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Rapid reversed-phase high performance liquid chromatographic method for the quantification of L-Dopa (L-3,4-dihydroxyphenylalanine), non-methylated and methylated tetrahydroisoquinoline compounds from Mucuna beans

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Abstract

A high-performance liquid chromatographic (HPLC) assay for the extraction and quantitative determination of L-Dopa, L-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline and 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline in *Mucuna pruriens* var. *utilis* seeds has been developed. All the three compounds, L-Dopa, CDTHIQ and MCDTHIQ in the white variety of whole (4.96 g 100 g^{-1}, 130.3 and 116.8 mg 100 g^{-1}) and dehulled (5.21 g 100 g^{-1}, 138.3 and 125.9 mg 100 g^{-1}) seeds have been found to be significantly ($P < 0.05$) higher than the black variety, respectively. The method makes use of a reversed-phase system with a C_{18}-bonded column and yields quantitative recovery of all the three compounds, L-Dopa (101.8%), CDTHIQ (98.2%) and MCDTHIQ (94.5%). This procedure is superior to other analytical techniques currently available because of its simplicity with regard to sample preparation, the sensitivity and rapidity of the method. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: L-dopa; L-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; HPLC; *Mucuna pruriens* var. *utilis*

1. Introduction

L-Dopa (L-3, 4-dihydroxyphenylalanine), L-3-carboxy-6, 7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (non-methylated) and 1-methyl-3-carboxy-6, 7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (methylated) (Fig. 1) are present in *Mucuna* spp. (Bell, Nulu & Cone, 1971; Daxenbichler, Kleiman, Weisleder, VanEtten & Carlson, 1972; Daxenbichler, VanEtten, Earle & Tallent, 1972). These compounds are pharmacologically active, L-Dopa, in particular, is being used for the symptomatic relief of Parkinson's disease (Amarasekara & Jansz, 1980; Pieris, Jansz & Dharmadasa, 1980). However, it is potentially toxic (Afolabi et al., 1985; Duke, 1981) if ingested in large amounts. When used to treat Parkinson's, L-Dopa has been reported to produce serious hallucinations and dyskinesias in addition to gastrointestinal disturbances like nausea, vomiting and anorexia (Da Prada, Keller, Pieri, Kettler & Haefely, 1984; Reynolds, 1989) and it has also been shown to be toxic in individuals with glucose-6-phosphate dehydrogenase deficiency in their erythrocytes, resulting in the induction of favism (Nechama & Edward, 1967). Recently, Takasaki and Kawakishi (1997) have reported that the oxidation products of Dopa conjugate with SH compounds of proteins (cysteine) forming a protein bound 5-S-cysteinyldopa cross links, leading to polymerisation of proteins and/or other protein. These three compounds (L-Dopa, non-methylated and methylated tetrahydroisoquinolines) are readily oxidisable at alkaline pH, high temperature (70–100°C) with more moist conditions and form dark coloured compounds (Siddhuraju & Becker, unpublished data).

Previous methods for the analysis of L-Dopa have involved a non-specific colorimetric test (Arnow, 1937;

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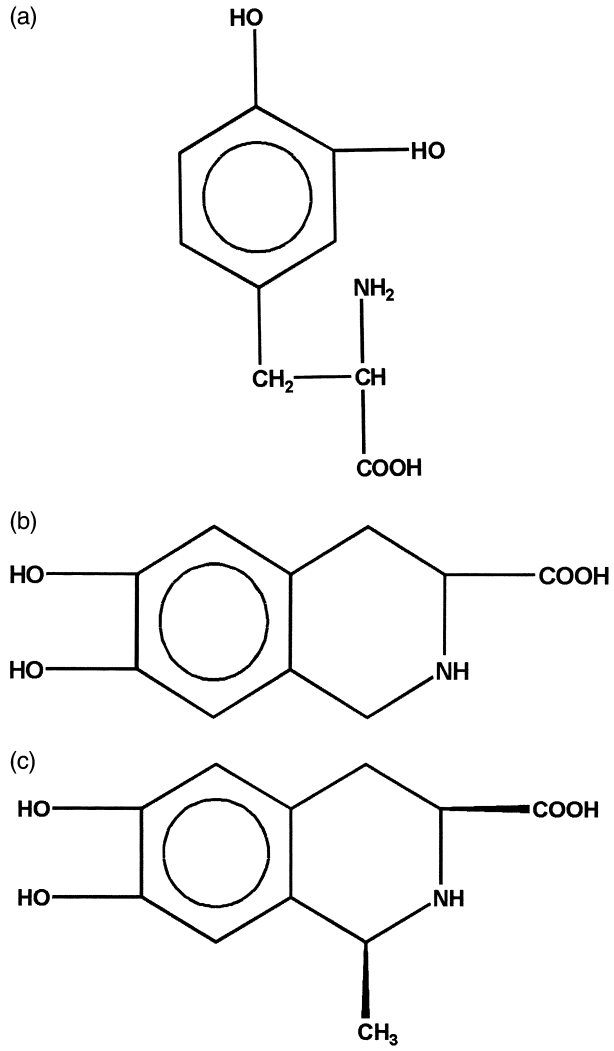

Fig. 1. Chemical structure of (a), L-3,4-dihydroxyphenylalanine (L-Dopa); (b), L-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (CDTHIQ) and (c), 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (MCDTHIQ).

Maggi & Cometti, 1972; Szent-Kiralyi, 1979). Separation of the compound using column chromatography followed by quantification using ultraviolet spectrophotometry (Daxenbichler, Kleiman, Weisleder, VanEtten & Carlson, 1972) and the recently developed method of estimation through an amino acid analyser (Bell & Janzen, 1971; Prakash & Tewari, 1999) are time consuming techniques and specific only for L-Dopa, whereas the other method developed by Marquardt and Frohlich (1981) is more specific for vicine, convicine and their hydrolytic products rather than L-Dopa. Furthermore, a procedure for the quantitation of L-Dopa and two other tetrahydroisoquinoline compounds from crude extract of mucuna seeds has not been reported in the literature.

Hence, this paper describes a simple and specific method involving the use of reversed-phase high-performance liquid chromatography (HPLC) for separating L-Dopa, L-3-carboxy 6,7-dihydroxy-1,2,3,4-tetrahydroiso-quinoline and 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline compounds. The method is capable of detecting these compounds at concentrations as low as 0.1 μg/ml. The analysis time for elution of these compounds is less than 8 min. No pre-treatment is required. Because of the method, its application may be directly utilised for routine screening of various species/cultivars/germplasms of mucuna beans including processed samples for the quantitative determinations.

2. Materials and methods

2.1. Samples

The seeds of the white and black varieties of *Mucuna pruriens* var. *utilis* (3 kg of each variety) were purchased from tribal people living in a village near Marthandam, Tamil Nadu, India in the month of January 1999. The dried seeds were cleaned thoroughly, and any broken and immature seeds were removed. The seeds were stored at room temperature (24°C). The seeds were cracked mechanically and the cotyledons were separated. Fine powder of both the whole and dehulled seed samples was stored in screw-capped bottles at room temperature (24°C) prior to the chemical analyses. The moisture content of samples was determined by oven drying to a constant weight at 105°C. Analyses were done in triplicate.

2.2. Apparatus

The chromatograph consisted of a Merk-Hitach model L-7100 HPLC pump, an L-7450 UV detection and photo diode array detector, an L-7200 autosampler with injector valve containing a 100-μl sample loop, a D-7000 interphase module and an LC organiser (Hitachi Instruments Inc., San Jose, USA). The analytical column was reverse phase C_{18} (nucleosil 120, mean particle diameter 5μm, 250×4.6 mm I.D., Macherey-Nagel GmbH & Co, Germany). A guard pre-column was packed with material, as in the main column. Absorbance was monitored at 282 nm and peak heights and areas were determined.

2.3. Chemicals

L-Dopa (L-3,4-dihydroxyphenylalanine) was obtained from Sigma (St. Louis, MO, USA). Analytical grade phosphoric acid, methanol, hydrochloric acid, and sodium hydroxide, formaldehyde, acetaldehyde and reagent grade acetic acid and petrolium ether were procured from Merk (Darmstadt, Germany).

2.4. Synthesis of L-3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (CDTHIQ)

L-Dopa (400 mg) was dissolved in water (20 ml) and 0.35 ml of 40% formaldehyde solution was added. The mixture was stirred and kept at 30°C for 72 h. The reaction mixture was then taken to dryness and the residue was recrystallized from 20% (v/v) acetic acid (Bell et al., 1971). In order to get more purified form, two additional recrystallizations were carried out. The yield was about 240 mg. The purity was checked by TLC and HPLC. Thin layer chromatography was performed on a tlc plate coated with Silica gel-60G (Merck) by using a developing solvent system, 1-butanol-acetic acid-water (4:1:3 v/v/v) and ninhydrin as spraying reagent.

2.5. Synthesis of 1-Methyl-3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (MCDTHIQ)

L-Dopa (400 mg) was dissolved in water (20 ml) and 0.35 ml of 40% acetaldehyde solution was added. The mixture was stirred and kept at 30°C for 72 h. The reaction mixture was then taken to dryness and the residue was recrystallized from 20% (v/v) acetic acid with two additional recrystallizations (Daxenbichler, Kleiman, Weisleder, VanEtten & Carlson, 1972) and the yield was about 230 mg. The purity of the standard was checked by tlc and HPLC techniques by adopting the aforementioned procedure.

2.6. Preparation of reagents

2.6.1. Standard preparation

Standard solutions of L-Dopa (200 μg/ml), CDTHIQ (240 μg/ml) and MCDTHIQ (240 μg/ml) were prepared by dissolving appropriate masses of the dried standards in 0.1N HCl. The stock solutions of these compounds were freshly prepared daily in water. Prior to analysis the stock solutions were diluted to the appropriate concentrations with 0.1N HCl. Stock solutions diluted in 0.1N HCl were prepared each day and were maintained at a temperature of between 2 and 5°C.

2.6.2. Preparation of sample

Finely ground and defatted whole seed and dehulled seed flour (50 mg) were placed in glass tubes and 5 ml of 0.1N HCl were added, after which the samples were mixed for 10 min at room temperature (22°C). The mixture was initially subjected to Ultra-turrax T25 homogenizer (20500 min^{-1}) for 30 s in an ice bath condition and subsequently it was kept on a magnetic stirrer for 1 h at room temperature. The supernatant was collected by centrifugation (13,000 rpm, 15 min). The extraction procedure was repeated twice and the supernatants of all three extractions were pooled and filtered through a 0.2-μm glass filter. The supernatants were diluted as required with 0.1N HCl and maintained 2–5°C until analysed. All samples were analysed within 8h of preparation.

2.6.3. Chromatographic conditions

Two solvents were used: the eluting solution (Solvent A) was made up of water, methanol and phosphoric acid in the ratio of 975.5:19.5:1 (v/v/v), pH 2.0, and washing solution (Solvent B) was 70% methanol. These solvents were stable at 22°C for 1 week. The gradient used was: start with 100% (A) and 0% (B) up to 12 min, next 5 min solvent (B) increase from 0 to 100% with 100 to 0% decrease of solvent A, increase A to 100% and decrease B to 0% in the next 5 min, and then the column is washed with solvent A alone in the next 15 min to adjust the column to the starting conditions (A 100% and B 0.00%). Isocratic elution was carried out. Separation was performed at room temperature (22°C) and the flow rate was 1.2 ml min^{-1}. All the aqueous containing solutions were passed through a 0.20-μm glass filter. Eluents were degassed by filtration or in an ultrasonic bath.

2.6.4. Statistical analysis

The data were subjected to a one-way analysis of variance and the significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$) using the Excel Programme (Statistica, Version 5.1).

3. Results and discussion

3.1. Optimal conditions

The chromatogram in Fig. 2 shows the separation of L-Dopa, CDTHIQ and MCDTHIQ compounds. All the three, ultraviolet absorbing compounds that are present in mucuna beans, are resolved into three distinct peaks. The injection solvent (0.1N HCl) is responsible for the small initial peak. The average elution time for all compounds was less than 8 min and that of CDTHIQ, MCDTHIQ and L-Dopa 5.2, 7.1 and 7.6 min, respectively (Fig. 2). Preliminary studies demonstrated that optimal resolution of CDTHIQ, MCDTHIQ and L-Dopa was achieved at low pH levels. Increasing the pH of the eluent to 3.0 decreased the elution times of all the three compounds by 10–20% and MCDTHIQ and L-Dopa co-eluted. Other results demonstrated that the elution patterns of CDTHIQ, MCDTHIQ and L-Dopa were variably influenced by the nature of the injecting solutions. Injection of all the three compounds in water, column eluting solution, 0.05% acetic acid and 0.1 N HCl yielded chromatograms with nearly identical elution times and peak

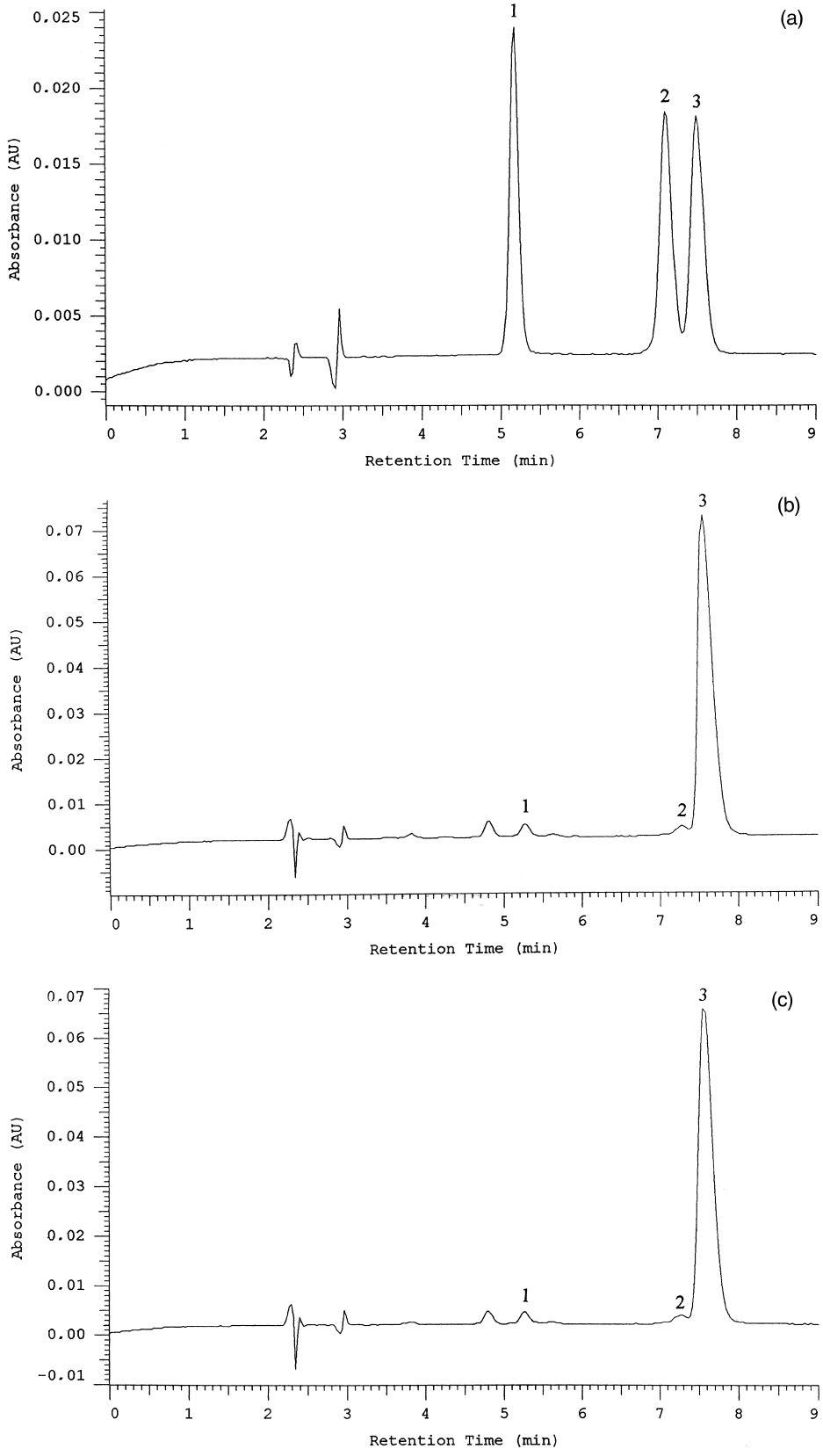

Fig. 2. Chromatogram of a standard solution (a) containing each 16 μg ml^{-1} of (1), CDTHIQ; (2), MCDTHIQ and (3), L-Dopa and (b) and (c) are an extract prepared from the white and black varieties of mucuna seeds, respectively (50 mg/25 ml). Compounds eluted in chromatogram B and C were CDTHIQ (1); MCDTHIQ (2); and L-Dopa (3). Mucuna seeds and standard solution, were either extracted or prepared in 0.1N HCl. Injection volume was 20 μl.

areas. However, modified elution patterns were obtained when the standards were injected in solutions that contained a 50:50 mixture of water and methanol or 1 N NaOH. Sodium hydroxide yielded distorted peaks and altered elution times. Solutions containing methanol greatly reduced the retention times of all compounds which caused the peaks to merge. Standards or unknown samples can, therefore, be prepared in the former, but not the latter solutions. The low pH of the eluting solvent does not cause destruction of the column, as a single column has been used for the analysis of more than 300 samples over a period of 4 months.

3.2. Stability of L-Dopa, CDTHIQ and MCDTHIQ

Preliminary studies demonstrated that aqueous solutions of all the compounds, when stored at 2°C, were stable for 2 days, or for a period of at least 8 h when incubated at 22°C. The above compounds also do not appreciably decompose when stored over a 3-day period at 2°C in 0.1N HCl or in eluting solution; increasing the temperature to 22°C however, decreases its concentration by approximately 10–15%. The average decrease in concentration of all the compounds in the presence of 0.1N HCl during an 8-h period would be 0% at 2°C and less than 5% at 22°C. These results would suggest that stock solutions of all the three compounds can be prepared at 3-day intervals in 0.1N HCl and that working solutions of these compounds should be maintained at 2°C and should be freshly prepared at least every 8 h. Unknown samples should be treated in a similar manner. On the basis of the above results, it may be concluded that 0.1N HCl can be used as an extractant and that filtrate samples can be injected directly into the column. Results under these conditions are identical to those obtained when samples were injected with the eluting solvent.

3.3. Linearity of response and reproducibility

The calibration curve for L-Dopa (at eight concentrations), CDTHIQ and MCDTHIQ (at five concentrations) over the ranges of from 100 to 2, 120 to 2 and 120 to 2 μg/ml, respectively were determined. Plots of peak height and area were linear for all compounds and the value of the intercepts were not significantly different from zero ($P < 0.05$). The correlation coefficients (r) between peak area and concentrations of the standard compounds were: L-Dopa 1.0000, CDTHIQ 0.9995 and MCDTHIQ 0.9990. Similar values were also obtained when the standards were prepared in water instead of 0.1N HCl. The lower limit of detection for all the compounds was about 0.1 μg/ml. The corresponding amount of sample injected into the column was 2 ng. This sensitivity is similar to that previously reported by Marquardt and Frohlich (1981) for L-Dopa analysis in faba beans through HPLC reported. No peaks interfered with the detection of L-Dopa, CDTHIQ and MCDTHIQ in mucuna seed samples with that of the seed components as can be seen in representative chromatograms in Fig. 2. The peak purity of corresponding L-Dopa, CDTHIQ and MCDTHIQ peaks in mucuna seed crude extracts was confirmed by analysing the peak spectra using a photodiode array detector and software. This further confirms the selectivity of the method.

The elution profile of seeds of mucuna beans (white and black varieties) demonstrated the presence of one major peak, L-Dopa, two minor peaks, CDTHIQ and MCDTHIQ and one unknown minor peak. The concentration of L-Dopa, CDTHIQ and MCDTHIQ in white and black varieties of whole seeds were 4.96 and 4.39%; 0.130 and 0.118%; 0.116 and 0.092%, respectively (Table 1). The concentration of all three compounds in the white variety were found to be significantly ($P < 0.05$) higher than the respective compounds in the black variety. However, dehulling did not improve the reduction of any of the above mentioned compounds in both the varieties. The percent recoveries of L-Dopa, CDTHIQ and MCDTHIQ when added at levels equal to these present in the white variety of mucuna bean extract were 101.8±0.26, 98.2±1.19 and 94.5±1.1, respectively.

Table 1
Determination of L-Dopa, CDTHIQ and MCDTHIQ concentrations in two varieties of *Mucuna pruriens* var. *utilis* seed samples (values on dry matter basis)[a]

Sample	L-Dopa[b] (g 100g^{-1})	CDTHIQ[c] (mg 100g^{-1})	MCDTHIQ[d] (mg 100g^{-1})
Whole seed			
White variety	4.96 a (± 0.13)	130.30 a (± 3.54)	116.80 a (± 3.15)
Black variety	4.39 b (± 0.05)	117.83 b (± 5.85)	92.00 b (± 2.78)
Dehulled seed			
White variety	5.21 a (± 0.12)	138.30 a (± 6.44)	125.88 a (± 2.81)
Black variety	4.66 b (± 0.11)	130.44 b (± 2.47)	102.95 b (± 2.54)

[a] Values are means of triplicate determinations±standard deviations. Means followed by different letters in a column denote statistically significant ($P < 0.05$) differences between white and black varieties of either whole or dehulled seeds.

[b] L-Dopa, L-3,4-dihydroxyphenylalanine.

[c] CDTHIQ, L-3-carboxy-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline.

[d] MCDTHIQ, 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline.

4. Conclusions

With the reversed-phase separation described, L-Dopa, CDTHIQ and MCDTHIQ can be readily quantified in mucuna beans. This procedure requires a simple sample preparation technique and is sensitive and rapid. We believe that this paper describes for the first time, a procedure for the quantification of L-Dopa along with two tetrahydroisoquinoline compounds from mucuna beans through a HPLC technique. From the nutrition point of view, L-Dopa and the two tetrahydroisoquinoline compounds seem to be toxic/food interfering substances (Duke, 1981; Infante et al., 1990), therefore, their quantification in both raw and processed samples of mucuna beans is important before advocating these for large scale consumption. This analytical technique may also be useful in future for routine tests in the germplasm/varietal variation screening programmes. Furthermore, to assess the toxicological and antinutritional consequences of these compounds, extensive and long-term animal feeding trials are required. Research work on the pharmacological activity of these two tetrahydroisoquinolines, which seem to be similar to the other tetrahydroisoquinolines currently used to reduce blood pressure, is also needed.

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