FINAL PROJECT REPORT

LATHYRUS IMPROVEMENT: AG CANADA/INDIA/MANITOBA

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Introduction

Legumes are versatile plants which are utilized as sources of food and animal feed. They are important components of crop rotation systems in many parts of the world. Lathyrus sativus L. otherwise known as lathyrus, grass pea, chickling vetch or khessari is a productive species in regions subject to adverse climatic conditions and marginal soil. It is a trailing vine, with a length up to 160 cm in good conditions, but averaging approximately 75 cm. It is similar to the field pea, Pisum sativum L., in growth habit, but has long grass-like leaflets. Its flowers resemble those of the sweet pea, L. odoratus L., but are smaller. They are normally borne singly in the axils of leaves, and have a colour range similar to the sweet pea. The seed pods are flat, 3.5 to 7.0 cm. long, dorsally-winged and contain from three to six seeds. The seeds, distinctively wedge-shaped and three to twelve mm. in size, are frequently white, brownish-grey or light cream in colour and often are speckled with black.

Studies are being conducted in Bangladesh, India, Nepal, Pakistan, Ethiopia, Syria, the United States and Canada to assess the potential and improve acceptability of this species as a crop. The seeds contain good quality protein with composition similar to that of other food legumes. Yields per hectare compare favourably with those of other legumes in a typical year, and are generally higher than for competitors if there is a drought. Reported yields range

from 1000 to 1500 kg ha^{-1} obtained in the United States (Duke et al. 1981).[1] However yields under plot conditions in Manitoba often range between 3000 and 4000 kg ha^{-1} with the maximum being over 9000 kg ha^{-1} (Campbell, unpublished data).

Within Asia and North Africa Lathyrus sativus could become established as one of the pulse grains of choice for humans and animals. Its protein content varies from 20-30% in seed meal (Duke et al. 1981, Padmanaban 1980).[2] As with most legumes, the seed is relatively rich in lysine, which is deficient in cereal proteins. However, grass pea seed protein is low in methionine and tryptophan (Duke et al. 1981). Lathyrus sativus possesses a good nodulation system and fixes atmospheric nitrogen, reducing the need for application of fertilizer. Lathyrus is easy to cultivate and is generally resistant to pests, factors which have made it a dominant food crop in countries such as Bangladesh.

Problems associated with the crop are common to many pulses, with the addition of the potential to produce the neurodegenerative disease, neurolathyrism. Trypsin inhibitors, tannins and non-protein amino acids are present in the seeds. Research on improving Lathyrus sativus as an agricultural crop has concentrated on eliminating or reducing the seed content of n-oxalyl-αß-

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[1]. J. A. Duke, C. F. Reed and J. K. P. Weder (1981). Handbook of Legumes of World Economic Importance. Plepum Press, N.Y. 107-110.

[2]. G. Padmanaban (1980). Toxic Constituents of Plant Foodstuffs. Academic Press, N.Y. pp. 239-263.

diamino-propionic acid (ODAP). This constituent is responsible for neurolathyrism when consumed as a major component of diet for prolonged periods. Toxicity may arise when the lathyrus grain comprises more than one third of the diet for four to six months. Trypsin inhibitors can be eliminated by breeding or processing and these factors and the tannin levels are low in several lines which have been selected for breeding new, safe lathyrus cultivars.

The objective of the Ag. Canada/India/Manitoba program is to eliminate or greatly reduce the concentration of ODAP in Lathyrus seed available for commercial development. The Lathyrus program at Morden has been established for about thirteen years, and the Lathyrus Improvement/Ag. Canada/India/ Manitoba project commenced in 1987 with establishment of the international breeding program with Raipur, India. The Faculty of Pharmacy at the University of Manitoba has been involved with development and application of analytical procedures. Training has been provided to visiting scientists from several countries involved in lathyrus research, and Dr. Arvind Geda from Raipur spent nine months in Winnipeg, returning to his own laboratories in July 1992.

There has been a need for improved analytical procedures to enable ODAP to be quantified at low concentration in small quantities of seed and to measure any ODAP residues in meat and milk. Farmers in some countries graze animals on young lathyrus plants as fodder, and ODAP would be consumed. Consequently there is a need for methods to assay the neurotoxicant levels in green plant

material, particularly as it has been shown by Lambein that lathyrus seedlings have high levels of non-protein amino acids. High performance liquid chromatography offers the best potential method for ODAP assay in both animal tissue and green plant tissue. The conventional spectrophotometric method is insufficiently sensitive for residue analysis and is susceptible to interference from plant pigments in young seedlings. The analytical method developed by Dr. Geda during his post doctoral experience in Winnipeg has many advantages of sensitivity and specificity for these assays.

Research Collaboration and Personnel

The Senior Investigator at the Faculty of Pharmacy (Dr. Briggs) continues to collaborate with the Principal Investigator on the plant breeding aspect, Dr. Clayton Campbell at the Morden Research Station. The IDRC project complements studies performed with Dr. F. Lambein at the University of Ghent, Belgium and the success of the analytical developments contributed to approval of a NATO grant for further collaboration on biochemical aspects of the lathyrus toxins. This travel grant enable Dr. Lambein to spend a week in Winnipeg in the summer of 1992 and Dr. Briggs visited the laboratory of Dr. Narayan and the University of Wales at Aberystwyth, the Welsh Agricultural Research Station and the University of Ghent, Belgium. The HPLC procedure developed with Dr. Geda has been used extensively in studies arising from these personnel exchanges. It also forms the analytical basis for the collaborative study with Dr. Castell at the

Agricultural Research Station, Brandon. This is the pig-feeding study described in the second paper cited in the appendix.

It is essential that a reliable method for assay of ODAP in animal tissues be available if lathyrus is to be licensed as a food and feed crop. A feeding study in mice has been conducted with Dr. E. Bruni of the Department of Anatomy, University of Manitoba. Tissues from these animals will be examined histologically and chemically for ODAP levels following maintenance on "high ODAP" and "low ODAP" lathyrus diets. This project is complete from the feeding study perspective, but chemical and histological evaluation will not be performed until technical assistance is available in the summer of 1994.

Problems experienced with extraction of ODAP from animal samples have been resolved by freeze drying the tissue prior to extraction of the fat with ether. The resulting dry tissue was powdered and extracted for ODAP residues. The procedure did not affect the results, as amino acids and ODAP are not soluble in ether. Recovery of ODAP from "spiked" samples gave consistent acceptable results. This method will be used in the mouse tissue analysis.

A summer research assistant was employed on the lathyrus plant project from May to August 1992. A research technician, Ms. Melanie Johnson, was appointed to provide research support to the end of 1993, and a second research assistant was employed for four months during the summer of 1993. Alternative financial support for this second assistant was obtained for three months. The primary

focus of their research was to apply the analytical method developed by Dr. Geda. These studies included detailed analysis of samples from the 1992 and 1993 crop years at Morden, and modification of the HPLC method for determination of residues in meat.

A graduate student from Iran was accepted to commence his program in September 1992. The acceptance was deferred to January 1993, but we had no further correspondence from the student and he did not join the project.

Availability of Analytical Standards and Lathyrus Samples

A report that the analytical standard used in the spectrophotometric assay, diaminopropionic acid, had been discontinued by the supplier was incorrect. It is available from the Sigma Chemical Company, Toronto, as DL-23, diaminopropionic acid monohydrochloride, catalogue number D-1502 (1992 catalogue). In addition this catalogue lists ß-N-oxalylamino L-alamine (BOAA), a synonym for ODAP. This product, catalogue number O-6255, had not previously been available from commercial sources. The fact that this neurotoxic component of lathyrus can now be purchased is a major convenience for research in the area.

Continuation of lathyrus research following termination of the IDRC contract for lathyrus improvement has been a concern, particularly with regard to availability of seed material. Following an approach for collaboration with Dr. Yu Jin Zhang, an agricultural research associate professor in Shoanxi province China, Dr. Clayton Campbell has confirmed that we will have reserve seed material available for the

foreseeable future. This would enable any necessary increases to be grown, and would provide a wide range of seed with varying ODAP content. In addition, we plan to expand our links with Professor Yao-Zu Chen of Lanzhou University, Lanzhou province. He is a prominent Chinese research worker and member of the Academic Council of the Chinese Academy of Science. His publications include numerous articles on agricultural, nutritional and chemical studies on lathyrus. Many of these investigations are complementary to those of Campbell, Lambein and the investigators in Raipur and Bangladesh.

Standardisation of Assay

Spectrophotometric assay procedures for ODAP in lathyrus samples vary in detail in different laboratories around the world. The Morden procedure (see Addendum I), adapted from the published method reported from the University of Manitoba, is receiving increased acceptance. An international comparison of results obtained in different laboratories for the analysis of the standard samples provided by the University of Manitoba was reported in the previous report. Results from the majority of laboratories were within an acceptable range, but one group obtained results which were unacceptably low. They have looked for possible cause for this anomaly. The study has been extended, and samples distributed to five laboratories for analysis. The reference material has been checked to ensure that ODAP levels remained constant during storage. Two laboratories in this study were suggested by Dr. Campbell, and were not

participants in the earlier survey. They have not previously taken part in international evaluations, and they were included because they wished to validate results which they report for plant breeding studies.

Results obtained from the different laboratories were more consistent than in the earlier report (July 1992) and none were unacceptably high or low. Results for five locations were

Tara Pea 0.021 ± 0.014; High ODAP Lathyrus 0.462 ± 0.386
Low ODAP Lathyrus 0.032 ± 0.010.

All results are expressed as % ODAP in Lathyrus seed.

As a result of discussions at the recent Lathyrus meeting in Dacca, it has been suggested that all laboratories should use water to extract ODAP from seed. This should eliminate one of the variables and make international comparison more reliable. However, from our studies, research results from any of the major laboratories working on Lathyrus should be acceptable and comparable with ±10% of the declared concentration. This would provide no problem when distinguishing between high and low lines, particularly as environmental and seasonal variables can make a much larger difference.

Near Infra-Red Assay. (NIR)

Although other workers have obtained good results with sophisticated NIR analytical equipment, we concur with Dr. Campbell in that the NIR technique cannot be recommended as a routine procedure for ODAP analysis in plants. It may have a place in advanced research laboratories, but results obtained by Dr.

Geda, Dr. Campbell and other using the Dickey-John equipment at Morden do not justify its adoption as a routine screening procedure.

Research in 1993

Results for all studies up to April 1993 have been presented in earlier reports. Assays of seed of interest from the 1992 crop were included in the year end report submitted in April 1993. Results from analysis of seed from irradiated Lathyrus were also summarised in that report.

The C_{18} SEP-PAKS were useful for forage sample clean-up, but subsequent studies with animal tissues were less successful. The technique appeared to have insufficient merit for inclusion in the routine procedure for ODAP residue analysis in animal samples.

Since the previous report, until the end of 1993, research was concentrated on the analytical aspects of animal samples. When the grant was approved, Morden required assistance with assays for the breeding program. They have developed an excellent facility which performs the majority of assays for the breeding program on site. This has left us greater flexibility in the application of our analytical experience. The potential animal residue problem with ODAP is an integral part of any grass pea breeding for feed or food use. For this reason, we have worked on development and use of the HPLC procedure for this purpose. This work is summarized in Addendum II which covers the analytical aspects of the Lathyrus pig feeding study. These will be reported in full in the paper cited.

Publications

The appendix includes three scientific publications which have arisen directly from the collaboration facilitated by the IDRC contract during the past year. The first one, by Geda, Briggs and Venkataram was included in pre-publication form in the last year-end report (April 1993). The second title by Castell et al. is a multi-disciplinary study on lathyrus feed for pigs. Seed samples, feed and animal tissue were analyzed for ODAP in our laboratories. The resultant paper has been accepted for publication by the Canadian Journal of Animal Science. The abstract by Briggs, Campbell and Castell is for a paper presented at the 2nd International Colloquium on Lathyrism in Bangladesh. This paper has been re-written and submitted to the conference organisers in Belgium for publication during 1994 in the conference proceedings. All three papers listed include reference to IDRC. The fourth paper was produced as a result of the NATO collaborative grant with Dr. Lambein. The work was not directly funded by IDRC, but arose during the collaboration as a consequence of the joint program with Morden, India, Bangladesh and Belgium. It is a good example of the extensive networking which has developed during the term of the IDRC funded "Lathyrus Improvement: Ag. Canada/India/Manitoba" Project.

Conclusion

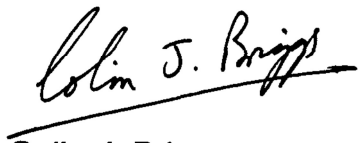
We will continue to investigate improved assay procedures for either general screening for ODAP or more detailed study of components of the plant. There is

particular interest in developing a method for routine use in the assay of lathyrus forage by HPLC.

Collaboration will continue with Dr. Campbell and Dr. Lambein, and it is anticipated that this will lead to selection and development of a Lathyrus sativus which will be suitable for widespread use in human and animal diets. The feeding studies with Drs. Bruni and Campbell have provided information to justify these uses for the seed developed. The Brandon study resulted in the recommendation that Lathyrus sativus seed could be used as a component of pig feed at a concentration of up to 15% of diet. The expectation is that the new low ODAP lines would be useful and safe at this level. Factors other than ODAP lead to the limitation, and funding is being sought to develop lines in which these will be eliminated.

The support of IDRC in this research has been appreciated. This will recognised when a new Lathyrus sativus line is registered as a valuable new crop for the prairies or as a major new food pulse to contribute to alleviation of food shortages in under developed countries.

Respectfully submitted,



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Wet chemistry method for estimation of B-N-oxalylamino-L-alanine (BOAA) also known as B-N-oxalyl-L-α-B-Diaminopropionic acid (ODAP) in Lathyrus Seeds.

Fast Screening Method

1. Preparation of Reagents

a. Potassium hydroxide (3N)

168.33 g KOH per litre distilled water

b. Potassium borate buffer
(shelf life of one month in a well stoppered bottle)

3.092 g potassium borate
3.728 g potassium chloride
3 pellets sodium hydroxide
999 mls distilled water

Adjust pH to 9.9 with concentrated sodium hydroxide solution.

c. O-phthalaldehyde reagent (OPT)
(make and use on same day)

100 mg O-phthalaldehyde
200 µl mercaptoethanol
1000 µl ethanol (95%)
99 ml potassium borate buffer

2. Standard Curve

(Diaminopropionic acid (DAP) is hygroscopic and also absorbs CO_2 from air, so it's stable salt, Diaminopropionic acid hydrochloride, which is commercially available, is normally used)

DAP stock solution = (400 µg DAP/ml of 60% ethanol)

Weigh 0.0054 g DAP hydrochloride and dissolve in 10 ml of 60% ethanol. (Conversion factor from DAP to DAP-hydrochloride = 1.35)
Make dilutions of the DAP stock sol., as in Table 1.

ODAP Analysis: Morden Procedure

Table 1.

Tube No.	DAP Stock Soln. (ml)	60% Ethanol (ml)	µg DAP in 2 ml soln.	Equivalent to µg DAP/ g seed	Equivalent to ODAP* or BOAA (%)
1	0.3	1.7	120	1200	0.203
2	0.6	1.4	240	2400	0.406
3	0.9	1.1	360	3600	0.609
4	1.2	0.8	480	4800	0.812
5	1.5	0.5	600	6000	1.015
6	1.8	0.2	720	7200	1.218

* Conversion factor from DAP to BOAA (ODAP) = 1.6916

- add 4 ml of 3 N KOH to each tube
- cap tightly and vortex
- place all tubes in boiling water bath for 30 minutes and vortex
- centrifuge at 4500 rpm for 15 minutes
- take spectrophotometric reading at 425 nm by setting absorbance of blank as zero
- construct a graph of absorbance of standards against % BOAA (ODAP) (Table 1).

3. **Extraction procedure**

- weigh 0.5 g powdered seed and place into test tube
- add 10 ml of 60% ethanol
- cap tightly and vortex
- shake tubes in shaker/mixer for 45 minutes
- centrifuge at 4500 rpm for 15 minutes
- decant ethanol extract (unhydrolyzed fraction) into prelabelled test tubes

4. **Hydrolysis**

(Conversion from BOAA (ODAP) in the sample to DAP takes place)

- pipette 2 ml of ethanol extract into screw top test tube
- prepare a blank tube by 2 ml of 60% ethanol instead of ethanol extract.
- add 4 ml of 3 N potassium hydroxide (KOH)
- cap tightly and vortex
- place all tubes in boiling water bath for 30 minutes
- centrifuge at 4500 rpm for 15 minutes

5. Colour Reaction

- pipette the following into screw top test tubes

  For all samples/standard curve tubes/blank tube

  - 250 µl hydrolyzed sample/standard/blank
  - 750 µl distilled water
  - 2000 µl OPT solution

- vortex all the tubes
- incubate for two hours at 40°C
- take spectrophotometric reading at 425 nm by setting absorbance of blank as zero
- Determine the % BOAA (ODAP) in the samples from the standard graph.

Addendum II.

The conventional ortho-phthaldehyde (OPT) method for analysis of neurolathyrogens has been used with animal tissues. Compared to HPLC procedures, it lacks sensitivity but could be applicable in situations where many samples have to be assayed or when the HPLC methods cannot be used, e.g. when monitoring meat for ODAP in third world countries.

Each of these techniques for analysis of ODAP requires further validation in situations where the compound might be present as a residue in animals fed diets containing lathyrus.

b) Development and Results

The preliminary investigation into the most appropriate method rejected electrophoresis as other techniques showed greater promise. Analysis of extracts using an amino acid analyzer, without hydrolysis of the ODAP, gave a peak which preceded (elution time of ~3 min) the amino acids in a reference mixture and readily detected 10 nm ODAP. However, HPLC appeared to have the greatest potential and was chosen to be the priority.

An improved method, using HPLC combined with spectrofluorometry, was developed for the detection and quantitative estimation of picomoles of ODAP in *Lathyrus* seed. The technique, subsequently published [Geda et al. 1993], represented a major advance over the spectrophotometric methods conventionally used to assay this compound in plant material. A copy of the paper is included in the appendix.

Applications with respect to animal tissues

The pig feeding trials at Brandon concluded in March, 1993 and provided the samples (pork chop and kidney) for subsequent investigations.

The applicability of the HPLC method to samples other than lathyrus seed is demonstrated in the following figures.

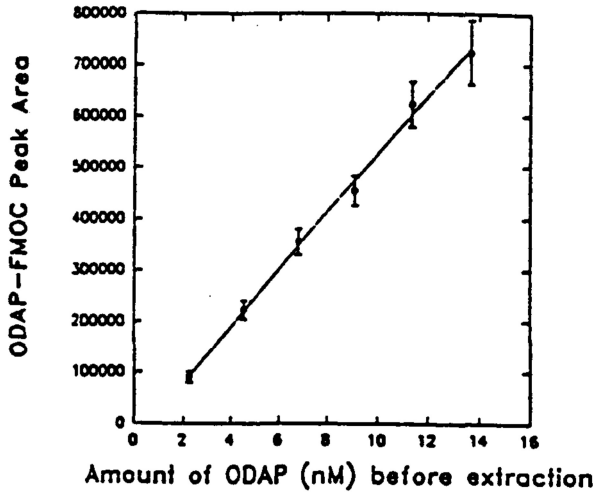


Figure 1. Calibration curve showing derivative peak area versus amount of ODAP used for extraction. (Slope=27544, intercept= -32.2; r=0.97)

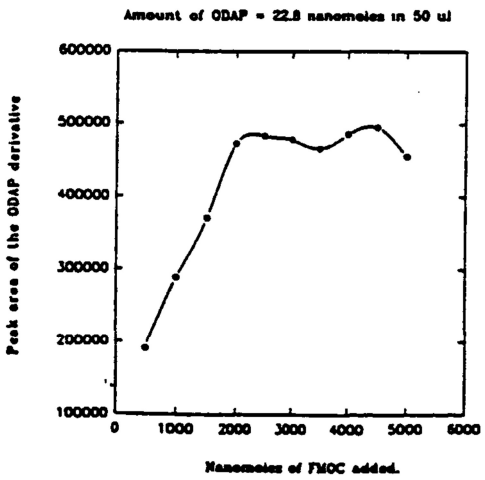


Figure 2. Optimization of the amount of FMOC required for complete derivatization of 22.8 nM ODAP.

Figure 1 shows the calibration curve for the assay and Fig. 2 emphasizes the importance of providing an excess of derivatizing reagent. Figure 3 illustrates the separation of derivatized ODAP from other amino acids in a mixture used as a reference standard for amino acid analysis in the Faculty of Agriculture, University of Manitoba. It shows conclusively that the ODAP-FMOC derivative separates clearly and comes off the column earlier than standard amino acids which are present in a protein hydrolysate. The ODAP-FMOC peak obtained with the pure compound is shown in Figure 4. Both runs were conducted using a μ-Bondapak C_{18} column; the mobile phase was sodium acetate buffer:acetonitrile (72:28) and the flow rate was 1 ml per minute.

Figure 3.
A typical chromatogram of the derivatized amino acid mixture and ODAP-FMOC.

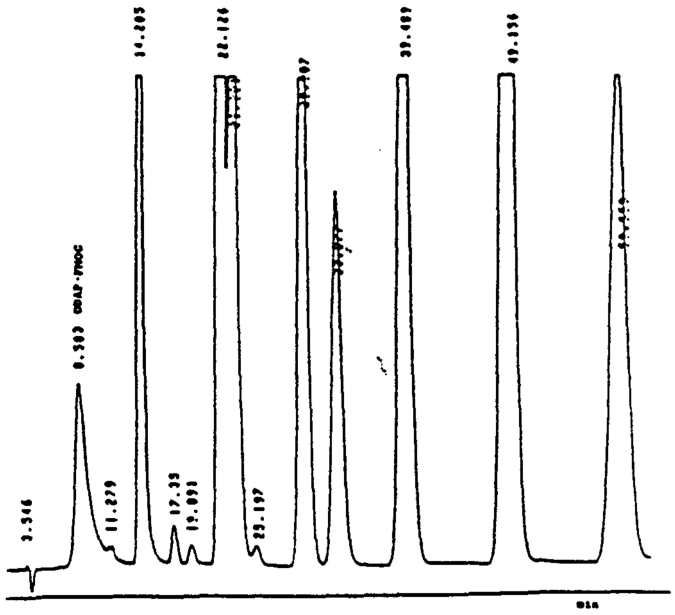
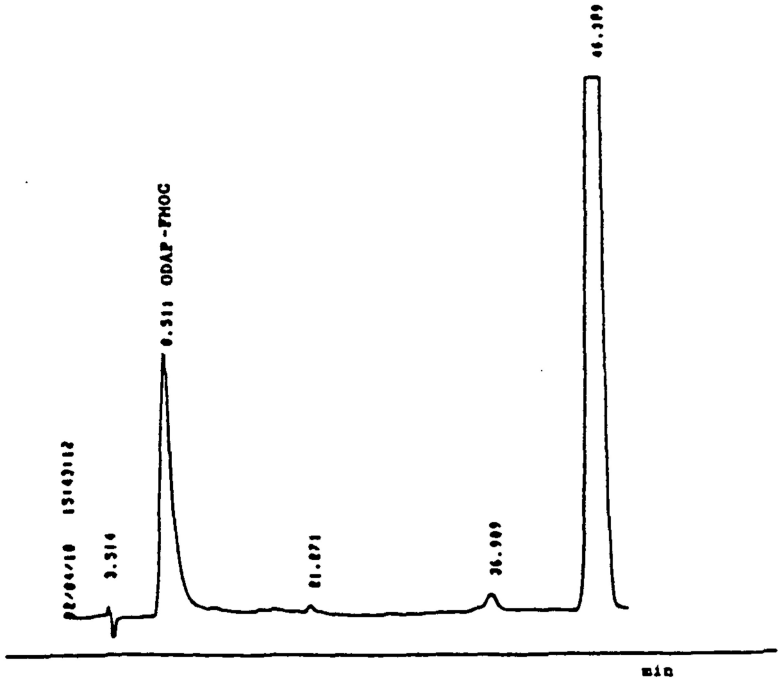


Figure 4.
A typical chromatogram of the ODAP-FMOC derivative.



The initial assays of pig samples produced inconsistent results following extraction apparently due to the fat present in the samples. This was solved by freeze-drying prior to ether extraction of the fat; this would not have compromised the assay since amino acids and ODAP are insoluble in ether. Analyses of samples processed in this way gave more consistent recoveries of ODAP from "spiked" samples.

Early assays also had to use ODAP which was obtained by isolation from seed material and characterization by reference to a standard synthesized sample provided by Dr. Nunn in London, U.K. In late 1992, a commercially available form became available from Sigma Chemical Co., Toronto [Ref. #06255].

The standard spectrophotometric assay, used to determine ODAP content in the diets fed to the pigs, was conducted on samples of raw kidney and pork chop and on defatted kidney and liver samples. In no case did the orthophthalaldehyde produce a quantifiable colour. While the OPT method is appropriate for the determination of microgram quantities of ODAP, it apparently lacks the sensitivity for measuring the compound in animal tissues.

Analytical procedure - HPLC

The published method [Geda et al. 1993] was applied to samples of kidney and pork muscle (*Longissimus dorsi*) from both female and castrated male pigs which had been fed the 0% LM (Control), 21% High-ODAP LM and 25% Low-ODAP diets in Expt. 3. The size of the freeze-dried, de-fatted sample was equivalent to 2 g of the raw tissue after thawing. To demonstrate recovery, samples from Control pigs were "spiked" with the equivalent of 100 or 300 ng ODAP per g prior to extraction. The chromatograms below show the effect of spiking tissue from an LM-fed pig.

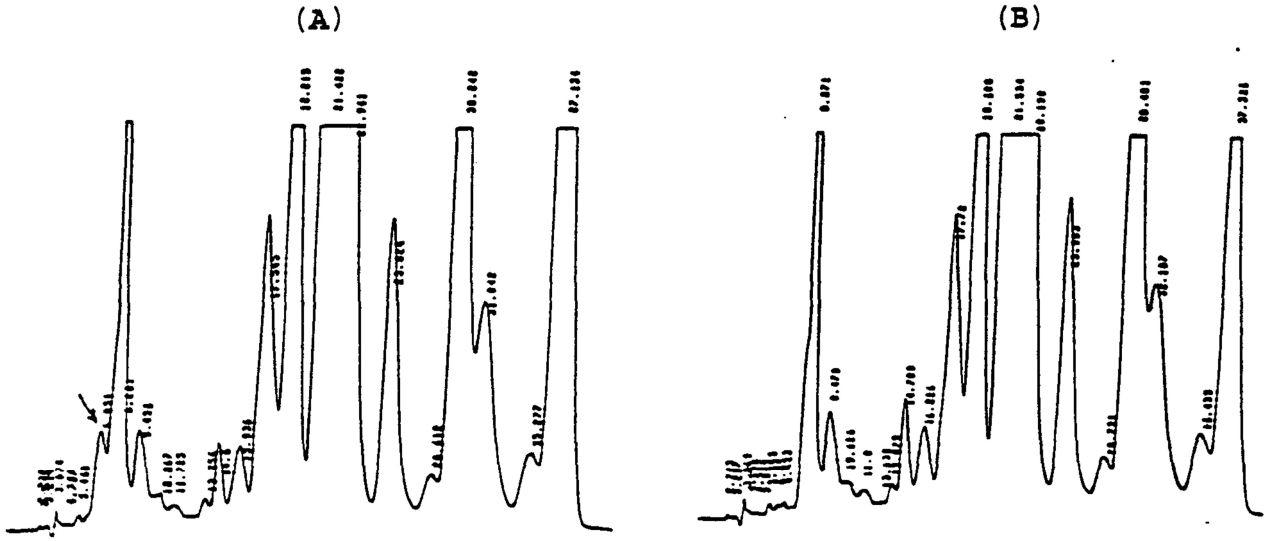


Figure 5. Chromatograms of analysis of defatted chop from male pig fed High-ODAP lathyrus diet: (A) arrow indicates peak when sample spiked with 5 µl ODAP, (B) shows un-spiked sample.

The assay could detect ODAP at a concentration equivalent to 100 ng per g of raw lean meat. It was noted that the traces showed variation in the retention time for the ODAP peak. This appeared to be due to temperature and storage conditions for the column. Thus, in proving the presence or absence of ODAP, care was taken to ensure equivalent equilibration of the column whenever tests were compared with standards. In no case was the neurolathrogen detected in the muscle from any of the lathyrus-fed pigs.

Aqueous extracts of the processed kidney samples contained excessive quantities of pigment and water-soluble extractives which inhibited the assay. Attempts to clarify the solution by filtration through silica or C_{18} reverse phase Sep-pak units were not successful since the resultant solutions still contained some of the interfering water-soluble extractives. It was subsequently found that treatment of the original extract with an equal volume of trichloracetic acid (10%), followed by centifugation (3000 g for 5 min), resulted in a clear solution which could be used to measure free ODAP. Controls were used to demonstrate that processing in this way caused no loss of ODAP. Addition of TCA (Final concentration = 5%) prior to aqueous extraction of the tissue denatured all the protein and enabled free and bound ODAP to be determined.

The extracts, following clarification by TCA, were analysed by the HPLC method. There was no indication of the presence of residual ODAP, determined at a level equivalent to 100 ng per g of raw kidney, from injecting up to 50 μl of derivatized extract. Examination of the chromatograms in the region of the retention time for ODAP found no difference between samples from control and lathyrus-fed pigs.

The Geda assay, which was developed with plant samples, is not as sensitive for animal tissue. To improve its application in meat assays, a concentration/purification step will be required for the ODAP after extraction. Ion exchange resins are probably suitable for this and it is planned to initiate further studies at the University of Manitoba. In addition, samples of all freeze-dried tissues have been sent to Dr. F. Lambein in Ghent, Belgium. His laboratories use an alternative procedure to assay ODAP and its analogues and the results will provide a valuable, independent assessment of ODAP residues in the pig tissues.

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Financial support for various aspects of this pig-feeding and residue analysis study were provided by Agriculture and Agri-Food Canada, IDRC and NATO.

APPENDIX

SCIENTIFIC PUBLICATIONS

1. **Geda, A., Briggs, C.J. and Venkataram, S. 1993.** Determination of the neurolathyrogen b-N-oxalyl-L-a,b-diaminopropionic acid using high-performance liquid chromatography with fluorometric detection. J. Chromat. 635:338-341. (Copy attached)

2. **Castell, A.G., Cliplef, R.L., Briggs, C.J., Campbell, C.G. and Bruni, J.E.** 1994. Evaluation of lathyrus (*Lathyrus sativus* L.) as an ingredient in pig starter and grower diets. Can. J. Anim. Sci. 74: [Manuscript submitted in October, 1993]

3. **Briggs, C.J., Campbell, C.G. and Castell, A.G. 1993.** Analysis of grass pea, *Lathyrus sativus*, and its evaluation as a component of animal feed. Abstr. #9 in Proc. 2nd International Colloquium on Lathyrism "*Lathyrus sativus* and Human Lathyrism, Progress and Prospects", held December 10-12 in Dhaka, Bangladesh.
[Full paper to be published in 1994]

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Short Communication

Determination of the neurolathyrogen β-N-oxalyl-L-α,β-diaminopropionic acid using high-performance liquid chromatography with fluorometric detection

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ABSTRACT

This paper describes a sensitive spectrofluorometric HPLC method suitable for determining picogram levels of β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), a neurotoxin. Aqueous extracts of powdered *Lathyrus sativus* seeds were treated with 9-fluorenylmethyl chloroformate (FMOC) and the ODAP–FMOC derivative analyzed by reversed-phase chromatography using a μBondapak C_{18} column. The excitation and emission wavelengths were 254 and 315 nm, respectively. The mobile phase was sodium acetate buffer (0.05 *M*, pH 6.35)–acetonitrile (72:28, v/v) at a flow-rate of 1 ml/min. This method represents a major advance over the standard spectrophotometric assays used currently.

INTRODUCTION

Lathyrism [1,2], a motor-neurone disease in humans, is associated with excess consumption of β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), also known as BOAA [1] or by the preferred IUPAC name L-2-amino-3-oxalyl-aminopropanoic acid [3–6]. This compound is present in the seed of *Lathyrus sativus* L., the grass pea, a legume cultivated in India, Bangladesh and Ethiopia. It grows in poor soil and is resistant to salt, flood and drought [7–9]. It is a staple food which is rich in protein. Lathyrus constitutes a health hazard when alternative food is in limited supply [4,10–12]. The concentration of ODAP in seed is genetically controlled and modified by environmental factors. It can range from 0.1–0.4% in the dry seed [13].

Several methods have been described for determination of ODAP including electrophoresis, spectroscopy and high-performance liquid chromatography (HPLC) [14–18]. In this paper a simple and precise method for the estimation of ODAP in Lathyrus seed is presented based on a HPLC–spectrofluorometric technique reported earlier [18] which used gradient chromatography. The method reported here is sensitive, reprodu-

* Corresponding author.

cible and utilizes precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC) [19] followed by reversed-phase HPLC with fluorescence detection. The system is suitable for determination of ODAP in seeds and could be used to assay ODAP in animal tissue at sub-microgram levels.

MATERIALS AND METHODS

The reference standard of ODAP was provided by Dr. P. Nunn, Kings College, London, UK. *Lathyrus sativus* seeds were obtained from Dr. C. Campbell, Agriculture Canada Research Station, Morden, Manitoba, Canada. A standard amino acid mixture was obtained from Sigma (St. Louis, MO, USA). FMOC was purchased from Pierce (Rockford, IL, USA). All other chemicals were analytical grade and solvents were HPLC grade from Fisher Scientific, Canada. Purified water was produced using a Millipore Milli-Q unit. Purified water was produced using a Millipore Milli-Q unit. Aqueous solvents were filtered through a 0.45-μm membrane prior to use.

HPLC analysis

All chromatographic studies utilized a Waters HPLC system with a M45 pump and a U6K injector. A 300 × 3.9 mm stainless steel μBondapak C_{18} column, 10 μm particle size, (Waters Chromatography Division, Millipore) was used. A Shimadzu RF-535 variable-wavelength spectrofluorometer with a CR 501 integrating recorder was used for collection of data and their analysis. The excitation and emission wavelengths were 254 and 315 nm, respectively. The FMOC derivatives of sample and standard were eluted using sodium acetate buffer (0.05 *M*, pH 3.65)–acetonitrile (72:28, v/v) as the mobile phase at a flow-rate of 1 ml/min at ambient temperature of 23°C.

Preparation of derivative

A known amount of ODAP or sample was derivatized by the addition of 1 ml borate buffer (0.025 *M*, pH 9.6), 1 ml acetone and 0.1 ml FMOC (0.1 *M*, freshly prepared in acetone). The tube was vortex mixed for 2 min and derivatization was complete in 30 min at room temperature. A 2-ml volume of hexane–ethyl acetate (1:1) was added, vortex mixed for 30 s and 1.3 ml of aqueous layer was collected. Since the ODAP–FMOC derivative is soluble in acetone and insoluble in hexane, diethyl ether, ethyl acetate and chloroform, the aqueous layer was taken for HPLC injection. To optimize the derivatization conditions, concentrations of FMOC up to 1.0 *M* were studied. Similarly, 2,3-diaminopropanoic acid (DAP) and a standard mixture of 17 amino acids were also derivatized and analyzed.

Preparation of lathyrus seed extract

Aqueous extract of seed material was prepared from 10–100 mg of seed powder. A 2-ml volume of water was added and the sample was placed on a mechanical shaker for 12 h. The aqueous solution was separated after centrifugation (10 min at 3000 *g* in an IEC clinical centrifuge) and subsequently filtered through a 5-μm membrane; 5–10 μl of the extract were used for derivatization and 10 μl of the product were taken for HPLC analysis.

RESULTS AND DISCUSSION

As shown in Fig. 1, the retention time for the ODAP–FMOC derivative was 8.4 min and for unreacted FMOC 48.4 min. Peak areas corresponding to ODAP–FMOC were recorded. The precision of the method was assessed by repeated analyses of samples containing known concentrations of ODAP. The relative standard deviation for within-day precision ranged from 6.3 to 8.7% ($n = 4$). The day-to-day variation observed in peak area was in the range of 8.1 to 11.5%. There was also some variation in the retention time of the ODAP–FMOC peak ranging from 8.1 to 8.6 min. Hence standard samples of known ODAP concentration were required on each day. The ODAP–FMOC derivative eluted before the DAP–FMOC or any of the amino acid–FMOC derivatives from a lathyrus extract or standard plant amino acid mixture. The reason for selecting DAP and the amino acid mixture was to identify any possible interference

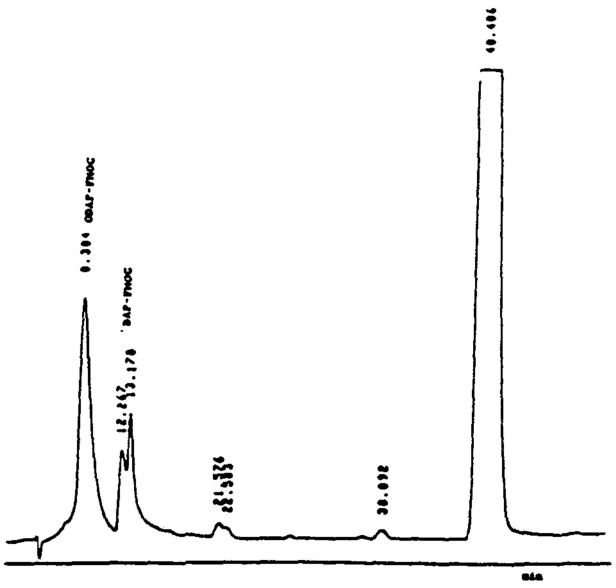

Fig. 1. A typical chromatogram of ODAP-FMOC and DAP-FMOC derivatives using a μBondapak C_{18} column.

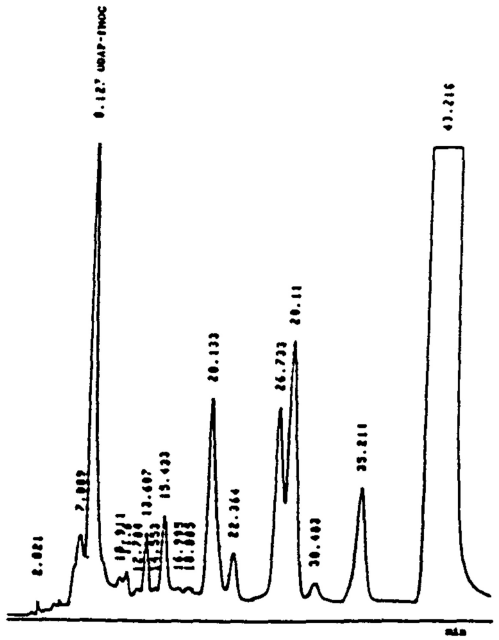

Fig. 2. A typical chromatogram of the FMOC-derivatized seed extract using μBondapak C_{18} column.

from these compounds with the ODAP–FMOC peak. Furthermore, DAP is the hydrolysis product of ODAP and any aqueous plant extract would contain amino acids as normal constituents. DAP exists in two isomeric forms and the doublet peaks at 12.3 and 13.2 min possibly correspond to their FMOC derivatives.

The amount of FMOC selected for derivatization was based on preliminary experiments which showed that the molar concentration of FMOC should be at least 100 times that of ODAP. Hence, 0.1 ml of a 0.1 *M* solution was used and found adequate for the reaction to be complete.

A typical chromatogram of the derivatized seed extract is shown in Fig. 2. It shows the presence of a number of peaks including the FMOC derivatives of amino acids, secondary metabolites and other normal constituents of an aqueous seed extract [20,21]. To reduce the total elution time of these compounds the column was flushed with pure acetonitrile 20 min after injection. The system was then re-equilibrated with 45 ml of the mobile phase before the next injection. Concentrations were determined by estimation of peak areas with reference to calibration curve for derivatized ODAP (correlation coefficient = 0.9745, intercept = −32.191 and the slope = 27543.6, linear up to 14 nmol of ODAP). The detection limit was found to be 15 pmol on column.

This HPLC–spectrofluorometric procedure showed that the average ODAP content of the *Lathyrus sativus* seed samples studied was 0.33 g/100 g of seed material. Breeding programs designed to eliminate ODAP from lathyrus seed may adopt this method for analysis of seed with very low levels of ODAP.

CONCLUSIONS

HPLC combined with spectrofluorometry can be used for the detection and quantitative estimation of pmol amounts of ODAP in Lathyrus seed. This technique is suitable for estimation of neurotoxic content of individual seeds from a single pod which is desirable for selection and breeding Lathyrus varieties with low ODAP content. This represents a major advance over spectrophotometric methods conventionally used to assay this compound in plant material.

ACKNOWLEDGEMENTS

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Second International Colloquium on Lathyrism in Bangladesh

LATHYRUS SATIVUS AND HUMAN LATHYRISM. PROGRESS AND PROSPECTS
10-12 December, 1993

PROGRAMME

Friday 10th December 1993

9.00-10.00	Registration
10.00-12.00	Inauguration (Venue: Dhaka Seraton)
12.00-15.00	Lunch
15.00-15.45	**Session I: CHEMISTRY & BIOCHEMISTRY** Chairman : Dr. S.L.N. Rao. Co-chairman : Dr. B. Abegaz Venue : Sheraton
15.00-15.15	**F. Ikegami, Faculty of Pharmaceutical Sciences, Chiba University.** Biosynthesis in vitro of some Lathyrus toxins.
15.15-15.30	**Y.-H Kuo, Laboratory of Physiological Chemistry, University of Ghent.** Biosynthesis in vivo of the neurotoxin β-ODAP in Lathyrus sativus.
15.30-15.45	**P.B. Nunn, Division of Biomedical Sciences, King's College, London.** Involvement of an imidazolidine derivative during the rearrangement of the neurotoxin β-ODAP.
15.45-17.00	**Session II : EPIDEMIOLOGY, SOCIO-ECONOMIC ASPECTS** Chairman : Dr. A. Haque. Co-chairman : Dr. R.T. Haimanot
15.:45-16.00	**R.T. Haimanot, Faculty of Medicine, Addis Ababa University.** Nutritional and neurotoxicological surveys of Lathyrus sativus consumption in Northwest Ethiopia.
16.00-16.15 :	**R.L. Pandey, Indira Gandhi Agricultural University.** Studies of Socio-economic strata, Lathyrus consumption and its effects on rural population in Madhya Pradesh, India.
16.15-16.30 :	**M. Hossain, Institute for Postgraduate Medicine and Research, Dhaka.** Epidemiological study of Lathyrism in north-western disiricts of Bangladesh.
16.45-17.00	**M.P. Dwivedi, National Leprosy Eradication Programme, Bhopal.** Legal status of Khesari consumption in India.
18.30-21.00	**Informal Reception**

Saturday 11th December 1993, Venue : IPGMR

08.30-09.00	**Arranging posters**
09.00-10.00	**Session III : ANALYSIS OF TOXICITY** Chairman : Dr. C.J. Briggs. Co-Chairman : Dr. H.K.M. Yusuf
09.00-09.15	**J.K. Khan, Laboratory of Physiological Chemistry, University of Ghent.** High-performance liquid chromatographic separation of the toxins and some nonprotein amino acids in Lathyrus sativus.
09.15-09.30	**C.J. Briggs, Faculty of Pharmacy, University of Manitoba.** Analysis of grass pea, Lathyrus sativus and its evaluation as a component of animal feed.
09.30-09.45	**G. Moges, Department of Analytical Chemistry, University of Lund, Sweden.** To be announced.
09.45-10.00	**B. Abegaz, Department of Chemistry, Addis Ababa University,** to be announced.
10.00-10.30	**Session IV: TOXICITY OF RELATED LEGUMES** Chairman: Dr. E. A. Bell. Co-chairman: Dr. F. Ikegami.
10.00-10.15:	**D. Enneking, CLIMA, University of Western Australia** Post-harvest detoxification. The key to alternative Vicia grain legumes?
10.15-10.30	**M.E. Tate, Waite Agricultural Research Institute, University of Adelaide.** Towards the detoxification of Vicia sativa L.
10.30-11.00	Tea break and Poster Session.

ANALYSIS OF GRASS PEA, LATHYRUS SATIVUS, AND ITS EVALUATION AS A COMPONENT OF ANIMAL FEED.

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Lathyrus sativus (L), the grass pea or khessari, is a widely cultivated, high yielding, drought resistant legume. The seeds are consumed as food in India, Bangladesh, Pakistan, Nepal, China, Ethiopia and nearby countries. The plant and seeds are also used to feed animals. The species contains a neurotoxin, ß-N-oxalyl- ∝ -ß diaminopropionic acid (ODAP) and nutrition - inhibiting anti-proteases. Cultivars are being developed in which ODAP has been reduced or eliminated. Subsequent crossing should give varieties suitable as approved food and feed crops.

Morden Research Station has developed several lines with potential for release. In addition to standard multigeneration agronomic assessment, studies were required to determine the suitablility of lathyrus meal for animal feeds. Low ODAP (0.68mg/g) and high ODAP (2.02mg/g) seed were selected using the routine σ-phthalaldehyde spectrophotometric assay used in Manitoba (Campbell Method). These were used in feeding studies with feeder pigs (25.6kg, 69days) taken to market weight (100kg). Seven quartets (2 male castrates and 2 unrelated females) were assigned to pens, replicated 3 times. Seven mash diets were prepared containing high or low ODAP lathyrus at O, 7, 14 or 21% of the mix. Performance on the diets was assessed and quality parameters determined after slaughter.

In a second study, pelleted extruded feed at 0, 7 and 14% high ODAP lathyrus meal was compared with 25% low ODAP pellets, alone and supplemented with 0.1 or 0.2% methionine. Samples of lean meat and kidney were assayed for ODAP residues by HPLC at the conclusion of the study.

Increasing the lathyrus content of feed significantly reduced daily weight gain, but pelleting resulted in higher growth rates. Lathyrus levels had no effect on conversion and methionine did not improve performance. Diet did not affect carcass grade and ODAP residues were not detected. Meat quality was mainly unaffected, but increased lathyrus levels produced heavier liver and kidneys, less-saturated backfat and an increase in loin fat. Neurotoxicity was not observed and there was no correlation between ODAP levels and performance. Antiprotease levels were reduced by pelleting.

Lathyrus meal could be an alternative to other pulses in pig feed, with inclusion rate limited to 10-20% to minimise adverse effects.

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NATURAL TOXINS 1:246–249 (1993)

Toxins in the Seedlings of Some Varieties of Grass Pea (*Lathyrus sativus*)

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Laboratory of Physiological Chemistry, University of Ghent (F.L., J.K.K., Y.-H.K.), Ghent, Belgium; Agriculture Canada (C.G.C.), Morden, Manitoba, Canada; Faculty of Pharmacy, University of Manitoba (C.J.B.), Winnipeg, Manitoba, Canada

ABSTRACT The major toxin present in the dry seeds and seedlings of *Lathyrus sativus* is the neurotoxin 3-N-oxalyl-L-2,3-diaminopropanoic acid (β-ODAP). The presence of one additional neurotoxin and an osteotoxin in the seedlings increases the overall toxicity. Isolation, purification, and detection of these toxins are described.

Key Words: β-ODAP, Isoxazolinones, Neurolathyrism, Osteolathyrism, Non-protein amino acids

INTRODUCTION

Grass pea (*Lathyrus sativus, Khesari* in India and Bangladesh, *Guaya* in Ethiopia, *pois carré* in France) is the well established cause of human lathyrism, an irreversible spastic paraparesis. While the ripe seeds containing the neurotoxin 3-N-oxalyl-L-2,3-diaminopropanoic acid (β-ODAP) are a popular food in those areas afflicted with lathyrism, the young tender shoots are often used as a vegetable. Among the toxic *Lathyrus* species the seedling is normally the most toxic stage containing higher concentrations of toxins than the ripe seeds [Ressler, 1964]. In addition to abundant free amino acids, including many non-protein amino acids, the seedlings of most *Lathyrus* species contain high concentrations of isoxazolin-5-one derivatives. These heterocyclic compounds are virtually absent in the ripe seeds but are formed rapidly during the early stages of germination sometimes reaching 10% of the dry matter in the young seedling axes [Lambein et al., 1976]. These isoxazolinones are unstable in alkaline solutions and are sensitive to UV-light. A mechanism for this UV-degradation was proposed recently [De Bruyn et al., 1992].

Two isoxazolin-5-one derivatives from *L. odoratus* have been reported to be toxic to experimental animals: α-amino-γ-(isoxazolin-5-on-2-yl)-butyric acid (compound VI), which produces symptoms identical to those produced by the neurolathyrogen α,γ-diaminobutyric acid (DABA), and 2-cyanoethyl-isoxazolin-5-one (compound VIII), which produces the symptoms of osteolathyrism in young rats and chicks in which it can be metabolised to the known osteolathyrogen β-aminopropionitrile (BAPN) [Lambein and De Vos, 1981].

Compound VI seems to be specific for the genus *Lathyrus*. In axes of 6-day-old seedlings of *L. odoratus* compound VI could reach 3.5% of the dry weight [Lambein et al., 1986]. In older plants the concentration of VI is much less but relatively high concentrations are still found in the roots, in the flower buds, and in immature seeds. Compound VIII is usually not found in the seed extracts but is found in small amounts in the imbibition water of *L. odoratus* [Kuo et al., 1987]. The highest concentrations are found in the reproductive parts of the plant, flower buds, petals, and immature pods. The root exudation by axenic etiolated seedlings of *Lathyrus odoratus* has been studied in detail [Kuo et al., 1982]. The main compounds released are compound VI and β-(isoxazolin-5-on-2-yl)-alanine (compound I), while compound VIII is found in highest concentration. As compound VIII is nonionic its solubility is very different from the amino acids I and VI. It is soluble in water, chloroform, and even slightly in hexane and probably it can pass freely through lipid membranes.

The presence of compound VIII in the dry seeds of *L. sativus* in low concentrations may be responsible for the minor skeletal lesions observed in some older lathyrism patients [Cohn, 1986]. Both compounds VI and VIII have been identified in seedlings of *L. sativus* [Lambein et al., 1992]. As these compounds may add to the overall

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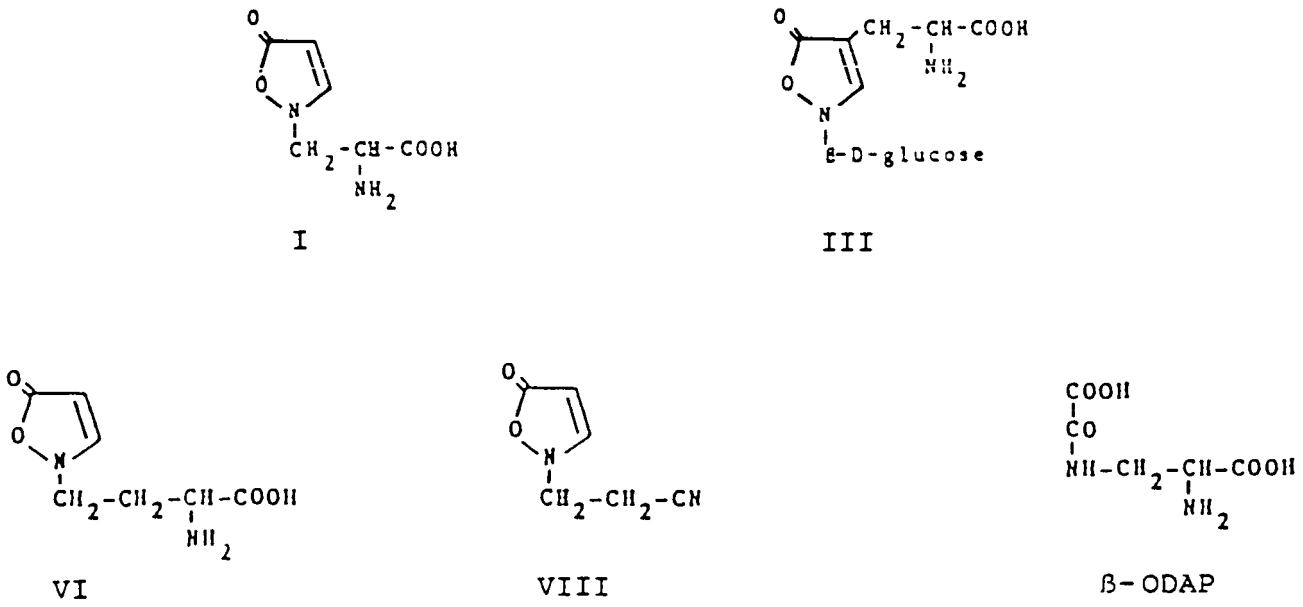

Scheme 1. Compounds I, III, VI, VIII, and β-ODAP.

toxicity of the shoots used as vegetable or as fodder, their occurrence in a number of varieties selected on the basis of the toxic levels in the seeds was studied.

MATERIALS AND METHODS

Plant Material and Chemicals

Seeds of *Lathyrus sativus* (Cv LS 87041, LS 8246, LS 82046, LS 8545(s), Nc8a 17/w, LS 90278, LS 90239, Nc8a 97/1, Nc8a 17/1, LS 8545(w)) were obtained from Agriculture Canada experimental station (Morden, Manitoba). ADES, Jamalpur, and Kathmandu are landraces originating respectively from Ethiopia, Bangladesh and Nepal. Hydrochloric acid (37%) from Merck, analytical grade; cation exchange resin, (AG 50W-X8) 200–400 mesh [H^+-form], and analytical grade anion exchange resin (AG1-X4) 200–400 mesh [Cl^--form] were obtained from Biorad.

Extraction and Isolation

For the isolation of α-amino-γ-(isoxazolin-5-on-2-yl)-butyric acid (VI) and 2-cyanoethyl-isoxazolin-5-one (VIII) 3-day-old seedlings of *Lathyrus sativus* grown in the dark at 25°C were used. Sixty-four grams of seedlings were extracted with 500 ml 70% ethanol and the extract was kept standing overnight at 4°C. It was then centrifuged at 17,750*g* for 20 min. The supernatant was concentrated under vacuum to approximately 200 ml. The extract was placed on a cation exchange column AG 50W-X8 [H^+-form]. Neutral compounds including compound VIII were eluted with 1 liter of water. The fractions were monitored at 254 nm (LKB Uvicord) and fractions of 20 ml were collected. A linear gradient of HCl (0–2 M) was then used to obtain compound VI from the Dowex 50 column. A total volume of 10 liters was used for a bed volume of 500 ml. After 3 liters of effluent a UV-absorbing peak was eluted containing β-(isoxazolin-2-glucosyl-5-on-4-yl)-alanine (III) [Lambein et al., 1970]. At 0.6 M HCl the β-(isoxazolin-5-on-2-yl)-alanine (I) peak eluted while compound VI eluted at 1.5 M HCl. The fractions containing UV-absorbing peaks were pooled and concentrated under vacuum. Further purification was done on an anion exchange column AG1-X4 [CH_3COO^--form] by eluting with water.

The fractions containing compound VI were pooled and concentrated under vacuum at 45°C and HCl was removed by repeated addition of aliquots of H_2O and drying. This material was further purified by passing over an anion exchange column AG1X4 [CH_3COO^--form] from which it was eluted with water. After concentrating under reduced pressure the compound was crystallized from ethanol/water. From 64 g of fresh seedlings 60 mg of crystalline compound VI and 0.05 ml of over 95% pure compound VIII as a clear colorless liquid were obtained. For estimation of β-ODAP in the dry seeds 100 mg of seed powder was extracted overnight at 4°C with 5 ml 70% ethanol. The extract was centrifuged at 17,750*g* for 20 min. The pellets were washed twice with 2 ml 70% ethanol. The supernatants were pooled, concentrated, and deproteinized with 5-sulphosalicylic acid before injecting into the amino acid analyzer.

Detection of α-Amino-γ-(Isoxazolin-5-on-2-yl)-Butyric Acid (VI) and 2-Cyanoethyl-Isoxazolin-5-One (VIII) by Liquid Chromatography

Instrumentation

Sykam S432 amino acid analyzer with on-line UV-detection (linear UV-106) followed by post-column ninhydrin reactor. Two C-R6A chromatopac (Shimadzu)

integrators were used for data acquisition of the signals at 254 nm before post column derivatisation and at 570 nm after post column derivatisation with ninhydrin.

Solvent System

Five lithium buffers were used. Buffer A (pH 2.75, 0.15 M Li^+) 33°C, 0–38 min; buffer B (pH 3.10, 0.15 M Li^+) 33°C, 38.1–71.4 min; buffer C (pH 3.50, 0.15 M Li^+) 33°C, 71.5–77 min and 74°C, 77.1–100.9 min; buffer D (pH 4.00, 0.60 M Li^+) 74°C, 101.0–123.9 min; and buffer E (pH 3.30, 1.4 M Li^+) 74°C, 124–200 min.

All the buffers contained Li-citrate (14.1 g/liter for A to C, 18.8 g/liter for D and E). Buffers A to C contained citric acid (5 g/liter), buffers D and E contained LiCl (16.9 g/liter and 50.7 g/liter respectively). Methanol (50 ml/liter) was added in buffers A and B. For all buffers 0.1 % EDTA and BRIJ (25%, 1 ml/liter) were included. The pH was adjusted with concentrated HCl (37%). After each analysis the column was regenerated with LiOH 0.3 M for 15 min at 74°C, followed by buffer A for 25 min at 33°C. Before injection the system was equilibrated for 15 min with buffer A and ninhydrin flow. Flow rate was 0.4 ml/min for the buffer and 0.2 ml/min for the ninhydrin.

Column

Amino acid column (15 cm × 4 mm) polystyrene-divinylbenzene (LCA K04), a strong cation-exchanger in lithium form, was used.

RESULTS AND DISCUSSION

In *Lathyrus sativus* seedlings the major toxin is 3-N-oxalyl-L-2,3-diaminopropanoic acid (β-ODAP), which is responsible for the neurological disorder called lathyrism [Haimanot et al., 1990]. On a dry-weight basis the concentration of β-ODAP in the seedlings is usually about two to three times higher than in the dry seeds. The presence of two additional toxic compounds makes the seedlings more toxic than the dry seeds in which these heterocyclic compounds are not detected.

Compounds VIII and VI can easily be identified by their sensitivity to UV-light and to dilute alkali. On TLC and paper chromatography compound VIII gives a distinct light-blue color after spraying with ninhydrin and heating. On the amino acid analyzer compound VI elutes from the column at pH 3.30 with retention time of 70 min and can easily be 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 with retention time of 9 min, but can only be detected at 254 nm as there is no free NH_2-group to react with ninhydrin.

The biosynthesis of isoxazolinone VI and VIII had been studied in vivo in *Lathyrus odoratus* with [^{14}C]-labelled precursors. When [carboxy-^{14}C]-S-adenosyl-methionine (SAM) labeled at the C—2 position of the methionine moiety is fed through the root of intact seedlings of *L. odoratus* part of the label was incorporated into compounds VI and VIII and γ-glutamyl-BAPN. It was postulated that compound VI is an intermediate in a biosynthesis pathway from SAM through compounds VI and VIII to BAPN and its γ-glutamyl derivative [Lambein et al., 1986]. Such a hypothetical pathway is corroborated by the distribution pattern of these compounds within the genus. Both compounds VI and VIII are present in species also containing BAPN or γ-glutamyl-BAPN, *L. venosus* being the only exception. Compounds VI and VIII are together in eight of 50 species and VIII without VI is present in three species. Further studies on the biosynthesis of the toxins in *Lathyrus sativus* are under way.

In several regions of Asia and Africa the seeds of *Lathyrus sativus* are a popular foodstuff. In some areas of Bangladesh the fresh green shoots are consumed as a vegetable. The concentration of various toxins in these shoots will depend on the age of the shoot and the potential danger of this habit remains to be evaluated. The current programs for selection of safe *Lathyrus* strains mainly or exclusively focus on the β-ODAP content of the dry seeds. The heterocyclic compounds described above add considerably to the overall toxicity of *L. sativus* seedlings and potentially of the shoots used as vegetable in some areas.

In Table I we compared the levels of the additional toxins VI and VIII in six low-toxin (i.e. low β-ODAP) lines and three high-toxin lines selected at the Morden Agricultural Station on the basis of their β-ODAP content in the dry seeds and in three land races from Bangladesh, Ethiopia, and Nepal. Although there probably is a biochemical link between β-ODAP and the additional toxins VI and VIII, this is not obvious from the data presented in Table I, which seem to be erratic.

Among the low-toxin lines two have markedly lower levels of compounds VI and VIII than the wild types while all others score equally or higher than the wild types. On the basis of the total toxin levels in the seedlings (β-ODAP + VI + VIII) the cultivars LS 82046 and LS 8545(s) are by far the most promising lines of *Lathyrus sativus* we have studied so far.

When comparing the levels of the toxic isoxazolinones VI and VIII (Table II), it is noteworthy that the ratio of the metabolically related compounds is around 2:1 [± 0.5] in most lines. In some low toxin lines however this factor is doubled.

The above results indicate a high biochemical variability in the species *Lathyrus sativus* and suggest that the characters for the different toxins present in the seedling are not genetically linked. The lines selected for low β-ODAP content are not necessarily low in the additional toxins VI and VIII. The techniques of breeding, mutation, and genetic engineering may be needed in order to produce safe lines of this promising but still risky crop.

TABLE I. Compounds VI and VIII in Different Lines of *Lathyrus sativus* With low and High β-ODAP Content in 3-day-old Seedlings

	β-ODAP	VI	VIII
Low toxin line			
LS8246	(0.66)	26.69	6.60
LS82046	(0.29)	6.66	1.28
LS8545(s)	(0.54)	5.11	2.85
LS8545(w)	(0.79)	13.82	8.72
LS90278	(0.34)	24.26	5.70
LS90239	(0.40)	29.81	4.90
Average		17.72	5.00
High toxin line			
Nc8a97/1	(6.30)	28.55	11.51
Nc8a17/1	(6.48)	49.19	23.36
Nc8a17/w	(3.99)	23.49	15.49
Average		33.74	16.78
Wild types			
ADES	(5.50)	18.16	9.49
Jamalpur	(5.92)	12.55	6.76
Kathmandu	(7.16)	17.98	8.51
LA-4	(7.15)	10.30	3.68
Average		14.74	7.11

The β-ODAP concentrations in the dry seeds are given in parentheses.
Values are expressed in mg/g dry weight.
Lines 8246 and 82046 are the same. 8246 has been stored for 2 years while 82046 are fresh seeds. Lines LS8545(s) and LS8545(w) are from the same origin but separated into white (w) and dark spotted (s) individual seeds.

TABLE II. Ratio of Compounds VI and VIII in Seedlings

	Ratio of VI/VIII
Low toxin line	
LS8246	4.04
LS82046	5.20
LS8545(s)	1.79
LS8545(w)	1.58
LS90278	4.25
LS90239	6.08
Average	3.82
High toxin line	
Nc8a 97/1	2.48
Nc8a 17/1	2.10
Nc8a 17/w	1.51
Average	2.03
Wild types	
ADES	1.91
Jamalpur	1.85
Kathmandu	2.11
LA-4	2.79
Average	2.16

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