Journal of Chromatography, 111 (1975) 165–170

CHROM. 8318

ISOLATION AND SEPARATION OF TALL FESCUE AND RYEGRASS ALKALOIDS*

LOWELL P. BUSH
Department of Agronomy, University of Kentucky, Lexington, Ky. 40506 (U.S.A.)
and
J. A. D. JEFFREYS
Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow C1 (Great Britain)
(Received January 28th, 1975)

SUMMARY

Bases were extractable from dried, powdered grass material by percolation with methanol. The bases were removed from the methanol by cation-exchange resins and subsequently removed from the resins. Isolation of bases by these methods eliminated the problems associated with the use of large quantities of chlorinated solvents and formation of emulsions found in previously described isolation methods. The bases were separated by paper and thin-layer chromatography (TLC). TLC and a spectrodensitometer were used to quantify the principal alkaloids in crude extracts of tall fescue samples. This procedure recovered all of the alkaloids in spiked samples, and the standard deviation for perlolidine and perloline in tall fescue samples was 3% or less of the mean value.

INTRODUCTION

The alkaloids of tall fescue (*Festuca arundinacea* Schreb.) and ryegrass (*Lolium perenne* L.) have been implicated in the etiology of animal diseases[1–3]. Alkaloids from plant tissue have been isolated by steeping or refluxing the tissue in dilute hydrochloric acid[3–6] or in aqueous alcohol[2,7]. Separation of the alkaloids from the crude extracts involved extraction of the alkaloids from an alkaline aqueous solution with chloroform or methylene chloride. Heavy emulsions formed at this step and were broken by centrifugation or standing for long periods of time. The emulsions and the large quantities of chlorinated solvent required for this step made this separation procedure very undesirable. Further purification of the alkaloid fraction necessitated repetition of this step and thus enhanced the objections to this procedure.

The quantitation of total alkaloids, usually as perloline, was done by

* Paper No. 75-03-17 from the Kentucky Agricultural Experiment Station and published with the approval of the Director.

titrimetric[8], colorimetric[9,10] or gravimetric[10] techniques. The titration procedure is not specific for alkaloids nor do the reagents combine with equal quantities of alkaloids, however, the quantitative estimation of total alkaloid was reported as perloline equivalents. The original colorimetric procedure[9] measured absorption of total alkaloids in chloroform at 470 nm. A significant modification of this procedure was the separation of the alkaloid fraction by thin-layer chromatography (TLC) and subsequent elution of the alkaloids from the sorbent and spectrophotometric determination of alkaloid concentration[10]. However, many of the tall fescue and ryegrass alkaloids are difficult to elute from TLC sorbents or chromatographic paper and the accuracy and precision of this procedure is not satisfactory.

Separation of tall fescue alkaloids into nine components by paper chromatography (PC) was reported by Yates[2]. The paper chromatograms were equilibrated for 6–16 h and developed for 16 h with freshly prepared *n*-butanol–acetic acid–water (10:1:3, v/v/v). Perloline had an R_F value of 0.46. Jeffreys[6] separated ryegrass alkaloids into 10 zones on Whatman No. 1 paper. The chromatograms were developed with ethyl acetate–acetic acid–water (75:9:9). Perloline, perlolyrine and perlolidine were found in R_F ranges of 0.08–0.15, 0.50–0.60 and 0.60–0.75, respectively[11].

Eleven alkaloid substances of tall fescue alkaloids were separated by TLC with silica gel G as the sorbent and developed in the upper phase of butanol–acetic acid–water (4:1:5)[10]. Festucine and perloline had R_F values of 0.10 and 0.32, respectively. This TLC method was much more rapid than the PC methods and more suitable to modification for quantitative analysis of many samples.

The objectives of our investigations were (1) to improve the techniques of isolating alkaloids from tall fescue and ryegrass and (2) to develop satisfactory qualitative and quantitative analytical procedures for the principal identified alkaloids.

PROCEDURES

Extraction of bases from the grasses was a modification of the procedure of Mattlocks[12]. Ryegrass or tall fescue stem and leaf tissue was dried at 70° and ground to pass a 2-mm screen. A slurry of the ground grass tissue and methanol was poured into a glass column (165 × 22 cm I.D.). By tamping the grass to remove air spaces we were able to put up to 15 kg of grass with a height of 150 cm into the glass column. The grass was steeped in the methanol overnight. After steeping, the methanol was percolated down through the grass and then passed through two cation exchange columns in the H^+ form (Dowex 50W-X8 or Amberlite IR 120). The first ion-exchange column held 1100 ml resin, four times the amount in the second column in the series. From the second ion-exchange column the methanol was pumped onto the top of the grass column, thus the methanol was continuously cycled with the bases being removed from the methanol by the ion-exchange resin. Methanol passed through the grass column at 4–5 l/h until 15 l/kg had percolated through the grass. Methanol percolation occurred only during the daytime; therefore, the grass was in contact with methanol for 3–5 days.

When percolation with methanol was stopped, the resin column was washed with fresh methanol and then water until no colored substances were being removed. The resin was transferred to a flask and treated with an excess of base (20 g sodium carbonate in 50 ml water per 100 ml resin). The liquid was decanted and the resin

washed three times with water. The aqueous fractions were combined, acidified with sulfuric acid and saved. The resin was washed five times with 50% aqueous methanol. The first liquid was decanted after 1 h contact time. The last four washings had 24 h contact time. The methanol fractions were combined, acidified with sulfuric acid and the methanol removed *in vacuo* in the presence of the previously combined aqueous fraction. The remaining aqueous solution was filtered through wet Whatman No. 1 filter paper, the filtrate was adjusted to pH 2 with sulfuric acid and extracted three times with methylene chloride. The methylene chloride was discarded. The aqueous material was made basic with sodium carbonate (*ca.* pH 10) and extracted three times with methylene chloride. The basic substances, alkaloids, were passed into 1 *N* sulfuric acid and back into methylene chloride. The methylene chloride fractions were combined, washed with water and dried over sodium sulfate. The dry methylene chloride was filtered through a sintered glass funnel and the methylene chloride removed *in vacuo* in the presence of methanol. The last portions of methanol were removed at atmospheric pressure to avoid loss of volatile bases. The residue consisted of the partially purified bases extracted from the grass.

The bases were also extracted by refluxing the grass in methanol–water–phosphoric acid (85:15:2)[7]. This procedure served as a control for the methanol percolation procedure.

The bases were further purified and quantified by PC and TLC. They were separated on Whatman No. 1 paper by descending chromatography with the solvent systems ethyl acetate–acetic acid–water (25:3:8) (upper phase) and butanol–acetic acid–water (10:1:3)[2]. TLC was done on silica gel plates with the upper phase of the solvent systems butanol–acetic acid–water (4:1:5) and ethyl acetate–acetic acid–water (25:1:8). All solvents were mixed and phases allowed to separate overnight before use.

Bases were located by color, fluorescence from UV light (maximum output at 365 nm) and by color development with potassium iodoplatinate. The iodoplatinate solution was prepared by mixing 3 ml of a 10% platinum chloride solution with 97 ml of water to which was added 100 ml of a 6% aqueous solution of potassium iodide. Most bases could be detected immediately after spraying as blue or black spots on a reddish background, but some bases were not observed for 24 h, *i.e.* after 24 h halostachyine appeared as a white spot.

Quantification of the bases on TLC chromatograms was done with a Schoeffel spectrodensitometer Model SD 3000. Samples and calibration curves for perloline, perlolidine and perlolyrine were run at 390, 340, and 390 nm, respectively. Perloline, perlolidine and perlolyrine in plant tissue were separated and quantified by TLC and spectrodensitometry. Plant tissue was dried at 70° for 24 h and ground to pass a 40-mesh screen. 1 g of sample powder, 1 g of glass beads, 0.3 g sodium bicarbonate and 2 ml water were placed in a mortar and ground for 2 min. Ten millilitres of chloroform–methanol (9:1) were added and the mixture was ground for 30 sec. The mixture was placed in a cold room at 4° for 15 min and then transferred to a centrifuge tube. The mixture was centrifuged 10 min at 25,000 *g*. The supernatant was decanted and 25 μl spotted on the TLC plates. The chromatograms were developed in butanol–acetic acid–water (4:1:5) (upper phase) and the perloline and perlolidine content measured. In most samples perlolyrine was not present in sufficient quantities to be detected by this routine method.

TABLE I

EXTRACTION OF BASES FROM DRIED POWDERED GRASS TISSUE

Extraction procedure	*mg Base per kg grass*	*Extraction procedure*	*mg Base per 100 ml resin*
Reflux with methanol–water–phosphoric acid	160	Percolation with fresh methanol one resin column	50
Percolation of grass column with fresh methanol	90	Percolation with recycled methanol first resin column (nearest the grass)	20
Percolation of grass column with recycled methanol	82	second resin column	36

RESULTS AND DISCUSSION

Extraction of large quantities of plant tissue in a column by percolation with methanol and subsequent adsorption of the bases on an ion-exchange column was successful. Fresh methanol added at the top of the column throughout the percolation step was more efficient (5 to 10%) for extraction of bases than was recycled methanol (Table I). However, the difference in the amount of bases recovered was not sufficient to justify distilling the methanol before adding it back to the top of the percolation column. The difference between the fresh and recycled methanol appeared to be caused by large amounts of lipids in the recycled methanol that physically interfered with adsorption of the bases on the ion-exchange column. To recycle the methanol during the percolation step, at least two resin columns in series (Dowex or Amberlite in both) are needed. The first column removed most of the pigment and some bases from the methanol and the second column removed principally bases from the methanol. Bases recovered from the resin following percolation with fresh or recycled methanol was 50% of that recovered by refluxing the grass with methanol–water–phosphoric acid (Table I). The problems associated with purification of the bases after extraction by refluxing are great enough to warrant use of a less efficient extraction procedure. All authentic bases applied to the ion-exchange resins were readily sorbed by the resin. Bases were not detected in methanol eluting from the resin. Removal of the bases from the resin, Dowex 50W-X8 or Amberlite IR 120, was the most time-consuming step in this extraction procedure. After the resin was treated with excess base (sodium carbonate) and washed with water, the resin was washed with 50% aqueous methanol or methanol with 24 h contact time. Four washings removed most of the bases from the resin but additional amounts of bases could be recovered with up to ten washings. Elution of the bases from the resin columns with 13 *N* sulfuric acid or 6 *N* hydrochloric acid was not satisfactory because of poor recovery (30%) and/or of the large amounts of acid solution required to get adequate recovery of bases from the resin[13]. Approximately 75% of the bases could be recovered from the resin with the volume of acid used to elute the column being twenty times the volume of the resin. 95% of the bases could be recovered with an acid to resin volume ratio of 50. Removal of the bases was not more efficient from Dowex 50W-X2 than the higher cross-linked resins.

Bases removed from the resin were separated by PC and TLC. Separations achieved by PC of purified alkaloids or synthetic bases are presented in Table II. The

TABLE II

SEPARATION OF ALKALOIDS BY DESCENDING PC

Butanol system: *n*-butanol–acetic acid–water, 10:1:3; ethyl acetate system: upper phase of ethyl acetate–acetic acid–water, 75:9:25.

Alkaloid	R_F *value*	
	Butanol system	*Ethyl acetate system*
Perlolidine	0.78	0.62
Perlolyrine	0.80	0.54
Halostachyine	0.60	0.44
Harman	0.80	0.33
β-Carboline	0.76	0.30
Perloline	0.65	0.13

plant material contained several other bases which were not identified. We did not have a festucine standard but the correct color with potassium iodoplatinate appeared at $R_F = 0.10$ which is the same R_F value as Yates[2] reported for festucine in this solvent system. The alkaloids were not separated satisfactorily in the butanol system, but the ethyl acetate system was more effective in separating these alkaloids. Harman and β-carboline were not resolved and halostachyine was difficult to resolve from harman and β-carboline with the ethyl acetate system. Festucine did not move from the origin in this system. By using the two solvent systems we resolved perloline, perlolidine, perlolyrine and festucine. Comparing the ethyl acetate solvent systems of Von Schmid *et al.*[14], Jeffreys[15], and ours (ethyl acetate–acetic acid–water, 75:9:9, 75:9:12, and 75:9:25, respectively), we observed progressively less migration of the alkaloids with greater water content in the solvent system.

Much greater resolution of these alkaloids was achieved by TLC (Table III). The butanol system did not resolve harman from halostachyine and poor resolution was obtained of perlolyrine from perlolidine. However, the ethyl acetate system resolved harman from β-carboline and perlolyrine from perlolidine.

TLC with the butanol system was the best procedure for separating the bases in crude extracts of tall fescue or ryegrass. Pigments that often interfered in the other chromatographic systems moved near the solvent front and the alkaloids could be

TABLE III

SEPARATION OF ALKALOIDS BY TLC WITH SILICA GEL

Butanol system: upper phase of *n*-butanol–acetic acid–water, 4:1:5; ethyl acetate system: upper phase of ethyl acetate–acetic acid–water, 25:1:8.

Alkaloid	R_F *value*	
	Butanol system	*Ethyl acetate system*
Perlolyrine	0.74	0.62
Perlolidine	0.70	0.40
Harman	0.57	0.09
β-Carboline	0.56	0.21
Halostachyine	0.48	0.02
Perloline	0.23	0.0

readily visualized. Because less interfering substances were present, the butanol TLC system was used for quantitation of the alkaloids in grasses.

The alkaloids were quantified on the TLC plates with the spectrodensitometer and by selecting the appropriate wavelength the poor resolution of perlolyrine and perlolidine proved not to be a critical problem. Two samples with differing amounts of perloline and perlolidine were extracted and quantitatively analyzed eight separate times. The standard deviation of the mean was 3% or less in all tests (Table IV). Recovery of perloline and perlolyrine added to cellulose samples was 100%. We have subsequently used this procedure for routine analysis of perlolidine and perloline content of tall fescue forage in the breeding program at the University of Kentucky[16,17].

TABLE IV

ANALYSIS OF TWO TALL FESCUE SAMPLES FOR PERLOLINE AND PERLOLIDINE BY SEPARATION WITH TLC AND QUANTITATION WITH A SPECTRODENSITOMETER

Perloline and perlolidine were measured at 390 and 340 nm, respectively. n, Number of samples; $\bar{x}$, mean value; s, standard deviation; s_x, standard deviation of the mean value.

Sample	*Perloline*				*Perlolidine*			
	n	$\bar{x}$	s	s_x	n	$\bar{x}$	s	s_x
805	8	1244	77	27	8	161	11	4
808	8	2787	271	96	8	492	22	8

ACKNOWLEDGEMENT

One of the authors (L.P.B.) gratefully acknowledges the opportunity to do much of this research in the Department of Pure and Applied Chemistry at the University of Strathclyde.

REFERENCES

1 L. P. Bush, J. A. Boling, G. Allen and R. C. Buckner, *Crop Sci.*, 12 (1972) 277.
2 S. G. Yates, *J. Chromatogr.*, 12 (1963) 423.
3 A. J. Aasen, C. C. J. Culvenor, E. P. Finnie, A. W. Kellock and L. W. Smith, *Aust. J. Agr. Res.*, 20 (1969) 71.
4 R. E. R. Grimmett and D. F. Waters, *N. Z. J. Sci. Technol., Sect. B*, 24 (1943) 151.
5 J. L. Mangan, *N.Z. J. Sci. Technol., Sect. B*, 33 (1952) 245.
6 J. A. D. Jeffreys, *J. Chem. Soc., London*, (1964) 4504.
7 L. P. Bush, C. Streeter and R. C. Buckner, *Crop Sci.*, 10 (1970) 108.
8 I. Reifer and N. O. Bathurst, *N.Z. J. Sci. Technol., Sect. B*, 24 (1942) 17.
9 N. O. Bathurst, I. Reifer and E. M. Clare, *N.Z. J. Sci. Technol., Sect. B*, 24 (1942) 161.
10 C. E. Gentry, R. A. Chapman, L. Hensen and R. C. Buckner, *Agron. J.*, 61 (1969) 313.
11 J. A. D. Jeffreys, *J. Chem. Soc., C*, (1970) 1091.
12 A. R. Mattlocks, *Nature (London)*, 191 (1961) 1281.
13 D. Herr, *Ind. Eng. Chem.*, 37 (1945) 631.
14 H. von Schmid, J. Kebrle and P. Karrer, *Helv. Chim. Acta*, 35 (1952) 1864.
15 J. A. D. Jeffreys, unpublished results.
16 R. C. Buckner, L. P. Bush and P. B. Burrus, II, *Crop Sci.*, 13 (1973) 666.
17 P. L. Cornelius, R. C. Buckner, L. P. Bush, P. B. Burrus, II and J. Byars, *Crop Sci.*, 14 (1974) 896.