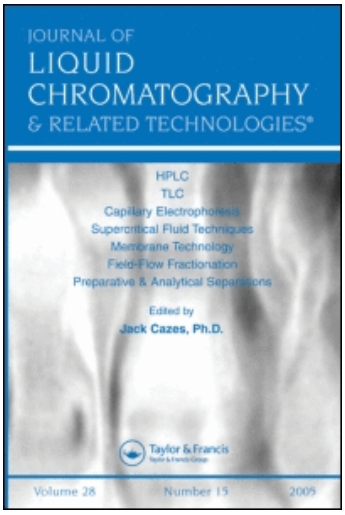

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DETERMINATION OF ERGOVALINE IN ENDOPHYTED SEEDS BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

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ABSTRACT

High performance thin layer chromatography (HPTLC) is used to rapidly determine the presence and amount of ergovalin in seeds of *Graminaceae* infested with *Neotyphodium Coenophialum.* Quinine is used as an internal standard. The mycotoxin is solubilized under acid conditions and extracted with chloroform under alkaline conditions. Samples are sprayed on silica gel plates and detected by fluorescence with λ_1 (excitation) set at 318 nm and λ_2 (emission) > 400 nm.

The method developed is specific for endophyte-infested seeds, linear from 3 to 12 μg/g and is both repeatable and reproducible. The limit of detection is 0.5 μg/g and the limit of determination (quantification) is 3.0 μg/g.

This technique was applied to several batches of seeds obtained from pastures that may or may not be infested with the fungus. Results were compared with results obtained using a standard High Performance Liquid Chromatography (HPLC) method.

INTRODUCTION

In the 70's, a relationship was established between the presence of endophyte-infected forage and various cases of poisoning in animals, later described as fescue toxicosis and rye grass staggers. The fungus responsible for these disorders belongs to the genus, *Neotyphodium,* Glenn, Bacon & Hanlin.[1] It grows between plant cells, especially around the reproductive organs and seeds, lacks a sexual stage of reproduction and, thus, disseminates only *via* the seeds of its host plant.

Two species of fodder grasses have been thoroughly studied as regards their endophytes: tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne).* The *Neotyphodium* species are considered to be synergistic because they improve the resistance of their host against abiotic stresses (drought, nitrogen, depletion) and through the presence of alkaloid mycotoxins, resistance to insects, nematodes, and herbivorous mammals.[2]

The mycotoxins produced belongs mainly to two classes. The lolitrems (indol diterpenoids) which are neurotoxins responsible for the "rye grass staggers," commonly encountered in animals grazing endophyted rye grass and the ergopeptines, the primary causative agent responsible for the "fescue toxicosis," but which can also be synthesised in endophyted rye grass.[3,4] The major recognised mycotoxin in this family is ergovalin.[5]

The ergopeptines interfere with prolactine secretion and body temperature regulation. They are responsible for gangrenes of the extremities "fescue foot disease,"[6,7,8] hypersalivation, rough hair, and lameness. Ingestion by animals of endophyted forage often results in a significant decrease in production level (reduced weight gain, reduced lactation, and low reproductive performances.[6,9]

Ergovaline (Figure 1) accounts for 85 to 97% of the ergot alkaloids identified in endophyte-infected plants and, can therefore, be considered as a major product responsible for these disorders.

A balance between agronomic advantages for the plant conferred by the endophyte and animal economical loss risks may be reached in certain cases. One prevalent condition is that if endophyted seeds have to be used, the level of mycotoxin production and, particularly of ergovaline, must be as low as possible.

Ergovaline (see Figure 1) accounts for 85 to 97% of the ergot alkaloids identified in endophyte-infected plants and is, therefore, considered as the major product responsible for these disorders.[6,9]

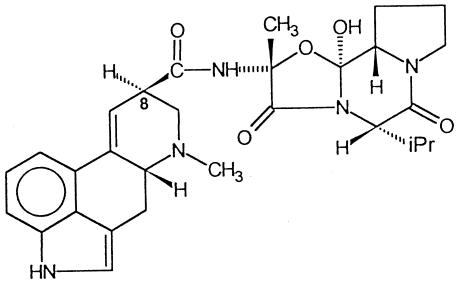

Figure 1. Structure of ergovaline.

The technique described herein is applied to the rapid analysis of seeds in order to offer a technical tool adapted for the estimation of the ergovaline content of seeds. Several HPLC techniques have already been described.[10-13] To our knowledge, there is no published technique using HPTLC. The major advantages of HPTLC are its simplicity and its rapidity. It may, therefore, be used as a screening technique to analyze several batches and cultivars for varieties of selection.

The purpose of this paper is to validate the method, i.e. to verify the specificity of the technique, its linearity, the percent recovery, repeatability, and reproducibility of analyses, and to determine the limit of detection, as well as, the limit of quantification.[14]

EXPERIMENTAL

Reagents

The solvents used are: pure chloroform (Chimie Plus, Denicé, France), ultra-pure ethanol (RPE-ACS), and HPLC-grade methanol (Carlo Erba, Milan, Italy). Deionized water is used throughout the method development.

Samples are acidified with pure, crystallized tartric acid (L+) and alkalinized with ultrapure ammonia solution at 25% (Merck, Nogent-sur-Marne, France).

Apparatus and Chromatographic Conditions

The HPTLC system (CAMAG Basel, Switzerland) used is composed of three parts: an automatic sampler (TLC3, version 2.05), a scanner (TLC

Scanner II) equipped with a mercury vapor lamp for fluorescence, and Cats® software (version 3.15) as driver.

Each sample or standard solution is sprayed automatically with high purity pressurized nitrogen gas (Carboxyque Française, Vénissieux, France) on a glass HPTLC plate, coated with silica gel (granulometry 60), 10 x 20 cm, with a film thickness of 0.25 mm and without a fluorescence indicator (Merck, Nogent-sur-Marne, France).

Plates are then eluted vertically in a standard glass chamber 21x21x10 cm^3.

Sample extracts are evaporated to dryness under vacuum with a rotary evaporator RE 111 (Büchi, Switzerland).

For each standard or sample solution, a volume of 10 µL is sprayed on the plate under high Nitrogen pressure (6,000 hPa), at 8 mm of the lower edge of the plate. The solutions are sprayed as 6x0.5 mm bands. Samples bands are separated by a 10-12 mm space. Plates are eluted in a mixture of chloroform (17.5 mL) and ethanol (2.5 mL). Plate development takes about 20 min (8 cm). After 10 min drying at room temperature, plates are read using fluorimetry with excitation wavelength (λ_1) of 318 nm and an emission wavelength (λ_2) > 400 nm, with a cut-off filter at 400 nm.

Preparation of Standard Solutions

Ergovaline is available as a tartaric acid salt. The degree of purity is 98% (Dr F. Smith, Auburn University, Alabama, USA).

A stock solution of 25 µg/mL free base ergovaline is prepared in methanol and immediately divided into fifty fractions of 400 µL each in conical tubes (i.e., 10 µg ergovaline per tube. Each fraction is evaporated to dryness under nitrogen flux and kept in darkness at –20°C).

The internal standard used (quinine hemisulfate) is available as a 90-95% pure salt (Sigma-Aldrich, Saint Quentin Fallavier, France). A mother solution at 100 µg/mL is prepared in methanol and diluted to prepare a daughter solution of 25 µg/mL.

Ergovaline mother solutions (25 µg/mL) are made-up, as necessary, after dilution of the dry tubes with 400 µL ethanol.

The working standard solution is prepared by combining 50 µL of each of the 25 µg/mL solutions (quinine and ergovaline) and adding 150 µL ethanol to obtain a final concentration of 5 µg/mL.

Origin and Preparation of Samples

Endophyte-infected perennial ryegrass and tall fescue seeds were obtained from the INRA Fodder breeding station of Lusignan (France) and the INRA plant breeding station of Crouelle (France).

Blank and spiked samples were prepared from non-endophyte infected seeds obtained from the same stations.

Seeds were milled in a Dangoumo ball-bearing miller.

Sample Extraction

0.2 to 0.5 g of milled seeds are placed in a 100-mL beaker. To each sample, 40 µL of internal standard solution (25 µg/mL) are added, with 0.5 g tartaric acid and 25 mL deionized water. After manual stirring, samples are filtered on Whatman 2V filter paper and alkalinized to pH> 10 with 25% ammonia.

The solution is transferred to a separatory funnel. A fixed volume of 75 mL chloroform is added. After mixing and separation, the lower chloroform phase is transferred to a 100-mL beaker and evaporated to dryness under vacuum at 70°C with a rotary evaporator. The dry residue is dissolved in three aliquots of 0.5 mL chloroform and kept in a 10-mL glass tube. Chloroform is evaporated under a nitrogen flux at 30°C and the final extract is dissolved in 200-500 µL ethanol.

Validation of the Analytical Procedure

Specificity is determined on non-endophyte infected ryegrass seeds. Two standard ergovaline solutions at 5 µg/mL, two spiked ryegrass samples (with ergovaline), two blank samples, and two ryegrass extracts are prepared.

Based on the noise level estimated on the blank samples, the limit of detection (Noise + 3 standard deviation SD) and the limit of quantification (noise + 10 SD) are determined theoretically.

Spiked samples are prepared and analyzed to obtain peaks corresponding to the above limits (check).

Linearity is evaluated on 5 points (3, 5, 8, 10, and 12 µg/mL) and three consecutive days on both standard and spiked samples. Concentration of internal standard is fixed at 5 µg/mL for all samples. Linear regression equations

are computed based on the ratio of areas between ergovaline and quinine, according to the theoretical concentration.

A Student's t-test is performed to check the Y-axis intercept and confirm that it is not significantly different from zero.

Homogeneity of variances within groups is verified based on a Cochran's C test. Y-axis intercepts and slopes are compared statistically. The existence of a significant slope and validity of the regression line is determined by means of analysis of variance.

Percent recovery for ergovaline and quinine hemisulfate are determined at each concentration.

Repeatability and reproducibility of the extraction procedure are determined on 6 samples from a homogeneous batch of endophyte-infected ryegrass seeds. This procedure is repeated over three days.

Variance homogeneity is verified by means of a Cochran's C test and coefficient of variation of repeatability (CVr) and of reproducibility (CVR) are computed.[14]

RESULTS AND DISCUSSION

Specificity

The densitograms obtained with blank seed samples (Figure 2c) are compared with standard solutions (Figure 2a). No interfering peak is observed in non-endophyte-infected seeds at Rf values corresponding to ergovaline (2, Rf 0.52) and quinine (1, Rf 0.25).

Five other ergot derivatives, available in our laboratory were tested:

Ergonovine, Rf : 0.13,

Ergotamine, Rf : 0.55,

Ergocryptine, Rf : 0.74,

Ergocrystine, Rf : 0.74,

Ergocornine, Rf : 0.74.

An unidentified compound is observed at a Rf of 0.45.

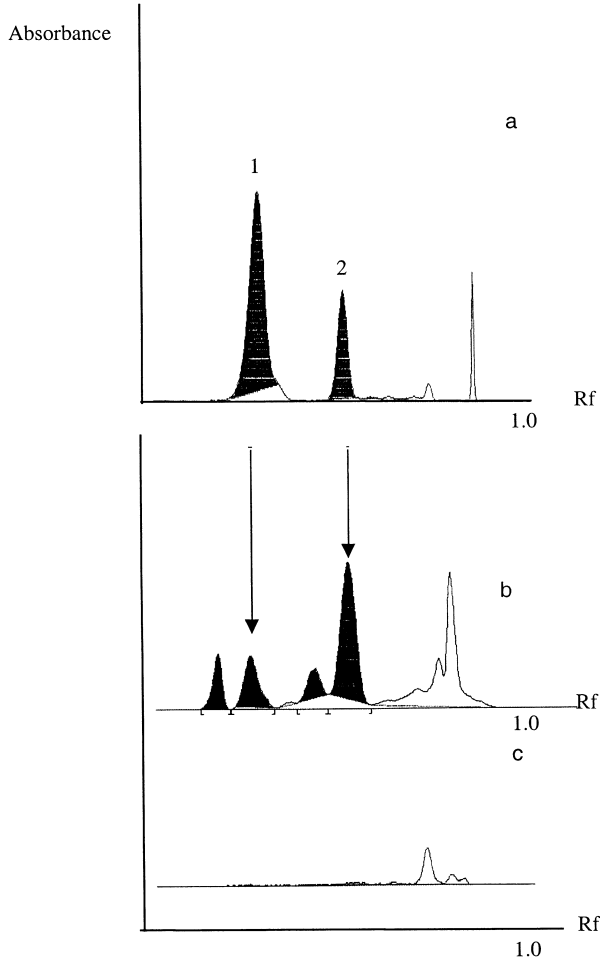

Figure 2. HPTLC densitograms of (a) standard solution at 5 μg/mL ergovaline (2) and quinine (1) (internal standard); (b) endophyte-infected seeds with *Neotyphodium Coenophialum* (test samples (E+) at 13 μg/g seeds); (c) non-endophyte-infected seeds (absorbance in arbitrary units, but all densitograms at the same scale).

Plates can also be read at 254 nm (UV) but the method is twice less sensitive.

Limit of Detection and Limit of Quantification

The mean noise level measured on non-endophyte infected seeds is 2.5 mm. Based on this value, the LOD is determined at 0.5 µg/g and the LOQ at 3.0 µg/g

Linearity-Percent of Recovery

Our results indicate that the regression lines obtained on standards and samples (Figures 3a and 3b) follow a linear regression model as follows: $y = ax + b$ between 3 and 12 µg/mL ($r^2 = 0.996$ for standard solutions and $r^2 = 0.995$ for spiked samples)

The Y-axis intercepts are not significantly different from zero (p=0.05) and do not differ, one from the other. Variance homogeneity among the five groups is confirmed at the 5% level (tabulated value at 0.05, 5, 2: 0.68 ; computed value : 0.32 for spiked samples and 0.40 for standard solutions).

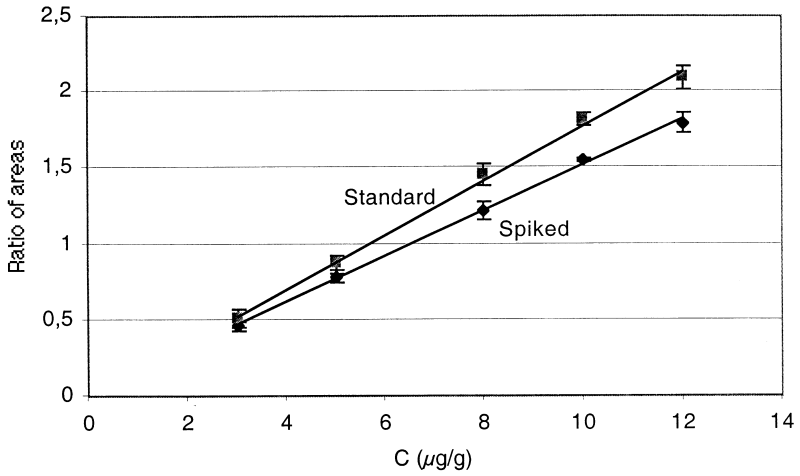

Figure 3. Calibration curves of standard and spiked sample solutions. Area = f (concentration) Y = 0.18 X – 0.010 (standard); Y = 0.15 X + 0.0210 (sample).

Analysis of variance of the linearity results indicate that there is a significant slope and the validity of the regression lines.

The mean percent of recovery on spiked samples varies between 79 and 88% for ergovaline and between 70 and 79% for internal standard.

The data obtained for the three study days are reported in Table 1.

Repeatability - Reproducibility

Concentration of ergovaline is determined in each sample based on the following formula:

$$\text{Concentration in } \mu g/g = \frac{\text{Area sample} \times \text{standard} \times (V) \text{ final dilution}}{\text{Area standard} \times \text{sample amount}}$$

Area sample or standard: area of the corresponding peak on the densitogram

(C) standard = concentration of ergovaline standards = 5 µg/mL

(V) dilution: final volume of dry sample in mL

Sample amount in g.

Variances obtained over the three study days are homogeneous (Cochran table value (0.05;3;6: 0.707; computed value 0.321).

Table 1

Estimation of Percent Recovery of Ergovaline in Spiked Perennial Ryegrass Seed Samples

Ergovaline Concentration (µg/mL)	Mean Ratio of Areas (Standards)	Mean Ratio of Areas (Spiked Samples)	Percent Recovery
3	0.46	0.51	74.6 %
5	0.78	0.88	90.1 %
8	1.22	1.45	90.5 %
10	1.55	1.82	80.5 %
12	1.79	2.09	81.7 %

Coefficients of variation are 5.2% (CVr) and 6.5% (CVR). Such values are highly acceptable in biological samples (< 10% each).[14]

Analysis of Batches

Ten samples of tall fescue and perennial ryegrass seeds have been analyzed according to the described protocol. They were all batches of different cultivars. These seeds came from endophyte-infected or non-endophyte-infected plants. Since only the seeds produced by endophyte-infected plants (E+) contained ergovaline, only their results are reported in Table 2.

All samples tested contained concentrations of ergovaline between 3 and 25 μg/g, compatible with the LOD and LOQ of our method.

These values compared well with results obtained by HPLC, using another published method,[10] as shown in Figure 4.

Table 2

Concentration of Ergovaline Determined in Tall Fescue and Perennial Ryegrass Seeds

Species	E	Conc. Ergovaline in μg/mg
Tall Fescue	+	9.63
Tall Fescue	+	5.36
Tall Fescue	+	8.05
Perennial Ryegrass	+	7.42
Perennial Ryegrass	+	7.94
Perennial Ryegrass	+	15.30
Perennial Ryegrass	+	25.04
Perennial Ryegrass	+	7.19
Perennial Ryegrass	+	7.21
Perennial Ryegrass	+	3.11

*E : endophyte (+ = infected).

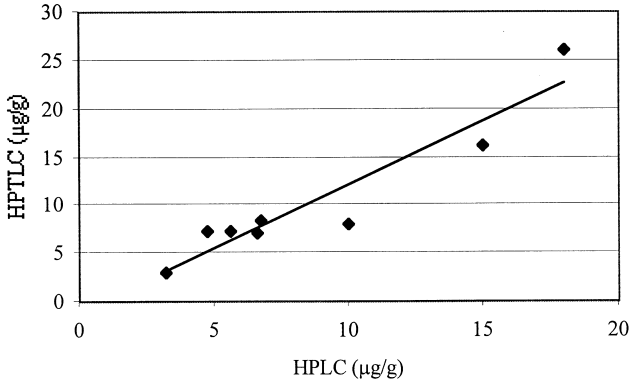

Figure 4. Comparison of results obtained with the HPTLC or HPLC[10] methods.

No statistical difference was found between the analytical results obtained, either with the published HPLC method[10] or with our HPTLC technique (paired t-test on ten samples, p=0.05). This suggests that the proposed HPTLC technique can be used as an alternative analytical technique to HPLC when the number of samples to be analyzed is important. Results obtained with any of these techniques are comparable, without any problem, for inter-laboratory comparison programs in QA/QC procedures.

CONCLUSION

The method proposed herein, and the parameters evaluated, indicate that HPTLC analysis of ryegrass seeds is feasible and valid. Extraction is simple, rapid, does not necessitate tedious purification processes, and is not expensive.

Our method is helpful as a screening tool to rapidly determine if plants are endophyte-infected, and to what extent the fungus species concerned synthesized ergovaline. It must be noted also that a low ergovaline content in seeds will result, in most cases, in a low level in forage. However, no direct quantitative relationship between seed and the corresponding forage ergovaline level exists, since the mycotoxinogenesis in growing plants can vary greatly according to the culture practices and environmental conditions (temperature, droughtness, season, fertilization, etc.). Based on analytical results, it is, however, possible to screen rapidly cultivars with low toxic potential for animals and, consequently, human consumers.

REFERENCES

1. A. E. Glenn, C. W. Bacon, R. Price, R. T. Hanlin, Mycologia, **88**, 369.

2. N. S. Hill, W. C. Stringer, G. E. Rottinghaus, et al, Crop Sci ., **30**, 156-161 (1990).

3. R. W. Hemken, L. P. Bush, in **Toxicants of Plant Origin, Vol.1, Alkaloids,** P. R. Cheeke, ed., CRC Press, Boca Raton, Ch.11, p.282 (1989).

4. J. K. Porter, in **Biotechnology of Endophytic Fungi of Grasses,** CRC Press, C. W. Bacon, J. F. White, eds., Boca Raton, Ch.8, p.103, (1994).

5. R. Brunner, P. L. Stütz, H. Tscherter, P. A. Stadler, Can. J. Chem., **57**, 1638 (1979).

6. A. M. Craig, D. Bilich, J. T. Hovermale, R. E. Welty, J. Vet. Diagn. Invest., **6**, (1994).

7. R. W. Hemken, J. A. Bull, J. A. Boline, et al., J. Anim. Sci., **49**, 641-649 (1979).

8. S. P. Schmidt, C. S. Hoveland, E. M. Clark, et al., J. Anim. Sci., **55**, 1259-1263 (1982).

9. P. C. Lyons, R. D. Plattner, C. W. Bacon, Science, **232**, 487 (1986).

10. G. E. Rottinghaus, G. B. Garner, C. N. Cornell, J. L. Ellis, J. Agric. Food Chem., **39**, 112-115 (1991).

11. A. M. Craig, D. Bilich, J. T. Hovermale, R. E. Welty, J. Vet. Diagn. Invest., **6**, 348-352 (1994).

12. G. E. Rottinghaus, L. M. Schultz, P. F. Ross, N. S. Hill, J. Vet. Diagn. Invest., **5**, 242 (1993).

13. N. S. Hill, G. E. Rottinghaus, C. S. Agee, L. M. Schultz, Crop Sci , **33**, 331 (1993).

14. J. Caporal Gautier, J. M. Nivet, P. Algrati, M. Guilloteau, M. Histe, M. Lallier, J. J. N'Guyen-Huu, R. Russotto, S. T. P. Pharma. Pratiques, **2**, 205 (1992).

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