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Countercurrent chromatographic isolation of lolitrem B from endophyte-infected ryegrass (*Lolium perenne* L.) seed

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Abstract

This paper describes a new method of purification of the Lolitrem B, a tremorgenic mycotoxin produced *in planta* by the endophytic fungus *Neotyphodium lolii*. The method is based on the large-scale isolation of the toxin by countercurrent chromatography (CCC). The lolitrem B content in endophyted ryegrass seed, 11 μg/g or 11 ppm, is extracted by stirring finely ground seeds with ethanol for 3 h at room temperature. The concentrated crude extract contains about 0.6 mg/g or 600 ppm of lolitrem B. It is then submitted to CCC purification with a biphasic four-solvent liquid system. A 160-fold enrichment was obtained in one step producing a raffinate containing 10% or 100 mg/g of the toxin. Further purifications were then performed by thin layer and low pressure liquid chromatography. Twenty-eight micrograms of lolitrem B with a 96% purity grade were obtained from 8 kg of seeds (yield 32%).

Keywords: Ryegrass seed; Lolitrem B; Countercurrent chromatography; Purification

1. Introduction

Lolitrem B is the major lolitrem neurotoxin isolated from perennial ryegrass (*Lolium perenne* L.) infected with the endophytic fungus *Neotyphodium lolii* [1,2]. This lipophilic indole-diterpene mycotoxin (Fig. 1) [3] is responsible for a nervous syndrome named "ryegrass staggers", widespread in ryegrass grazing animals in New Zealand and Australia, and also occurring but to a lower extent in Europe [4]. In the European countries, the clinical impact of this disease is rather low because of the feeding systems that are commonly used [5], and/or the low levels of toxin in the forages. However, the possible presence of residues of lolitrem B in the animal products (milk, meat, . . .) remains actually difficult to estimate because of the lack of pure or at least concentrated lolitrem solutions and this situation seriously hampers research in animal studies (i.e. pharmaco- and toxicokinetics, distribution and biotransformation). Moreover, it may be useful to evaluate the risk for human health of the toxin and its residues.

The only large-scale method of isolation of lolitrem B was performed by Miles et al. [6]. In order to provide sufficient amounts of the toxin to conduct pharmacological studies, we have undertaken the present study to develop a method using countercurrent chromatography (CCC). This liquid–liquid separation technique relies on the continuous partitioning of a solute between two immiscible liquid phases. Today, CCC is performed in hydrodynamic apparatuses in which a complex centrifugal field (inducing successive mixing and demixing

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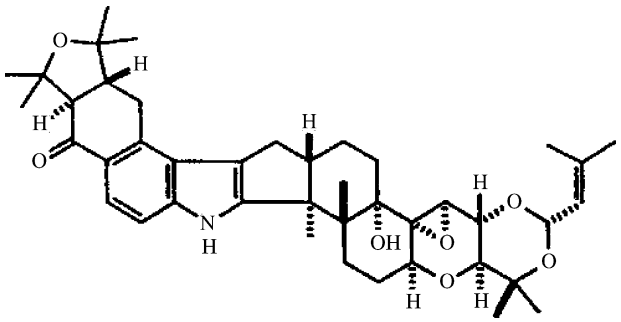

Fig. 1. Structure of lolitrem B.

zones of the two liquid phases) is created by the planetary motion of coiled spools. This method has proved recently to be very effective for the separation and even large-scale production of numerous natural products [7–9].

2. Experimental

2.1. Chemicals

The solvents used for extraction and CCC purification were all analytical grade. Ethanol, methanol, HPLC grade acetonitrile and methylene chloride were obtained from Carlo Erba RS-plus (Milan, Italy). Ethyl acetate and heptane were provided by Aldrich (Saint Quentin Fallavier, France). Water was prepared using a Milli-Q plus system Millipore, (Molsheim, France). An analytical standard of lolitrem B (Fig. 1) was purchased from Dr. N.R. Towers (AgResearch, New Zealand).

2.2. Extraction

Eight kilograms of highly endophyte-infected ryegrass seed (*turfgrass cultivar*) containing about 11 mg/kg lolitrem B (determined by HPLC analysis) were finely ground (0.5 mm diameter or less) in a grinding mill (Cyclotec, Tecator, Höganäs, Sweden). Sixteen batches of 500 g were successively extracted each with 2500 ml 95% ethanol by stirring for 3 h at room temperature. Then, for each aliquot, the liquid phase was filtered on a Buchner and the solvent was removed under vacuum, to obtain finally a brown extract. This extract was kept overnight at 4 °C. An oily precipitate formed at cold temperature and was identified as a lipidic, ethylether soluble, lolitrem-free fraction and discarded by centrifugation. The supernatant was concentrated under vacuum to give 99.2 g of a brown oily residue (crude extract).

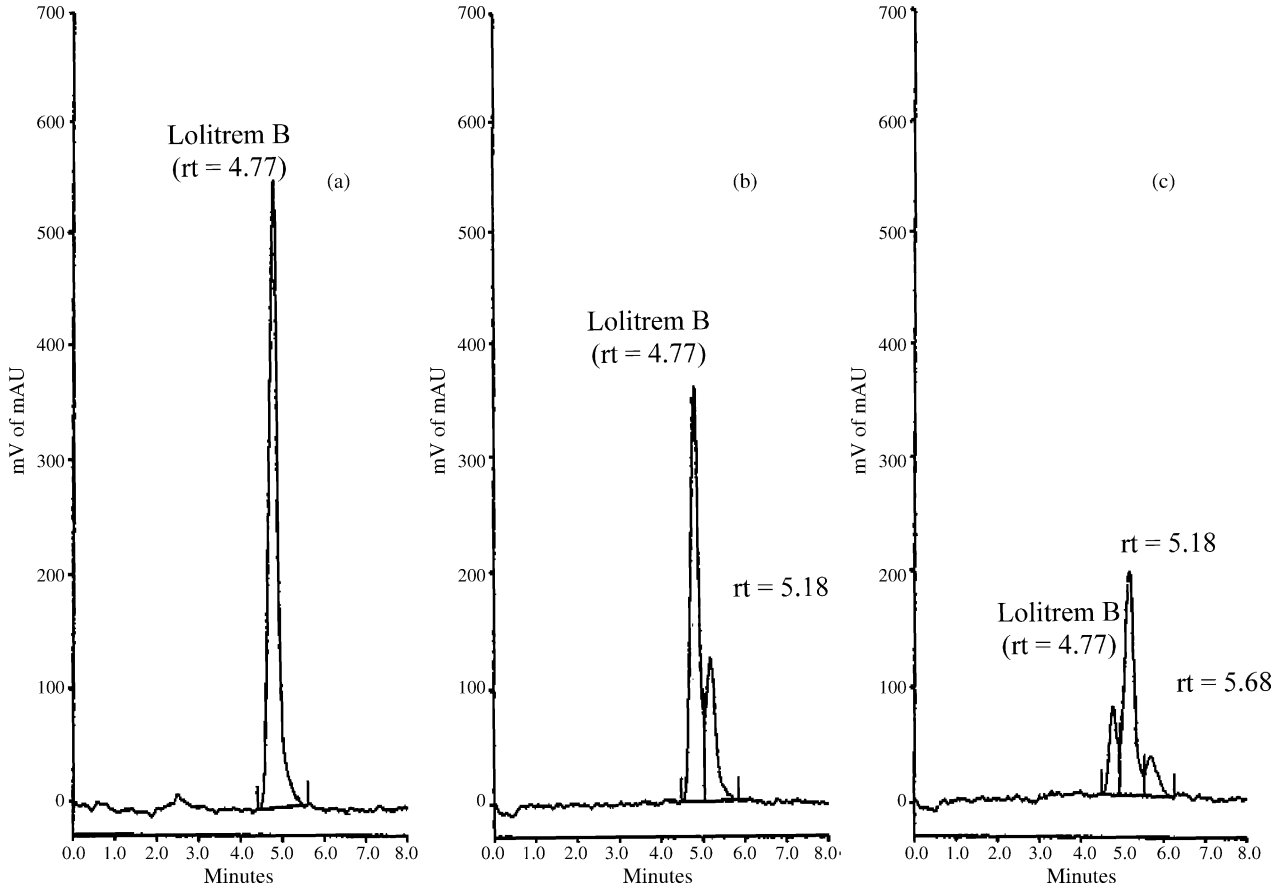

Fig. 2. HPLC chromatograms of Pool 1 (a), Pool 2 (b) and Pool 3 (c) after CCC purification of an ethanol crude extract of ground endophyted ryegrass seed. HPLC conditions: isocratic elution with methylene chloride/acetonitrile (85/15 (v/v)); flow rate: 0.8 ml/min; column: Nucleosil N3-15QK (Interchim, France); fluorimetric detection: excitation $\lambda = 268$ nm, emission $\lambda = 440$ nm.

2.3. Purification

2.3.1. Countercurrent chromatography

The CCC apparatus employed in the present study was of hydrodynamic type (designed by CPC-Kromaton, Angers, France[1]). It had a total volume of 1070 ml. Two 530 ml spools, each prepared by winding a 250 m long single piece of 1.6 mm i.d. polytetrafluoroethylene tubing, were hold in a rotor. A gear arrangement allowed producing a planetary motion of the spools rotating around their own axis when the rotor was spinning at 300 rpm around its central axis. The average multilayer coil radius (r) was 10 cm, the rotation radius (R) was 15 cm and the beta value (r/R) was 0.67. A biphasic liquid system, consisting of heptane, ethyl acetate, methanol and water in the volume percentages of 33, 33, 24 and 10, respectively, was used for the CCC purification. About 6.2 g of the brown oily ethanolic extract (crude extract from 500 g ground seed) were dissolved in 30 ml of light and 30 ml of heavy phase. The mixture was introduced into the CCC apparatus using an injection valve with a 65 ml loop. The injection was done in about 15 min pumping the mobile phase at 4.5 ml/min flow rate. In a first step (4 h), the stationary phase was the light phase (62.5, 36.5, 0.7 and 0.3 (v/v) of heptane, ethyl acetate, methanol and water, respectively) and the mobile phase was the heavy one (3.2, 30, 46.8 and 20 (v/v) of heptane, ethyl acetate, methanol and water, respectively). The heavy phase was pumped in the tail to head direction at 4.5 ml/min. Between 75 and 80% of the light liquid phase were retained at 300 rpm by the machine. At equilibrium, the stationary phase volume was between 800 and 850 ml. The mobile phase volume was between 220 and 270 ml. About twice this volume of mobile phase was then passed in the first step to remove the most polar compounds contained in the extract. These polar compounds appeared in the form of a bright orange band after about 500 ml elution with the heavy liquid phase (∼2 h at 4.5 ml/min). They were discarded. In a second step, the phase role was permuted. The apolar light phase was used as the mobile phase and pumped in the head to tail direction and the heavy polar liquid phase was the stationary one. Again, about 450 ml (∼2 h at 4 ml/min) of the light liquid phase allowed eluting the less apolar compounds in the form of a dark green fraction rapidly followed by the desired fractions containing the lolitrems.

Fractions of 18 ml (4 min elution intervals) were collected in glass tubes. Sixteen CCC runs were needed to purify the 99.2 g of crude extract. The fractions containing lolitrem B were identified using the described HPLC method. The fractions containing only one fluorescent peak at the expected retention time of lolitrem B were pooled together for all the 16 CCC purifications (Pool 1, 528 mg after solvent evaporation, Table 1). The fractions containing 1 or 2 additional fluorescent peaks close to the expected lolitrem B retention time were also pooled and identified as Pool 2 (lolitrem B and one peak) and Pool 3 (lolitrem B and two peaks), respectively. After evaporation under vacuum of the solvents, the toxin was quantified by HPLC in each concentrated aliquot.

2.3.2. Thin-layer chromatography and low pressure column chromatography

The purification of lolitrem B was completed using preparative thin-layer chromatography (TLC) and low pressure liquid chromatography (LC). The dry residues of the CCC fractions containing the toxin (Pools 1, 2 and 3) were diluted with a minimum volume of methylene chloride. The resulting organic solution was divided into aliquots of 4 ml (corresponding to 8 mg of the CCC-extract and containing about 500 μg lolitrem B). They were deposed on silica gel preparative plates (60 F 254, 1 mm, ref 1.13792, Merck, Darmstadt, Germany,) and eluted with a methylene chloride/acetonitrile (90/10 (v/v)) mobile phase. The plates were visualized under UV at 254 nm and the brown lolitrem B bands were scraped off, then dissolved with a methylene chloride/acetonitrile (80/20 (v/v)) mixture. The resulting organic solution was filtered and evaporated under vacuum. The dry residue was diluted with a minimum volume of methylene chloride and finally purified by low pressure column liquid chromatography (LC).

LC was performed on glass columns (24 cm × 2 cm) packed with 20 g silica gel (Grade 9385, 60 Å, 50 μm, Merck, Darmstadt, Germany). About 12 mg of the previous residue, containing about 6 mg of lolitrem B, were applied at the top

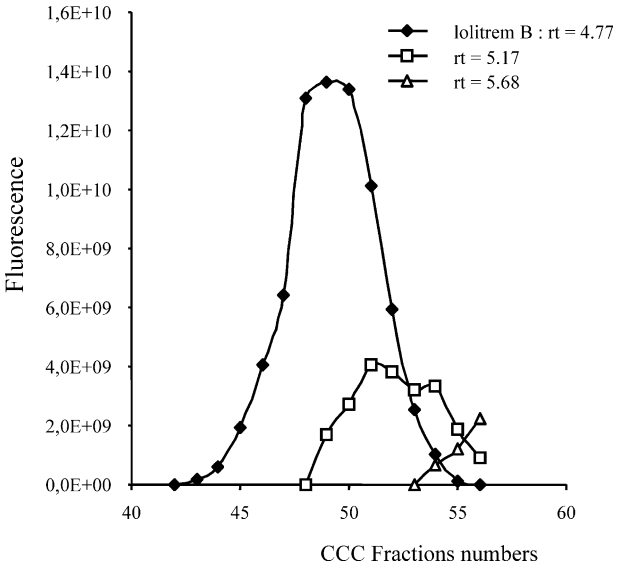

Fig. 3. Distribution of lolitrem B among the CCC fractions of an ethanol crude extract of ground endophyted ryegrass seed (500 g) (lolitrem B injected: 4516 μg; lolitrem B detected: 3980 μg; recovery: 88%). CCC conditions: revolution radius (R) = 15 cm, β = 0.67, revolution speed: 300 rpm; biphasic liquid system: heptane/ethylacetate/methanol/water (33/33/24/10 (v/v/v/v)); flow rate: 4.5 ml/min; stationary phase: light phase first, heavy phase second; (♦) lolitrem B, rt = 4.77 min; (□) rt = 5.17 min; (△) rt = 5.68 min.

[1] This device was sold from 1989 to 1994. It is no more available. Dynamic Extractions (Brunel Entreprise Center, Uxbridge, Middlesex UB8 3PH, UK) is marketing equivalent machines.

Table 1
Successive steps of the extraction of lolitrem B from 8 kg of ground endophyted ryegrass seeds

Process	Ground seeds of ryegrass (0.5 mm)	Extraction	Precipitation	Countercurrent chromatography (CCC)	Purification thin-layer chromatography	Purification column liquid chromatography	Purification
	8 kg	Ethanol 95% stirring for 3 h (20 °C)	4 °C, 24 h	3 fluorescent fractions:	Silica TLC fractions a, b, c eluent	Silica column	MeOH/acetone (1/1) discarding of the precipitate
		Filtration and vacuum concentration	Discarding of the oily precipitate	(a) Loli B (b) Loli B + 1 peak (c) Loli B + 2 peaks	CH_2Cl_2[a]/ACN[a] (90/10) (elimination of colored contaminants)	Fractions a, b, c: eluents CH_2Cl_2/ACN (97/3), CH_2Cl_2/ACN (94/6) Fractions b, c: separation of other fluorescent compounds CH_2Cl_2/EtOAc[a] (95/5)	
Volumes of solvent used (l)		EtOH (40)		EtOAc (7) Heptane (7) MeOH (5)	CH_2Cl_2 (2) ACN (0.5)	CH_2Cl_2 (1) ACN (0.1)	<0.1
Weight of residue (mg)			99.2	528	70.4	38.4	29.1
Lolitrem B (mg)	**88**	**69.6**	**60.8**	**52.8**	**38.4**	**29.6**	**28**
Recovery process (%)	–	79	87	87	73	77	94.6
Cumulated (%)		79	69	60	43.6	33.6	32
Lolitrem B concentration (% (w/w))	0.0011		0.061	10	55	77	96

[a] ACN: acetonitrile; CH_2Cl_2: methylene chloride; EtOAc: ethyl acetate; EtOH: ethanol; MeOH: methanol.

of the column on the silica gel. Two compositions of methylene chloride/acetonitrile mobile phases were successively added. First, the 97/3 (v/v) (130 ml) composition was used to remove impurities of low polarity. Next, a 94/6 (v/v) (130 ml) composition allowed to elute the lolitrem B. The LC fractions containing the toxin were combined together and the solvents were removed under vacuum to obtain a pale yellow solid. Pools 1, 2 and 3 were all submitted to the same purification protocol. In order to separate lolitrem B from the other fluorescent components, Pools 2 and 3 were eluted by LC on silica gel with a 95/5 (v/v) methylene chloride/ethyl acetate mobile phase. The fractions containing only lolitrem B were identified by HPLC.

A comparative LC–MS–MS analysis of the final product and of the analytical standard of lolitrem B was performed to control the identity and the purity of the isolated toxin. An unequivocal proof of the compound identity was obtained by NMR spectroscopy.

2.4. Analysis

2.4.1. HPLC analysis

At each purification step, the lolitrem B was identified and quantified by HPLC, using a method derived from Gallagher et al. [10]. An HPLC system (Thermo Separation Product, Les Ulis, France) with a P 1000 pump, a FL 2000 fluorimetric

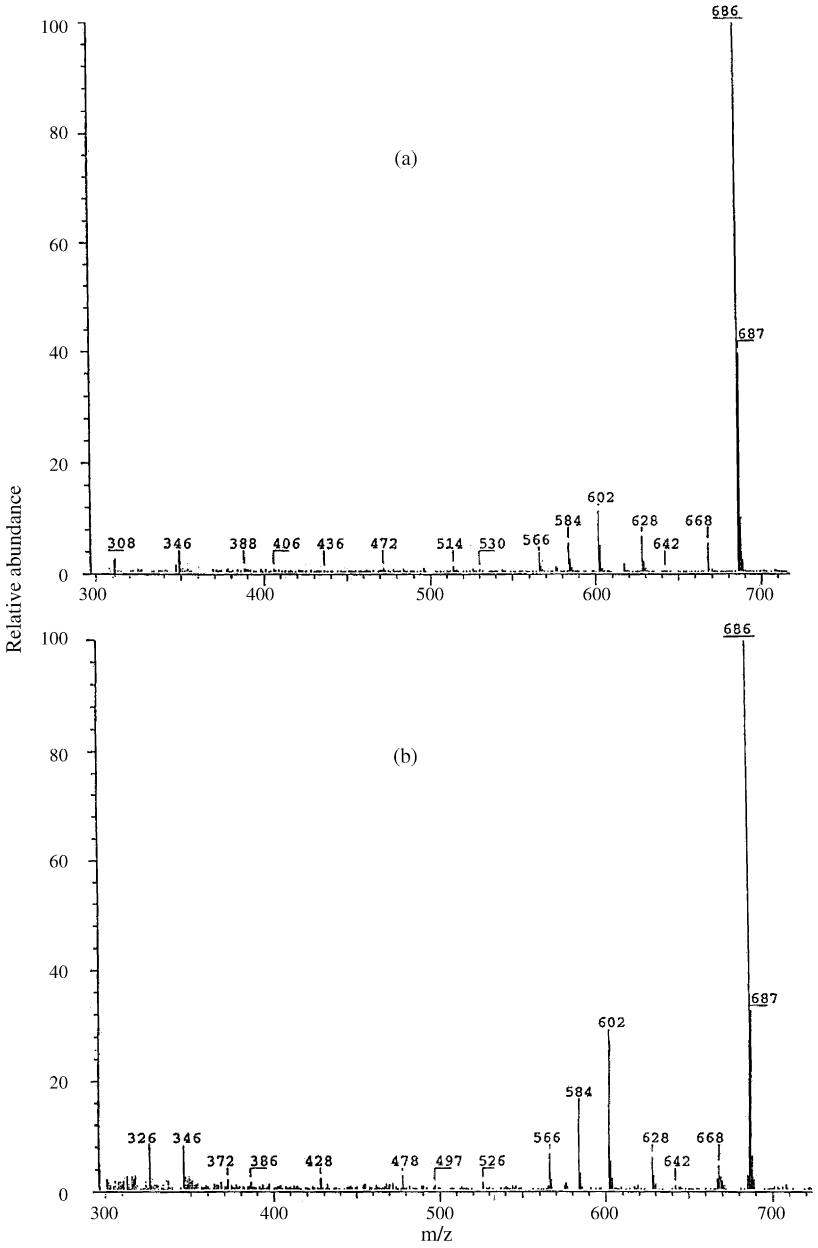

Fig. 4. Full scan HPLC–MS spectra of lolitrem B: (a) standard, (b) seed extracted molecule.

detector, and a Rheodyne injection valve fitted with a 20 μl loop was used. The control of the HPLC system and the integration of the chromatographic peaks were made using a computer system controller with the PC 1000 (v.2.5) software.

Chromatographic separations were performed under the following conditions: an isocratic elution of methylene chloride/acetonitrile (85/15 (v/v)) at a flow rate of 0.8 ml/min allowed to elute the lolitrem isomers in less than 8 min with a Nucleosil N3-15QK column (150 mm × 4.6 mm i.d., 3 μm bare silica particles, Interchim, Montluçon, France). The analytical column was maintained at +4 °C. Lolitrem B can be selectively detected since it is fluorescent around 440 nm. The fluorimetric detector was set with an excitation wavelength of 268 nm, and an emission wavelength of 440 nm.

2.4.2. LC–MS and LC–MS–MS analysis

At the end of the whole separation and purification process, lolitrem B was identified by LC–MS and LC–MS–MS. A second HPLC system (Spectra Physics) with a P 4000 pump, a Nucleosil C18 column (150 mm × 4.6 mm i.d.; 3 μm octadecyl silane silica bonded particles, Interchim, Montluçon, France) and a Rheodyne injection valve fitted with a 20 μl loop was connected through a heated APCI interface to a TSQ 700 mass spectrometer (Finnigan MAT, Les Ulis, France). The separations were performed with an isocratic 100% acetonitrile elution, at a flow rate of 0.65 ml/min. The column effluent was continuously infused through the APCI interface heated at 450 °C in the APCI source with nitrogen (5.6 kg/cm^2 or 80 psi; 5 l/min) sheath gas and the spectra were recorded in the positive ion mode. The full scan (LC–MS) and the daughter-ion scans (LC–MS–MS) of *m/z* 686 (lolitrem B) were obtained at a collision energy of 30 eV and a collision gas pressure of 2.2 mTorr.

2.4.3. NMR analysis

The purified lolitrem B was identified by ^{1}H (500.13 MHz) and ^{13}C (125.77 MHz) NMR spectroscopy. Eight milligrams of sample were dissolved in 450 μl of $CDCl_3$, then analysed with 1D (proton and carbon) and 2D HSQC experiments. A Bruker DRX500 NMR spectrometer was used to perform all NMR analyses at 25 °C. Chemical shifts where measured relatively to an internal TMS reference.

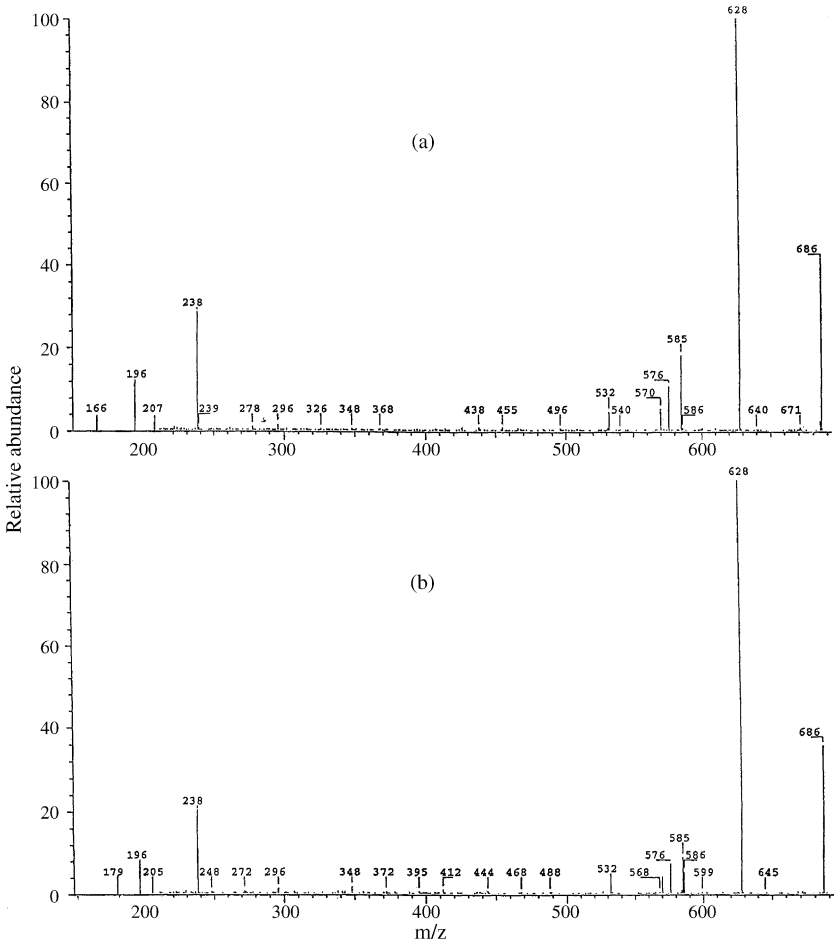

Fig. 5. HPLC–MS–MS spectra of the parent-ion (*m/z*: 686) and characteristic daughter-ions (*m/z*: 628, 586, 585, 576, 532, 348, 296, 238 and 196) of lolitrem B: (a) standard, (b) seed extracted molecule.

3. Results and discussion

3.1. Extraction and CCC isolation of lolitrem B

The 99.2 g of crude extract produced (Table 1) from the ethanolic extraction of 8 kg of infected seed were analyzed for lolitrem B. The HPLC analysis gave a 0.061% content for lolitrem B (60.8 mg of lolitrem B in the 99.2 g of extract) (Table 1). The chromatogram showed three peaks, one corresponding to lolitrem B and the two others corresponding probably to other lolitrems. The peak retention times were 4.8, 5.2 and 5.7 min, respectively. This chromatographic profile was in accordance with those previously described with similar analytical HPLC conditions [6]. The ethanol extraction yield of lolitrem B from seed before CCC was 79%.

HPLC analyses of the CCC fractions were done to locate the toxin. About five fractions contained a lolitrem B peak only (Fig. 2a), whereas one additional peak was found in each of the next five fractions (Fig. 2b). In the last fractions, a very small quantity of lolitrem B was still present and two additional peaks were indicating other compounds (Fig. 2c). The repartition of lolitrem B in the CCC fractions is shown in Fig. 3.

All the fractions of the 16 CCC runs containing only the lolitrem B peak were pooled together, concentrated to give 528 mg of a brown oily residue and called Pool 1. The

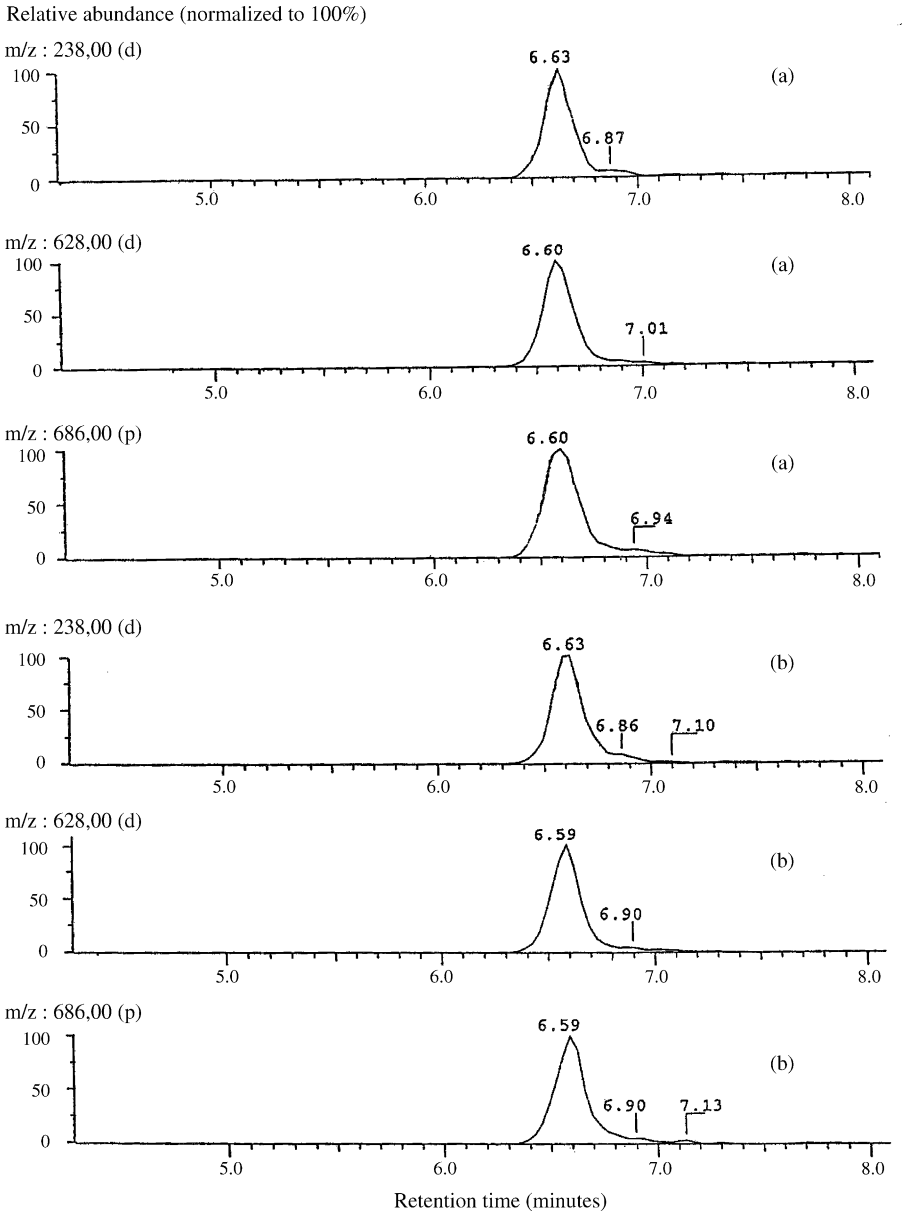

Fig. 6. Reconstituted chromatograms of characteristic ions of lolitrem B obtained by LC–MS–MS (a: standard, b: seed extracted molecule). From bottom to top are displayed the ions of *m/z* 686 (parent-ion: p), *m/z* 628, 238 (daughter-ions: d).

same protocol was applied for the CCC fractions showing two or three peaks (Pools 2 and 3). The average yield of recovery of lolitrem B from the ethanol extract during the CCC is estimated to be 87%. The total extraction yield of lolitrem B after the CCC step was estimated to be about 60% (Table 1).

3.2. Thin-layer chromatography and column chromatography purification

3.2.1. Thin-layer chromatography

As described previously, the extracts obtained after the CCC step were first purified by TLC. Among several yellow or brown bands, a colorless strip was revealed under 254 nm UV light at Rf = 0.32. The HPLC analysis of this strip revealed that it contained about 50% lolitrem B. However, the two unidentified additional peaks observed by HPLC analysis in Pools 2 and 3 were not separated yet at this stage of purification.

3.2.2. Low pressure liquid chromatography

After purification on a silica column as described previously, Pool 1 gave a yellow pale residue containing 11 mg lolitrem B. Pools 2 and 3 were mixed together and processed by the same LC purification steps. Then, lolitrem B was isolated by an extra LC chromatography with a methylene chloride/ethyl acetate (95/5 (v/v)) mobile phase. Pools 2 and 3 provided a purified amount of 18.6 mg lolitrem B.

The whole lolitrem B obtained from these successive chromatographic purifications (29.6 mg) was dissolved in a minimum volume of methanol/acetone (1/1 (v/v)). After discarding the non-soluble fraction and evaporating the solvent, 29.1 mg of a lightly cream-colored solid was obtained. It contained 28 mg of lolitrem B which was characterized by LC–MS, LC–MS–MS and NMR. The final yield of the whole process was 32% (Table 1).

3.3. Identification

3.3.1. LC–MS and LC–MS–MS analysis

Using the conditions listed in the experimental section, the extracted toxin and the standard lolitrem B showed the spectra (full scan) displayed in Fig. 4. These spectra were in accordance with the fragmentation pathways previously proposed for the toxin [2] with a molecular ion of *m*/*z* 686 (characteristic ions of *m*/*z* 602 and 583).

In the daughter-ion scans obtained from the *m*/*z* 686 parent-ion (Fig. 5), the characteristic ions *m*/*z* 686, 628, 348 (indicative of ring A–E of a lolitrem type structure [3]) and 238 were found. Reconstructed chromatograms of the characteristic daughter-ions 628 and 238 of lolitrem B were drawn. Fig. 6 compares the two sets of reconstituted ion chromatograms obtained with the lolitrem B standard (Fig. 6a) and the extracted products (Fig. 6b). The peak retention times as well as the mass spectra are both in full agreement.

3.3.2. NMR analysis

The chemical shifts of carbons were extracted from the carbon spectra. The chemical shifts of attached protons were deduced from the HSQC 2D experiment. The obtained table is in total agreement with the reference values given by Munday-Finch et al. [11] for lolitrem B. For all carbon and proton resonances, the maximum deviation between the purified compound and the reference reaches only 0.1 ppm. Although numerous chemical shifts of the four possible isomers are very close, the chemical shifts of nucleus at positions 31–40 differ significantly, so the structure of the extracted and purified product can be identified unambiguously as lolitrem B.

4. Conclusion

The proposed new isolation method is highly original by the use of countercurrent chromatography for the extraction and purification of lolitrem B from endophyte-infected perennial ryegrass seed. The first ethanol extraction step used 40 l of solvent and produced a 60-fold enrichment. The CCC process used only 19 l of solvent (Table 1) to produce a 160-fold enrichment, showing the method capabilities. Five hundred and twenty-eight milligrams of a raffinate with a 10% lolitrem B content was obtained in two weeks. Further purifications using TLC and LC allowed to obtain 28 mg of 96% pure lolitrem B, unambiguously identified by LC–MS and NMR, from 8 kg of contaminated seed. It corresponds to a total recovery of 32%, which represents a better yield than that of Gallagher et al. [1] and is roughly equivalent to that of Miles et al. [6], both groups using three times more solvent volumes than in the proposed method. The quantity and the purity (96%) of the extracted lolitrem B are sufficient to provide enough substrate for further pharmacokinetic investigations of the toxin and of its metabolism in ruminants.

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