CHROM. 4175

PHYTOCHEMICAL INVESTIGATION OF MANITOBA PLANTS

II. A GAS–LIQUID CHROMATOGRAPHIC SCREENING TECHNIQUE FOR THE IDENTIFICATION OF THE ALKALOIDS OF *PHALARIS* SPECIES

R. C. S. AUDETTE, J. BOLAN, H. M. VIJAYANAGAR AND R. BILOUS
School of Pharmacy, University of Manitoba, Winnipeg 19, Manitoba (Canada)
AND
K. CLARK
Plant Science Department, University of Manitoba, Winnipeg 19, Manitoba (Canada)

(Received May 6th, 1969)

SUMMARY

A gas–liquid chromatographic screening technique has been developed to identify the alkaloids of *Phalaris* species. Hordenine, and the following nine indole alkaloids, gramine, N,N-dimethyl-5-methoxy-tryptamine, N,N-dimethyl tryptamine, 5-methyl tryptamine, N-methyl tryptamine, tryptamine, 5-methoxy-tryptamine, 5-methoxy-N-methyl tryptamine and bufotenine were separated on three columns. Crude alkaloid fractions of three varieties of *Phalaris arundinacea* separated into seven components on the three columns employed.

INTRODUCTION

The deleterious effects including alkaloidal-type lesions on livers of cattle, examined in post-mortem, produced in livestock grazing on *Phalaris arundinacea* prompted us to investigate the alkaloids of these species[1]. Severe neurological disorders, the "staggers syndrome", and in an acute form, sudden deaths, have occurred among livestock grazing upon a related species, *Phalaris tuberosa*, in Australia[2]. This species has been reported to contain several N,N-dimethylated indolealkylamines, notably, N,N-dimethyl tryptamine, 5-methoxy-N,N-dimethyl tryptamine and 5-hydroxy-N,N-dimethyl tryptamine[3].

Australian grown *P. arundinacea* has been reported to contain two indolealkylamines, gramine and 5-methoxy-N-methyl tryptamine, and the phenylethylamine, hordenine[4]. In addition to gramine and hordenine, a new indole alkaloid, 2,9-dimethyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline has been tentatively identified from *P. arundinacea* var. Ottawa synthetic C grown in Manitoba[1].

The present study was undertaken to establish an effective means of identifying the alkaloid constituents of *Phalaris* species and to serve as a potential screening method for determining the alkaloidal components of *P. arundinacea*.

EXPERIMENTAL

A Beckman model GC-5 gas chromatograph, equipped with a flame ionization detector and a Beckman 1 mV linear potentiometric recorder was used. Helium was used as the carrier gas.

Preliminary experiments showed that supports which had been acid washed and treated with dimethyldichlorosilane gave the best results. The columns used by HOLMSTEDT AND LINDGREN[5] were tested, but the retention times and peak symmetry were found to be dependent on the amount of sample injected. Base washed supports were also tested, but were found to produce excessive tailing, especially with hydroxylated compounds. Glass columns were tried, but Teflon[6] was found to give comparable results, and was used throughout the work.

The column packings used were 1% diethyleneglycol succinate (DEGS) on Chromosorb W, AW-DMCS, 80–100 mesh; 0.5% 1-hydroxy-2-heptadecenyl imidazoline (Amine 220) on Chromosorb G, AW-DMCS, 60–80 mesh and 0.4% cyclohexanedimethanol succinate (CHDMS) on Chromosorb G, AW-DMCS, 60–80 mesh.

The Chromosorb G packings were prepared by soaking, with stirring, one part by weight of the support, in five parts by volume of a solution containing double the required percentage of liquid phase in a suitable solvent. After 15 min, the mixture was vacuum filtered, and the damp packing was dried, in a stream of dry nitrogen preheated to 55°, in a fluid bed dryer. The Chromosorb W packing was prepared by soaking 20 g. Chromosorb W, AW-DMCS in a solution containing 0.2% DEGS and subsequently evaporating the mixture to dryness in a vacuum rotary evaporator. After drying, the packings were screened to remove fines which had formed during the coating of the supports.

The columns, 6 ft. long, 1/4 in. O.D., 1/8 in. I.D., Teflon 6 tubes, were packed before coiling. The columns were conditioned at the maximum usable temperature for the relevant liquid phase, while helium was passed through at 100 ml/min until a satisfactory base line was obtained. A glass liner was used at the injection port.

In order to minimize tailing of the compounds, 10 μl Silyl 8 (Pierce Chem. Co.) was injected onto the Amine 220 and DEGS columns. This also had the effect of reducing the retention times of some compounds.

The alkaloids, as the free base, were dissolved in a suitable solvent and injected with a Hamilton 0–10 μl syringe. Gramine and 5-methoxy-tryptamine were dissolved in chloroform, all other standards were dissolved in peroxide-free ether. The free bases of 5-methyl tryptamine, tryptamine, hordenine, 5-methoxy-N-methyl tryptamine and bufotenine were prepared by dissolving the corresponding salt in water, making alkaline with saturated sodium carbonate solution and extracting by shaking with the relevant solvent. The extracts were dried over anhydrous sodium sulphate for 30 min, filtered, and the solvent evaporated to dryness. The residues were redissolved in 10–50 μl of the relevant solvent. Of each standard 1–5 μl were injected individually on the three columns. Mixtures of all the standard compounds were also tested and satisfactory separation was obtained on the columns employed.

Extracts of *P. arundinacea* L. var. Ottawa synthetic C, Ottawa synthetic F (Grove) and Frontier, were prepared from the minced, dried grass by a slight adaptation of the method of CULVENOR *et al.*[4].

Of each variety 20 g were individually extracted with ethanol for 24 h in a

Soxhlet apparatus. The ethanol was removed in a vacuum rotary evaporator and the dry residue was dissolved by shaking with alternate portions of chloroform and dilute sulphuric acid. The chloroform portions were mixed and exhaustively extracted by shaking, in a separatory funnel, with several portions of dilute sulphuric acid. The acid portions were mixed and washed with chloroform to remove traces of coloured material. The acid part was saturated with sodium chloride and made alkaline with ammonium hydroxide solution (pH 9). This mixture was extracted by shaking with several portions of chloroform, until an aliquot of the chloroform extract, spotted onto a TLC Silica Gel G plate, gave negative reactions when sprayed with Van Urk's reagent (1% *p*-dimethylaminobenzaldehyde in hydrochloric acid–95% ethanol, 1:1) and Dragendorff's spray reagent[7]. The chloroform portions were mixed, dried over anhydrous sodium sulphate for 1 h, filtered and evaporated to dryness in a vacuum rotary evaporator.

Each residue was dissolved in 0.5 ml chloroform. All three extracts were injected individually on the three columns and identification of the major components was attempted by comparison with the retention times of the standard compounds, and by admixing individually with the extracts, those compounds having a similar retention time to a component of the extracts.

GLC fractions of Ottawa synthetic C were collected by placing an all glass stream splitter at the outlet end of the column. The stream splitter was constructed by inserting one arm of a glass T piece into the end of the column and connecting the other two arms to the detector and collection outlets with two pieces of 1 mm I.D. glass capillary tubing. Constrictions were made in the capillary tubing so that one-tenth of the carrier gas was carried to the detector, and nine-tenths to the collection outlet. Make-up helium gas was added to the hydrogen gas flowing to the detector in order to optimise flame sensitivity. A glass collection vessel, containing chips of glass, was cooled in liquid nitrogen before and during collection, while dry nitrogen was passed through it, prior to collection, to prevent the condensation of oxygen in the tube. Fractions were collected by connecting the collection vessel to the collection outlet when the relevant peak was observed on the recorder. When the compound had been eluted, the vessel was removed, and a different vessel was connected as the next peak appeared.

RESULTS

Hordenine and the following nine indole alkaloids were studied: gramine, N,N-dimethyl-5-methoxy-tryptamine, N,N-dimethyl tryptamine, 5-methyl tryptamine, N-methyl tryptamine, tryptamine, 5-methoxy-tryptamine, 5-methoxy-N-methyl tryptamine and bufotenine. Anthracene was tested as a reference standard but it did not separate from some of the components of the extracts. The standards were resolved on all the columns (Figs. 1–3), with the exception of 5-methoxy-N-methyl tryptamine and 5-methoxy-tryptamine on the DEGS column, N-methyl tryptamine and tryptamine on the CHDMS column, and N-methyl tryptamine, tryptamine and 5-methyl tryptamine on the Amine 220 column. All these compounds had overlapping bases.

Bufotenine was not detected on the Amine 220 column at 165° but it was eluted at 180° (Table I). Retention times for the standards are shown in Table I.

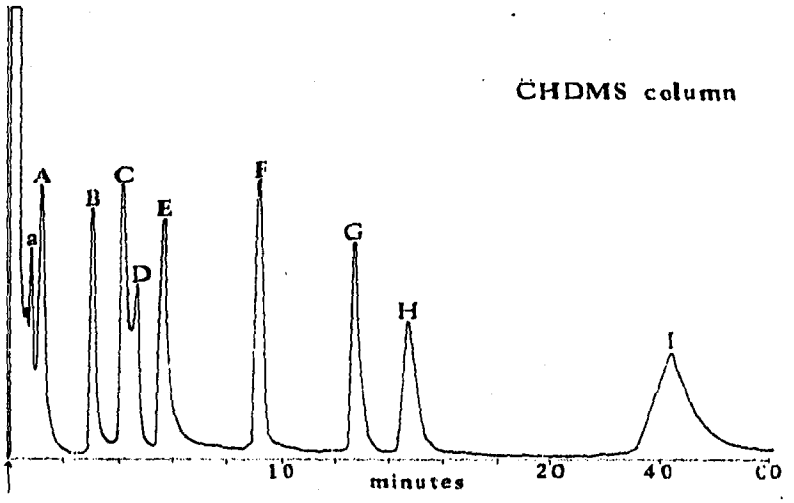

Fig. 1. Gas chromatographic analysis of tryptamine alkaloids and hordenine. a = gramine; A = hordenine; B = N,N-dimethyl tryptamine; C = N-methyl tryptamine; D = tryptamine; E = 5-methyl tryptamine; F = N,N-dimethyl-5-methoxy-tryptamine; G = 5-methoxy-N-methyl tryptamine; H = 5-methoxy-tryptamine; I = bufotenine.

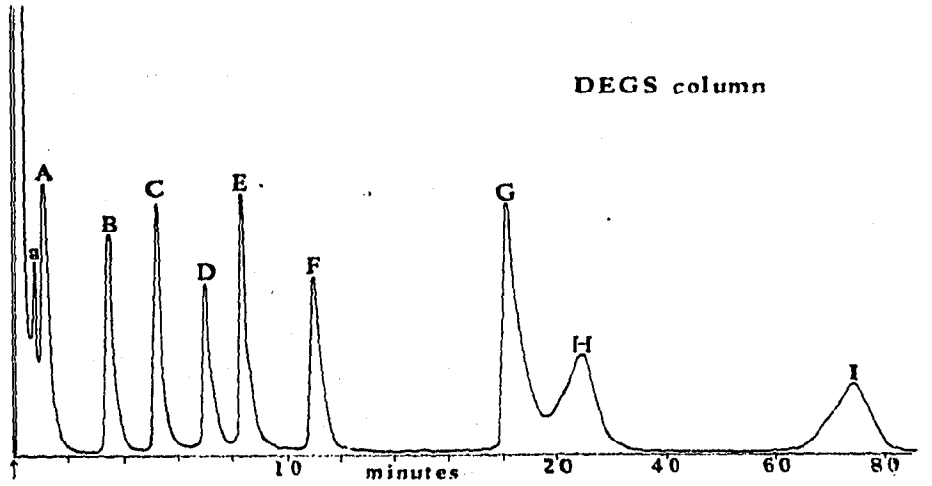

Fig. 2. See Fig. 1 for legend.

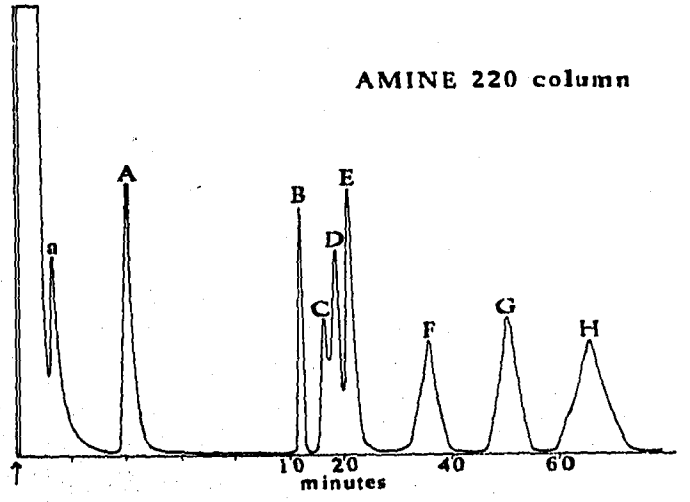

Fig. 3. See Fig. 1 for legend.

TABLE I

RETENTION TIMES OF COMPOUNDS

Compound	*Column*		
	Amine 220[a]	*CHDMS*	*DEGS*[a]
Gramine	1′31″	51″	52″
Hordenine	4′04″	58″	1′08″
Dimethyl tryptamine	12′08″	3′03″	3′29″
5-Methyl tryptamine	20′09″	5′44″	8′13″
Tryptamine	18′35″	4′28″	6′58″
N-Methyl tryptamine	15′40″	4′00″	5′09″
N,N-Dimethyl-5-methoxy-tryptamine	36′05″	9′16″	10′50″
5-Methoxy-tryptamine	66′04″	14′41″	24′48″
5-Methoxy-N-methyl tryptamine	50′04″	12′42″	18′07″
Bufotenine	[b]	42′16″	74′00″
Column temperature (°C)	165	195	180
Inlet temperature (°C)	210	230	220
Detector temperature (°C)	250	260	255
Helium flow rate at outlet (ml/min)	100	100	100
Column length (ft.)	6	6	6

[a] Columns silanised with 10 μl Silyl 8 after conditioning.
[b] Bufotenine was not detected at 165°, but at 180° it was eluted in 82½ min.

GLC of gramine indicated that gramine decomposes quite rapidly in solution. A saturated solution of gramine in chloroform was injected on to the CHDMS column and two peaks were recorded (Fig. 1). After 3 h at room temperature, the solution was again injected and it was found that the major peak had decreased in size, while the smaller peak had increased. It was assumed that the major peak was gramine. Pure gramine gives a single peak on the DEGS and Amine 220 columns (Figs. 2 and 3).

Gas chromatographic analysis indicated that some interfering compounds were present in the crude alkaloidal extracts, causing the recorder pen to take an excessive time to return to zero after the solvent had been eluted, but further purification of the extracts[1] minimized this effect.

The major component of a purified fraction of the crude alkaloidal extract from Ottawa synthetic C was collected from the Amine 220 column, at the collection outlet, in a glass tube containing 250 mg potassium bromide powder. The tube was stored in a desiccator between injections and after three injections of 30 μl, sufficient material had been collected for an IR analysis. The IR spectrum (Fig. 9), was identical with that of reference hordenine.

The major component (peak 1, Figs. 4–8) in all three extracts was found to be hordenine. When admixed with pure hordenine it did not separate from it in any of the chromatographic systems.

Peak 2 (Figs. 4–8) in all three extracts corresponded to gramine, having the same retention time on all three columns as reference gramine; because of the rapid decomposition of gramine, isolation by fraction collection was not successful.

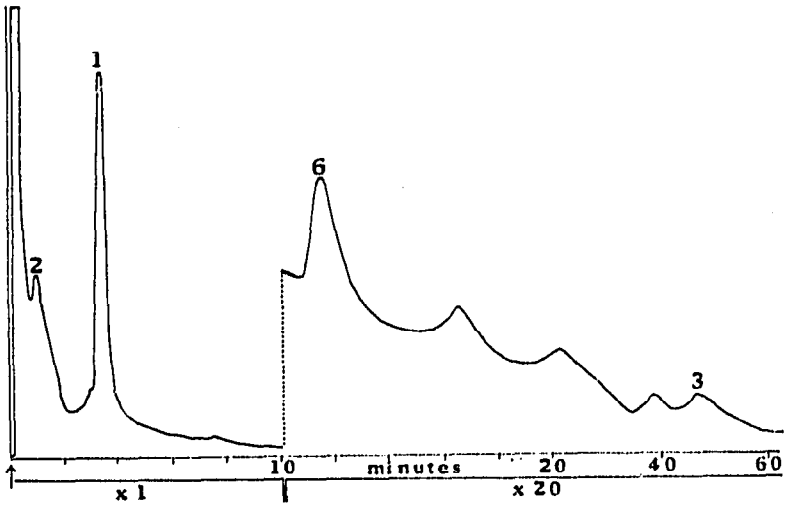

Fig. 4. Gas chromatogram of a crude alkaloidal extract from *Phalaris arundinacea* L. var. Frontier, on a 0.5% Amine 220 column.

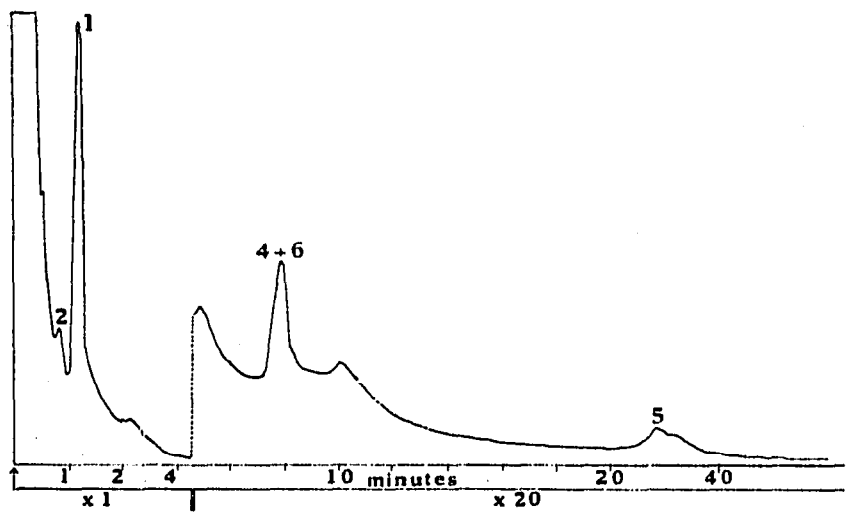

Fig. 5. Gas chromatogram of a crude alkaloidal extract from *Phalaris arundinacea* L. var. Ottawa synthetic F, on a 1% DEGS column.

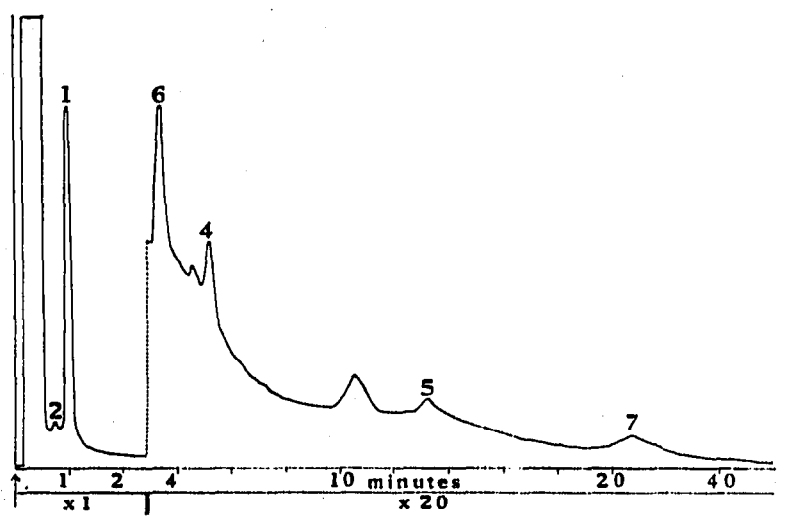

Fig. 6. Gas chromatogram of a crude alkaloidal extract from *Phalaris arundinacea* L. var. Ottawa synthetic C, on a 0.4% CHDMS column.

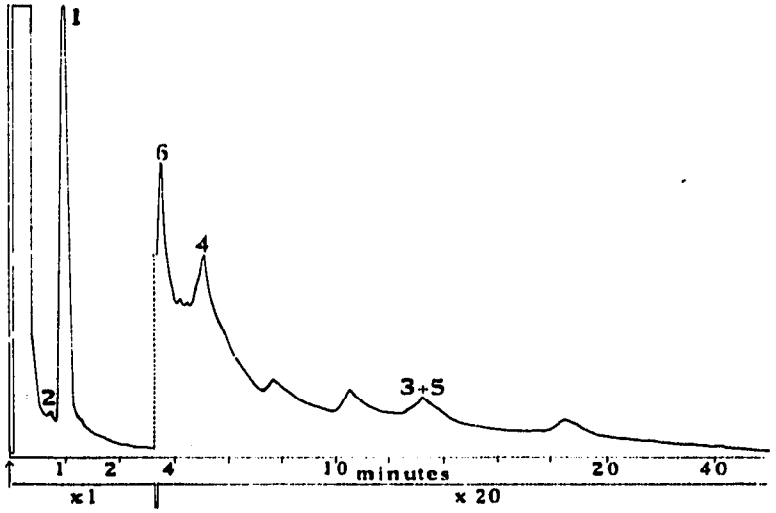

Fig. 7. Gas chromatogram of a crude alkaloidal extract from *Phalaris arundinacea* L. var. Frontier, on a 0.4% CHDMS column.

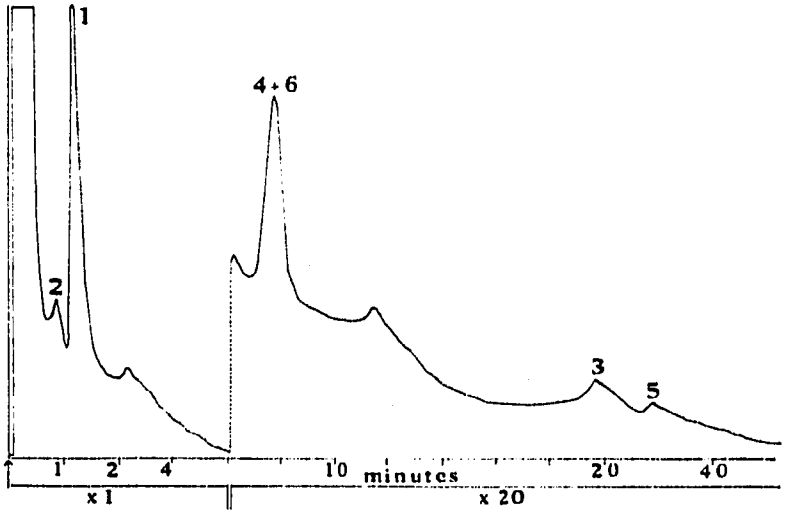

Fig. 8. Gas chromatogram of a crude alkaloidal extract from *Phalaris arundinacea* L. var. Frontier, on a 1% DEGS column.

The retention times of peaks 4 and 5 (Figs. 5–8) in all three extracts corresponded to the retention times of 5-methyl tryptamine and 5-methoxy-tryptamine respectively on the CHDMS and DEGS columns. However, when 5-methyl tryptamine and 5-methoxy-tryptamine were added to the extracts, they separated from peaks 4 and 5.

Peak 3 (Figs. 4, 7 and 8) corresponded to the retention time of 5-methoxy-N-methyl tryptamine in Frontier, and did not separate from it on any of the columns.

The second major component (peak 6, Figs. 4–8) which is present in all the extracts, does not correspond to any of the reference compounds and is at present unidentified. This component is not resolved from peak 4 on the DEGS column. The component of peak 6 has been isolated from a purified fraction of the crude alkaloidal extract from Ottawa synthetic C, by fraction collection from the CHDMS column using the technique described above. The compound was eluted from the collection

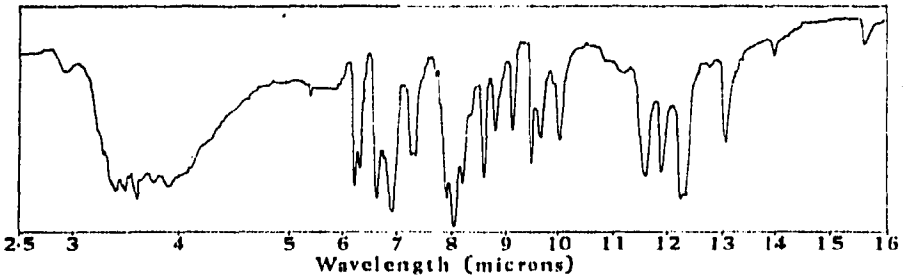

Fig. 9. IR spectrum of hordenine isolated from *Phalaris arundinacea* by GLC fraction collection (potassium bromide disc).

vessel with ether. The ether was evaporated to a small volume and the solution was applied to Silica Gel G thin-layer plates, which were developed in chloroform–methanol (80:20). The compound had an R_F value of 0.90 and gave a blue colour with Van Urk's reagent[1].

The compound, peak 7 (Fig. 6) present in Ottawa synthetic C and F has been isolated from Ottawa synthetic C and identified as 2,9-dimethyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline (ref. 1).

The plant extracts were stored at 0° when not in use, and signs of decomposition were first noticed about two weeks after preparation. The minor components appear to break down more readily than the major components. Best results were obtained using freshly prepared chloroform extracts, from which the chloroform was removed when not in use.

For screening purposes plant samples of 1 g or less may be employed to obtain a suitable gas chromatographic pattern on the three columns described. The fact that active breeding projects are developing many new lines at several research stations in Canada and the U.S.A. necessitates a more complete knowledge of the biochemistry of the *Phalaris* species. The development of a suitable screening method for the alkaloids of these new lines then is essential. The GLC techniques described above can be readily applied for this purpose.

ACKNOWLEDGEMENTS

We wish to thank M. BOLAN for his technical advice. We also wish to thank Dr. S. WILKINSON for the sample of 5-methoxy-N-methyl tryptamine HCl.

We wish to acknowledge the financial support received from the Northern Studies Committee, University of Manitoba, the Faculty of Graduate Studies of the University of Manitoba, the Canada Department of Agriculture and the National Research Council of Canada.

REFERENCES

1 R. C. S. AUDETTE, H. M. VIJAYANAGAR, J. BOLAN AND K. W. CLARK, *Can. J. Chem.*, in press.
2 R. M. MOORE AND R. J. HUTCHINGS, *Australian J. Exptl. Agr. Animal Husbandry*, 7 (1967) 17.
3 R. M. MOORE, J. D. WILLIAMS AND J. CHIA, *Australian J. Biol. Sci.*, 20 (1967) 1131.
4 C. C. J. CULVENOR, R. DAL BON AND L. W. SMITH, *Australian J. Chem.*, 17 (1964) 1301.
5 B. HOLMSTEDT AND J. E. LINDGREN, in D. H. EFRON (Editor), *Ethnopharmacologic Search for Psychoactive Drugs*, U. S. Department of Health, Education and Welfare, Washington, D.C., 1967, pp. 339–373.
6 M. BOLAN AND J. W. STEELE, personal communication.
7 K. MACEK, in E. HEFTMANN (Editor), *Chromatography*, 2nd ed., Reinhold, New York, 1967, p. 607.