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BIOLOGICALLY ACTIVE SECONDARY METABOLITES OF BARLEY. III. A METHOD FOR IDENTIFICATION AND QUANTIFICATION OF HORDENINE AND GRAMINE IN BARLEY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract—A method was devised for the extraction and quantification of hordenine and gramine from barley (*Hordeum vulgare*) tissue using HPLC techniques. Quantification was by peak area, the relationship between peak area and concentration of authentic standards being linear for both hordenine and gramine. Significant differences in the ability of three lines of barley to produce hordenine and gramine were detected using this method.

Key Words—Barley, *Hordeum vulgare*, allelochemicals, hordenine, gramine, quantification, HPLC, cultivars.

INTRODUCTION

Hordenine (*N*,*N*-dimethyltyramine) and gramine (*N*,*N*-dimethylindolemethylamine) have been isolated from barley (Bowden and Marion, 1951; Leete et al., 1952; Leete and Marion, 1953; Rabitzsch, 1959; Steinhart et al., 1964; Schneider et al., 1972; Hanson et al., 1981; Lovett and Liu, 1987; Liu and Lovett, 1990) and have been implicated in being, at least in part, responsible for barley's vigor in competition with other species (Overland, 1966; Liu and Lovett, 1990). However, Hanson et al. (1981) failed to detect gramine in all barley cultivars they investigated, and others (Kirkwood and Marion, 1950) found that *N*-meth-

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yltyramine (the immediate precursor of hordenine) was present in greater quantities in the roots of some cultivars than hordenine itself.

Working on the tryptamine-based alkaloids of reed canary grass (*Phalaris arundinacea*) (of which gramine is one), Marum et al. (1979) found that the inheritance of gramine was controlled by two recessive genes, and they postulated that it would be possible to breed lines capable of producing this alkaloid. In a series of papers published in the 1950s and 1960s, a Canadian group (e.g., Massicot and Marion, 1957; Gower and Leete, 1963) elucidated the pathways of gramine and hordenine synthesis in barley. From this work, and on the basis of "one gene–one enzyme," it should also be possible to breed barley lines with a high alkaloid content, a desirable end if a defensive role for such compounds is accepted (Lovett et al., 1989).

To facilitate the identification of possible parents with a high alkaloid content, a rapid, reliable method of estimating the quantity of gramine and hordenine in plant samples is necessary.

To date, high-performance liquid chromatography (HPLC) has been used extensively to identify and quantify alkaloids of many types, including hordenine and gramine (Kohl et al., 1983; Renaudin, 1984; van der Heijden et al., 1987; Liu and Lovett, 1990), both quickly and accurately. Liu and Lovett (1990) isolated and quantified hordenine and gramine from hydroponic solutions in which barley (cv. Triumph) had been grown, and sample purification and concentration was achieved by partitioning against chloroform, a relatively time-consuming, expensive, and hazardous method. Further, the HPLC quantification method used a mobile phase of relatively high molarity and pH, both of which shorten column life. Johansson and Schubert (1990) described a method for the quantification of hordenine by HPLC but expressed dissatisfaction with the detection component. We have found no published method for the quantification of gramine by HPLC and no sample preparation method using sample preparation cartridges, e.g., the Water's Sep-Pak range.

We describe in this paper methods for the extraction of hordenine and gramine from barley root and shoot tissue, extract purification and concentration, and some amendments to the methods of HPLC quantification that have been previously reported.

METHODS AND MATERIALS

Standard solutions of authentic hordenine and gramine hemisulfate (Sigma Chemicals) of 5, 10, 15, and 20 ppm and 2.5, 5.0, 7.5, and 10.0 ppm respectively, were prepared.

Barley seedlings, cvv. Skiff and Proctor and line CI400117, were grown to the stage of one fully expanded leaf in a growth cabinet (12-hr day, 8–18°C

temperature regime). Fifty grains of each variety were sown in sand in 20-cm pots replicated three times on three separate occasions (blocks A, B, and C). The seedlings were separated into roots and shoots and the remains of the grain discarded. The alkaloids were extracted from the frozen material by grinding approximately 1.5-g samples in a mortar with 30 ml 0.01% acetic acid (Renaudin, 1985).

After standing for 24 hr at room temperature, the extracts were then filtered through glass wool, the pH adjusted to 9.15 and centrifuged at 3000 rpm for 5 min. Ten-milliliter aliquots of the extracts and 1-ml aliquots of the standards were applied to prepared Sep-Pak C_{18} cartridges (Waters Associates). Two replicates of each standard, pH adjusted to 9.15, were processed. The method of purification for gramine and shoot samples was based on that of van der Heijden et al. (1987) with minor modifications, viz.

Acetonitrile (ACN) 2 ml

↓

0.001 M KH_2PO_4 pH 7 2 ml

↓

aliquot applied

↓

0.05 M KH_2PO_4 pH 9.5/isopropanol (70:30) 2 ml

↓

0.05 M KH_2PO_4 pH 9.5/isopropanol (95:5) 2 ml

↓

0.05 M KH_2PO_4 pH 2.3/isopropanol (70:30) 1.5 ml

Hordenine was not adequately retained using this method and the following procedure was developed:

ACN 2 ml

↓

0.001 M KH_2PO_4 pH 7 2 ml

↓

aliquot applied

↓

0.05 M KH_2PO_4 pH 9.5/isopropanol (70:30) 2 ml

↓

0.05 M KH_2PO_4 pH 2.3/isopropanol (70:30) 1.5 ml

The final eluates were evaporated to dryness under nitrogen at 40°C and taken up in 1 ml mobile phase. Recovery of alkaloid from each batch of root and shoot extracts purified was checked by concurrently "purifying" a 10-ml aliquot of 1 ppm hordenine and gramine, respectively.

Ten-microliter aliquots of each purified standard and plant sample were injected into a Waters HPLC system consisting of an M40 pump, flow rate 2 ml/min; U6K injector; UV/VIS spectrophotometer, wavelength 221 nm, and the results recorded as peak areas using a Waters 745 data module. The column used was a Water's μBondapak Phenyl 10 μ (3.9 mm × 30 cm) and the mobile phase was 0.025 M KH_2PO_4 + 0.1% TEA (triethylamine), pH 7.15/ACN (2:1 for hordenine and root samples) (6:4 for gramine and shoot samples). Duplicate injections were made for each sample. Identification of hordenine and gramine in plant samples was by retention time relative to authentic compound and confirmed by coelution of representative samples with authentic compound. The presence of hordenine and gramine in barley material has previously been confirmed by GC-MS (Liu and Lovett, 1990).

RESULTS AND DISCUSSION

Methanol and ethanol have commonly been used for the extraction of alkaloids from plant tissue (Massicot and Marion, 1957; Schneider et al., 1972; Renaudin, 1984); however, when compared with extraction with 0.01% acetic acid, very low recovery rates of the alkaloids were achieved by us using this method. For example, when root tissue was extracted in either methanol or 0.01% acetic acid spiked with 2 ppm hordenine, the proportion of the hordenine spike recovered from the methanol extraction was 0.5%, whereas that from acetic acid extraction was 75%. Recovery from unspiked root tissue was similarly related. Reextraction of the samples in their respective solvents failed to increase total recovery by more than 5% and extraction in double the quantity of solvent gave similar results. It is probable, therefore, that small quantities of hordenine remained adsorbed on the root tissue and that, in the case of methanolic extraction, hordenine was not retained on the Sep-Pak cartridges. Data on the recovery of gramine and hordenine standards from the sample preparation protocol are presented in Table 1. Recovery of hordenine ranged from 97.9% to 109% and of gramine from 87.7% to 96%. These figures are consistent with the literature (Renaudin, 1985; van der Heijden et al., 1987) and are considered acceptable.

TABLE 1. RECOVERY OF HORDENINE AND GRAMINE FROM SEP-PAK PROCEDURES

Hordenine			Gramine		
Concn. (ppm)	Peak area	Recovery (%)	Concn. (ppm)	Peak area	Recovery (%)
5.0	102,936	109.0	2.5	233,098	87.7
	100,948	106.9		236,722	89.1
10.0	199,672	105.8	5.0	506,664	95.3
	186,629	98.9		493,887	92.9
15.0	277,231	97.9	7.5	722,800	90.7
	382,474	100.1		728,126	91.3
20.0	374,728	98.2	10.0	1,020,395	96.0
	370,886	98.2		1,004,719	94.5
10.0 Untreated std.	188,788[a]		3.0 Untreated std.	317,926[a]	

[a] Mean of four injections.

Liu and Lovett (1990) successfully used HPLC techniques to quantify hordenine and gramine released by barley into hydroponic solution; however, a Waters μBondapak 10 μ C_{18} column and a mobile phase consisting of 0.05 M KH_2PO_4 + 0.1% TEA, pH 7.65/ACN (60:40) were used. When this method was modified by using the μBondapak Phenyl 10 μ column (3.9 mm $\times$ 30 cm) recommended by Waters for the analysis of basic compoundsand a mobile phase with the molarity and pH of the buffer reduced to 0.025 M and 7.15, respectively, resolution of peaks at a flow rate of 2 ml/min was close to the baseline (Figure 1) quantification of both hordenine and gramine and, therefore, was acceptable. An added advantage of this method was reduced retention times (e.g., 3 min as opposed to 4.5 min for hordenine) (Liu, 1991). Up to 1500 injections of these alkaloid samples have been processed through one column, which is considered in the upper range for a system using buffered mobile phases.

When standards of authentic hordenine and gramine were injected directly into the HPLC, the relationship between concentration and peak area was found to be linear over the ranges tested (r = 0.9994 and r = 0.9999, respectively) as was the relationship with peak height. Regression analysis of the data presented in Table 1 for the recovery of the alkaloids from the purification protocols, excluding the untreated standards, confirmed this relationship for both hordenine and gramine (r = 0.9993 and r = 0.9989, respectively). As the purification protocol was in each case carried out across a batch of eight car-

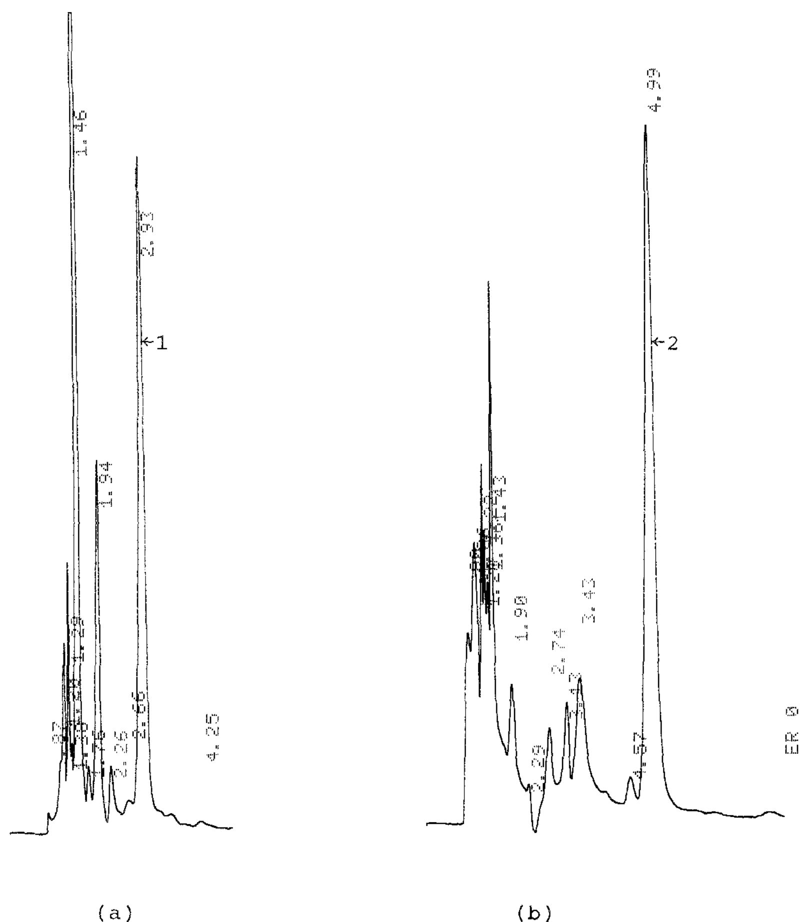

FIG. 1. Chromatographs showing separations of hordenine (a) and gramine (b) from barley root and shoot material respectively. 1 = hordenine, retention time 2.93 min; 2 = gramine, retention time 4.99 min.

tridges, the results were indicative of the homogeneity of the cartridges and the reliability of the protocol in general.

UV absorption by gramine was great enough to allow its quantification down to 1.0 μg/ml (20 μg/g dry wt. approx. of plant sample) with an accuracy of less than 5% variation between dual injections. This figure compares favorably with the lower detection limit of 30 μg/g dry wt quoted by Hanson et al. (1981).

In comparison with gramine, hordenine absorbs considerably less UV light,

resulting in a lower detection limit, in the order of 2.5 μg/ml. This figure is higher than that achieved by Johansson and Schubert (1990) using electrochemical detection and of the same order as they achieved using UV detection at 275 nm. (We found both gramine and hordenine to absorb more strongly at 221 nm than at 275 nm and, therefore, used this wavelength.) Johansson and Schubert, however, reported a run time of 35 min/injection and contamination of the electrodes used with electrochemical detection, which led to downtime. The method reported here, while not as sensitive, has a run time of 8 min and no downtime associated with electrode contamination. Further, given the concentration of hordenine found by us in barley roots (Table 2), we would argue that, for our purposes, quantification to lower levels is unnecessary.

Two major problems in quantifying hordenine in plant material were encountered. The first of these concerns the time scale of hordenine production by barley plants. A trial experiment (data not presented here) with glasshouse-grown plants of cv. Skiff indicated that hordenine content of the roots peaked and declined within the space of three days, the peak roughly coinciding with expansion of the first leaf. This is consistent with data obtained for cv. Triumph (Lovett and Liu, 1987). The apparent disparity with the data presented in Figure 1 of the preceding paper of this series (Liu and Lovett, 1993) may be explained by the sampling intervals of 5–10 days used in this work, resulting in averaging of production over a minimum of five days. The data presented here (Table 2) are for 8-day-old plants, an age chosen to accommodate the slower growth of cv. Proctor and line CI400117.

Duncan's multiple-range test was applied to the data to determine if there was a block effect; line CI400117 contained significantly (5% level) less hordenine in block B than in block C, while no differences were found for the other cultivars. Analysis of variance of the complete data showed no block effect but

TABLE 2. MEAN[a] HORDENINE AND GRAMINE CONTENTS OF THREE BARLEY LINES GROWN ON THREE OCCASIONS

	Hordenine (μg/g fresh weight)				Gramine (μg/g fresh weight)		
Block	Proctor	Skiff	CI400117	Block	Proctor	Skiff	CI400117
A	33[cde]	14[ab]	26[bcd]	A	5	1	975
B	37[de]	14[ab]	21[abc]	B	3	3	900
C	42[e]	11[a]	37[de]	C	8	2	962
Mean	37	13	28	Mean	5	2	946

[a]Mean of three replicates. LSD (hordenine) (5%) = 6.67. Data followed by the same letters do not differ at the 5% level.

indicated that all cultivars differed in their hordenine content at the 5% level (LSD 6.67). This indicates a potential difficulty in comparing the hordenine production capacity of different cultivars and, indeed, the lower levels of hordenine production by cv. Skiff may reflect a greater physiological age and hence declining hordenine production rather than a lesser capacity to produce the compound. It may be more appropriate to analyze plants at the stage when their first leaf is fully expanded rather than at a common chronological age.

The second problem was the difficulty in retaining hordenine on the Sep-Pak cartridges during sample purification. Considerable quantities of the plant matrix remained, but sufficient was removed that adjustment of the pH of the mobile phase to 7.15 permitted the resolution of peaks close to the baseline, thus allowing satisfactory quantification (Figure 1a). Considerable upward adjustment of the pH of the mobile phase is still possible before reaching pH 7.5 where silica starts to dissolve from the column at the parts per million level (Runser, 1981).

No such problems were encountered in quantifying gramine. Subsequent analyses of further lines have confirmed that barley lines either produce large quantities of gramine or virtually none at all (Table 3). The results for cv. Proctor are consistent with those of Hanson et al. (1981), who considered that there were two possible reasons for the lack of gramine production—lack of capacity to synthesize the compound or the capacity to degrade it faster than it is made. The detection of trace amounts of the compound by us suggests a third possibility: that the capacity to produce gramine is present in all lines but that breeding for standard agronomic traits has led to increased competition within the plant from other metabolic pathways for substrate(s) required for gramine synthesis.

TABLE 3. GRAMINE CONTENT OF BARLEY LINES OF VARIOUS GENETIC BACKGROUNDS

Name/number	Background	Gramine production
H. agriocrithon	ancestral	High
H. spontaneum	ancestral	High
cv. Prior	English (old)	Medium
cv. Proctor	English (1952)	Low
cv. Triumph	English (1985)	Low
cv. Grimmett	Australian (1982)	Low
cv. Skiff	Australian (1988)	Low
CI400117[a]	Ethiopian	High
CI490224[a]	Syrian	Low

[a] These are lines obtained by the Australian Winter Cereals Collection from ICARDA and represent germplasm from closer to the crop's centre of origin. High = 1000–1500 μg/g fresh weight; medium = 300–1000 μg/g fresh weight; low = 0–25; μg/g fresh weight.

CONCLUSIONS

The alkaloids hordenine and gramine can be isolated from plant material and quantified by using Sep-Pak C_{18} cartridges to prepare the samples, followed by reversed-phase liquid chromatography. This method is faster and easier to use than methods hitherto reported and is capable of detecting differences between cultivars in their ability to produce both hordenine and gramine.

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