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Identification and quantitation of phenylalanine, tyrosine and dihydroxyphenylalanine in the thoracic nervous system of the locust, *Schistocerca gregaria*, by gas chromatography–negative-ion chemical ionisation mass spectrometry

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ABSTRACT

Phenylalanine, tyrosine and dihydroxyphenylalanine (DOPA) were identified unambiguously and quantitatively determined in single ventral thoracic nerve cords from the locust, *Schistocerca gregaria*, by gas chromatography–negative-ion chemical ionisation mass spectrometry. Deuterium-labelled analogues of each compound were added to a single ventral thoracic nerve cord in hydrochloric acid; the tissue was homogenised and the suspension centrifuged. The remaining hydrochloric acid was eliminated azeotropically by repeated additions of acetonitrile followed by evaporation under a stream of nitrogen and the resultant residue derivatised by reaction with hexafluoroisopropanol and pentafluoropropionic anhydride. Under negative-ion chemical ionisation conditions, the hexafluoroisopropanol–pentafluoropropionyl derivatives produced characteristic ions which were sufficiently abundant to be suitable for selected-ion monitoring. This method is highly specific and gave a limit of detection below the nanogram level. The amounts of phenylalanine, tyrosine and DOPA in a single ventral thoracic nerve cord were, respectively, 194 ± 81, 347 ± 88 and 11 ± 11 ng per tissue.

INTRODUCTION

It has been proposed that *p*-octopamine, dopamine and 5-hydroxytryptamine (5-HT) are neurotransmitters in the central nervous system of insects [1–4]. In our earlier work [5,6] we established unequivocally that the principal amines in cockroach brain and locust ventral thoracic nerve cords are *p*-tyramine, *p*-octopamine, 5-HT and noradrenaline. In addition we also identified and quantified the N-acetylated metabolites of *p*-tyramine, *p*-octopamine, dopamine and 5-HT in the thoracic nervous system of the locust [7]. These compounds (and the pathways involved in their biosynthesis and metabolism) are of considerable interest

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per se and also in relation to the systematic development of insecticides. To further study the biosynthesis of biogenic amines a suitable technique was required which was sufficiently specific and sensitive for the analysis of their proposed amino acid precursors.

Early attempts to identify free amino acids in the nervous system of the cockroach, *Periplaneta americana*, involved the use of non-specific radiolabelled dansyl derivatives [8] and the use of ion-exchange chromatography followed by colorimetric assay [9]. More recently, high-performance liquid chromatography (HPLC) has been used to report the presence of dihydroxyphenylalanine (DOPA) in larval blowflies [10] and nematode parasites [11]. Phenylalanine and tyrosine have been determined in human plasma by gas chromatography–negative-ion chemical ionisation mass spectrometry (GC–NICIMS) using pentafluorobenzyl–trifluoroacetyl derivatives [12]. However, a new method reported for the determination of L-DOPA in plasma and cerebrospinal fluid which combines N,O-acetylation of amino acids in aqueous media, preparation of pentafluorobenzyl esters under anhydrous conditions and subsequent analysis by GC–NICIMS [13] failed to work in our hands.

We report here a highly sensitive and specific GC–NICIMS technique [using hexafluoroisopropanol–pentafluoropropionyl (HFIP–PFP) derivatives] which allows for the first time the simultaneous, unambiguous measurement of phenylalanine, tyrosine and DOPA in individual nerve cords.

EXPERIMENTAL

Gas chromatography–mass spectrometry

GC–MS in the NICI mode was carried out using a Hewlett-Packard 5988A gas chromatograph–mass spectrometer interfaced with an HP RTE-6/VM data system. The following MS conditions were used: the instrument was tuned in the NICI mode to the ions at m/z 452, 595 and 633 from the perfluorobutylamine (PFTBA) calibrant; source temperature was 140°C, electron energy 200 eV, and methane reagent gas was introduced to give a source pressure of approximately 0.9 Torr. The gas chromatograph was fitted with an SGE BP-1 (12 m x 0.2 mm) capillary column, and helium carrier gas was used with a head pressure of 0.54 bar.

The GC conditions were as follows: injector temperature 250°C, transfer line temperature 280°C; the oven temperature was maintained at 100°C for 1 min, then programmed at 10°C/min to 300°C. Injections were made using a Grob splitless injection system.

Materials and reagents

All solvents used in extraction were HPLC grade (Rathburn, Walkerburn, U.K.). Chemicals were obtained from the following sources: DL-tyrosine, DL-DOPA, trifluoroethanol (TFE), pentafluoropropionic anhydride (PFPA),

[$^{13}C_2$]glycine, glycine (Aldrich, Gillingham, U.K.); HFIP (Fluorochem, Glossop, U.K.); DL-β-phenylalanine (Fisons, Loughborough, U.K.). Deuteriated internal standards were synthesised as previously described in the literature: [2,3,4,5,6-$^2H_5$]phenylalanine [14], [3,5-$^2H_2$]tyrosine and [2,5,6-$^2H_3$]DOPA [15].

The composition of deuteriated phenylalanine was determined by GC-NICIMS of the HFIP–PFP derivative using $[M-CO]^-$ (*m/z* 433–438), which indicated $[^2H_5]M^-$ (100%) and $[^2H_4]M^-$ (53%). The remaining ions (*m/z* 433–436) were absent from the spectrum. The composition of deuteriated tyrosine was determined by GC–NICIMS of the HFIP–PFP derivative using $[M-C_2F_5COH]^-$ (*m/z* 475–477), which indicated $[^2H_2]M^-$ (100%): none of the corresponding monodeuteriated or non-deuteriated compound was present. The composition of deuteriated DOPA was determined by GC–NICIMS of the HFIP–PFP derivative using $[M-HF]^-$ (*m/z* 765–768) which indicated $[^2H_3]M^-$ (100%) and $[^2H_2]M^-$ (42%): none of the corresponding monodeuteriated or non-deuteriated compound was present.

Extraction and derivatisation

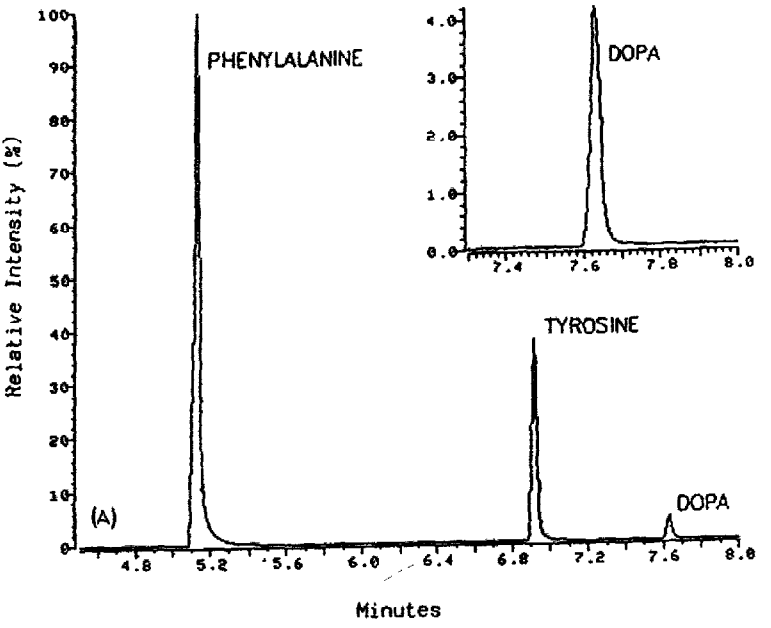
Ventral thoracic nerve cords were dissected from male and female two- to seven-day-old adult locusts, *Schistocerca gregaria*, obtained from crowded laboratory cultures fed on wheat seedlings and were immediately frozen on dry ice. They were transported overnight in dry ice from the University of Cambridge and stored at −20°C prior to analysis. Prolonged storage did not appear to have any adverse affects on the concentrations of amino acids in the locust nerve cords.

A standard solution (100 μl; equivalent to 100 ng each of [$^2H_5$]phenylalanine (Phe-d_5), [$^2H_2$]tyrosine (Tyr-d_2) and [$^2H_3$]DOPA (DOPA-d_3)) was added to hydrochloric acid (0.1 *M*, 0.5 ml) containing a single ventral thoracic nerve cord in a ground-glass homogeniser. The tissue was homogenised and the homogenate centrifuged for 30 min at 2500 *g* (4500 rpm). The supernatant was transferred to a screw-capped vial (1 ml) and the remaining hydrochloric acid was eliminated azeotropically by repeated additions of acetonitrile followed by evaporation under a stream of nitrogen.

Dried tissue extracts or standards were heated with HFIP (5 μl) and PFPA (50 μl) for 1 h at 100°C in a screw-capped vial. The excess reagents were evaporated under a stream of nitrogen and the residue was dissolved in ethyl acetate (100 μl). For the formation of trifluoroethanol–pentafluoropropionyl (TFE–PFP) derivatives, the extracts or standards were heated with TFE (5 μl) and PFPA (50 μl) at 100°C for 1 h and the excess solvent was removed under a stream of nitrogen. The residue was dissolved in ethyl acetate (100 μl) prior to analysis by GC–NICIMS.

RESULTS AND DISCUSSION

Fig. 1 shows typical GC–MS responses to the HFIP–PFP derivatives of phenylalanine (*m/z* 433), tyrosine (*m/z* 475) and DOPA (*m/z* 765) in a standard mix-



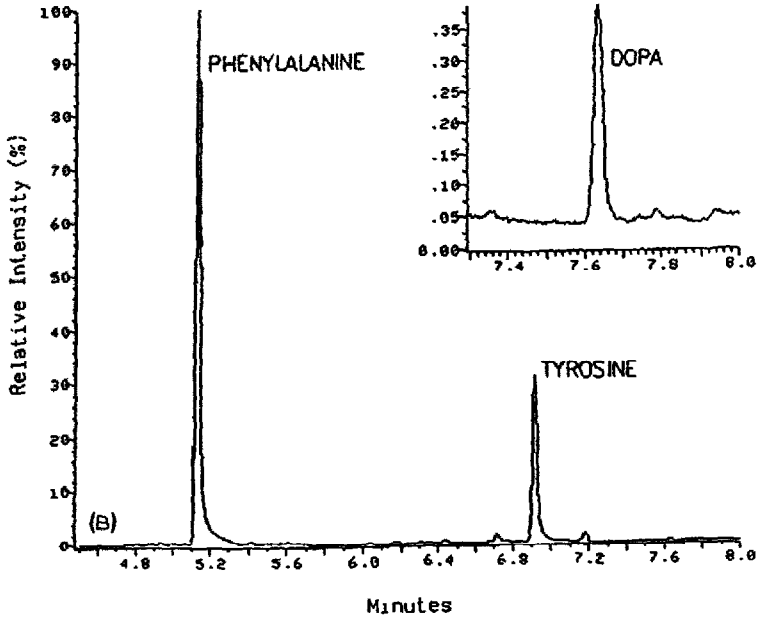


Fig 1 (A) Selected-ion monitoring (SIM) trace of a standard mixture (20 ng) of phenylalanine (*m/z* 433, retention time 5.13 min), tyrosine (*m/z* 475, retention time 6.91 min) and DOPA (*m/z* 765, retention time 7.63 min) as their HFIP–PFP derivatives. (B) SIM trace of phenylalanine (*m/z* 433, retention time 5.13 min), tyrosine (*m/z* 475, retention time 6.91 min) and DOPA (*m/z* 765, retention time 7 63 min) in an extract of a single locust ventral thoracic nerve cord, as their HFIP–PFP derivatives

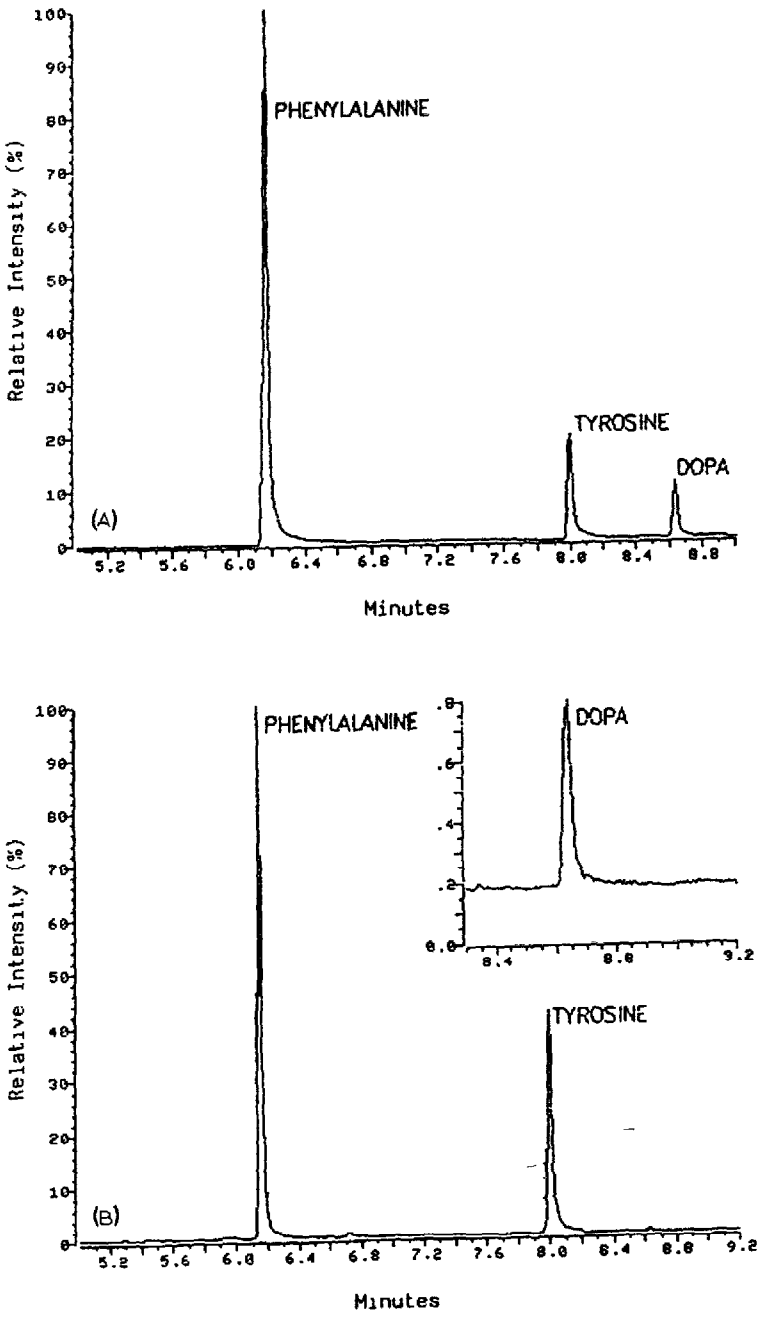


Fig 2 (A) SIM trace of a standard mixture (20 ng) of phenylalanine (*m/z* 373, retention time 6.14 min), tyrosine (*m/z* 407, retention time 7 99 min) and DOPA (*m/z* 569, retention time 8 64 min) as their TFE–PFP derivatives. (B) SIM trace of phenylalanine (*m/z* 373, retention time 6 14 min), tyrosine (*m/z* 407, retention time 7.99 min) and DOPA (*m/z* 569, retention time 8 64 min) in an extract of a single locust ventral thoracic nerve cord, as their TFE–PFP derivatives

ture (A) and those from a preparation of a single locust ventral thoracic nerve cord (B). Identification of phenylalanine, tyrosine and DOPA in locust nervous tissue was carried out by establishing that the retention times (phenylalanine, 5.13 min; tyrosine, 6.91 min; DOPA, 7.63 min) and the selected ions (phenylalanine, $[M-CO]^{\cdot-}$, *m/z* 433; tyrosine, $[M-C_2F_5COH]^-$, *m/z* 475; DOPA $[M-HF]^{\cdot-}$, *m/z* 765) of the HFIP–PFP derivatives of endogenous phenylalanine, tyrosine and DOPA were identical to those of the derivatised authentic standard substances. The same identification procedure was carried out on the corresponding TFE–PFP derivatives by establishing that the retention times (phenylalanine, 6.14 min; tyrosine, 7.99 min; DOPA, 8.64 min) and the selected ions (phenylalanine, $[M-HF]^{\cdot-}$, *m/z* 373; tyrosine, $[M-C_2F_5COH]^-$, *m/z* 407; DOPA, $[M-C_2F_5COH]^-$, *m/z* 569) of the TFE–PFP derivatives were identical to those of the derivatised authentic standard substances (Fig. 2A and B). This method of double derivatisation provides unequivocal confirmation of phenylalanine, tyrosine and DOPA in locust ventral thoracic nerve cords since the retention times and *m/z* values of the characteristic ions are changed in a predictable manner. Quantitation was carried out by determining the ratio of the selected characteristic ion (phenylalanine, *m/z* 433; tyrosine, *m/z* 475; DOPA, *m/z* 765) of the HFIP derivative of the endogenous compound in the derivatised biological extract to that of the selected ion of the added deuteriated internal standard (Phe-d_5, *m/z* 438; Tyr-d_2, *m/z* 477; DOPA-d_3, *m/z* 768). Day-to-day instrumental variation was corrected for by measuring the corresponding ratio obtained from a derivatised 1:1 mixture of the deuteriated isotopomers and non-deuteriated standard compounds. Calibration curves were linear over the range examined (correlation coefficients >0.99). Blank samples were processed prior to each analysis in order to ensure that reagents were not contaminated by standard solutions and thoracic nerve cords were also processed without the addition of deuteriated internal standards, to rule out any possibility that these might contribute significantly to the endogenous phenylalanine, tyrosine and DOPA present.

Table I lists the concentrations of phenylalanine, tyrosine and DOPA in single ventral thoracic nerve cords of the locust, *Schistocerca gregaria*.

TABLE I

CONCENTRATIONS OF PHENYLALANINE, TYROSINE AND DOPA IN A SINGLE LOCUST VENTRAL THORACIC NERVE CORD (AS THEIR HFIP–PFP DERIVATIVES)

Compound	Concentration found (mean ± S.D., $n=10$) (ng per tissue)
Phenylalanine	194 ± 81
Tyrosine	347 ± 88
DOPA	11 ± 11

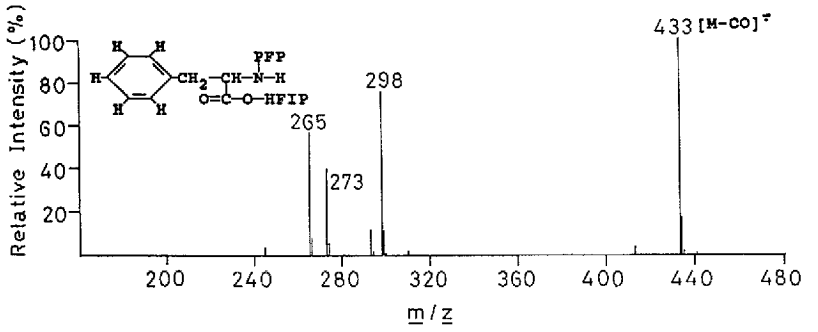


Fig. 3. NICI mass spectrum of the HFIP–PFP derivative of phenylalanine

The HFIP–PFP derivative of phenylalanine gave the NICI spectrum which is illustrated in Fig. 3. Under NICI conditions the mass spectrum of derivatised phenylalanine contained four principal fragment ions. The base peak (*m/z* 433) was due to the loss of 28 a.m.u. from the molecular ion, this presumably arose via the loss of CO from the molecular ion. The ions of *m/z* 298 and 265 probably arose via the loss of PFP-NH_2 and HFIP-O-COH, respectively, from the molecular ion. The mass spectrum of the corresponding derivative of [$^2H_5$]phenylalanine produced an identical pattern of ions but with a mass increment of 5 a.m.u

The mass spectra of derivatised glycine and [$^{13}C_2$]glycine exhibited a similar pattern of ions but with a loss of 29 a.m.u. ([$M - {}^{13}CO]^-$) from the molecular ion of [$^{13}C_2$]glycine (Fig 4). This supports the possibility that the loss of 28 a.m.u. from the HFIP–PFP derivative of phenylalanine was due to loss of CO.

The mass spectrum of derivatised tyrosine (Fig. 5) exhibited a base peak of *m/z* 475 due to the loss of 148 a.m.u. ([$M - C_2F_5COH]^-$) from the molecular ion. The ion of *m/z* 460 probably arose via loss of PFP-NH_2 from the molecular ion. The loss of 28 a.m.u. from the molecular ion was less marked (57%) than in the mass spectrum of HFIP–PFP phenylalanine. The mass spectrum of the corresponding

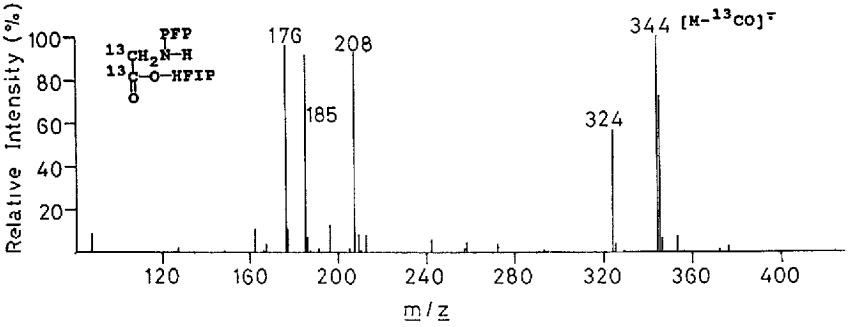


Fig. 4 NICI mass spectrum of the HFIP–PFP derivative of [$^{13}C_2$]glycine.

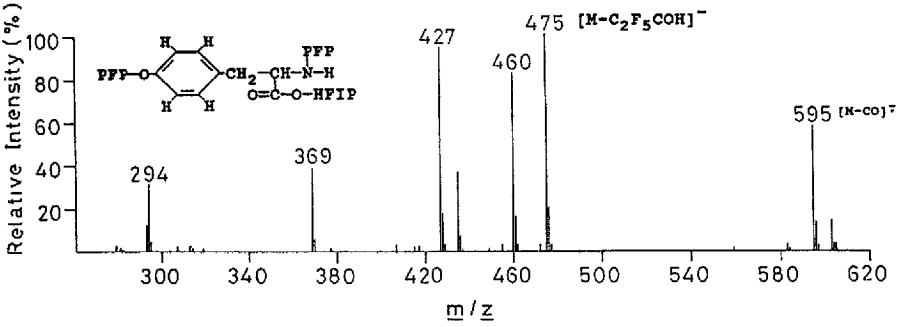


Fig. 5 NICI mass spectrum of the HFIP–PFP derivative of tyrosine.

derivative of [$^2H_2$]tyrosine exhibited a similar pattern of ions but with a mass increment of 2 a.m u.

The HFIP–PFP derivative of DOPA gave a mass spectrum (see Fig. 6) which showed a base peak of *m/z* 765 ([M − HF]$^-$) due to the loss of 20 a.m.u. from the molecular ion. Once again the loss of 28 a.m.u. from the molecular ion was less marked (4%) than that observed in the mass spectra of the HFIP–PFP derivatives of phenylalanine and tyrosine. The mass spectrum of the corresponding derivative of [$^2H_3$]DOPA exhibited an identical pattern of ions but with a mass increment of 3 a.m.u.

The TFE–PFP derivative of phenylalanine gave a mass spectrum which produced a base peak of *m/z* 373, due to loss of 20 a.m.u. ([M − HF]$^-$) from the molecular ion. The mass spectrum of the corresponding derivative of [$^2H_5$]phenylalanine exhibited an identical pattern of ions but with a mass increment of 5 a.m.u. There was a peak of low intensity (0.7%) due to the loss of CO from the molecular ion, similar to that observed in the spectrum of the corresponding HFIP–PFP derivative.

The TFE–PFP derivatives of tyrosine and [$^2H_2$]tyrosine afforded spectra

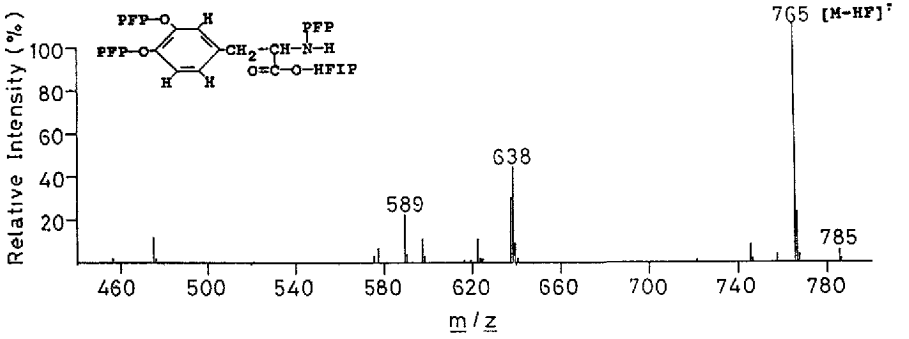


Fig 6 NICI mass spectrum of the HFIP–PFP derivative of DOPA

TABLE II

RETENTION INDICES AND NICI MASS SPECTRAL DATA FOR HFIP–PFP AND TFE–PFP DERIVATIVES OF PHENYLALANINE, TYROSINE AND DOPA

Compound	Kovats index	Base peak *(m/z)*	Other significant ion *(m/z)*
HFIP–PFP derivative			
Phenylalanine	1091	433	298 (76%)
Tyrosine	1301	475	427 (94%)
DOPA	1386	765	638 (44%)
TFE–PFP derivative			
Phenylalanine	1444	373	230 (59%)
Tyrosine	1518	407	301 (28%)
DOPA	1548	569	697 (21%)

which exhibited base peaks of *m/z* 407 and 409, respectively. These were due to loss of 148 a.m.u. ($[M - C_2F_5COH]^-$) from the molecular ion.

The TFE–PFP derivatives of DOPA and [$^2H_3$]DOPA produced mass spectra which showed base peaks of *m/z* 569 and 572, respectively. These were due to loss of 148 a.m.u. ($[M - C_2F_5COH]^-$) from the appropriate molecular ion, similar to that observed in the spectrum of the tyrosine TFE–PFP derivative. Table II lists the principal ions and the Kovats' indices of the HFIP–PFP and TFE–PFP derivatives of amino acids.

In view of their selectivity and sensitivity for the analysis of phenylalanine, tyrosine and DOPA the HFIP–PFP derivatives were chosen for the identification and quantitation of amino acids in biological samples.

In vertebrates phenylalanine and tyrosine are not only important constituents of proteins but are of considerable interest as precursors for the synthesis in the body of substances (*e.g.* catecholamines) having important regulatory actions, such as neurotransmitters and hormones [16]. It has been proposed that, in vertebrates, octopamine is synthesised from tyrosine by initial decarboxylation to tyramine and subsequent β-hydroxylation to octopamine [17]. It is presumed that, in the nervous system of insects, a similar metabolic pathway occurs [18] where tyrosine would be metabolised sequentially to tyramine and octopamine in nervous tissue of the locust [19,20] and cockroach [21,22].

Early attempts to quantify amino acids in the nervous system of the cockroach, *Periplaneta americana*, involved the use of a microdansyl procedure on pooled pieces of nervous tissue [8]. Quantitative values for phenylalanine and tyrosine were reported as 0.31 ± 0.10 and 1.17 ± 0.02 μmol/g wet weight of tissue, respectively. However, estimates of phenylalanine and tyrosine levels in single thoracic nerve cords were obtained by the use of ion-exchange chromato-

graphy and colorimetric detection [9] and gave values of 30 and 152 ng per thoracic nerve cord (assuming a wet weight of 2.7 mg for a thoracic nerve cord [23]) for phenylalanine and tyrosine, respectively. GC analysis of the heptafluorobutyrate derivatives of isoamyl esters of the amino acids was used to report phenylalanine (4.8 nmol/mg of protein) and tyrosine (11 nmol/mg of protein) in the brain of the locust, *Schistocerca gregaria* [24]. The presence of tyrosine (11 nmol/mg of protein) was also reported in the thoracic nerve cord but, in all cases, quantitation was achieved using an external standard and the results were based on pooled tissues from at least twenty insects. Jabbar and Strang [25] reported that phenylalanine (0.55 ± 0.09 μmol/g wet weight of tissue; equivalent to 227 ng per tissue assuming a wet weight of 2.5 mg for a thoracic nerve cord [23]) and tyrosine (0.97 ± 0.40 μmol/g wet weight of tissue; equivalent to 439 ng per tissue) were present in locust thoracic nerve cord using a double-isotope dansylation procedure. Estimates were made on pooled tissues of at least six insects.

A direct comparison between these and the results reported here is not possible due to the difference in quantitative units; however, the relative values compare with those in previous reports on insects in that the concentration of tyrosine is approximately double that of phenylalanine. To the best of the our knowledge, there are no previous reports concerning the concentration of DOPA in the locust nervous system.

Thus a highly sensitive, specific and convenient method for the identification and quantification of phenylalanine, tyrosine and DOPA in biological systems has been developed. Variation in derivatisation reagent allowed predictable shifts in the m/z values of characteristic ions and retention times of a given compound. These permit their unequivocal identification, in contrast to methods such as HPLC which rely on retention time alone.

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