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Short communication

Rapid determination of the mycotoxin lolitrem B in endophyte-infected perennial ryegrass by high-performance thin-layer chromatography
A validated assay

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Abstract

A method based on high-performance thin-layer chromatography with UV detection, is described for the rapid determination of the mycotoxin lolitrem B in perennial ryegrass infected with the endophytic fungus *Acremonium lolii*. The method, which requires a clean-up of the crude extract on C_{18} Sep-Pak prior to chromatographic analysis, has been validated according to the criteria established by the *Journal of Chromatography B*. Its preliminary application to samples of seven perennial ryegrass cultivars infected with *A. lolii* and cultivated in France, revealed the presence of lolitrem B at concentrations ranging between 0.1 and 0.94 μg/g dry mass. These results are of epidemiological importance for studying the "ryegrass staggers", a neurotoxic syndrome which is induced in herbivorous animal species mainly by lolitrem B.

Keywords: Ryegrass; Lolitrem B; Mycotoxins; Neurotoxins

1. Introduction

Lolitrem B is the major lolitrem neurotoxin isolated from perennial ryegrass (*Lolium perenne* L.) infected with the endophytic fungus *Acremonium lolii* [1,2]. This lipophilic indole-diterpene mycotoxin (Fig. 1) [3–5] is thought to be the principal causative agent of the "ryegrass staggers", a nervous disorder of sheep, cattle, horses and deer grazing endophyte-infected perennial ryegrass-dominant pastures [1–3,6,7]. The toxic syndrome, characterized by severe incoordination, hypersensitivity to external stimuli and a consistent lack of observable specific lesions, has been largely described in New Zealand, Australia and the UK [8]. It seems also to exist in

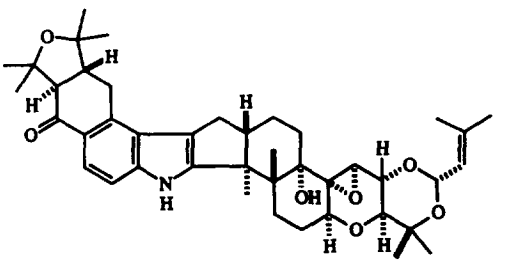

Fig. 1. Structure of lolitrem B.

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continental Europe, although very few certain clinical cases [9] and identification [10] of mycotoxins – particularly lolitrem B – in endophyted forages have already been reported. Because of this lack of information, the present study was undertaken to corroborate the biosynthesis of lolitrem B (considered as a toxicological biomarker), in *A. lolii* infected perennial ryegrass cultivars harvested in France.

It was therefore of great interest to validate an analytical method, convenient for a rapid screening determination of the involved mycotoxin. Some assays, based on HPLC, have been already described for identification and quantification of lolitrem B in perennial ryegrass [8,11]. The use of a fluorescence detection was particularly suitable for determining the neurotoxin in the whole plant, the seed or the dissected plant components, in the ppm to sub-ppm range [11,12]. Nevertheless, some authors demonstrated recently that, for routinely performed analysis of toxic substances, the HPTLC technique, because of its simplicity and rapidity, should be regarded as a favourable alternative to HPLC [13]. The second aim of the present study was therefore to validate an HPTLC method, available for a screening analysis of lolitrem B in ryegrass hay and seeds.

2. Experimental

2.1. Apparatus

An HPTLC system was used, whose components were an automatic TLC sampler III, and a TLC scanner II (Camag, Switzerland). The spraying system of the automatic sampler was operated under high-purity pressurized nitrogen gas (Carboxyque Française, Vénissieux, France). The scanner, equipped with a deuterium lamp, was driven by Cats software (Camag).

The commercially glass precoated plates used, were nano HPTLC RP_{18} plates (Merck, Nogent sur Marne, France), 20×10 cm, 2–10 μm particle size. The thickness of the layer, containing a fluorescent indicator, was 0.2 mm. The plates were eluted in a small (internal dimensions: 23×7 cm) elution chamber (Camag).

Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA) were used for rapid sample clean-up.

2.2. Reagents and lolitrem reference solutions

Solvents used were analytical-grade chloroform, methanol, acetonitrile and dichloromethane (Carlo Erba, RS-plus, Milan, Italy).

Lolitrem B, extracted and purified from ground endophyte-infected perennial ryegrass seeds [14], was graciously provided by Dr. N.R. Towers (AgResearch, New Zealand). A lolitrem B primary standard solution (15.2 μg/ml) was made up in 10 ml dichloromethane, then immediately divided into 20 aliquots of 500 μl, which were put in 1-ml conical glass tubes. Each fraction was evaporated to dryness at 20°C under a nitrogen stream, before being stored in the dark at −20°C. Prior to each assay, the convenient volume of the primary lolitrem B standard solution was obtained, by dissolving the lolitrem B contained in each conical glass tube with 500 μl of dichloromethane. Solutions (7.6, 3.8, 1.9, 0.95 and 0.47 μg/ml) were prepared by appropriate dilutions of the primary lolitrem B standard solution, for plotting the standard calibration curve.

2.3. Sample preparation

Samples of seven perennial ryegrass cultivars ("NUI", A, B, C, D, E, F), 80–100% infected (E+) or not (E−), were used. Among the studied cultivars, some (A, B) were available in the (E−) and (E+) forms. (E+) and (E−) "NUI" were kindly provided by AgResearch (New Zealand). Some cultivars (D, E, F), commercially available in the (E−) form, were artificially inoculated according to the method of Latch and Christensen [15] with a strain of *Acremonium* endophyte able to sporulate, and isolated from *Lolium perenne L.* The C genotype corresponded to an experimental cultivar. All the cultivars were harvested at the beginning of July 1996. Except for "NUI" (oven-dried overnight at 60°C), the plants were immediately frozen in liquid nitrogen and lyophilized (−50°C, 13 Pa). (E−) and (E+) F genotype seeds were also analyzed.

All samples were milled (Cyclotec mill, 30 mesh screen) and stored in the dark at −20°C until

analyzed. The non-infected samples were used as blanks for the validation procedures.

2.4. Analytical procedure

2.4.1. Sample extraction

Sample extraction was performed in accordance with a method adapted from Gallagher et al. [11]. Each sample (0.5 g) was introduced into a 30-ml centrifugation glass tube, and 10 ml of chloroform–methanol (2:1) were added. The tube was stoppered, shaken gently for 1 h on an orbital shaker, and centrifuged (800 *g*, 15 min). The supernatant, dispensed into a glass vial, was evaporated to dryness under a nitrogen stream at 40°C, then the dry residue was redissolved into 500 μl of methanol, before being purified. The spiked samples were extracted using the same method.

2.4.2. Extract clean-up

A C_{18} Sep-Pak was pre-washed with successively 2 ml of methanol and 2 ml of water, using a 5-ml glass syringe. The extract was then loaded onto the Sep-Pak, and was eluted with 1 ml water into waste. This was followed by a further elution with 3 ml acetonitrile. The cartridge eluate from this latter step was collected into a glass tube, prior to chromatographic analysis. The eluate was evaporated to dryness under a nitrogen stream in a waterbath at 40°C, and the residue was dissolved in 250 μl methanol for analysis (concentration factor: 2).

2.4.3. HPTLC analysis

Using the automatic TLC sampler, 5 μl of each sample or lolitrem B standard solutions were sprayed on a plate as a thin band (4×0.5 mm) with pressurized nitrogen gas (6000 hPa), 1.0 cm apart from the border of the plate. Samples bands were separated by a 3–5 mm space. After spraying, the plate was eluted with 10 ml dichloromethane–acetonitrile (9:1). The elution chamber being saturated for 5–10 min with the solvent, development (8 cm) took approximately 30 min at room temperature. The plate was then removed, and allowed to dry for 5–10 min. Reading was performed with the TLC scanner at 268 nm, peaks were then integrated, and an UV spectrum (220–380 nm) was plotted for each detected peak on the plate. Each substance was therefore characterized both by its R_F and UV absorption spectrum.

2.4.4. Validation procedure

Spiked samples were prepared to determine the extraction percent recovery as follows: 250, 125, 62.5, 31.2 and 15.1 μl of the reconstituted lolitrem B (15.2 μg/ml) standard solution were each added to a 0.5 g sample of dried and milled (E−) "NUI" ryegrass. The theoretical concentrations of the spiked samples were therefore 7.6, 3.8, 1.9, 0.95 and 0.47 μg/g, since the final volume used was 500 μl (instead of 250 μl as in naturally contaminated samples). The same operation was repeated three times. The volume was later reduced to 250 μl, because available results indicated that there were no interferences in the ryegrass samples, and therefore, the extracts could be concentrated to lower the limit of detection (LOD) and the limit of quantification (LOQ). A set of spiked samples was tested with both volumes, and results did not differ (data not shown).

The three previous spiked samples series (0.47–7.6 μg/g) were used for plotting five points calibration curves. For each point, three measurements were made to improve repeatability of the procedure. Reproducibility was checked with three plates on three different days. For each point, data were averaged, and the calibration curve was determined by means of a linear regression model (least square regression).

3. Results and discussion

3.1. Validation of the analytical method

3.1.1. Specificity

The specificity of the method was determined with (E−) ryegrass samples. It appeared that the HPTLC chromatograms of the corresponding extracts, compared with that of standard lolitrem B (Fig. 2), exhibited no interfering peaks that could be misleading (i.e., the specificity is close to 100%). Moreover, the R_F value for the mycotoxin (0.57) was found to be repeatable and reproducible from one plate to another [relative standard deviation (R.S.D.), less than 5%]. Lastly, lolitrem B had a specific solid-phase UV spectrum (Fig. 3) which was, in the

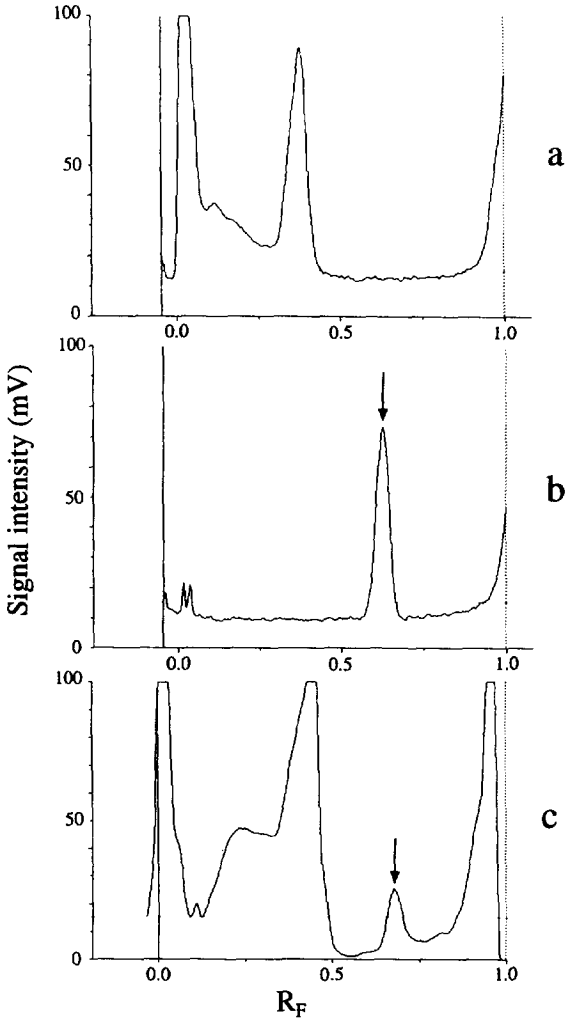

Fig. 2. HPTLC chromatograms obtained with a non-infected ryegrass sample (a), standard lolitrem B (15.2 μg/ml) (b), and a ryegrass sample infected with *Acremonium lolii* (B cultivar, containing lolitrem B at the concentration of 0.9 μg/g dry mass) (c).

selected experimental conditions, characterized by two maximum absorption wavelengths, at 268 and 295 nm, and troughs at 230 and 285 nm. These data were in accordance with those of Gallagher et al., who recorded maximum absorbances at 267 and 290 nm in methanol [3], and at 265 and 290 nm in dichloromethane–acetonitrile (70:30) [8]. An alternative fluorescence scanning of the plate was performed with a mercury lamp (excitation wavelength 366 nm, reading at λ>400 nm with an UV filter).

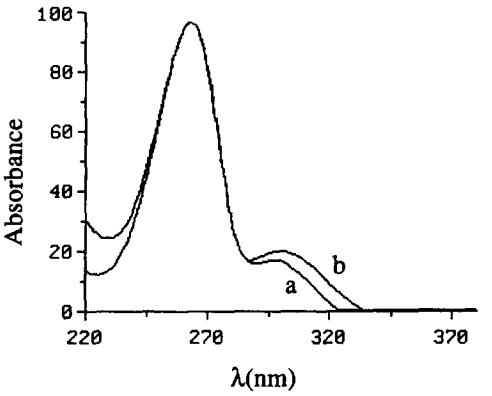

Fig. 3. Comparison between the UV spectrum of standard lolitrem B (a), and that of lolitrem B extracted from a ryegrass sample infected with *Acremonium lolii* (b).

The results were not improved, neither in sensitivity nor in specificity, therefore it was decided to validate the UV-detection method.

3.1.2. Extraction

The percent recovery was found to vary between 89 and 102% (R.S.D.<10%) for all spiked samples, from 0.47 to 7.2 μg/g.

3.1.3. Limit of detection

The noise level was estimated on a (E−) "NUI" ryegrass sample, and the mean noise level plus three standard deviations was used to estimate the LOD [16]. This last parameter was determined, in the experimental conditions, as 0.1 μg/g dry sample.

3.1.4. Quantitation – linearity, repeatability and reproducibility

The LOQ, calculated as the mean noise level plus 10 standard deviations [16] was determined as 0.4 μg/g for spiked ryegrass samples, and 0.2 μg/g for naturally infected samples (concentration factor: 2). Data were shown to follow a linear model between 0.47 and 7.6 μg/g ($r^2 \geq 0.99$) (Fig. 4), and since the R.S.D.s were less than 10% within plates and between plates, the method appeared both repeatable and reproducible. Also, the accuracy was good, since the concentrations measured in analyzing the spiked samples were found to be within 10% from the actual concentrations added, for five levels ranging from 0.47 to 7.6 μg/g. The linear regression equa-

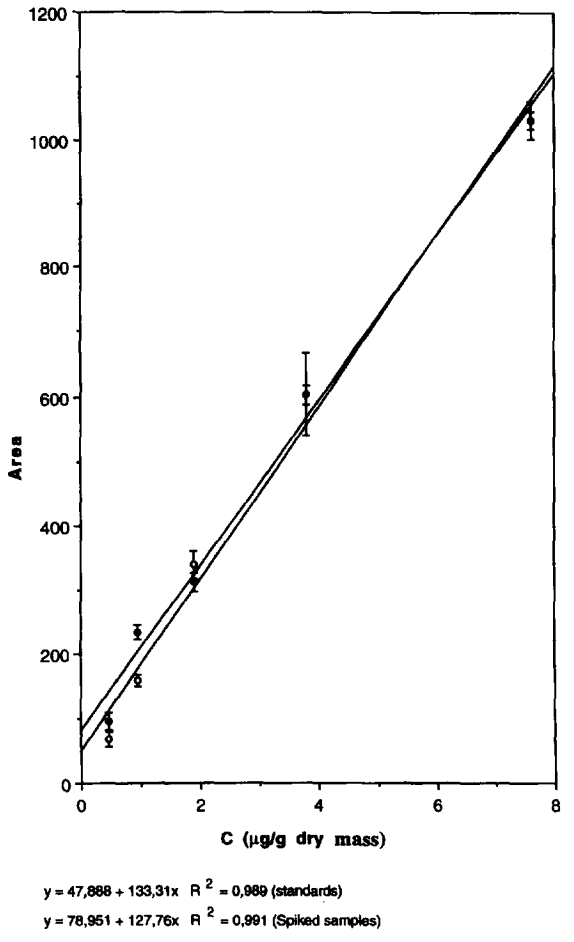

Fig. 4. Calibration curves plotted for the quantification of lolitrem B in standards (open circles) and spiked samples of ryegrass hay (black circles).

tions of standards and spiked samples were compared, and the slopes and origins of the lines of the spiked samples were not found to be statistically different from those obtained with standards [17]. The validation data are summarized in Table 1.

3.2. Determination of lolitrem B in ryegrass samples

Lolitrem B was found in all samples (dried or lyophilized grasses) infected with the endophytic fungus *Acremonium lolii*, which were analyzed in comparison with the corresponding (E−) samples. The mycotoxin isolated using HPTLC was identified

Table 1
Recapitulation of the validation data for lolitrem B analysis in ryegrass samples, by HPTLC

Parameter	Value	R.S.D. (%)
R_F (n=6)	0.57±0.01	1.7
%Recovery (0.47 µg/g, n=3)	89%	7.3
%Recovery (0.95 µg/g, n=3)	98%	5.6
%Recovery (1.9 µg/g, n=3)	104%	1.5
%Recovery (3.8 µg/g, n=3)	103%	3.6
%Recovery (7.2 µg/g, n=3)	97%	2.9
LOD (µg/g dry sample)	0.1	–
LOG (µg/g dry sample)	0.2	–
Linearity (0.47–7.2)r^2	0.99	–
Repeatability (0.95 µg/g, n=6)[a]	–	6.3
Reproducibility (0.95 µg/g, 3 days)[b]	–	7.2

[a] Six standards.
[b] Mean of 3 standards per day over three days.

both by its R_F and UV spectrum, compared with those of the standard (Figs. 2 and 3). Moreover, the data obtained with the validated HPTLC method were qualitatively confirmed with a previously described HPLC method, using fluorescence detection [11]: the chromatograms obtained with the second method, analyzing samples extracted with the same procedure than for HPTLC, showed presence of a peak at a t_R identical to that of the standard lolitrem B.

The neurotoxin concentrations found in grass and seed samples, using the validated HPTLC method, were 0.1–0.94 µg/g and 0.3 µg/g, respectively (Table 2). These values were in accordance with

Table 2
Lolitrem B concentration in several (E+) cultivars of ryegrass harvested in France

Ryegrass cultivar	Mean±S.D. (n=3) (µg/g dry mass)
NUI	0.58±0.06
A	0.65±0.05
B	0.94±0.14
C	0.16±0.04[a]
D	+
E	0.23±0.05
F	0.35±0.04
F (seeds)	0.30±0.04

[a] One value was below the quantification limit and assumed to be 0.1, i.e., the detection limit.
+ The value obtained was between the limit of detection (0.1) and the limit of quantitation (0.2).

those, ranging between 0.23 and 1.68 μg/g in whole plants, determined with HPLC by Gallagher et al. [11]. It could be of interest to note that the three experimental cultivars (D, E, F), all artificially inoculated with the same sporulating strain of an endophyte, which probably belongs to the taxonomic group named Lp-TG2 by Christensen et al. [18], produce low levels of lolitrem B. However, the lolitrem B concentration detected in each of these three cultivars, vary from a very low value (below the LOD) to 0.35±0.05. This suggests that plant genotype could have an influence on the lolitrem B biosynthesis. Therefore, the mycotoxin production seems to be controlled by both plant and endophyte genotype. Such a remark is in accordance with the conclusions already published by Roylance et al. [19], who showed similar controls on ergovaline and peramine productions, in endophyte-infected tall fescue.

4. Conclusions

According to its characteristic parameters, the tested bioanalytical method can be considered to be valid, i.e., acceptable for its intended purpose. The main advantages found for HPTLC were: (1) the possibility to scan a large number of samples in a short time period (up to twenty extracted samples were scanned and quantitatively analyzed within 30 min). (2) The low cost of the method: the running cost per sample was divided by six, comparatively to HPLC. (3) The technical easiness to perform the assay. (4) The same sample could be identified both by its solid-phase UV spectrum and its fluorescence, if necessary.

The preliminary applications of the validated HPTLC method indicated the presence of lolitrem B in various samples of ryegrass, infected with *Acremonium lolii* and cultivated in France. Such results, which have never been obtained up to now, are of epidemiological importance for the study of the "ryegrass staggers" syndrome in France. Additionally, the variability of the mycotoxin production found in this work, suggests the possibility to search for low lolitrem B plant/*Acremonium* associations, in order to avoid animal toxicosis.

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References

[1] R.T. Gallagher, E.P. White and P.H. Mortimer, N.Z. Vet. J., 29 (1981) 189.
[2] R.T. Gallagher, A.G. Campbell, A.D. Hawkes, P.T. Holland, D.A. McGaveston, E.A. Pansier and I.C. Harvey, N.Z. Vet. J., 30 (1982) 183.
[3] R.T. Gallagher, A.D. Hawkes, P.S. Steyn and R. Vleggaar, J. Chem. Soc., Chem. Commun., (1984) 614.
[4] P.S. Steyn and R. Vleggaar, Progr. Chem. Org. Nat. Prod., 48 (1985) 25.
[5] R.M. Ede, C.O. Miles, L.P. Meagher, S.C. Munday and A.L. Wilkins, J. Agric. Food. Chem., 42 (1994) 231.
[6] R.T. Gallagher, R.G. Keogh, G.C.M. Latch and C.S.W. Reid, N.Z. J. Agr. Res., 20 (1977) 431.
[7] P.H. Mortimer, L.R. Fletcher, M.E. Di Menna, I.C. Harvey, G.S. Smith, G.M. Barker, R.T. Gallagher and E.P. White, Recent Advances in Ryegrass Stagger, Proc. Ruakura Farmer's Conf., 34 (1982) 71.
[8] R.T. Gallagher and A.D. Hawkes, J. Chromatogr., 322 (1985) 159.
[9] J. Le Bars and P. Le Bars, Vet. Res., 27 (1996) 383.
[10] P. Dapprich, V.H. Paul and K. Krohn, in K. Krohn and V. H. Paul (Editors), Proceedings of the 2nd International Conference on Harmful and Beneficial Microorganisms in Grassland, Pasture and Turf, IOBC Wprs Bull., Vol. 19, 1996, p. 103.
[11] R.T. Gallagher, A.D. Hawkes and J.M. Steward, J. Chromatogr., 321 (1985) 217.
[12] R.G. Keogh, B.A. Tapper and R.H. Fletcher, N.Z. J. Agric. Res., 39 (1996) 121.
[13] P.J. Berny, T. Buronfosse and G. Lorgue, J. Anal. Toxicol., 19 (1995) 576.
[14] C.O. Miles, S.C. Munday, A.L. Wilkins, R.M. Ede, A.D. Hawkes, P.P. Embling and N.R. Towers, in D.E. Hume, G.C.M. Latch and H.S. Easton (Editors), Proceedings of the 2nd International Symposium on Acremonium/Grass Interaction, Palmerston North, New Zealand, 4–6 February 1993, p. 85.
[15] G.C.M. Latch and M.J. Christensen, Ann. Appl. Biol., 107 (1985) 17.
[16] J.M. Green, Anal. Chem., 68 (1996) 305A.
[17] J. Caporal-Gauthier, J.M. Nivet, P. Algranti, M. Guilloteau, M. Histe, M. Lallier, J.J. N'Guyen-Huu and R. Russotto, S.T.P. Pharma Pratiques, 2 (1992) 205.
[18] M.J. Christensen, A. Leuchtmann and B.A. Tapper, Mycol. Res., 97 (1993) 1083.
[19] J.T. Roylance, N.S. Hill and C.S. Agee, J. Chem. Ecol., 20 (1994) 2171.