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Simplified Extraction of Ergovaline and Peramine for Analysis of Tissue Distribution in Endophyte-Infected Grass Tillers

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An improved extraction and cleanup procedure for quantitative analysis of ergovaline in *Neotyphodium*-infected grass tissues by high-performance liquid chromatography was developed, utilizing aqueous 2-propanol−lactic acid as extraction solvent. Losses of sample material and time requirements were significantly reduced, handling procedures simplified, and ergovaline and internal standard ergotamine recovered with similar efficiency from extracts. Analyses can be carried out on very small amounts (2−5 mg of dry weight) of samples and another endophyte-alkaloid, peramine, determined in the same extracts. Calibration curves with 2-propanol−lactic acid were linear over the range 0.004−0.938 μM ergovaline (= 2−500 ng/mL) in extracts, corresponding to 0.04−10 μg/g in samples. The distribution of ergovaline in the plant was extremely heterogeneous, indicating low *in-planta* mobility and strong regulation of accumulation by the internal plant environment. In contrast, peramine was much more uniformly distributed. These results clearly demonstrate very large differences in the tissue specificities of ergovaline and peramine.

KEYWORDS: Ergovaline; peramine; HPLC; ergot alkaloids; *Neotyphodium* endophytes

INTRODUCTION

The ergot (ergopeptine) alkaloid ergovaline (**Figure 1**) is produced in the agriculturally important grasses tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) infected by species of endophytic fungi of the genus *Neotyphodium* [formerly *Acremonium* (*1*)]. Ergovaline in endophyte-infected grasses has been implicated in the strong toxic effects these grasses have on mammals (*2*, *3*), causing high economic losses to agriculture (*4*, *5*). However, endophyte infection significantly enhances plant persistence by protecting endophyte-infected grasses from pests and improving drought tolerance and competitive performance (*6*−*10*). Because of the benefits of endophyte infection, current research is aimed at improving animal health while maintaining endophyte-mediated grass protection, for example, by selecting low-toxin endophyte−grass associations. Ergovaline levels in infected grasses are affected by the environment and the genotype of plant and/or endophyte (*11*−*15*). Little is known, however, about factors controlling ergovaline production in the internal plant environment. The objective of this study was therefore the development of sensitive methods for ergovaline analysis to investigate its accumulation in different tissues from an individual grass plant (tiller).

To examine the tissue specificity of ergovaline accumulation, fine fractionation of tissues from grass tillers is required. Available methods for ergovaline analysis were developed mainly for determination of levels in whole pastures and typically call for 50−100 mg of grass tissues (*14*, *16*−*19*), requiring sampling and dissection of a very large number of tillers. To significantly reduce sample and time requirements, a new method was needed for extraction from grass samples of <10 mg of dry weight, with sufficient sensitivity to measure ergovaline at levels down to 0.1 μg/g.

Ergovaline can be quantified in extracts from endophyte-infected grasses by high-performance liquid chromatography (HPLC) with fluorescent detection (*20*, *21*), providing high sensitivity and selectivity in the determination. Ergovaline quantification is usually performed with another ergopeptine (e.g., ergotamine; **Figure 1**), which is not typically produced by grass endophytes, as an internal standard (*14*, *16*−*19*). Solvent extractions from grass material are carried out with the pH adjusted low [e.g., with organic acids (*18*, *22*)] or high [e.g., with NaOH or ammonia (*14*, *16*, *17*)], to ensure good solubility of the ergopeptines in extracts. However, ergopeptines are susceptible to degradation due to oxidation and isomerization/epimerization (*20*, *21*), especially under the conditions of high or low pH. Consequently, recovery of the ergopeptines from grass extracts is always <100% (*14*, *17*−*19*). Therefore, to ensure accuracy in the quantification, both ergovaline and internal standard must be recovered from extracts with similar efficiencies.

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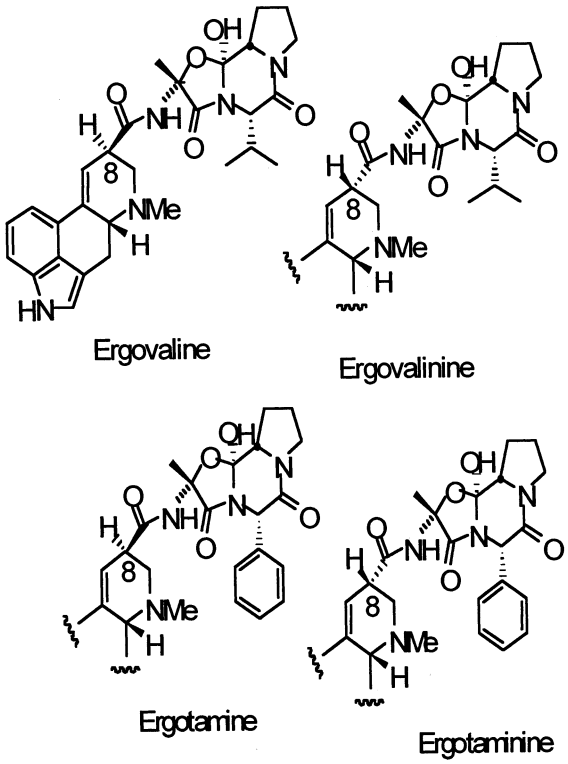

Figure 1. Ergovaline, internal standard ergotamine, and their respective C-8 stereoisomers ergovalinine and ergotaminine. (Wavy lines indicate remainder of structure identical to ergovaline.)

These aspects have been taken into consideration in the development of a new microextraction method for ergovaline extraction from grass samples of 2−5 mg of dry weight. This new method utilizes lactic acid, previously found to have good qualities for ergopeptine extraction (*22*), and 2-propanol, which is less hazardous and more compatible with plastic labware used in extractions than chloroform. This solvent system also allows coextraction of peramine, another important endophyte alkaloid. The utility of the new method for assessing ergovaline and peramine distribution within grass tillers was tested on fractionated tissues from endophyte-infected perennial ryegrass.

MATERIALS AND METHODS

Materials. All solvents used in extractions or HPLC elution were of analytical grade or HPLC grade, respectively (Analar, BDH, Poole, U.K.). Deionized ultrapure water was prepared by reverse osmosis, followed by filtration through carbon and ion-exchange cartridges (Milli-Q, Millipore Corp., Bedford, MA).

Two grass samples (cut from the basal regions of the grass plant = pseudostem), referred to as high-ergovaline (from an experimental seed line of *Lolium perenne* cv. Grasslands Pacific, infected by a high-ergovaline-producing *N. lolii* strain, AR5) and low-ergovaline [*L. perenne* × *multiflorum*, infected by *N. lolii*, strain Lp19 (*23*)], were used for method development. Additional materials including leaf and pseudostem samples from *L. perenne* GA66 infected by the *Neotyphodium* strain Lp1 (*23*), a high-ergovaline-producing species, and *L. perenne* cv. Grasslands Nui infected by *N. lolii* Lp19 and corresponding uninfected plants were utilized for calibration studies. Ergovaline and peramine distribution in a grass tiller were determined on an Lp19-infected genotype of Grasslands Nui. After collection, each sample was freeze-dried for 24 h and ground in a coffee grinder, except for tissues dissected to determine alkaloid distribution, which were ground in microcentrifuge tubes to minimize losses (see below). Samples were agitated vigorously by shaking for 10 s immediately prior to use to remix the ground particles, because during storage separation into different-sized particles occurred.

Ergopeptine Standard Solutions. A standard solution of ergovaline in methanol was prepared from the free base (estimated purity = 90−95%; kindly provided by Dr. F. Smith, Pharmacal Sciences Department, Auburn University, Auburn, AL; 97.6 μM). Internal standard ergotamine in methanol was prepared from ergotamine hemitartrate salt (Sigma Chemical Corp., St. Louis, MO; 172 μM). Standard solutions and diluted working solutions of the internal standard (17−34 μM) in methanol/water (1:1 v/v) were stored for up to 2 weeks at −20 °C. This period was found to have no effect on ergopeptine stability under the conditions specified. In all experiments, 20 μL of working solution [containing a recorded amount of (3.4−6.9) × 10^{-4} μmol of ergotamine standard] was added to each sample. For calibration studies, solutions were prepared in aqueous 2-propanol−1% (w/v) lactic acid (1:1 v/v) with a range of concentrations of ergovaline (0.004−0.938 μM) and a constant concentration of ergotamine (1.9−2.6 μM) by dilution from the ergovaline and ergotamine standard solutions.

Ergovaline Analysis by HPLC. The column was a 150 × 4.6 mm i.d., 5 μm, Prodigy C_{18} (Phenomenex, Torrance, CA) equipped with an RP-18 Brownlee Newguard precolumn (Perkin-Elmer Analytical Instruments, Norwalk, CT), and the column temperature was 28 °C. Sample extracts (volumes between 10 and 30 μL) were injected with an 851 AS autosampler (Jasco Corp., Tokyo, Japan) onto the column. Multilinear binary gradients for the separation of ergovaline, ergotamine, and their respective isomers in the HPLC were run as follows: 0 min, 95% (v/v) solvent A, 5% (v/v) solvent B; 20 min, 80% solvent A, 20% solvent B; 35 min, 50% solvent A, 50% solvent B; 40 min, 30% solvent A, 70% solvent B; 47 min, 30% solvent A, 70% solvent B; 52 min, 95% solvent A, 5% solvent B. Solvent A was acetonitrile−aqueous ammonium acetate (0.1 M) (1:3 v/v); solvent B was acetonitrile−aqueous ammonium acetate (0.1 M) (3:1 v/v). The flow rate was 1 mL/min, controlled by a PU-980 pump (Jasco Corp.). Each compound was identified and measured by fluorescent detection (λ_{ex} = 310 nm, λ_{em} = 410 nm, RF 551 detector, Shimadzu, Kyoto, Japan).

Sample Extraction with Chloroform—Methanol Ammonia. The extraction was carried out essentially as described by Barker et al. (*16*). In brief, 50 mg of dried and ground grass samples was weighed into a small glass vial. One milliliter of chloroform−methanol ammonia (75:25:2 v/v) and 20 μL internal standard solution were added to each sample, and the mixture was agitated on a vortex mixer for 10 s. Samples were extracted standing overnight at room temperature in the dark. After extraction, the solute was separated by aspiration through an immersion filter and the residual plant material rinsed twice with 0.5 mL of the extraction solvent. The solvent was evaporated (1−2 h) under reduced pressure in 10 mL vials. The dried residue was suspended in and partitioned between carbon tetrachloride (0.5 mL) and a methanol−25 mM aqueous tartaric acid solution (0.5 mL, 1:1 v/v). Upon standing, two phases separated; the upper (aqueous) fraction was separated and filtered (0.2 μm filter) and transferred into a glass vial for HPLC analysis.

Sample Extraction with 2-Propanol—Lactic Acid. Dried and ground grass samples (20 mg) were weighed into 2.0 mL plastic vials (Sarstedt, Nürnbrecht, Germany) and extracted in 0.5 mL of aqueous 2-propanol (50% v/v) with 1% (w/v) lactic acid, unless indicated otherwise. 2-Propanol−lactic acid containing the internal standard ergotamine was added to each vial, and the vials were agitated for 60 s at setting 5 in a cell disrupter (FP 120 Savant FastPrep; BIO 101 Inc., La Jolla, CA). All samples were extracted standing in darkness for 2 h (unless indicated otherwise), at room temperature. Particulate material in the extracts was removed by replacing the vial cap with a modified cap containing a porous 3.2 mm thick, 70 μm pore size polyethylene filter (Bel-Art Products, Pequannock, NJ) fitted onto a second extraction vial and filtering the extracts into the second vial by centrifugation at 6000*g* for 5 min (Megafuge 10, Heraeus Sepatech, GmbH, Osterode, Germany). The filtered extracts were transferred into HPLC vials. The extracts were either immediately loaded onto the HPLC or stored at −20 °C for no longer than 24 h before loading. Handling of extracts in this way ensured that no detectable degradation of the ergopeptines occurred between collection and analysis.

The effect of 2-propanol solvent concentration on ergopeptine extraction was determined on high-ergovaline grass samples extracted with 20, 30, 40, 50, 60, or 70% (v/v) aqueous 2-propanol−1% (w/v)

lactic acid. After extraction for 1 h, extracts were collected as described above and analyzed by HPLC. Relative recoveries (= percent of recovery in the first extraction) of the ergopeptines were determined by re-extracting plant material overnight with 0.5 mL of fresh solvent of the same 2-propanol concentration as in the first extraction.

A time course of ergopeptine extraction was performed with high-ergovaline grass samples; extracts from three designated samples were collected at each time point. Ergovaline analysis of small sample quantities was tested with high- and low-ergovaline grass samples of 2, 5, 10, 20, and 50 mg dry weight, extracted in 0.5 mL (20 and 50 mg samples) and 0.3 mL (2, 5, and 10 mg samples) of 2-propanol−lactic acid.

Extraction with Acetic Acid. The solvent system (20% v/v acetic acid in water) was adapted from that of Shelby and Flieger (*18*). Ergopeptine extraction efficiency in acetic acid was directly compared with 2-propanol−lactic acid; sample material and amount of internal standard added to each sample were identical. Extractions (for 1 h) and re-extractions were performed as described for 2-propanol−lactic acid.

Calibration. The effect of the plant matrix on relative recoveries of added ergopeptine standards with 2-propanol−lactic acid was determined for a range of standard concentrations. The calibrations were performed with solvent containing standards and dried, ground plant material or no plant material. Plant material infected by endophyte having a range of endogenous concentrations of ergovaline 0.7−5.7 μg/g and endophyte-free plant material (nil) were used. Both pseudostem and leaf blade materials were examined. Plant samples (50 mg) were extracted in 1 mL of 2-propanol−lactic acid with added standards, giving a range of concentrations of ergovaline (corresponding to 0.04−10 μg/g of plant material) and a constant concentration of ergotamine (22−30 μg/g of plant material). To determine the recovery of added ergotamine standard from grass material, 400 ng of ergotamine standard in 0.5 mL of 2-propanol−lactic acid solvent was added to extraction vials with 20 mg grass samples and to vials without grass material. Ergotamine recovery from extracts with grass material was determined as percent recovery relative to extracts without added plant material.

Limit of Detection and Limit of Quantification. The lower limit of detection for ergovaline was determined as the concentration giving a signal exceeding the noise in the blank by a factor of 3 and the lower limit of quantification as 3 times the limit of detection. Accuracy was determined by the addition of known amounts of ergovaline standards to plant material before extraction (above) and reproducibility by repeated measurements of samples.

Plant Dissection for Determination of Alkaloid Distribution. The perennial ryegrass plants used in the dissection were non-reproductive; that is, no stem elongation for formation of seed heads had occurred. Tillers excluding the roots were collected and dissected under a stereomicroscope (Olympus, Tokyo, Japan). The small basal region of the tiller bearing the leaves and descendent tillers was identified as the true stem and dissected into stem (~3−8 mm in length) and stem apex (the stem region above the youngest mature leaf, ~2−3 mm in length). The leaves (comprising sheath and blade) from each tiller were dissected in separate layers: mature leaves (i.e., fully expanded leaves, with a fully developed ligule at the collar) were separated into first (the youngest), second, and third (oldest) leaf. Emerging leaves were young, growing leaves without a fully developed ligule. The mature leaves were separated at the ligule into sheath and blade, and both sections were further divided by a median cut into lower and upper parts; the sheath and blade of emerging leaves were separated at the immature ligule (identified under the stereomicroscope at 10× magnification by a small protuberance at the adaxial side of the leaf). Leaf meristems (1.5 cm in length; from sheath or blade, depending on developmental stage of the leaf) consisted of the basal section of the emerging leaf. Due to small tissue quantities, each dissected and freeze-dried sample was ground to a fine powder in liquid nitrogen with a micropestle (Eppendorf, Hamburg, Germany) in a 1.5 mL microcentrifuge tube (Sarstedt).

Quantification of Ergovaline in Grass Samples. Under the extractive conditions used, ergovaline (analyte) and ergotamine (standard) progressively form the two isomers ergovalinine and ergotaminine (**Figure 1**), respectively (*18*). To account for isomerization in the quantification of ergovaline, measured peak areas of the "-inine" isomers were included in the calculation.

Ergovaline was quantified on the basis of plant dry mass (DW) by the equation

$$\frac{\mu\text{g ergovaline}}{\text{g of DW sample}} = \frac{\text{MW of ergovaline}}{\text{MW of ergotamine}} \times \frac{\text{mass of standard}}{\text{mass of analyte}} \times \frac{\text{peak area of ergovaline} + \text{peak area of ergovalinine}}{\text{peak area of ergotamine} + \text{peak area of ergotaminine}} \quad (1)$$

Calculations were performed using digital data collection with Class GC 10 software (Shimadzu).

Analysis and Quantification of Peramine. The efficiency of peramine extraction with 2-propanol−lactic acid was tested by serial extractions on endophyte-infected grass samples and gave results equivalent to those obtained with the method of Barker et al. (*16*) (data not shown). A standard amount of the nitrate hydrogen salt of homoperamine [3-(-4-guanidinylbutyl)-2-methylpyrrolo[1,2-*a*]pyrazin-1(2*H*)-one] [AgResearch Grasslands, ~5.7×10^{-3} μmol (=1.5 μg) of the free base in 0.5 mL of MeOH] was added to the extraction solvent as internal standard. Chromatography of peramine and homoperamine was carried out according to a modification of procedures described by Barker et al. (*16*) and Cox and Stout (*24*), utilizing a column-switching procedure for initial ion-exchange separation for removal of interfering compounds. This was facilitated by a six-port electrically switched controlled valve system (Valco Instruments, Houston, TX). An aliquot of extract (15−30 μL) was injected (SIL-9A autoinjector, Shimadzu) into a stream of 0.4 mL/min (M-6000A pump, Waters Associates, Milford, MA) of methanol−water−ammonium hydroxide (33%), 670:330:6 v/v, onto a 7.5 × 4.6 mm, 7 μm, Macrosphere 300 WCX All-Guard silica-based weak anion exchange cartridge (Alltech Associates, Deerfield, IL) for 2 min. Peramine and the internal standard homoperamine were selectively retained in the cartridge while other UV-absorbing compounds were washed through to waste. The cartridge was back-flushed at 1 mL/min with a solvent of 50 mM ammonium acetate, 5 mM guanidinium carbonate, and 0.2% (v/v) acetic acid in water−methanol (4:1 v/v) with an LC Pump 250 (Perkin-Elmer) onto a 250 × 4.6 mm i.d., 5 μm, Sphereclone silica HPLC column (Phenomenex) for the separation of peramine and homoperamine. Peramine and homoperamine peaks were detected by UV absorption at 280 nm (UV-970 detector, Jasco Corp.). Peramine concentration (micrograms per gram) in the grass sample was determined as for ergovaline, by the above eq 1, with homoperamine as internal standard.

Statistical Analyses. To determine statistical significance of treatments and alkaloid distribution patterns, data were analyzed by one-way analysis of variance (ANOVA). Tests for significant differences between means were performed with Fisher's test of least significant difference.

RESULTS

Comparison of 2-Propanol—Lactic Acid Extraction with Standard Ergovaline Extraction Methods. To test whether 2-propanol−lactic acid is generally suitable for extracting ergovaline, a comparison with standard methods was performed. In a first experiment with low-ergovaline grass material, samples were processed with the 2-propanol−lactic acid method (extraction for 1 h) or with a published chloroform−methanol ammonia method (*16*, *17*). As shown in **Figure 2**, both methods gave similar chromatograms with well-separated and prominent ergopeptine peaks with no detectable interference from the grass sample. The concentration of ergovaline in the plant material estimated by the 2-propanol−lactic acid extraction method (0.48 ± 0.04 μg/g; mean ± 1 SD, n = 6) was not significantly different from the estimate by the chloroform−methanol ammonia method (0.52 ± 0.08 μg/g; n = 8). 2-Propanol−lactic acid was also compared with an aqueous acetic acid extraction method described by Shelby and Flieger (*18*). The estimated ergovaline content was nearly 2-fold higher in the samples

Table 1. Estimated Ergovaline Concentration in the Sample, Chromatographic Peak Areas of Ergovaline and Internal Standard Ergotamine, and Percent Recovery of Ergovaline and Standard in Re-extractions in Samples Extracted with 2-Propanol–Lactic Acid or Acetic Acid

solvent	estimated ergovaline concn in the sample[a] (μg/g)[b]	peak area (lysergyl units)		amount in re-extractions (% of first extraction)	
		ergovaline $\times 10^4$	internal standard $\times 10^5$	ergovaline	internal standard
2-propanol–lactic acid	2.27 ± 0.05	9.67 ± 0.37	6.72 ± 0.13	17.0	17.0
acetic acid	3.94 ± 0.16	9.19 ± 0.19	3.69 ± 0.10	21.6	36.0

[a] High-ergovaline grass material (see Materials and Methods for details) was used. [b] Mean ± 1 standard deviation ($n = 3$).

Table 2. Chromatographic Peak Areas, Estimated Ergovaline Concentration in the Sample, and Isomerization of Internal Standard at Various Concentrations of Aqueous 2-Propanol with 1% (w/v) Lactic Acid

2-propanol concn (%, v/v)	peak area (lysergyl units)		estimated ergovaline concn in the sample[b] (μg/g)	peak areas in re-extractions (% of first extraction)		isomerization (% ergotaminine)
	ergovaline $\times 10^4$[a]	internal standard $\times 10^5$		ergovaline	ergotamine	
30	5.57[1c]	3.93[1]	2.33[1]	18.3[1]	25.9[1]	1.71[1]
40	6.87[1]	4.83[2]	2.27[1]	nd[d]	nd	1.88[1]
50	8.31[2]	5.06[2]	2.63[2]	nd	nd	2.90[2]
60	6.70[1]	4.91[2]	2.19[3]	nd	nd	3.62[2]
70	6.73[1]	4.69[2]	2.28[1,3]	6.6[2]	7.7[2]	4.77[3]

[a] Means from three replicate extractions. [b] High-ergovaline grass material was used. [c] Means followed by the same number in a column are not significantly different from each other ($P < 0.05$). [d] nd = not determined.

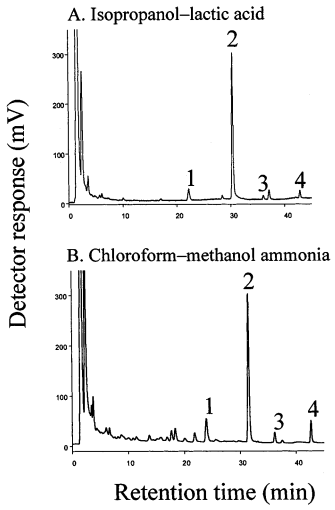

Figure 2. HPLC chromatograms of endophyte-infected grass samples (pseudostem) extracted with 2-propanol–lactic acid or chloroform–methanol ammonia. Numbers above peaks indicate ergopeptines and isomers: 1, ergovaline; 2, ergotamine; 3, ergovalinine; 4, ergotaminine.

extracted with acetic acid (**Table 1**). However, amounts of recovered ergovaline in extracts were nearly identical in acetic acid and 2-propanol−lactic acid, as indicated by the chromatographic peak areas (**Table 1**). In contrast, recovery of the internal standard was significantly (nearly 2-fold) greater with 2-propanol−lactic acid than with acetic acid (**Table 2**). Thus, the higher estimate of ergovaline content in the acetic acid-extracted samples was due to a lower recovery of internal standard in this solvent rather than a higher recovery of ergovaline. This conclusion was supported by data from re-extractions. Relative recoveries of ergovaline and internal standard on re-extraction with 2-propanol−lactic acid were practically identical. With acetic acid, the relative recovery of internal standard on re-extraction was much greater than that of ergovaline (**Table 1**), providing further evidence of a large differential in the recoveries of ergovaline and standard in acetic acid. Isomerization (**Figure 1**) of internal standard was 8.3% in acetic acid but only 2.2% in 2-propanol−lactic acid, suggesting greater losses of the standard in acetic acid.

Variation of 2-Propanol Concentration in the Solvent. Ergovaline and internal standard were more efficiently extracted at 50% 2-propanol than at the higher and lower 2-propanol concentrations (**Table 2**). However, estimated ergovaline concentrations in samples were very similar overall, and re-extractions demonstrated similar recoveries of analyte and internal standard over the range of different 2-propanol concentrations (**Table 2**; for 50% 2-propanol, see also **Table 1**). Variation in lactic acid concentration, ranging between 1 and 10% (w/v) (in 50% 2-propanol), gave no significant effects on ergopeptine extraction (data not shown).

Time Course of Extraction of Ergovaline and Internal Standard in 2-Propanol—Lactic Acid. Extracted ergovaline increased rapidly initially (15−120 min; **Figure 3A**), and subsequent changes were <5%. Internal standard also showed an initial increase but less than for ergovaline. For both analytes variation between replicates was greater during the first 60 min of extraction (**Figure 3B**). At 2−4 h, levels of analyte and standard stabilized, giving stable estimates for ergovaline concentration in the sample (**Figure 3C**).

Quality Criteria. Calibration curves with the 2-propanol−lactic acid solvent and ergovaline standard were linear between 0.004 and 0.938 μM ergovaline in extracts, corresponding to 0.04−10 μg/g ergovaline in plant samples [slope, 0.948 (SD = 0.006); intercept, 0.014 (SD = 0.015), standard error of the regression, 0.044 μg/g, correlation coefficient, 0.9995; duplicate measurements at six concentrations of standard ergovaline, including blank]. For nil-endophyte samples, regression intercepts were not significantly different from zero, and for endophyte-infected material, regression intercepts were not significantly different from estimates of endogenous ergovaline in unspiked samples. Recoveries of ergotamine standard from grass material averaged 82% (range = 77−86%), similar to recoveries reported in the literature (*14*, *16*, *17*). Mean recovery of added ergovaline relative to the ergotamine standard was 99% (range = 93−105%). For ergovaline standard alone, and after addition to nil-endophyte plant material, the mean standard deviation of duplicate measurements was 0.017 μg/g and the

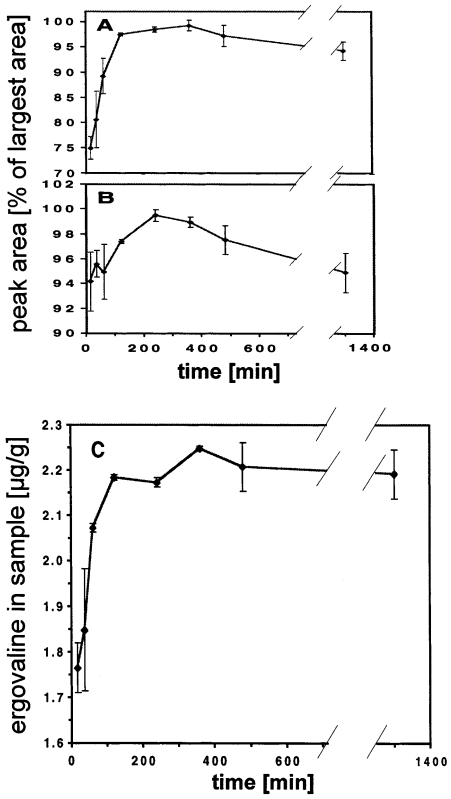

Figure 3. Time course of ergopeptine extraction. Indicated are chromatographic peak areas of ergovaline (A) and internal standard (B) and estimated ergovaline concentration in the sample (C). High-ergovaline grass material was used in this experiment. Error bars show ± 1 standard deviation ($n = 3$).

coefficient of variation (CV), 4.5%. For measurements of endophyte-infected material (with and without added standards) the mean standard deviation of duplicate measurements was 0.13 μg/g and the CV 2.2%. In the latter case, variation of endogenous ergovaline in the sample appeared to contribute to most of the variation between the measurements.

The lower limit of detection of the ergovaline peak estimated from the instrument noise was 0.01 μg/g and the lower limit of quantification, 0.04 μg./g. In practice, the limit of quantification can be affected by minor interferences with ergovaline or ergovalinine, observed in both endophyte-free and endophyte-infected grass samples, and we consider the practical limit of quantification for this assay to be 0.1 μg/g.

Extraction and Quantification of Ergovaline from Small Sample Quantities. Estimates of ergovaline concentrations in low-ergovaline plant material were similar for samples in the range of 5−50 mg of dry weight (ranging from 0.52 ± 0.01 to 0.59 ± 0.02 μg/g; mean ± SD, $n = 3$) but significantly ($P < 0.05$) lower in the 2 mg samples (0.36 ± 0.08 μg/g; $n = 3$). The lower estimate in the 2 mg samples was due to small peak areas for ergovaline, approaching the limit of quantification in the HPLC (data not shown). In high-ergovaline samples, peak areas of ergovaline were well above the limit of quantification in the HPLC, even at the lowest sample quantity (= 2 mg, data not shown). However, with this sample material, the 25-fold reduction in sample dry weight resulted in a significant ($P < 0.05$) but minor (~10%) decline in estimated ergovaline concentration (50 mg sample, estimate = 2.41 ± 0.07 μg/g; 2 mg sample, estimate = 2.19 ± 0.09 μg/g; $n = 3$). Recovery of ergovaline remained constant over the range of sample dry weights, but recovery of internal standard increased slightly as sample dry weight decreased (data not shown).

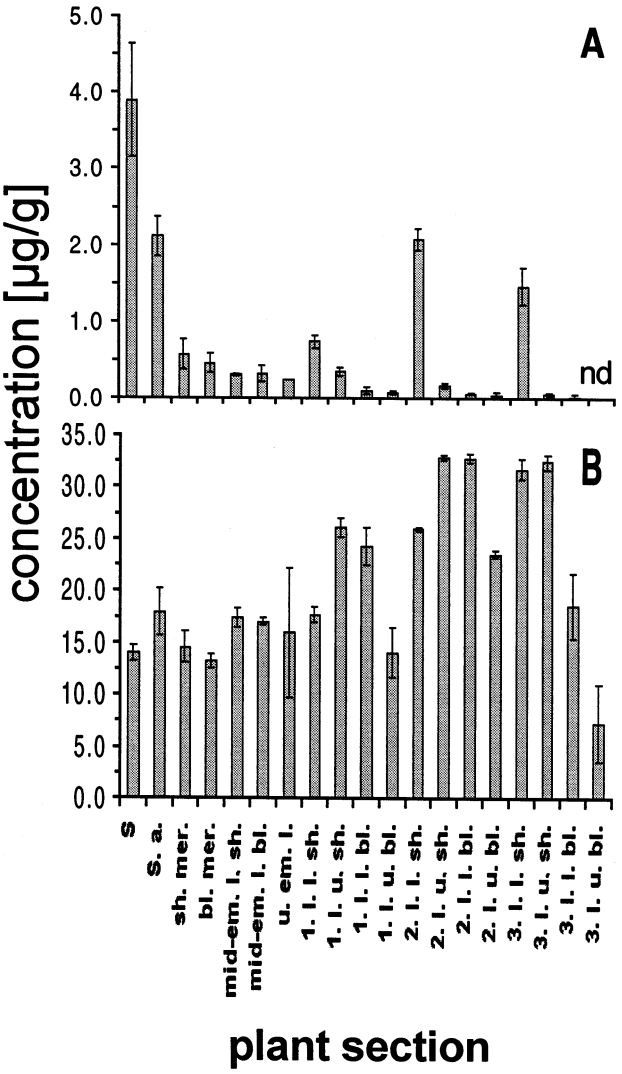

Figure 4. Distribution of ergovaline (A) and peramine (B) in an *N. lolii*-infected perennial ryegrass tiller determined with the 2-propanol–lactic acid solvent method. Leaf tissues were dissected as specified under Materials and Methods into stem (S), stem apex (S. a.), sheath meristem (sh. mer.), blade meristem (bl. mer.), mid-emerging leaf sheath (mid-em. l. sh.), mid-emerging leaf blade (mid-emerg. l. bl.), upper emerging leaf (u. em. l.), first leaf lower sheath (1. l. l. sh.), first leaf upper sheath (1. l. u. sh.), first leaf lower blade (1. l. l. bl.), first leaf upper blade (1. l. u. bl.), second leaf lower sheath (2. l. l. sh.), second leaf upper sheath (2. l. u. sh.), second leaf lower blade (2. l. l. bl.), second leaf upper blade (2. l. u. bl.), third leaf lower sheath (3. l. l. sh.), third leaf upper sheath (3. l. u. sh.), third leaf lower blade (3. l. l. bl.), and third leaf upper blade (3. l. u. bl.). Ergovaline and peramine were determined on the same extracts (see Materials and Methods) from tissue samples taken from 5–12 tillers per replicate plant. Indicated are means from two replicates; error bars show ± 1 standard deviation; nd = not detected.

Distribution of Ergovaline and Peramine in a Grass Tiller. Ergovaline concentrations in tissues from ryegrass tillers were determined with the 2-propanol−lactic acid method, using tissue amounts of 4−10 mg (stem tissues) and 10−30 mg (leaf tissues). The distribution of peramine was determined by a separate HPLC analysis procedure (see Materials and Methods), using the same extracts as for the ergovaline analysis.

Ergovaline accumulation was highly tissue-specific, with highest levels in the true stem tissues and greater amounts in mature lower leaf sheath sections (**Figure 4**). A steep gradient in ergovaline concentration from lower to upper leaf sheath was

observed, particularly in the second and third leaves where the ergovaline concentration was significantly higher in lower than in upper sheath ($P < 0.05$).

The detailed tissue fractionation revealed differences in peramine levels between different tissues (**Figure 4**), but these were generally much smaller than for ergovaline, indicating little or no tissue specificity of peramine accumulation. There was no basal−apical concentration gradient in the leaf sheaths as seen for ergovaline. Rather, peramine concentration peaked in the upper sheath and lower blade of younger leaves ($P < 0.05$).

DISCUSSION

Several studies on composite tissues (e.g., pseudostem consisting of whole leaf sheaths of different age and various amounts of true stem) have indicated differences in ergovaline concentration in different parts of the grass tiller (*2*, *14*, *25*), suggesting considerable tissue specificity of ergovaline accumulation. To examine this in greater detail, a new ergovaline extraction procedure for endophyte-infected grass samples of small (2−50 mg) dry weight was developed. This new method has the added advantage that it allows measurement of both ergovaline and another important grass-endophyte alkaloid, peramine, in a single extract.

We chose a lactic acid−2-propanol mixture as extraction solvent, for good ergopeptine solubility at low pH, with better extraction efficiency than aqueous lactic acid as used in a previous study (*22*), and compatibility with the plastic vials used for sample extraction and cleanup. Moreover, the alkaloid peramine is also extracted efficiently with this solvent mixture (*26*, *27*). Sample losses during transfer and in extractions were minimized by extracting and filtering samples in the plastic vials, giving extracts ready for HPLC. Alternatively to the filtration step used here, we have found that centrifugation of the extracts with grass samples can also efficiently remove grass material from extracts (not shown).

Ergovaline estimates obtained with the 2-propanol−lactic acid solvent were practically identical to estimates obtained with a chloroform-based solvent but with considerably less handling during sample processing. Moreover, 2-propanol−lactic acid was clearly superior to an acetic acid extraction solvent (*18*). The internal standard, ergotamine, was recovered more efficiently with 2-propanol−lactic acid than with acetic acid. More importantly, recoveries of ergovaline and internal standard were practically identical in 2-propanol−lactic acid, even with different percentages of 2-propanol in the solvent. In contrast, whereas recoveries of ergovaline were similar to those in 2-propanol−lactic acid, recovery of ergotamine was much lower in acetic acid. The higher estimates of ergovaline concentration reported by Shelby and Flieger (*18*) for acetic acid extractions compared to chloroform−methanol NaOH thus appear to be due to poor recovery of the internal standard rather than better extraction of ergovaline. Evidently ergotamine is not a satisfactory internal standard for acetic acid extractions.

2-Propanol−lactic acid extraction thus provides a rapid and simple sample preparation for accurate and sensitive quantification of ergovaline. A 2 h extraction time was found to be sufficient for efficient extraction of ergovaline and recovery of the internal standard. In practice, <3 h is required to prepare 20 samples with the new procedure, allowing quantification of ergovaline in grass samples down to very low levels (0.1 μg/g). In addition, maceration of whole grass tissues can be carried out in plastic vials with a cell disrupter for extraction and analysis of ergovaline and peramine (M.J.S. and G.A.L., unpublished results).

With the new procedure, ergovaline content could be measured on very small quantities of grass tissues; only 2 mg (if ergovaline content is ≥ 2 μg/g) or 5 mg (if ergovaline content is ≤ 0.5 μg/g) of grass sample is needed in the determination, compared to 50−100 mg required in the other methods (*14*, *16*−*19*). This reduction in the quantity of sample material has made it possible for the distribution of ergovaline and peramine in the grass tiller to be mapped with much greater resolution than performed in previous studies.

Consistent with earlier studies on broader composite tissue fractions (*2*, *14*, *25*), concentrations of ergovaline were found to be higher in the basal regions of the tiller. Concentrations in the lower leaf sheath were well above levels that can cause adverse physiological effects in mammals (*28*). High concentrations of ergovaline were also found in the stem apex, and very high levels were found in the true stem, which, in terms of mass, constitutes only a small part of the tiller. In addition, there was a very steep gradient of ergovaline concentration within leaf sheaths, indicating significant differential accumulation even within single leaf tissues. By contrast, the distribution of peramine in the grass tiller was found to be relatively uniform, consistent with findings on other *N. lolii*−perennial ryegrass associations (*29*, *30*). There are clearly considerable differences in the regulation of accumulation of the two alkaloids within the plant. These may include differences in the site of production in the tiller, rates of production, release from endophyte mycelium, degradation, and/or translocation. Peramine is more water-soluble than the other alkaloids commonly found in the *N. lolii*−perennial ryegrass symbiosis (*31*), and this may be a major factor in its more even distribution within the tiller.

To further address alkaloid accumulation in the grass tiller, studies on a range of plant genotypes are now underway to elucidate the relationship between the characteristic patterns of alkaloid accumulation and the distribution of fungal mycelium. This is being carried out by combining the sensitive alkaloid analysis method developed here with molecular techniques for precise mapping of the endophyte in the plant (*32*−*34*). Ultimately, this should lead to the identification of key factors and compounds regulating alkaloid accumulation in the endophyte-infected grass plant, potentially providing methods for more effective control of alkaloid levels in endophyte-infected pastures to provide pest-resistant pastures that are safe for grazing livestock.

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