New Quantitative High-Performance Liquid Chromatography Method for Analysis of Gramine in Cereal Leaf Tissue

Alister D. Muir,* Neil D. Westcott, and Christopher F. Hinks

Agriculture Canada Research Station, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada

Gramine was extracted from fresh and freeze-dried cereal leaf tissue according to a simplified methanol/ammonia extraction procedure and quantified by reversed-phase HPLC without further sample preparation. Chromatography was achieved using a modified Pico-Tag solvent system with gradient elution. Quantitative recovery (76–78%) of gramine (17–871 μg/mL) from plant samples was achieved. Recovery of 3-(aminomethyl)indole (AMI), *N*-methyl-3-(aminomethyl)indole (MAMI), and tryptamine, intermediates in the biosynthesis of gramine, was also demonstrated.

INTRODUCTION

Secondary plant compounds can be both desirable and undesirable in agronomically important plants. Gramine [*N*,*N*-dimethyl-3-(aminomethyl)indole], an indole alkaloid derived from tryptophan, occurs in some barleys (*Hordeum vulgare* L. and *H. spontaneum* Koch) (Hanson et al., 1981; Argandona et al., 1987), reed canarygrass (*Phalaris arundiacea* L.) (Majak et al., 1979), and some lupins (Leete, 1975). In forage grasses such as reed canarygrass, the presence of gramine is considered an undesirable trait which is detrimental to ruminant animals. Leaf concentrations exceeding 2000 μg/g of dry weight resulted in reduced palatability (Simons and Marten, 1971) and animal performance (Gallagher et al., 1964; Marten et al., 1973, 1976, 1981; Coulman et al., 1977). In barley and other cereal crops, studies have demonstrated that gramine appears to act as a feeding deterrent to aphids (Corcuera, 1984; Zuniga et al., 1985, 1988; Lohar, 1989). Gramine has also been shown to have antibacterial properties (Sepulveda and Corcuera, 1990). Growth and survival of grasshoppers fed a powdered wheat leaf diet were inversely proportional to the concentration of added gramine (Westcott et al., 1992). In this study, significant effects to grasshoppers were recorded at gramine concentrations equivalent to those found in some cultivars of barley by Hanson et al. (1981). In barley, 70% of the gramine was found in the mesophyll parenchyma and 30% in the epidermal tissue (Argandona et al., 1987), where its presence would potentially act as a feeding deterrent to chewing insects such as grasshoppers. The present studies were initiated to develop a rapid, quantitative assay for gramine that could be used to screen barley germplasm for plants producing gramine.

Existing methods for analysis of gramine in plant samples require large quantities of plant material (Simons and Marten, 1971) and are not readily adaptable to automation (Coulman et al., 1976; Hanson et al., 1981). Paper chromatographic methods used previously (Simons and Marten, 1971) are not able to resolve complex alkaloid mixtures which are occasionally present. The method described in this paper utilized a simplified extraction procedure which permitted quantitative analysis of gramine in 30 single-leaf samples per day. This method was also used to analyze for intermediates in the biosynthesis of gramine including 3-(aminomethyl)indole (AMI) and *N*-methyl-3-(aminomethyl)indole (MAMI).

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Liquid Chromatography. A Waters liquid chromatograph equipped with an M600E gradient module, an M700 autosampler, and a 991 photo diode array detector (PDA) was used [Millipore (Canada) Ltd., Mississauga, ON, Canada]. The PDA software was used to integrate the signal. Gramine levels were determined by chromatography on a Pico-Tag RP column [3.9 × 300 mm, Millipore (Canada) Ltd.] or a LiChrosorb C-18 column (7 μm, 4.0 × 250 mm, E. Merck, Darmstadt, Germany) equipped with a 4.0 × 4.0 mm C-18 guard column. Gramine and related compounds were eluted with the following gradient: $T = 0$, solvent A = 10%, solvent B = 90%; $T = 10$, A = 20%; $T = 22$, A = 34%; $T = 24$, A = 100%; $T = 30$, A = 100%; $T = 32$, A = 10%. All gradient changes were linear. The column flow rate was 1.00 mL/min and 8 min of equilibration time was allowed between injections. Detection was at 270 nm. All solvents were degassed under a constant stream of helium. UV spectra from 240 to 300 nm were recorded for each peak.

Reagents. *HPLC Solvent A.* Sodium acetate trihydrate (19 g), triethylamine (0.5 mL), and 1 mL of 0.4 mg/mL Na_2EDTA were dissolved in 1 L of HPLC grade water and adjusted to pH 6.2 with glacial acetic acid.

HPLC Solvent B. Acetonitrile (600 mL) and HPLC grade water (400 mL) containing 1 mL of 0.4 mg/mL Na_2EDTA were measured separately, mixed, and filtered. Solvent B was prepared daily.

Methanol and acetonitrile were of spectroscopic grade purchased from BDH Canada Inc. (Toronto, ON, Canada). Triethylamine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Gramine and tryptamine were purchased from Sigma Chemical Co. (St. Louis, MO). AMI and MAMI were a gift from Dr. W. Kurtz, Plant Biotechnology Institute, Saskatoon, SK, Canada. All other chemicals were of analytical grade purchased from BDH Canada Inc.

Plant Material. *H. vulgare* cvs. Abee, Samson, Compana, and Arimar were grown in a growth cabinet at 25 or 30 °C (15 °C nights) under a constant 16 h/8 h day/night regime.

Extract Preparation. Fresh leaf samples (whole leaf 50–600 mg) were chopped into small pieces (0.5–1 cm in length) and extracted in 4 mL of $MeOH/NH_4OH$ (99:1) for 1 h at room temperature. Freeze-dried powders (50 mg) were also extracted in 4 mL of $MeOH/NH_4OH$ (99:1) for 1 h without further grinding. The samples were centrifuged, and the supernatant was recovered, reduced to dryness under reduced pressure, taken up in 0.5 mL of 0.1 M HCl, and subjected to HPLC analysis. Gramine standards, fresh and freeze-dried samples of cvs. Abee, Arimar, Compana, and Samson spiked with gramine, were processed using this protocol. Freeze-dried powders (100 mg) of cv. Abee were also spiked with AMI, MAMI, and tryptamine (5 μmol/g of dry weight) and extracted. Gramine standards (10 mmol) used to spike plant samples were prepared in $MeOH/NH_4OH$ (99:1). All other standards were prepared as 5 or 10 mM solutions in 0.1 M

0021-8561/92/1440-1836$03.00/0 Published 1992 by the American Chemical Society

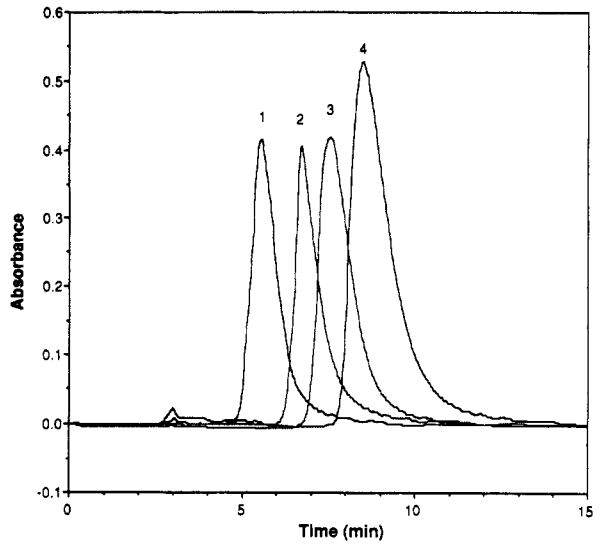

Figure 1. HPLC separation of gramine and related compounds on a reversed-phase Pico-Tag column. Peak identity: 1, 3-(aminomethyl)indole (AMI); 2, *N*-methyl-3-(aminomethyl)indole (MAMI); 3, tryptamine; 4, gramine. Chromatographic conditions as per text.

HCl. Standards prepared in HCl were stable for several months and showed no evidence of decomposition.

RESULTS AND DISCUSSION

HPLC analysis of authentic standards of gramine, AMI, MAMI, and tryptamine demonstrated that these compounds could be resolved (Figure 1). The HPLC traces of cultivars Arimar (A), Abee, and Abee spiked with gramine (174 μg) (B) are illustrated in Figure 2. The absence of gramine in cv. Abee is consistent with our previous unpublished data. HPLC traces of extracts of other barley cultivars, shown by classical analytical procedures to be deficient in, or have less than 10 μg/g gramine, did not contain peaks coeluting with gramine. The calibration for authentic gramine was linear over the range 17–871 μg ($Y = 0.022X$, $R^2 = 0.999$, $n = 12$). The UV spectrum of the peak corresponding to gramine in the cv. Arimar exhibited the same profile as gramine. Comparable separations were achieved on Lichrosorb C-18 and Resolvex C-18 (Fisher Scientific) columns with similar gradients.

The mean percent recoveries of added gramine (17, 87, 174, and 871 μg) from the barley cultivars Abee, Samson, Compana, and Arimar freeze-dried leaf powders and fresh leaf material were 78.5 ± 3.2 (±SE, $n = 24$) and 76.7 ± 2.6 (±SE, $n = 32$) respectively. These results compare favorably with those obtained by Hanson et al. (1983), who achieved an average recovery rate of 70% determined as gramine equivalents by classical extraction and purification methods.

Concentrations of gramine in fresh leaf samples of cv. Arimar, a high gramine barley, were 13 494 ($n = 2$) μg/g of dry weight. Extracts (five), prepared from an unrelated freeze-dried sample of cv. Arimar obtained by pooling material from several plants, were each analyzed five times by HPLC (20 μL). The mean gramine concentration was 11138 ± 27.7 μg/g (±SE, $n = 25$, CV = 0.0124). These values are significantly higher than those previously reported for Arimar (1710–6879 μg/g of dry weight; Hanson et al., 1981) but fall within the range of values reported for high gramine barleys (Hanson et al., 1983; Salas and Corcuera, 1991).

The sample size requirements for this method are dependent upon the gramine level of the plant tissue.

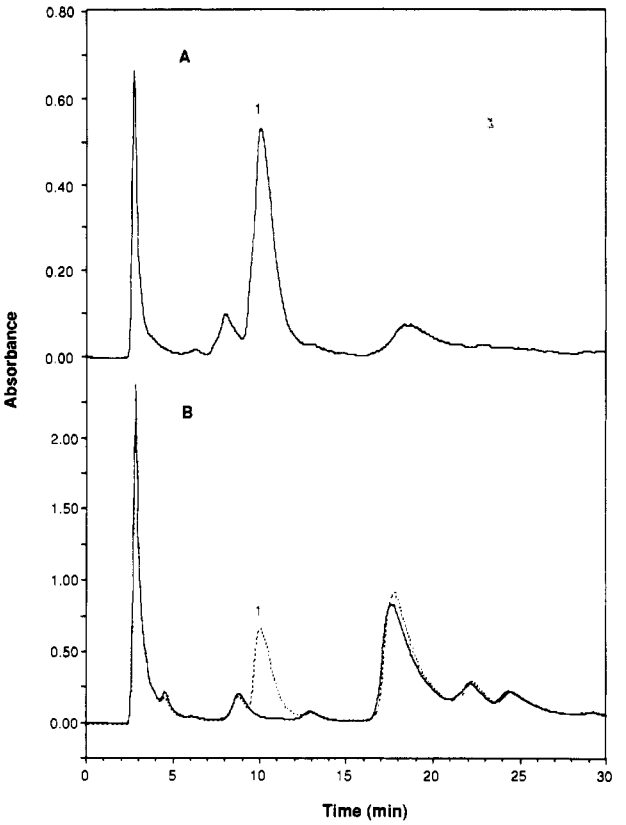

Figure 2. HPLC analysis of methanol/ammonia (99:1) extracts of freeze-dried powders (50 mg) of the barley cultivars Arimar (20-μL injection) (A) and Abee (100-μL injection) (B). (···) Gramine spike (174 μg) was added to Abee sample prior to extraction. Peak identity: 1, gramine. Pico-Tag column and chromatography as per text.

Gramine concentrations in plants with high endogenous gramine levels can be reliably determined in 5–10 mg of dry weight samples or the equivalent amount of fresh plant material. In plants expressing low levels of gramine, a larger sample (100–200 mg) is required to detect gramine. An additional advantage of this method over the classical methods used by Hanson et al. (1981) is the smaller tissue sample requirement. In the Hanson et al. (1981) protocol, two to six shoots are pooled, whereas our method requires only a single leaf. The advantage of the small sample size is that gramine concentrations are at their highest 6–10 days after seeding, at which time there is often not enough plant material available to permit analysis of single plants by classical methods.

ACKNOWLEDGMENT

We thank K. A. Bassendowski, R. Power L. Braun, S. F. Northrup, D. Moore, and R. M. Costain for technical assistance. We also thank Dr. W. Kurtz for the gift of AMI and MAMI.

LITERATURE CITED

Argandona, V. H.; Zuniga, G. E.; Corcuera, L. J. Distribution of gramine and hydroxamic acids in barley and wheat leafs. *Phytochemistry* **1987**, *26*, 1917–1918.

Corcuera, L. J. Effects of indole alkaloids from Gramineae on aphids. *Phytochemistry* **1984**, *23*, 539–541.

Coulman, B. E.; Woods, D. L.; Clar, K. W. Identification of low alkaloid genotypes of reed canary grass. *Can. J. Plant Sci.* **1976**, *56*, 837–845.

Coulman, B. E.; Clark, K. W.; Woods, D. L. Effects of selected reed canary grass alkaloids on in vitro digestibility. *Can. J. Plant Sci.* **1977**, *57*, 779–785.

Gallagher, C. H.; Koch, J. H.; Moore, R. M.; Steel, J. D. Toxicity of *Phalaris tuberosa* for sheep. *Nature* **1964**, *204*, 542–545.

Hanson, A. D.; Traynor, P. L.; Ditz, K. M.; Reicosky, D. A. Gramine in barley forage—effects of genotype and environment. *Crop Sci.* **1981**, *21*, 726–730.

Hanson, A. D.; Ditz, K. M.; Singletary, G. W.; Leland, T. J. Gramine accumulation in leaves of barley grown under high-temperature stress. *Plant Physiol.* **1983**, *71*, 896–904.

Leete, E. Biosynthesis and metabolism of gramine in *Lupinus hartwegii*. *Phytochemistry* **1975**, *14*, 471–474.

Lohar, M. K. Effect of an indole alkaloid, gramine, on the feeding behaviour of the aphids, *Schizaphis graminum* (R) and *Rhopalosiphum padi* (L.). *Pak. J. Zool.* **1989**, *21*, 261–266.

Majak, W.; McDiarmid, R. E.; van Ryswyk, A. L.; Broersma, K.; Bonin, S. G. Alkaloid levels in reed canary grass *Phalaris arundinacea* on wet meadows in British Columbia. *J. Range Manage.* **1979**, *32*, 322–326.

Marten, C. G.; Barnes, R. F.; Simons, A. B.; Wooding, F. J. Alkaloids and palatability of *Phalaris arundinacea* L. grown in diverse environments. *Agron. J.* **1973**, *65*, 199–201.

Marten, C. G.; Jordan, R. M.; Hovin, A. W. Biological significance of reed canarygrass alkaloids and associated palatability variation to grazing sheep and cattle. *Agron. J.* **1976**, *68*, 909–914.

Marten, C. G.; Jordan, R. M.; Hovin, A. W. Improved lamb performance associated with breeding for alkaloid reduction in reed canarygrass. *Crop Sci.* **1981**, *21*, 295–298.

Salas, M. L.; Corcuera, L. J. Effect of environment on gramine content in barley leaves and susceptibility to the aphid *Schizaphis graminum*. *Phytochemistry* **1991**, *30*, 3237–3240.

Sepulveda, B. A.; Corcuera, L. J. Effect of gramine on the susceptibility of barley leaves to *Pseudomonas syringae*. *Phytochemistry* **1990**, *29*, 465–467.

Simons, A. B.; Marten, G. C. Relationship of indole alkaloids to palatability of *Phalaris arundinacea* L. *Agron. J.* **1971**, *63*, 915–919.

Westcott, N. D.; Hinks, C. F.; Olfert, O. Dietary effects of secondary plant compounds on nymphs of *Melanoplus sanguinipies* (Orthoptera: Acrididae). *Ann. Entomol. Soc. Am.* **1992**, *85*, 304–309.

Zuniga, G. E.; Salgado, M. S.; Corcuera, L. J. Role of an indole alkaloid in the resistance of barley seedlings to aphids. *Phytochemistry* **1985**, *24*, 945–947.

Zuniga, G. E.; Varanda, E. M.; Corcuera, L. J. Effect of gramine on the feeding behaviour of the aphids *Schizaphis graminum* and *Rhopalosiphum padi*. *Entomol. Exp. Appl.* **1988**, *47*, 161–165.

Received for review March 12, 1992. Accepted July 20, 1992.

Registry No. AMI, 22259-53-6; MAMI, 19293-60-8; gramine, 87-52-5; tryptamine, 61-54-1.