATIVES OF pGLU, GLU, GLN, ASP AND ASN

The amounts of the reagents were 15 μl of MTBSTFA, 15 μl pyridine and 1 μl triethylamine.

Reacting groups (μmoles)*	Ratio	Relative molar response				
		pGLU	ASP	GLU	ASN	GLN
0.175	400	0.917	1.043	0.888	0.955	0.823
0.700	100	0.862	1.055	0.878	1.008	0.873
1.400	50	0.862	0.962	0.817	0.974	0.715
2.800	25	0.948	1.037	0.882	1.038	0.702

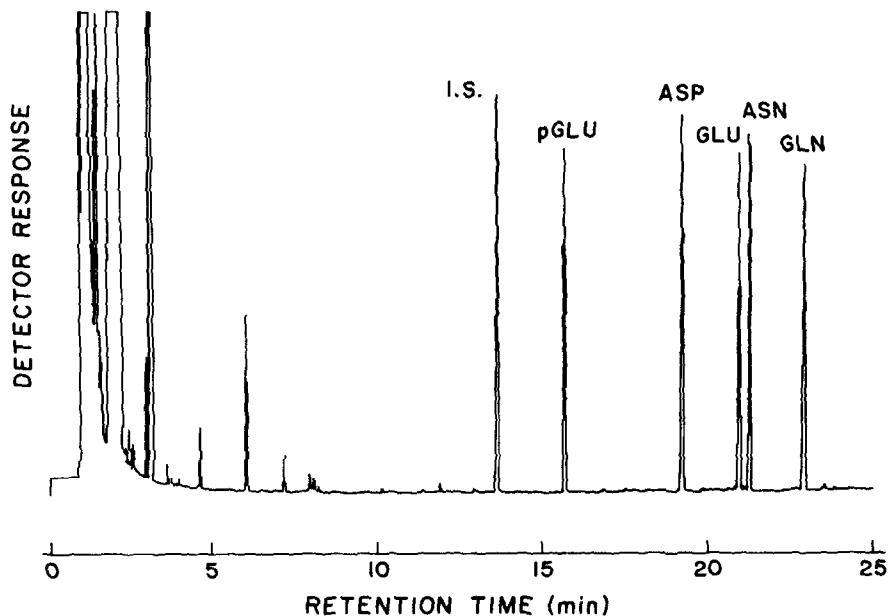* The theoretical number of reacting groups was confirmed by mass spectrometry.

Fig. 3. Chromatogram illustrating resolution of the dimethyl-*tert.*-butylsilyl derivatives of pyroglutamic acid, glutamic acid, glutamine, aspartic acid and asparagine.
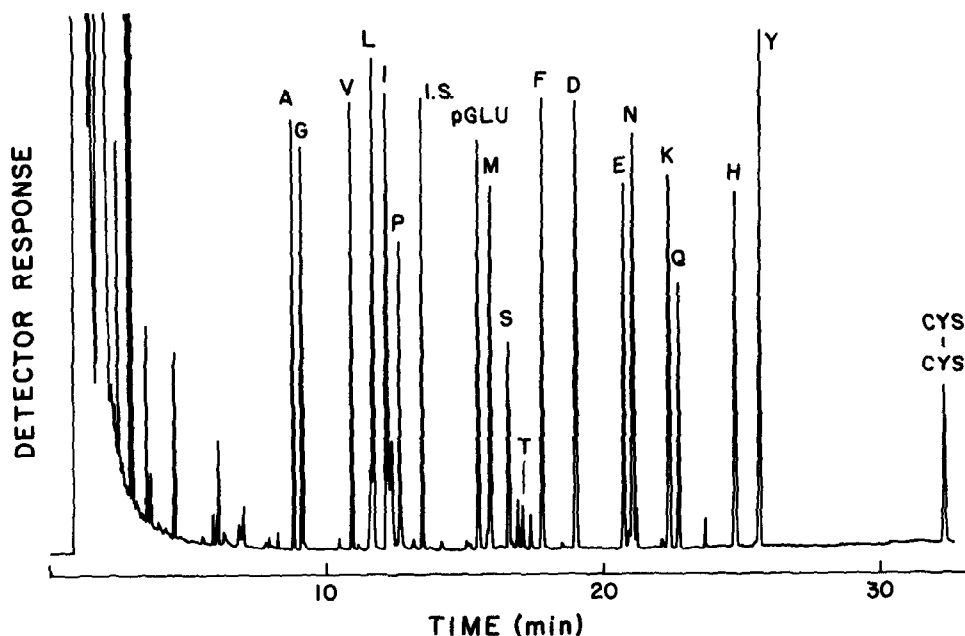

Fig. 4. Chromatogram illustrating resolution of the dimethyl-*tert.*-butylsilyl derivatives of pyroglutamic acid, glutamic acid, glutamine, aspartic acid and asparagine from other proteic amino acids. The letters represent the standard single-letter convention for the amino acids.
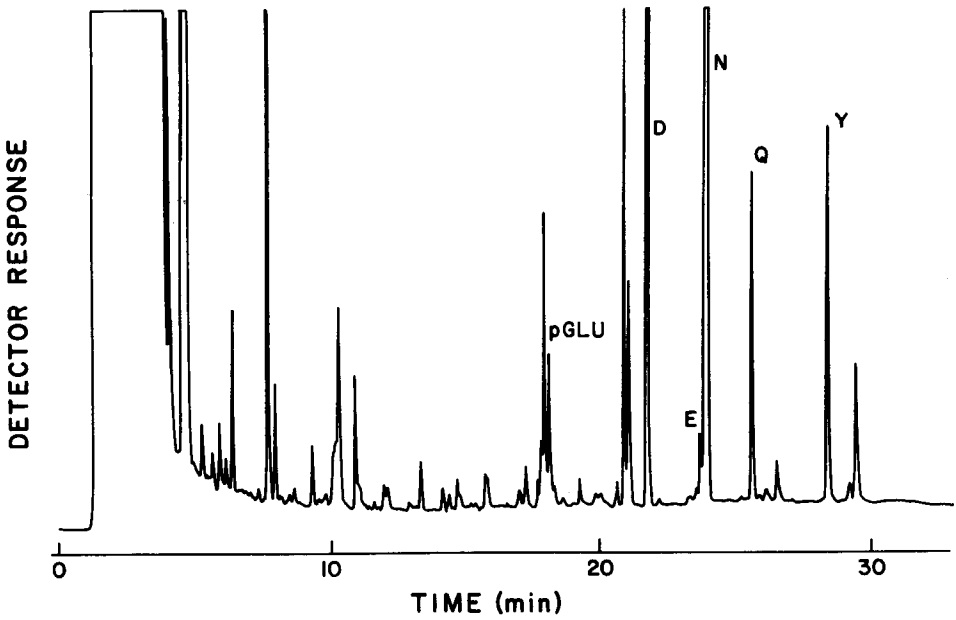
Fig. 5. Chromatogram illustrating identification of asparagine, aspartic acid, pyroglutamic acid, glutamine and glutamic acid in soybean xylem sap. The letters represent the standard single-letter convention for the amino acids.
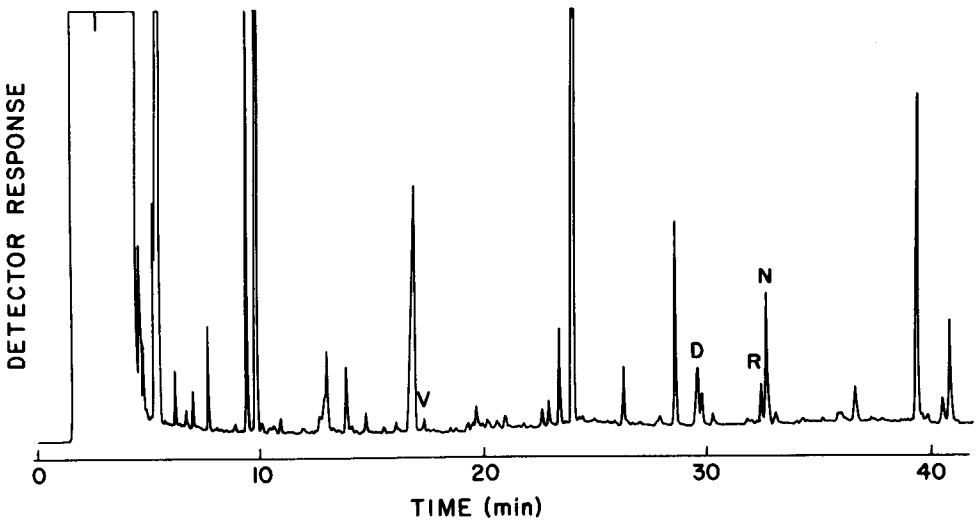

Fig. 6. Chromatogram illustrating identification of asparagine, aspartic acid, pyroglutamic acid, glutamine and glutamic acid in pea xylem sap. The letters represent the standard single-letter convention for the amino acids.

The effect of varying the proportion of reagent to the amino acids present in the standard sample is shown in Table III in which the ratio is calculated on the basis of the number of reactive groups to be derivatised. There is a significant decrease in the RMR of GLN at ratios of 50 or less. The low values for the RMR of GLN at a ratio of 400 and for GLU at a ratio of 50 cannot be explained at present.

Chromatography

The chromatographic resolution of the DMBS derivatives of pGLU, GLU, GLN, ASP and ASN is illustrated in Fig. 3. Further, their resolution from the DMBS derivatives of the other proteic amino acids on a single column is shown in Fig. 4. The resolution is more than adequate for assaying not only GLN and ASN but also pGLU and all the other proteic amino acids.

Physiological sample analysis

The application of the micro procedure to physiological fluids is illustrated in Figs. 5 and 6. The soybean xylem sample (Fig. 5) contained no detectable GLN and an insignificant amount of pGLU probably reflecting the fact that allantoin and allantoic acid are the major nitrogen-transport vehicles of nitrogen-fixing soybean. The pea xylem sap (Fig. 6) contained predominantly ASP and ASN but also a substantial amount of GLN. In addition, there was a peak at the location of pGLU but its identity was not confirmed. The xylem sap analyses were based on 100 μl of the original fluid with only the equivalent of 25 μl being used for the derivatisation and a portion of that measure (1/30) being applied to the chromatographic column. The quantities of amino acids present in the sample were such that a ten-fold reduction in scale could readily be achieved, thus making analysis of physiological samples at the sub-micro level entirely feasible.

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