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Note

Gas–liquid chromatographic assay for asparagine and glutamine*

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The amides of glutamic and aspartic acids play an important role in cell metabolism and nitrogen transport in multicellular organisms. In addition, they are common constituents of proteins. However, the amide group is acid-labile and thus is completely destroyed under the strongly acidic conditions required to hydrolyse proteins. The amide group is likewise hydrolysed in any procedure involving acid-catalysed esterification, although not necessarily completely. Thus, assay methods for amino acids based on their conversion to volatile derivatives for analysis by gas–liquid chromatography (GLC)[1,2] cannot be used directly to assay glutamine and asparagine.

A number of modifications to derivatisation procedures for GLC analysis of amino acids have been described. These are based on the principle of minimising the destruction of glutamine and asparagine under precisely controlled conditions so that conversion of the amides to the corresponding acids is reproducable. Appropriate conversion factors can then be calculated. For example, Hediger *et al.*[3] modified the direct esterification procedure of Roach and Gehrke[4] by reducing the time and acid concentration required for complete formation of the amino acid *n*-butyl esters. Samples were esterified for precisely 7 min at which time the ratio of the amide to the acid forms was a maximum at 0.8 for aspartate and 1.8 for glutamate. This method was applied to enzymic hydrolysates of proteins. An analysis of an acid hydrolysate was required to obtain the total amounts of glutamic and aspartic acids.

Collins and Summer[5] also used the procedure of Roach and Gehrke[4], and observed that the proportion of glutamic acid converted to pyroglutamic acid depended on time, temperature and acid concentration. However, if these factors were constant, the ratio was independent of the glutamine concentration. By using the same strategy as Hediger *et al.*[3], they were able to determine the amount of glutamine and asparagine with an error of 5% or less.

Young and Desiderio[6] converted glutamic and aspartic acids and their amides to the corresponding thiazolinone derivatives and concluded that hydrolysis of the amide side chains was avoided. However, inadequate information was provided to enable duplication of the procedure.

All of the above procedures use indirect means to assay glutamic and aspartic

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acids and their amides. When all four components are present in the sample, as in a free amino acid mixture from a physiological source, the assay becomes rather complicated.

Wolfensberger *et al.*[7] developed a direct assay for glutamine or glutamic acid by reacting the sample with pentafluoropropionic anhydride–hexafluoroisopropanol. Derivatization of glutamic acid produced two derivatives of pyroglutamic acid in addition to the glutamic acid derivative. The constancy of the relative proportions of these artefacts was not established so that considerable error could occur in the assaying of glutamic acid.

At present, there is no simple, direct GLC method available for assaying glutamine and asparagine either simultaneously or in the presence of the parent acids. This report addresses this problem and describes the development of a GLC assay for glutamine and asparagine which does not rely on conversion ratios.

EXPERIMENTAL

Reagents

N-Methyl-N-(*tert.*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, U.S.A.). All other reagents and solvents were of analytical grade.

Sample preparation and derivatisation

Physiological samples were "cleaned-up" by absorption on Dowex-50 ion-exchange resin in 0.01 *N* hydrochloric acid followed by elution of the amino acids using 2 *N* ammonia.

A TECAM, Model DB-3H Driblock heater was used to heat samples during derivative preparation.

The mixture of amino acids (5 μl of a solution containing 25 μmol/ml) was dispensed into a 1-ml Reactivial (Pierce) and excess solvent was evaporated at 50°C using a stream of dry nitrogen (100–200 ml/min). A molar equivalent of octadecane was added and the solvent evaporated. After 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. Alternatively, the reagents were evaporated and the residue dissolved in hexane.

Chromatography

All chromatography was performed using a Varian Vista 6000 gas chromatograph 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 splitless mode. The carrier gas flow was 60 cm/sec.

Mass spectrometry

Mass spectra of the derivatives of glutamine and asparagine were obtained using a Finnigan Model 3000 mass spectrometer operated in the chemical ionization (CI) mode using methane as the reagent gas.

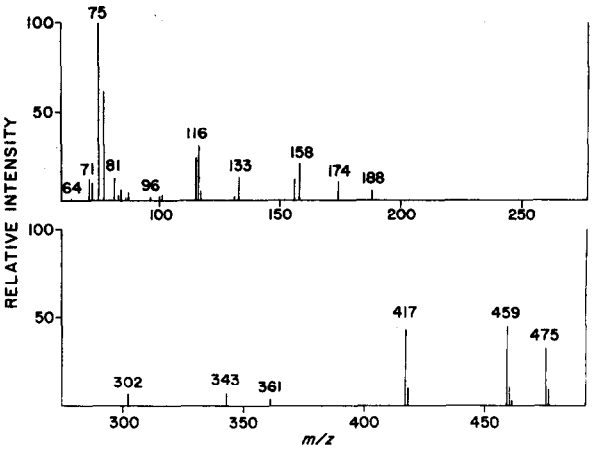

Fig. 1. Methane chemical ionization mass spectrum of N(O)-*tert.*-butyl dimethylsilyl asparagine.

RESULTS AND DISCUSSION

Mass spectra

The identity of the derivatives was established by CI mass spectrometry. The CI mass spectrum of the asparagine derivative is illustrated in Fig. 1. The molecular mass of the derivative was 474 as indicated by the protonated molecular ion at *m/z* 475. This mass corresponds to the addition of three *tert.*-butyl dimethylsilyl moieties to asparagine indicating that no degradation of the asparagine occurred. The adduct ions [M + C_2H_5] and [M + C_3H_5] were not observed but the identity of the molecular ion could be inferred with confidence from the [M − 15] ion at *m/z* 459, an ion characteristic of silylated compounds. The presence of the *tert.*-butyl moiety was indicated by the ion at *m/z* 417, [M − 57]. The spectrum of the asparagine derivative corresponds to the formation of a tri(*tert.*-butyl dimethylsilyl) derivative.

The CI mass spectrum of the glutamine derivative is illustrated in Fig. 2. The

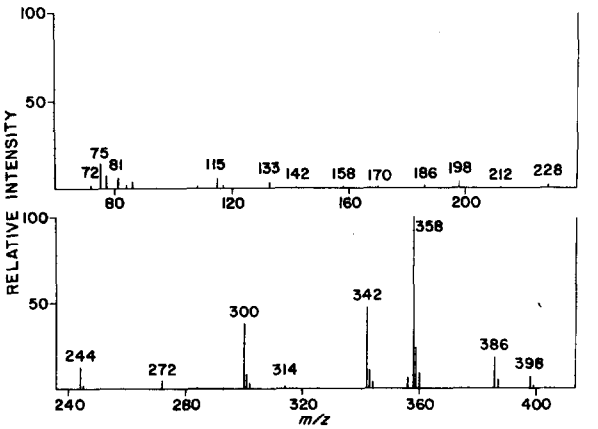

Fig. 2. Methane chemical ionization mass spectrum of N(O)-*tert.*-butyl dimethylsilyl glutamine.

TABLE I

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

Abbreviations: DMBSi denotes the dimethyl-*tert.*-butylsilyl moiety.

m/z	*m/z*	
	Asparagine	*Glutamine*
[M + C_3H_5]	–	398
[M + C_2H_5]	–	386
[M + H]	475	358
[M]	474	357
[M – CH_3]	459	342
[M – C_4H_9]	417	300
[M – (C_4H_9 + C_4H_8)]	361	244
[M – ODMBSi]	343	–
[M – (ODMBSi – 2H)	–	228
[M – (C_4H_9 + DMBSi)]	302	–
[M – (C_4H_9 + DMBSi – 2H)]	–	186
[M – (DMBSi + DMBSi + C_4H_9)]	188	–
[M – (ODMBSi + DMBSi + C_4H_8)]	174	–
[M – (COODMTBSi + CONHDMBSi – H)]	158	–
[COODMBSiH_2]	133	133

molecular mass of the derivative was 357 as indicated by the protonated molecular ion at *m/z* 358 and the adduct ions at [M + 29] and [M + 41] (*m/z* 386 and 398 respectively). This mass and the other ions in the spectrum are consistent with the conversion of glutamine to pyroglutamic acid.

The spectra of the asparagine and glutamine derivatives are described in more detail in Table I.

Derivatisation

The effect of reaction time and temperature was studied to determine the op-

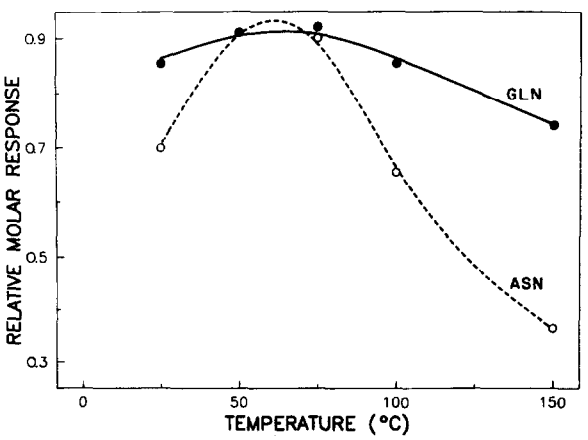

Fig. 3. Effect of reaction time and temperature on the formation of N(O)-*tert.*-butyl dimethylsilyl asparagine and glutamine.

TABLE II

EFFECT OF REACTION TIME AND TEMPERATURE ON RELATIVE MOLAR RESPONSE OF GLUTAMINE AND ASPARAGINE *tert.*-BUTYL DIMETHYLSILYL DERIVATIVES*

Abbreviations: RMR = relative molar response; S.D. = standard deviation, n = 3; C.V. = coefficient of variation.

Time (min)	*Temperature (°C)*	*Glutamine*			*Asparagine*		
		RMR	*S.D.*	*C.V.*	*RMR*	*S.D.*	*C.V.*
30	25	0.855	0.008	0.95	0.703	0.013	1.84
45	25	0.947	0.007	0.76	0.821	0.009	1.09
10	50	0.760	0.003	0.38	0.671	0.013	1.04
20	50	0.904	0.006	0.62	0.771	0.004	0.57
30	50	0.911	0.007	0.81	0.906	0.005	0.66
10	75	0.873	0.006	0.72	0.760	0.015	1.99
20	75	0.871	0.003	0.29	0.783	0.015	1.93
30	75	0.925	0.006	0.70	0.899	0.011	1.22

* Values are relative to octadecane = 1.

timum values of these experimental variables for quantitative derivatisation. Because of the lability of the amide bond, it was particularly desirable to minimise the reaction temperature.

The effect of temperature for a reaction time of 30 min is illustrated in Fig. 3. The relative molar responses decrease significantly at temperatures greater than 75°C indicating that degradation of the amides occurred, the effect being more pronounced for asparagine. However, the reduced conversion did not result in formation of the corresponding acids.

More detailed results are shown in Table II in which the molar responses relative to octadecane are a direct measure of the degree of conversion of the amides to the silylated derivatives. There was no significant difference in the optimum values

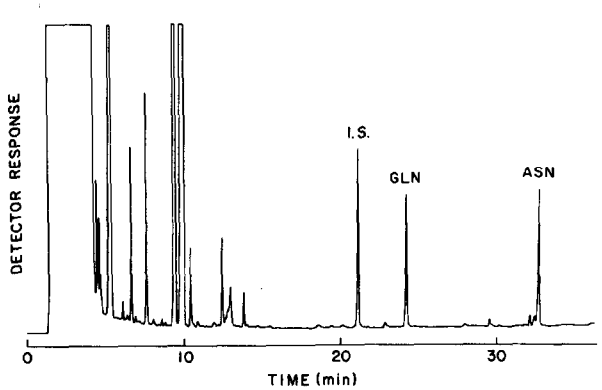

Fig. 4. Chromatogram illustrating formation of N(O)-*tert.*-butyl dimethylsilyl asparagine and glutamine. The oven temperature was programmed from 100°C to 250°C at 4°C/min. All peaks eluting before 15 min are derived from the reagent or solvents.

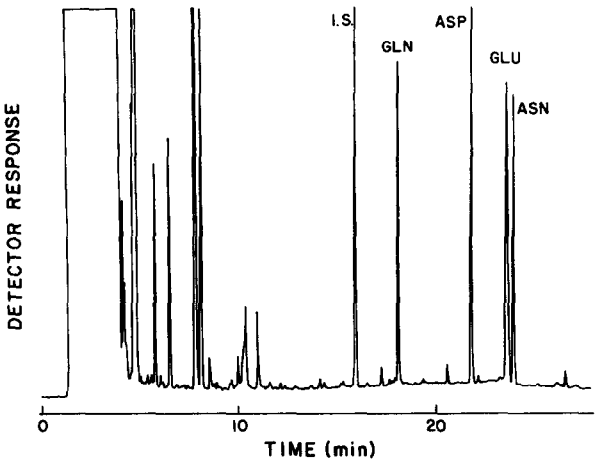

Fig. 5. Chromatogram illustrating the resolution of the N(O)-*tert.*-butyl dimethylsilyl derivatives of asparagine, glutamine, aspartic acid and glutamic acid. The oven temperature was programmed from 100°C to 260°C at 6°C/min.

obtained at 50°C and 75°C. Therefore, derivatisation for 30 min at either of these temperatures would be appropriate for routine use. These conditions represent the best compromise, between the conflicting demands of a short reaction time and a minimum reaction temperature, for the purpose of derivatising and assaying both asparagine and glutamine simultaneously.

Initial attempts to form the *tert.*-butyl dimethylsilyl derivative of asparagine were largely unsuccessful since only a small peak was obtained. However, increasing the basicity of the reaction medium by the addition of a small amount of triethylamine promoted the reaction. Ordinarily, pyridine is not recommended as a solvent for the silylation of primary amino groups. However, derivative formation was limited unless pyridine was added, presumably because of the limited solubility of asparagine and glutamine in most organic solvents.

The derivatisation reaction produces only traces of compounds other than the derivatives described above (Fig. 4). It is particularly important that no significant

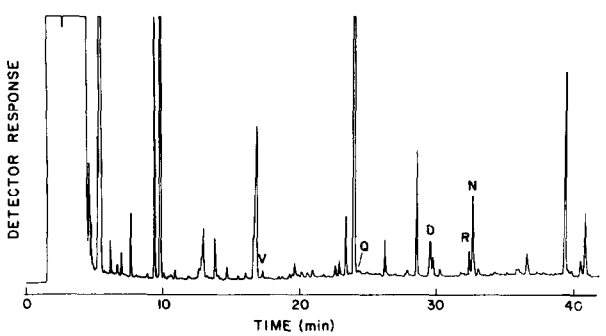

Fig. 6. Chromatogram illustrating identification of asparagine, glutamine and aspartic acid in soybean xylem. The letters represent the standard single-letter convention for the amino acids. The oven temperature was programmed from 100°C to 260°C at 4°C/min.

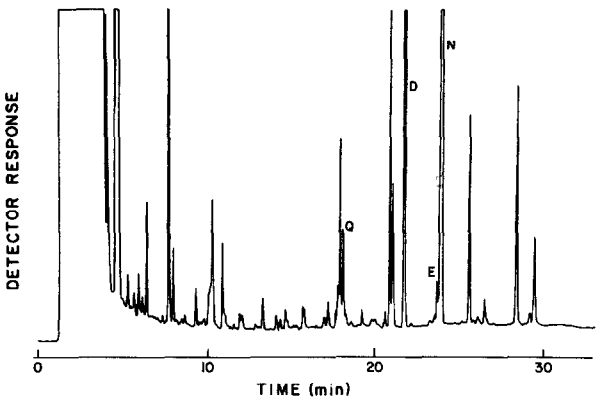

Fig. 7. Chromatogram illustrating identification of asparagine, glutamine, aspartic acid and glutamic acid in pea xylem. The letters represent the standard single-letter convention for the amino acids. The oven temperature was programmed from 100°C to 260°C at 6°C/min.

amounts of aspartic and glutamic acids are formed. Thus, no conversion factors or elaborate reaction conditions are required. In addition, the concentrations can be calculated by routine internal standardisation methods.

Chromatography

The chromatographic elution of the derivatives of a mixture containing glutamic and aspartic acids and the corresponding amides is shown in Fig. 5. No significant tailing is evident. All four derivatives are also adequately resolved from the derivatives of the other proteic amino acids (see Fig. 8).

Physiological sample analysis

The analysis of a soybeam xylem fluid is illustrated in Fig. 6. The major components were not identified but these did not correspond to any of the proteic amino acids. Asparagine and aspartic acid were well resolved from the other components of the sample. Only traces of glutamic acid and glutamine were detected. The former elutes just before arginine. The lack of glutamine may reflect the fact that allantoin

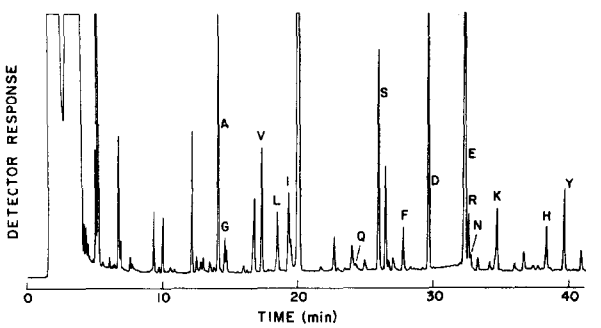

Fig. 8. Chromatogram illustrating identification of asparagine, glutamine, aspartic acid and glutamic acid in free amino acids extracted from Black Spruce leaves. The letters represent the standard single-letter convention for the amino acids. The oven temperature was programmed from 100°C to 260°C at 4°C/min.

and allantoic acid are the major nitrogen-transport vehicles of nitrogen-fixing soybeans[8].

Asparagine and aspartic acid were the major components of pea xylem (Fig. 7) but small amounts of glutamine and glutamic acid and traces of valine and leucine were also detected. This sample was analysed directly without ion-exchange purification.

Black spruce [*Picea mariana* (Mill.) BSP] needle free amino acids (Fig. 8) contained only traces of asparagine and glutamine. This result is perhaps expected since the sample was obtained during the dormant phase[9].

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