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AMINO ACID ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY USING A NITROGEN-SELECTIVE DETECTOR*

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SUMMARY

A nitrogen-specific gas-chromatographic detector has been used to analyse amino acids in the low picomole range as their N-heptafluorobutyryl isobutyl esters. The amino acid derivatives were studied over the range 3–60 pmol and a coefficient of variation of $<4\%$ was obtained for the relative molar responses of most of the monobasic amino acids. A detection limit of *ca.* 250 fmol was established for the monobasic amino acids. No simple relation could be established between detector response and the atomic composition of the amino acids. The specificity of the detector allowed the nitrogenous components of complex physiological samples to be analysed without prior fractionation of the samples.

INTRODUCTION

The use of a specific detector in gas–liquid chromatographic (GLC) analysis presents the analyst with the potential for enhanced selectivity and sensitivity. Nitrogen-selective detectors have most frequently been based on the phenomenon of the emission of positive ions from heated alkali-metal surfaces, hence the commonly used descriptors alkali-flame detector and flame-thermionic detector. This principle has also been used to develop detectors selective for phosphorus-, sulphur-, arsenic- and lead-containing compounds, but the present discussion is restricted to the context of nitrogen selectivity.

Since the first application of an alkali-flame detector to the detection of organic nitrogen-containing compounds[1], detectors of this nature have been widely applied. However, there have been very few applications to the analysis of amino acids.

Butler and Darbre[2] studied N-trifluoroacetyl (TFA) amino acid methyl esters in the range 0.19–1.9 μg using a packed column and reported the relative molar responses (RMR) for fourteen amino acids. The response of the detector was dependent on the rate of hydrogen, air and make-up gas flow and the position of the detector was critical.

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Adams *et al.*[3] studied N-acetyl *n*-propyl amino acid esters in the range 50 pmol–5 nmol using a 50 m × 0.27 mm capillary column. The nitrogen-selective detector was 56 to 180-fold more sensitive than a flame ionization detector and the detection limit of the system was *ca.* 5 pmol. No data on the RMR values were reported.

Chauhan *et al.*[4] studied the N-heptafluorobutyryl (HFB) isobutyl esters[5,6] of amino acids using a capillary column in conjunction with a Pye Series 104 Model 24 nitrogen-selective detector. Detector linearity was established over the range 4 pmol–1 nmol and RMR values were reported for 23 amino acids. The coefficient of variation (C.V.) was < 2 except for arginine (6.0) and histidine (6.1). It was concluded that, to obtain maximum sensitivity, the detector should be used with high flow-rates of air and nitrogen and that probe distance and hydrogen flow-rate should be adjusted to give optimum detector response.

Gamerith[7] studied the N-TFA amino acid *n*-propyl esters using a packed column and a Hewlett-Packard nitrogen–phosphorus selective detector. RMR values were reported for about 40 amino acids with the C.V. ranging from 1.1 (leucine) to 11.7 (histidine) when 750 pmol of each amino acid were injected. Generally, the variability was significantly greater than that observed by Chauhan *et al.*[4]. Furthermore, it appears that the use of an ethylene glycol adipate (EGA) stationary phase prevented reproducible analysis of the basic amino acids.

The earlier studies of the use of a nitrogen-selective detector for the analysis of amino acid derivatives were conducted using different derivatives, columns and detectors and covered different ranges of amino acid amounts. Also, the purpose of the study was not necessarily solely to explore the range and effectiveness of the detector. In addition, the detectors produced by different manufacturers are not identical in design and operating characteristics and are not necessarily identical in sensitivity and capability.

For these reasons, we studied the application of the Hewlett-Packard nitrogen–phosphorus selective detector to the analysis of N-HFB isobutyl amino acid esters using a capillary column and with an emphasis on the low picomole range.

EXPERIMENTAL

Derivative preparation

The N(O,S)-HFB amino acid isobutyl esters were prepared as previously described[5,6] except that the final volume of solvent was adjusted to accommodate a capillary column and detector sensitivity.

Chromatography

The chromatography was performed using a Hewlett-Packard Model 5710A gas chromatograph equipped with a nitrogen–phosphorus selective detector[8,9], a capillary column injection system, and a fused-silica column (12.5 × 0.2 mm I.D.) coated with OV-101. The injection system was operated in the splitless mode. The carrier gas (helium) flow was typically 20 cm/s. The detector was provided with air (50–60 ml/min) and helium containing 8.5% (v/v) hydrogen was used as the make-up gas at a flow-rate of 25–30 ml/min. The injector and detector were maintained at 250°C. Unless otherwise indicated in the text, the oven temperature was programmed from 100 to 260°C at 8°C/min.

Samples were injected using a 1-μl syringe, the appropriate sample volume being drawn completely into the barrel of the syringe before injection. Syringes were covered when not in use and the outside of the needle was cleaned just before use. Otherwise, a significant number of extraneous peaks was observed. Interfering peaks have been attributed to the condensation of nitrogenous compounds in the laboratory air on the syringe needle[8].

The detector was operated at a 10% offset.

The data were acquired using a Hewlett-Packard 3354 Laboratory Data System and each sample was analysed at least in triplicate. All calculations were based on the internal standard method.

RESULTS AND DISCUSSION

As a preliminary to this work, we attempted to operate the nitrogen–phosphorus detector in conjunction with a packed column. However, the resolution of the proteic amino acids as the N-HFB isobutyl ester derivatives requires the use of a 3-m column. Thus, despite a selectivity of $>35\,000$ with respect to carbon, baseline rise precluded combination of the nitrogen–phosphorus detector with a packed column for temperature-programmed chromatography.

Detector gas flow-rates were maintained as recommended by Hewlett-Packard. However, the height of the detector probe above the flame jet was varied to determine the optimum position. The recommended procedure for adjusting detector height relies on a series of equidistant ridges built in to the top of the collector. This scale was found to be unreliable for precise, reproducible location of the collector. Therefore, a tool based on a micrometer was constructed (Fig. 1). The effect of collector height on detector response is illustrated in Fig. 2, in which the collector height is represented as a nominal value. The alkali salt bead is located inside the collector assembly in such a way that measurement of its distance from the jet is difficult. Thus, the distance represented in Fig. 2 is the distance that the collector assembly was moved from a position of contact with the jet assembly. Detector response was significantly reduced if the collector was too near the jet, but did not increase at distances greater than 0.075 in. This conclusion agrees with the results of Chauhan *et al.*[4]. Since there can be no assurance that the alkali halide bead is located a precise and reproducible distance from the bottom of each collector assembly, it is recommended that each collector be adjusted for optimal response.

We have consistently obtained a detector lifetime of several hundred analyses.

The column was operated in the splitless mode to optimize the estimate of the amount of sample actually entering the column, although there seems to be general agreement that better precision is obtainable in the split mode. The injection is subject to error biased in the direction of material loss. Therefore, estimates of detector sensitivity and detection limits are probably better than those indicated by the data that follows.

A typical chromatogram of a standard proteic amino acid mixture is illustrated in Fig. 3. The profile is different from that obtained using a flame ionization detector[5,6], particularly with respect to the response of the basic amino acids.

The relative molar responses (RMR) of a standard proteic amino acid mixture, injected in amounts covering the range 3–60 pmol, are shown in Table I. Most of the

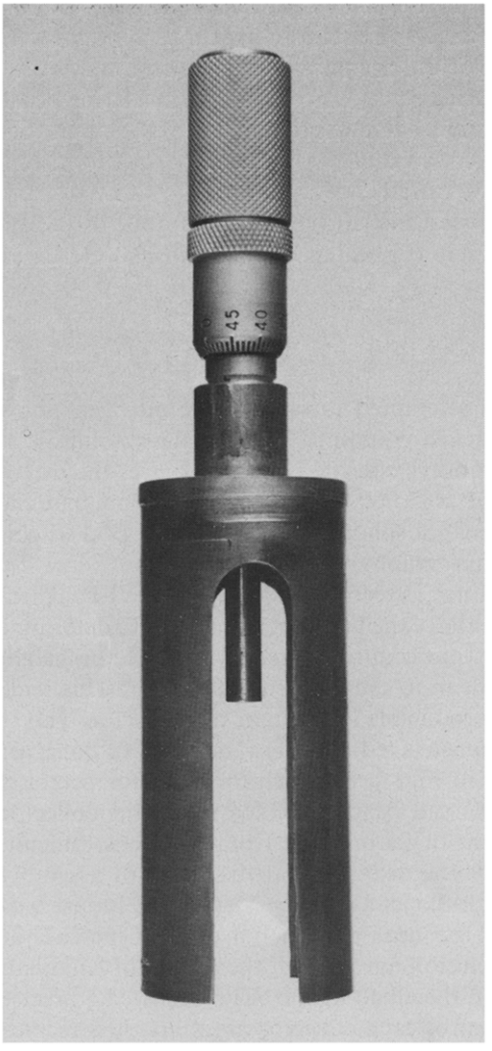

Fig. 1. Photograph of tool used to measure height of collector assembly above jet assembly.

values were constant over the range 15–60 pmol, except for threonine which decreased at the lower end of the range, and histidine and cystine which gave atypically low values when 60 pmol were injected. The data at the 3-pmol level showed considerably more variation. A decrease in RMR was observed for most of the amino acids, with the decrease being greatest for lysine, tyrosine, arginine and cystine. The RMR values for leucine, isoleucine, phenylalanine and proline were constant over the entire range. Only a small decrease was observed in the RMR values of glycine, valine, threonine, serine, methionine, aspartic acid, glutamic acid and histidine.

The RMR was close to unity for most of the monobasic amino acids, except for alanine, glycine, proline, phenylalanine and glutamic acid. The response for lysine

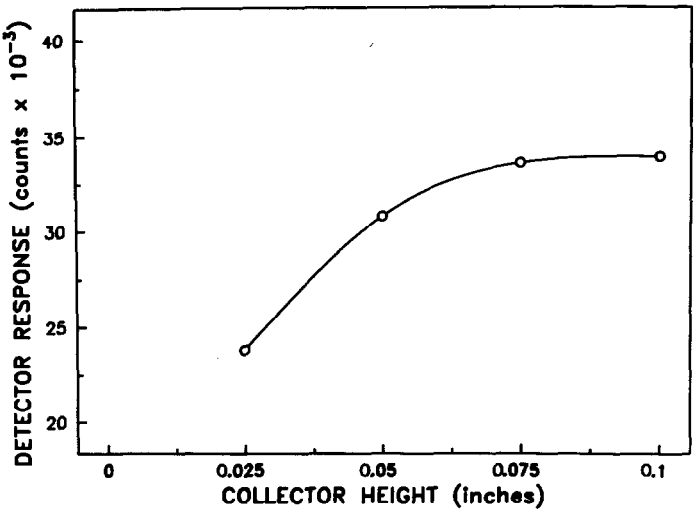

Fig. 2. Effect of detector assembly height above jet assembly on detector response.

reflected the presence of two nitrogen atoms per mole but that for arginine was considerably lower than expected. The possibility that arginine had not been derivatized in the usual amounts was discounted by checking the samples using flame ionization detection (FID). It is also possible that there were losses of arginine in the chromatographic system, which would not be detected in the amounts normally

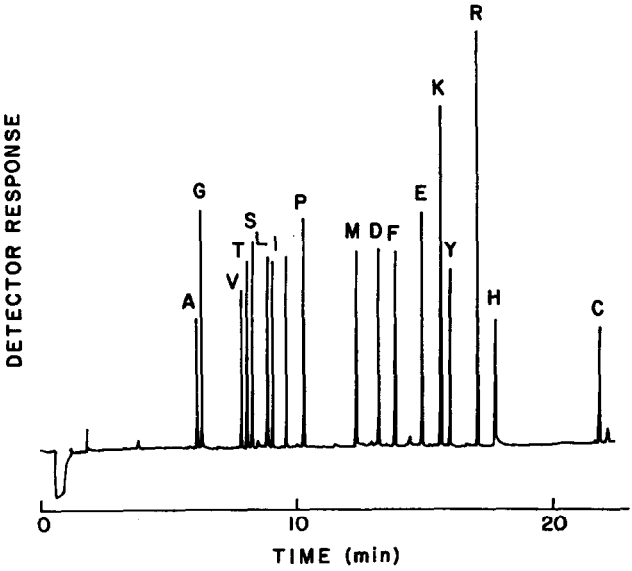

Fig. 3. Chromatogram illustrating resolution and response of amino acid N-heptafluorobutyryl isobutyl esters to a nitrogen-selective detector. The injector was operated in the splitless mode and the oven temperature was programmed from 90 to 240°C at 8°C/min.

TABLE I

RELATIVE MOLAR RESPONSES OF N(O,S)-HFB ISOBUTYL AMINO ACID ESTERS USING A NITROGEN-SELECTIVE DETECTOR

The molar responses are expressed relative to norleucine. Values are means of three assays.

Amino acids	*Amount (pmol)*					*Ref. 4*
	60	*45*	*30*	*15*	*3*	
Ala	0.82	0.80	0.79	0.80	0.71	0.82
Gly	1.26	1.23	1.24	1.26	1.22	1.03
Val	0.96	0.94	0.94	0.95	0.91	0.45
Thr	0.97	0.96	0.96	0.94	0.92	0.77
Ser	1.02	1.02	1.02	0.99	0.93	0.85
Leu	1.02	1.02	1.02	1.02	1.01	1.04
Ile	1.01	0.99	0.98	1.02	0.99	0.88
Pro	1.24	1.25	1.25	1.23	1.22	1.05
Met	1.08	1.09	1.13	1.07	1.05	1.14
Asp	1.09	1.10	1.10	1.09	1.05	0.96
Phe	1.13	1.17	1.16	1.15	1.14	1.15
Glu	1.36	1.40	1.37	1.39	1.32	0.98
Lys	2.22	2.31	2.29	2.21	2.05	1.99
Tyr	1.13	1.13	1.13	1.11	0.89	0.98
Arg	2.48	2.51	2.50	2.53	2.01	2.32
His*	0.85	1.15	1.12	1.17	1.14	2.31
Cys	1.63	1.86	1.80	1.70	1.44	1.92
Cys						

* No coinjection with acetic anhydride.

injected for FID. However, a greater variation would be expected in the RMR for arginine over the range studied if a constant proportion were degraded on the column or injector. If this factor contributes to a loss of arginine, it is evident only in the sample containing 3 pmol. Since the decrease in the RMR for arginine at this level is only *ca.* 20% relative to the average values at the other injection levels, it would appear that *ca.* 0.6 pmol of arginine is lost on the column. Therefore, system losses do not appear to contribute significantly to the low RMR for arginine at greater injection levels.

The RMR of histidine also does not give the results expected on the basis of nitrogen content. When using a packed column, losses of the unstable HFB derivative of the imidazole ring are observed, unless the more stable acetyl derivative is formed by coinjection with acetic anhydride[6]. In this instance, coinjection was deliberately not performed to assess the possibility of simplifying the injection procedure. The results indicate that the capillary column is sufficiently inert to permit reproducible assaying of histidine without an on-column acylation.

The RMR values were in good general agreement with the only other study of the N-HFB isobutyl amino acid esters using a N-specific detector[4], but differences were observed for valine, threonine, serine, isoleucine, glutamic acid and histidine. It is particularly interesting to note that Chauhan *et al.*[4] obtained a comparable RMR for arginine, suggesting that the values represent the specific response of the

detector to the molecular architecture of the nitrogen in arginine. Furthermore, these authors did not obtain a value of 3 for the RMR of histidine, although the sample was acetylated before analysis.

The precise mechanism of the detector response is not known[8,9]. However, the data in Table I and that reported by others[4] would suggest that the response is primarily to primary amino nitrogen and that secondary nitrogen elicits a lesser molar response.

The RMR values of some non-proteic amino acids are shown in Table II. No simple relationship appears to exist between the proportion of nitrogen in the molecule and the RMR. The RMR values of members of a homologous series show similarities (proline and pipecolic acid) and differences (amino-heptanoic and amino-octanoic acids) which cannot be interpreted readily. The RMR of homoserine is significantly lower than that of serine but that difference can be attributed to the lack of stability of the HFB isobutyl ester of homoserine[10].

The data obtained for structural isomers is also conflicting. *Allo*-threonine, *allo*-leucine and norvaline have RMRs similar to, respectively, threonine, leucine and valine, but the RMR of cycloleucine is significantly less than was obtained for leucine.

The results for sulphur-containing amino acids suggest that the simpler the derivative the more likely the RMR is to accurately reflect a response only to nitrogen. S-Methyl and S-ethyl cysteine both have RMR values close to unity and the

TABLE II

COEFFICIENTS OF VARIATION OF RELATIVE MOLAR RESPONSES OF N(O,S)-HFB ISOBUTYL AMINO ACID ESTERS USING A NITROGEN-SELECTIVE DETECTOR

Values are means of three assays.

Amino acid	*Amount (pmol)*					*Ref. 4*
	60	*45*	*30*	*15*	*3*	
Ala	0.72	1.08	1.04	0.80	3.06	1.2
Gly	0.47	0.77	0.86	0.06	0.76	2.9
Val	0.82	0.36	1.10	0.97	1.20	2.2
Thr	0.61	0.26	0.49	0.93	0.93	1.3
Ser	0.81	0.41	0.80	0.86	1.55	1.2
Leu	0.32	0.37	0.77	0.28	1.40	0.9
Ile	0.52	0.15	0.42	0.28	0.81	2.3
Pro	0.72	0.72	0.49	0.93	0.93	1.9
Met	0.66	0.32	0.82	0.60	2.19	2.6
Asp	0.81	1.00	1.96	1.04	3.35	1.0
Phe	0.98	1.15	0.39	0.56	2.27	0.9
Glu	1.71	1.37	1.23	1.43	2.07	2.0
Lys	3.36	1.91	0.57	1.02	3.15	2.0
Tyr	1.49	1.63	0.82	0.95	4.08	2.0
Arg	3.02	3.63	0.56	2.28	5.37	6.0
His*	0.84	5.33	7.53	14.28	17.20	6.0
Cys | Cys	3.02	5.20	3.43	4.66	6.83	2.6

* No coinjection with acetic anhydride.

structure of each molecule precludes further replacement of hydrogen on the sulphur atom. S-Carboxymethyl cysteine allows the addition of more mass to the derivative via esterification and its lower RMR would suggest that the response is in some way related to the additional mass. Similarly, penicillamine would be S-acylated and have considerably more mass per N atom than valine and the added complication of S-acylation which includes the incorporation of seven electron-withdrawing fluorine atoms. Likewise, the RMR of sarcosine is significantly greater than that of valine. That the RMR values of the "simpler" molecules is greater than unity may be simply a reflection of the fact that the internal standard, norleucine, is a somewhat complicated molecule.

Clearly, the relationship between detector response and molecular structure is not a simple one and requires more detailed study.

The coefficients of variation (C.V.s) of the RMR values of the proteic amino acids are shown in Table III. Except for histidine and cystine, the values are $< 5\%$ for all the amino acids over the range 15–60 pmol and, even for the 3-pmol samples, the C.V.s are significantly greater than 4% only for arginine, histidine and cystine. The values are similar to those obtained by Chauhan *et al.*[4] over a range of 4 pmol–1 nmol.

Detector response, exemplified for glutamic acid and norleucine in Fig. 4, was linear over the range examined. Thus, in those cases where the RMR changes over the entire range, an inherent lack of linearity in detector response is probably not responsible.

In a study focusing on the lower limits of operation of this particular nitrogen-specific detector, it was important to establish the detection limits. Analysis of a sample containing 3 pmol (Fig. 5) of each amino acid yielded average peak-area

TABLE III

RELATIVE MOLAR RESPONSES OF N-HFB ISOBUTYL ESTERS OF NON-PROTEIC AMINO ACIDS USING A NITROGEN-SELECTIVE DETECTOR

The molar responses are expressed relative to norleucine.

Amino acid	*RMR*	*Amino acid*	*RMR*
Mono-amino acids			
2-Aminoadipic acid	0.931	*Allo*-leucine	0.903
2-Aminoheptanoic acid	1.002	Cycloleucine	0.512
2-Aminooctanoic acid	0.761	Homoserine	0.653
2-Aminopimelic acid	0.790	Norvaline	0.990
Allo-threonine	0.932	Pipecolic acid	1.158
Mono-amino sulphur-containing acids			
Cystathionine	1.627	Sarcosine	1.317
Methionine sulphone	0.707	S-Carboxymethyl cysteine	0.929
Methionine sulphoxide	0.502	S-Ethylcysteine	0.974
Penicillamine	0.428	S-Methylcysteine	1.103
Diamino acids			
2,6-Diaminopimelic acid	1.077	5-Hydroxylysine	0.761
Homoarginine	0.722	Ornithine	0.946

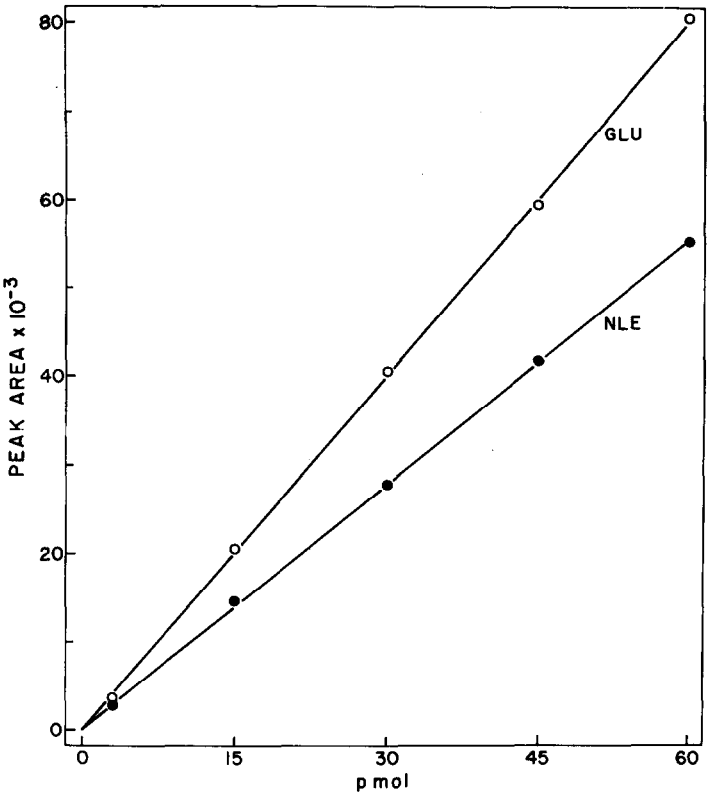

Fig. 4. Graph illustrating the linearity of the nitrogen–phosphorus detector response to the N-heptafluorobutyryl isobutyl esters of glutamic acid and norleucine over the range 3–60 pmol.

counts of 950 for the mononitrogenous amino acids. Analysis of a control under the same experimental conditions gave a mean peak size of 80 counts. Arbitrarily setting the criterion for the detection limit at 3 × background, corresponds to a detection limit of *ca.* 250 fmol of a monobasic amino acid. Thus, the detector appears to be

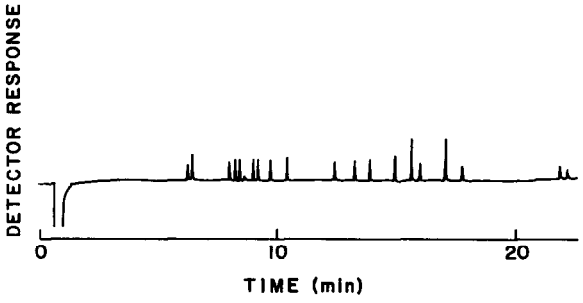

Fig. 5. Chromatogram illustrating detection of 3 pmol of each amino acid in a standard calibration mixture as the N-heptafluorobutyryl isobutyl esters. The column (12.5 m × 0.2 mm I.D.) was coated with OV-101 and operated in the splitless mode. The oven temperature was programmed from 90 to 240°C at 8°C/min.

considerably more sensitive for the detection of amino acids than any other which has so far been reported. Increasing the detector offset introduced a constant factor and thus did not increase the effective detection limit, but did produce chromatograms in which the peaks were proportionally larger and thus more visible. The detection limits for polybasic amino acids are reduced in proportion to their RMR values, thus corresponding to *ca.* 125 and 100 fmol, respectively, for lysine and arginine. There is considerable potential for further improvement of the detection limits by derivatizing the amino acids with a nitrogen-containing moiety.

In an earlier report[5], we demonstrated the feasibility of assaying a proteic amino acid mixture in a packed column in less than 15 min using a temperature program rate of 16°C/min. The primary advantage of a capillary column compared with a packed column is the potential for improved resolution. However, if the resolution is adequate on a packed column, the use of a short capillary column, in conjunction with an elevated oven temperature program rate, offers the possibility of a reduced analysis time. By programming the oven temperature at 32°C/min, a standard amino acid mixture can be assayed in less than 7 min, as illustrated in Fig. 6. If the starting temperature were higher, the analysis could no doubt be completed in less than 5 min.

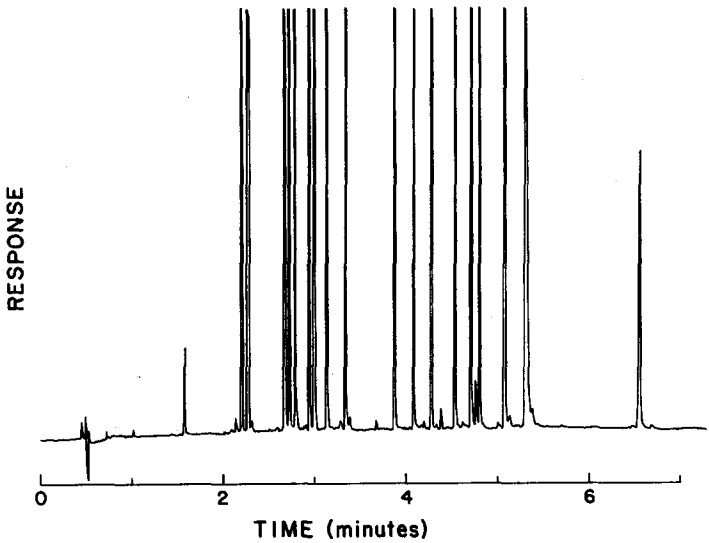

Fig. 6. Chromatogram illustrating the rapid analysis of a standard amino acid mixture as the N-heptafluorobutyryl isobutyl esters on a 10 m × 0.2 mm I.D. OV-101-coated capillary column. The column was operated in the splitless mode and the oven temperature was programmed from 90 to 240°C at 32°C/min.

A further advantage of using a specific detector is that the need for sample clean-up may be reduced when only nitrogenous compounds are of interest. The low response to carbon allows direct analysis of complex samples while avoiding clean-up procedures such as ion-exchange during which the recovery of specific individual compounds may be incomplete. In addition, the identification of nitrogenous compounds is useful for interpreting mass spectra of unknown compounds by allowing

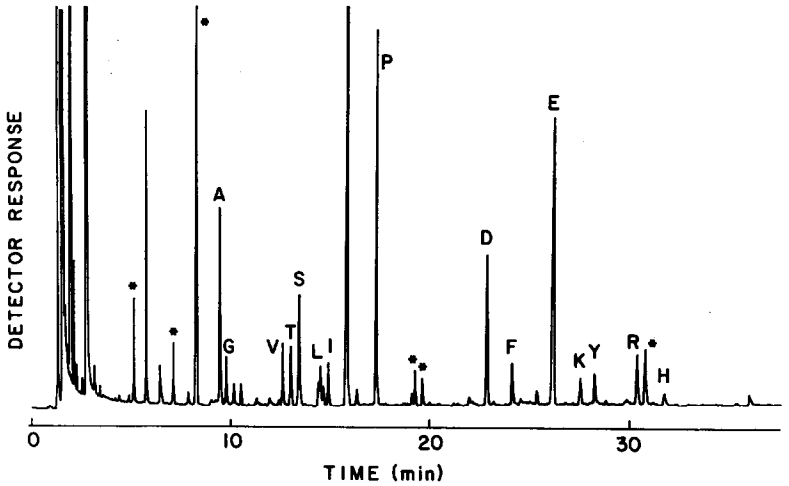

Fig. 7. Chromatogram illustrating analysis of an extract of White Spruce leaves using a 10 m × 0.2 mm I.D. OV 101-coated fused-silica capillary column and a flame ionization detector. The injector was operated in the splitless mode and the oven temperature was programmed from 90 to 260°C at 4°C/min. The peaks marked by an asterisk did not contain nitrogen.

a clear distinction to be made between N-acylation and O-acylation in combination with unsaturation. Analysis of a physiological sample without prior clean-up is illustrated for White Spruce free amino acids[11] (Fig. 7). The chromatogram shown was obtained using FID but another analysis of the same sample using an nitrogen–phosphorus detector allowed the identification of those peaks which did not contain nitrogen. These are indicated by an asterisk.

An analysis of pea xylem sap using the nitrogen–phosphorus detector is shown in Fig. 8. The major component was aspartic acid (includes asparagine) but the sample also contained significant amounts of glutamic acid, arginine and homoserine.

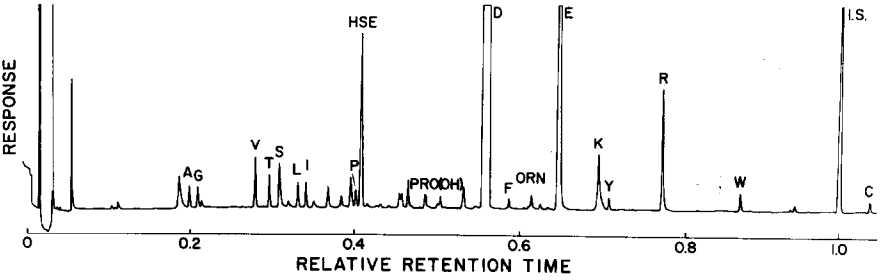

Fig. 8. Chromatogram illustrating detection of nitrogenous compounds in pea xylem sap using a 15 m × 0.2 mm I.D. bonded SE-30 fused-silica capillary column. The injector was operated in the splitless mode and the oven temperature was programmed from 90 to 260°C at 4°C/min.

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