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Determination of non-protein amino acids and toxins in *Lathyrus* by high-performance liquid chromatography with precolumn phenyl isothiocyanate derivatization

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Abstract

A simple procedure for the precolumn derivatization of toxic and non-toxic non-protein amino acids occurring in the legume crop *Lathyrus sativus* and other *Lathyrus* species with phenyl isothiocyanate and an HPLC method for the separation of the derivatives in the nano- and picomole range are reported. The results are compared with those of conventional ion-exchange chromatography for the separation of non-protein amino acids with postcolumn ninhydrin reaction. The relative standard deviations for the two methods are compared.

1. Introduction

Automated amino acid analysis was pioneered by Moore and Stein in the early 1950s [1]. They developed an amino acid analyser that automatically coupled the amino acid separation on anion-exchange columns with quantification based on the ninhydrin reaction. This has been the standard method for amino acid analysis since then, with the main variations being in the choice of buffers: sodium citrate-based buffers for the rapid determination of protein amino acids and lithium citrate buffers for the high-resolution analysis of physiological fluids or the determination of non-protein amino acids. Amino acid determination by reversed-phase HPLC after precolumn derivatization with Edman's reagent, phenyl isothiocyanate (PITC), is now a well established method [2,3]. The chromatographic separation of the amino acid derivatives is a relatively fast procedure, and applications in medical research for the rapid determination of some selected amino acids in brain, plasma and urine have been reported [4,5]. However, for the identification of unknown amino acids, more information is available on the separation of unusual amino acids by ion-exchange methods than by the more recent HPLC methods.

Lathyrus sativus (khesari in India and Bangladesh, guaya in Ethiopia, san li dow in China, pois carré in France) is a popular drought-tolerant crop and foodstuff for several hundred million people in drought-prone areas of Africa and Asia. The overconsumption of *L. sativus* seed can cause an upper motor neurone degenerative disease known as neurolathyrism. The major neurotoxin present in the seeds and seedlings has

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been determined as 3-N-oxalyl-L-2,3-diaminopropanoic acid (β-ODAP, sometimes referred to as BOAA or β-oxalylaminoalanine) [6]. Recently, a chromatographic method to determine the neurotoxin β-ODAP and to differentiate it from the non-toxic α-isomer has been developed [7]. Higher plants produce a great diversity of non-protein amino acids, the physiological and ecological importance of which is poorly understood. The genus *Lathyrus* is especially rich in unusual amino acids, some of which contain a heterocyclic ring [8]; several have toxic properties [9]. The neurotoxin β-ODAP and homoarginine are the major amino acids in the seeds of *Lathyrus sativus*, while in the seedlings homoserine appears as a major free non-protein amino acid along with some heterocyclic amino acids derived from the isoxazolin-5-one ring [10]. BIA or β-(isoxazolin-5-on-2-yl)alanine (compound I) is the precursor for the neurotoxin β-ODAP [11–14]. The higher homologue of BIA is ACI or 2-(3-amino-3-carboxypropyl)isoxazolin-5-one (compound VI), which is found only in the genus *Lathyrus*. When ACI is given to young chicks along with food or by intraperitoneal injection, the symptoms of neurotoxicity develop [15]. ACI can be hydrolysed or photolysed by UV radiation with the formation of the neurotoxic compound 2,4-diaminobutanoic acid (DABA) [16] found in *Lathyrus sylvestris*. Another *Lathyrus* metabolite, CEI or 2-cyanoethylisoxazolin-5-one (compound VIII), can be hydrolysed, photolysed by UV radiation or metabolized with the formation of β-aminopropionitrile (BAPN), the osteolathyrogen from *L. odoratus* [16]. These toxic substances in the seedlings of *L. sativus* indicate that, while the seeds of *L. sativus* are mainly neurolathyritic (β-ODAP), the seedlings also contain an osteolathyritic toxin compound VIII in addition to a second neurolathyrogen compound VI [10,17]. Lathyrine or β-(2-aminopyrimidin-4-yl)alanine, which is metabolically linked to homoarginine, is another heterocyclic amino acid that is specific to *Lathyrus* species [18].

In order to determine more correctly the overall toxicity of *Lathyrus* species, we have developed an HPLC method that can simultaneously determine these various metabolites, and which is more sensitive and much faster than the conventional ninhydrin method.

2. Experimental

2.1. Materials

Phenyl isothiocyanate (99%) and triethylamine (99+%) were purchased from Aldrich, lithium citrate, citric acid and HCl from Merck, lithium chloride, lithium hydroxide and EDTA from Sigma and ammonium acetate from UCB. Acetonitrile and methanol were of HPLC grade. HPLC-grade water was obtained with an Elgastat UHQPS deionizing system. β-ODAP was purified from plant extracts [12] and also obtained by chemical synthesis [19]. The isoxazolinone compounds I, VI and VIII and lathyrine were purified from appropriate *Lathyrus* species. Standard amino acids were obtained from Pierce and Sigma.

2.2. Chromatographic systems

A Sykam S432 amino acid analyser with a postcolumn ninhydrin reactor was used for conventional amino acid analysis. The instrument was connected with an on-line UV detector (Linear UV-106), followed by the postcolumn ninhydrin reactor and then by detection at 570 nm. Two Chromatopac C-R6A (Shimadzu) integrators were used for data acquisition of the signals at 254 nm before postcolumn derivatization and 570 nm after postcolumn derivatization with ninhydrin. For the Sykam amino acid analyser an amino acid column (150 × 4 mm I.D.) packed with sulphonated polystyrene–divinylbenzene (LCA KO4), a strong cation exchanger, in the lithium form was used. This set-up and the buffers used [17] can be considered as a newer version of the system used previously for the identification and determination of UV-absorbing non-protein amino acids [20].

For the determination of precolumn-derivatized amino acids a Waters–Millipore Model 625 LC system, equipped with a column oven (Wa-

ters) to optimize the separation conditions was used. This HPLC system was connected with a Waters Model 991 photodiode-array detector with a scanning range from 200 to 800 nm to detect compounds of interest which did not react with PITC, and allowing identification of some peaks from the absorption spectrum. Data acquisition and integration for the Waters system were effected with Millennium 2010 chromatography manager software. For reversed-phase HPLC an Alltima C_{18} column (250 × 4.6 mm I.D.; 5-μm particle size) from Alltech was used. A guard column cartridge (Alltima C_{18}, 5 μm) was directly connected to the column.

2.3. Precolumn derivatization procedure

The derivatization reagents were freshly prepared every day by mixing methanol–water–triethylamine–PITC (7:1:1:1, v/v). Volumes of 100 μl of standard amino acids or samples were dried under vacuum. The residue was dissolved in 40 μl of the coupling buffer methanol–water–triethylamine (2:2:1, v/v/v), immediately dried under vacuum and then mixed with 60 μl of PITC derivatization reagent and allowed to stand at room temperature for 20 min. Excess reagent was then removed in vacuo. Before analysis the PITC derivatives were dissolved in 1 ml of buffer A, centrifuged for 10 min at 47 000 *g*, and filtered through a 0.45-μm Millipore filter.

2.4. Preparation of standard solutions

The standard amino acid solution for the ion-exchange amino acid analyser contained 0.5 m*M* of each amino acid in lithium buffer (pH 2.2) (injection buffer). A 40-μl volume was used per injection, containing 20 nmol of each amino acid. For the PITC derivatization, a stock solution, containing 6.25 m*M* of each amino acid in water, was used. After PITC derivatization and dissolution in buffer A, 20 μl were used per injection, containing 12.5 nmol of most amino acids, except aspartic acid, tyrosine and tryptophan (5 nmol each) and compound VIII (0.08 nmol). A standard mixture of ten non-protein amino acids occurring in *Lathyrus* species, i.e., β-ODAP, compounds I, VI and VIII, Hse, Har, GABA, DAPRO (L-2,3-diaminopropanoic acid), BAPN and DABA was derivatized to evaluate the reproducibility and linearity of the method (Table 1).

2.5. Mobile phases

Solvent A consisted of 0.1 *M* ammonium acetate and was prepared freshly every other day. Solvent B was 0.1 *M* ammonium acetate in acetonitrile–methanol–water (46:10:44, v/v/v). Both buffers were adjusted to pH 6.5 with glacial acetic acid, filtered through a 0.22-μm membrane filter and degassed by purging with helium. The column temperature was optimized at 43°C. The buffers for the amino acid analyser were prepared as described previously [17].

3. Results and discussion

Standard non-protein amino acids were derivatized using the procedure described for precolumn derivatization. The separation of 30 compounds is shown in Fig. 1. Most of the amino acids in the standard were clearly resolved. Only phenylalanine and DABA were not separated under the conditions used and co-eluted at 45.3 min in peak 27. GABA and threonine, which co-eluted in peak 13, were only separated with a new column under optimum conditions. An unnatural amino acid, DL-allylglycine, was included as an internal standard, eluting at 34.5 min and not interfering with any of the compounds used.

The same standard solution was also analysed with the conventional amino acid analyser using postcolumn ninhydrin reaction and detection at 570 nm. The relative standard deviations for peak areas and retention times of the PITC amino acids are compared with the data for the ninhydrin reaction of ten amino acids in Table 1. The regression coefficients were calculated for twelve derivatizations of amino acid standards, ranging in amount from 100 pmol to 15 nmol, and they indicate a good linearity in this range.

All the selected non-protein amino acids,

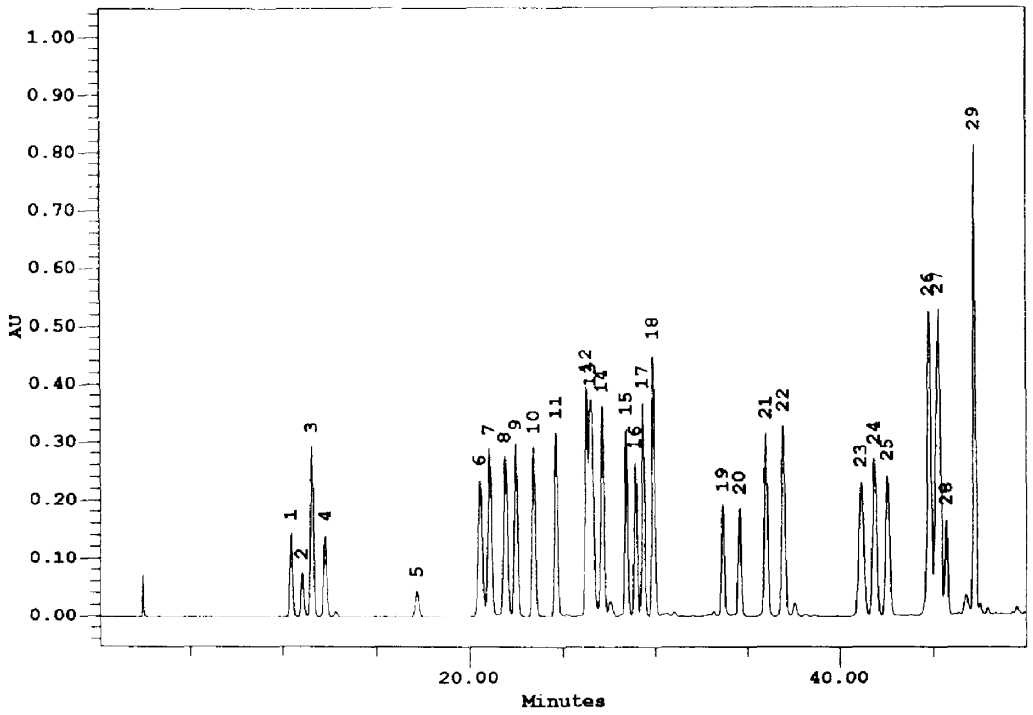

Fig. 1. Chromatogram of an amino acid standard mixture derivatized with PITC. Column temperature, 43°C. Peaks: 1 = Asp; 2 = compound VIII; 3 = β-ODAP; 4 = Glu; 5 = α-aminoadipic acid; 6 = Ser; 7 = Asn; 8 = Gly; 9 = Gln; 10 = Hse; 11 = His; 12 = Arg; 13 = Thr + GABA; 14 = Ala; 15 = Har; 16 = compound I; 17 = lathyrine; 18 = compound VI; 19 = Tyr; 20 = allylglycine (internal standard); 21 = Val; 22 = Met; 23 = BAPN; 24 = Ile; 25 = Leu; 26 = DAPRO; 27 = DABA + Phe; 28 = Trp; 29 = Lys.

Table 1
Relative standard deviations for peak areas and retention times (RT) of PITC amino acids compared with ninhydrin reaction

Amino acid	R.S.D. (%) for ninhydrin peak area ($n = 8$)	R.S.D. (%) for PITC peak area ($n = 10$)	R.S.D. (%) for ninhydrin RT (min) ($n = 8$)	R.S.D. (%) for PITC RT (min) ($n = 10$)	Regression coefficients for PITC ($n = 12$)[a]
β-ODAP	6.72	1.72	0.40	1.28	0.99826[b]
Compound I	9.35	2.07	1.85	0.73	0.92606
Homoserine	11.59	2.50	0.72	1.13	0.98782
Compound VI	3.68	1.99	0.54	0.63	0.98632
GABA	12.85	2.08	0.17	1.22	0.99824
DAPRO	3.87	3.01	0.19	0.33	0.99885
DABA	4.18	2.38	0.199	0.17	0.99112
BAPN	–	2.67	–	0.45	0.99953
Compound VIII[c]	–	3.66	–	1.24	0.98035
Homoarginine	11.29	2.60	0.37	0.56	0.97600

[a] Regression coefficients are from twelve derivatizations between 100 pmol and 15 nmol, except where indicated.
[b] Linear regression between 20 pmol and 3 nmol.
[c] Compound VIII does not react with PITC but can be detected at 254 nm.

Table 2
Gradient program for PITC amino acid analysis

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
15	90	10
30	60	40
40	50	50
50	0	100
55	0	100
57	100	0
65	100	0

except the isoxazolinone derivative compound VIII, reacted with PITC under alkaline conditions to yield PITC derivatives. The reaction is rapid and quantitative and can take place at room temperature. The gradient programme used for the chromatographic separation of the selected non-protein amino acids, toxins and some protein amino acids is given in Table 2. Higher temperatures decreased the retention times and altered some peak resolutions; optimum resolution for this method was achieved at 43°C.

With the conventional amino acid analyser the amino acids in *Lathyrus sativus* seedlings were separated on the cation-exchange resin column (Li^+ form) using stepwise elution with five lithium buffers of increasing pH until the fourth buffer, and then by increasing the ionic strength. Selectivity effects in this system are primarily due to changes in the ionization of the amino acids and the general order of elution is acidic < neutral <basic amino acids. Li^+ buffers show a better selectivity than Na^+ buffers [21].

On the amino acid analyser compound VI elutes from the column at pH 3.30 and can be easily detected because of its absorbance at 254 nm before reacting with ninhydrin and at 570 nm after reacting with ninhydrin. Compound VIII is slightly retained and elutes at pH 2.75. Compound I, homoserine and homoarginine elute at

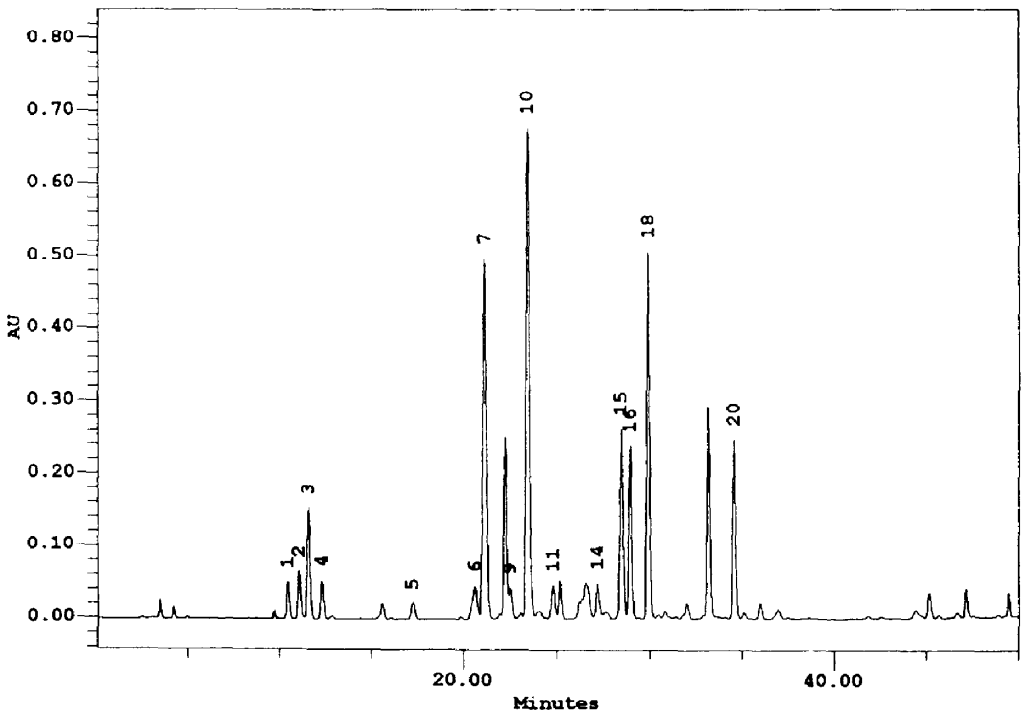

Fig. 2. Free amino acids and toxins in 3-day-old seedlings of *Lathyrus sativus*, without cotyledons, after PITC derivatization. Peak numbers as in Fig. 1.

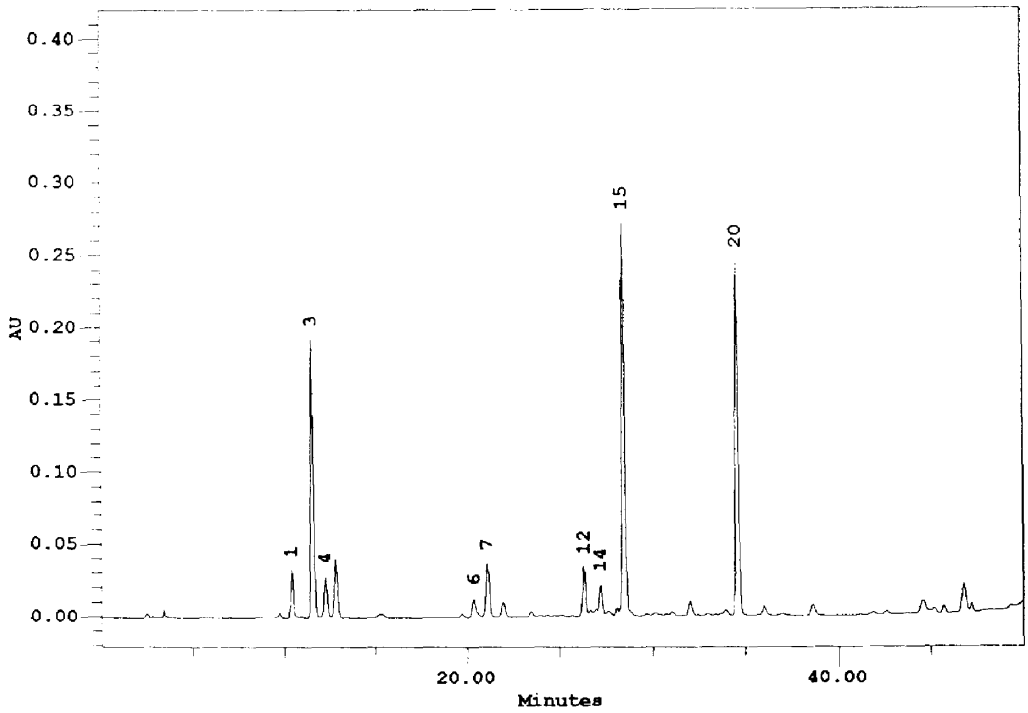

Fig. 3. Free amino acids in dry seeds of *Lathyrus sativus* after PITC derivatization. Peak numbers as in Fig. 1.

pH 2.75, 3.10 and 3.30, respectively. Homoarginine has a retention time of 195 min on the amino acid analyser with the buffer system used for high resolution, whereas on a reversed-phase column with PITC derivatization the retention time is 28.4 min. Compound VIII has a retention time of 11 min on the reversed-phase column. Although compound VIII does not react with PITC, it can be easily detected at 254 nm owing to the isoxazolinone ring. Compound I–PITC, VI–PITC and the osteotoxic β-aminopropionitrile (BAPN–PITC) have retention times of 28.9, 29.9 and 41.1 min, respectively. In addition to the shortened time of analysis, the relative standard deviation being 2–3% ($2.47 \pm 0.56\%$) for PITC and 4–13% ($7.94 \pm 3.80\%$) for the ninhydrin method, the reproducibility of quantification is superior to the ion-exchange method followed by ninhydrin reaction. The reproducibilities of the retention times are similar (Table 1).

Compound VIII is present in the seedlings and, when fed to vertebrates, it can be metabolized to BAPN [15]. Skeletal lesions that have been reported in neurolathyrism patients have recently been linked to the habit of consuming the young shoots of *Lathyrus sativus* and the presence of compound VIII [22,23].

Recent advances in the understanding of neuronal degeneration caused by overactivation of excitatory amino acid receptors has raised intriguing questions regarding additive or synergistic actions of compounds present in *Lathyrus sativus*. This excitotoxic process is perhaps not confined to the action of β-ODAP on neurones but may also extend to the toxic action of α-amino adipic acid on astrocytes [24].

Homoarginine in the seeds of *Lathyrus sativus* (up to 0.7%) [10] has been considered as a positive factor because it can be converted into the essential amino acid lysine by the mammalian liver. However, it is also a precursor of nitric oxide (NO), and as NO mediates glutamate neurotoxicity [25], a new direction for

research on and understanding of neuronal damage on consumption of the seeds of *Lathyrus sativus* is opened up.

The method reported here for the rapid determination of all the metabolites that may play a role in this toxicity should be useful for determining the overall toxicity of the seeds and seedlings (Figs. 2 and 3) of the many varieties of *Lathyrus sativus* and related *Lathyrus* species available, and of culinary preparations made from *Lathyrus*.

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References

[1] S. Moore and W.H. Stein, *J. Biol. Chem.*, 192 (1951) 663.
[2] R.L. Heinrikson and S.C. Meredith, *Anal. Biochem.*, 136 (1984) 65.
[3] B.A. Bidlingmeyer, S.A. Cohen and T.L. Tarvin, *J. Chromatogr.*, 336 (1984) 93.
[4] S. Gunawan, N.Y. Walton and D.M. Treiman, *J. Chromatogr.*, 503 (1990) 177.
[5] R.A. Sherwood, A.C. Titheradge and D.A. Richards, *J. Chromatogr.*, 528 (1990) 293.
[6] S.L.N. Rao, P.R. Adiga and P.S. Sarma, *Biochemistry*, 3 (1964) 432.
[7] J.K. Khan, N. Kebede, Y.H. Kuo, F. Lambein and A. De Bruyn, *Anal. Biochem.*, 208 (1993) 237.
[8] F. Lambein, Y.-H. Kuo and A. De Bruyn, *Phytochem. (Life Sci. Adv.)*, 11 (1992) 145.
[9] F. Lambein, Y.-H. Kuo, G. Ongena, F. Ikegami and I. Murakoshi, in K. Takai (Editor), *Frontiers and New Horizons in Amino Acid Research*, Elsevier, Amsterdam, 1992, p. 99.
[10] F. Lambein, J.K. Khan and Y.H. Kuo, *Planta Med.*, 58 (1992) 380.
[11] F. Lambein, G. Ongena and Y.H. Kuo, *Phytochemistry*, 29 (1990) 3793.
[12] Y.H. Kuo and F. Lambein, *Phytochemistry*, 30 (1991) 3241.
[13] Y.H. Kuo, J.K. Khan and F. Lambein, *Phytochemistry*, 35 (1994) 911.
[14] Y.H. Kuo, F. Lambein, L.C. Mellor, R.M. Adlington and J.E. Baldwin, *Phytochemistry*, in press.
[15] F. Lambein and B. De Vos, *Arch. Int. Physiol. Biochim.*, 89 (1981) 66.
[16] A. De Bruyn, G. Verhegge and F. Lambein, *Planta Med.*, 58 (1992) 159.
[17] F. Lambein, J.K. Khan, Y.H. Kuo, C.G. Campbell and C.J. Briggs, *Nat. Toxins*, 1 (1993) 246.
[18] E.G. Brown and N.F. Al-Baldawi, *Biochem. J.*, 164 (1977) 589.
[19] F.L. Harrison, P.B. Nunn and R.R. Hill, *Phytochemistry*, 16 (1977) 1211.
[20] Y.H. Kuo, F. Lambein, F. Ikegami and R. Van Parijs, *Plant Physiol.*, 70 (1982) 1283.
[21] R.L. Cunico and T. Schlabach, *J. Chromatogr.*, 266 (1983) 461.
[22] D.F. Cohn and M. Streifler, *Arch. Suisses Neurol. Neuroch. Psychiat.*, 128 (1981) 151.
[23] A. Haque, M. Hossain, F. Lambein and E.A. Bell, *Nat. Toxins*, in press.
[24] R.J. Bridges, C. Hatalski, S.N. Shim and P.B. Nunn, *Brain Res.*, 561 (1991) 262.
[25] V.L. Dawson, T.M. Dawson, E.D. London, D.S. Bredt and S.M. Snyder, *Proc. Natl. Acad. Sci. U.S.A.*, (1991) 6368.