Journal of Chromatography, 321 (1985) 217–226
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,384

RAPID DETERMINATION OF THE NEUROTOXIN LOLITREM B IN PERENNIAL RYEGRASS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

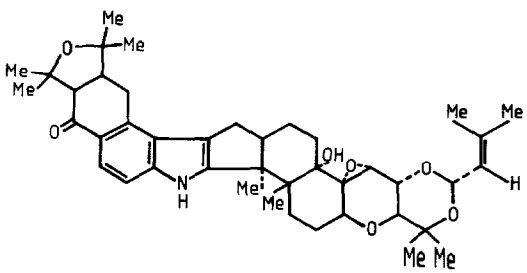
REX T. GALLAGHER*, ALLAN D. HAWKES and JAN M. STEWART
Ruakura Animal Research Station, Ministry of Agriculture and Fisheries, Private Bag, Hamilton (New Zealand)
(Received November 8th, 1984)

SUMMARY

A rapid, sensitive and quantitative method, based on high-performance liquid chromatography with fluorescence detection, is described for the determination of the neurotoxin lolitrem B in perennial ryegrass, in the ppm to sub-ppm range. The method, which requires a minimal clean-up step prior to chromatographic analysis, is suitable for the routine analysis of large numbers of ryegrass samples, and is currently being used in New Zealand to study the livestock disorder ryegrass staggers. The method is suitable for determining lolitrem B in the whole plant, the seed, and dissected plant components.

INTRODUCTION

Potent neurotoxins, called lolitrems, have recently been isolated from toxic perennial ryegrass (*Lolium perenne* L.) and ryegrass seed[1,2]. These lipophilic neurotoxins have been implicated as the causative toxins of ryegrass staggers, a nervous disorder of sheep, cattle, horses, and deer grazing perennial ryegrass-dominant pastures[1–7]. The structure of the major lolitrem neurotoxin, lolitrem B, of molecular weight 685 and formula $C_{42}H_{55}NO_7$, has been determined[8] and shown to be a complex substituted indole (1).



(1)

0021-9673/85/$03.30

Perennial ryegrass, a major pasture species in New Zealand, is also an important pasture grass in areas of Australia (especially Victoria and Tasmania) and the United Kingdom, where outbreaks of ryegrass staggers occur[5,7–11]. Grazing trials and plant breeding programmes with this grass in New Zealand, aimed at understanding and controlling ryegrass staggers, require a rapid, reliable, and sensitive assay for the lolitrem neurotoxins.

The lolitrems were originally assayed using a mouse bioassay, following solvent partitioning and chromatographic purification of ryegrass extracts[1,2,12]. The bioassay was based on a visual rating scale for tremor assessment of neurotoxin-dosed mice, and allowed a useful indication of animal neurotoxicity of herbage on a sample scale much reduced from that of the grazing animal. However, the bioassay required a 25-g dry weight sample of grass per single determination and the complete assay results were not known for several days. Furthermore, apart from a lack of sensitivity and repeatability inherent in the assay, it was unsuitable for large numbers of replicated samples. Subsequent investigations utilising high-performance liquid chromatography (HPLC) with stopped-flow UV spectral scanning, showed that the lolitrems could be detected in ryegrass plant and seed extracts by HPLC with UV detection at 268 nm[13]. The unique UV absorption spectra of the lolitrems[1,8,13] allowed confirmation of suspect lolitrem peaks by the stopped-flow UV spectral scanning technique. Unfortunately, even with the availability of this technique, the presence of many strongly UV-absorbing compounds in the ryegrass extracts interfered with the reliable detection of the lolitrems at the low levels (ppm to sub-ppm range) found in toxic grass, unless substantial prior clean-up of sample extracts was carried out. This latter requirement worked against development of a rapid method.

We wish to report here an improved, rapid, reliable and sensitive method for the determination of lolitrem B in herbage and seed, based on HPLC with fluorescence detection. The development and success of this improved analysis procedure for lolitrem B resulted from our very recent discovery that the lolitrems are strongly fluorescent molecules[14].

EXPERIMENTAL

Apparatus

The liquid chromatograph system has been described previously[13]. In the present investigation, a 150-μl sample loop was fitted to the Rheodyne injector. The fluorescence detector was a Shimadzu RF-530 Fluorescence Spectromonitor with a 75-W xenon lamp light source. This detector features diffraction grating monochromators for both the excitation and emission light paths, with excitation and emission band widths of 18 nm and 22 nm, respectively. The flow-cell was a square cross-section quartz cell with a volume of 12 μl. The detector was placed in-line before the UV detector, and the analogue outputs from these two detectors were connected to a dual-pen Omniscribe chart recorder.

Sep-Pak silica cartridges (Waters Assoc., Milford, MA, U.S.A.) were used for rapid sample clean-up.

An orbital shaker (Model SS70, Chiltern Scientific, U.K.) with a platform able to accommodate 50-ml conical glass flasks, was used for solvent extraction of grass samples.

Reagents and lolitrem reference solutions

Solvents used were analytical grade chloroform and methanol (Analar, BDH, Poole, U.K.) and Ajax Unichrom (Australia) HPLC-grade dichloromethane and acetonitrile. All solvents were distilled under vacuum in a Buchi rotary evaporator before use.

Lolitrem B was available from the authors research programme. Standard solutions of lolitrem B were made up in the HPLC mobile solvent (dichloromethane–acetonitrile, 80:20) and stored in glass containers in the dark at −18°C. A lolitrem B primary standard solution (10 μg/ml) was found to be stable for at least 3 months under these conditions. Working solutions of lolitrem B were prepared by appropriate dilutions of the primary standard in the same solvent; such solutions were used for determining the fluorometric response curve, and for external standards run daily.

Reference grass samples

Samples of "Ellett" perennial ryegrass (*Lolium perenne* L.) with high lolitrem B content ("high-toxin grass") and low lolitrem B content ("low-toxin grass") were obtained from a local site in Hamilton. The grass samples were oven-dried (45°C overnight), milled (Wiley mill, 40 mesh screen), and homogenised by thorough mixing, to give bulk reference samples of grass with high and low toxin content. These samples were stored at −18°C in the dark.

Analytical procedure

Chromatographic conditions. The column used was a Zorbax Silica (25 cm × 4.6 mm I.D.), particle size 5–6 μm (DuPont, Wilmington, DE, U.S.A.). Column temperature was room temperature, 25 ± 4°C. The solvent system used was dichloromethane–acetonitrile (80:20), run isocratically at 1.8 ml/min. For routine analyses, the fluorometric detector was set with an excitation wavelength of 268 nm and an emission wavelength of 440 nm; the UV detector was set at 268 nm. The chart recorder was run at 1.0 cm/min. Quantitation was by measurement of peak height.

Grass extraction. Oven-dried, milled grass was weighed (1.0 g) in a 50-ml Quickfit conical flask and 15 ml of chloroform–methanol (2:1) was added. The flask was glass-stoppered and then shaken gently for 1 h on an orbital shaker. After shaking, the flask was left to stand for several minutes to allow the grass to settle in the extraction solvent. An aliquot (1.0 ml) of solvent was removed using a Socorex autopipette with a disposable plastic tip and dispensed into a glass vial. The sample was evaporated to dryness under a stream of dry nitrogen.

Extract clean-up. The silica Sep-Pak was pre-washed with 2 ml of dichloromethane using a 5-ml glass syringe. The dried extract above was dissolved in four 0.5-ml rinsings of dichloromethane and these were loaded onto the Sep-Pak using a Socorex autopipette fitted with a disposable plastic tip. The Sep-Pak was eluted with 1 ml of dichloromethane–acetonitrile (80:20). This was followed by a further elution with 3 ml of the same solvent. The cartridge eluate from this latter step was collected in a glass vial and plastic-capped. prior to 100-μl injections onto the column.

Lolitrem B exhaustive recovery experiment

Solvent extraction of grass was carried out as in the procedure described above, but the time of each extraction step was 30 min.

After each 30-min shaking period the contents of the extraction flask were transferred to a 30-ml Nalgene plastic centrifuge tube. This was spun in an I.E.C. Model Centra-7R refrigerated centrifuge (International Equipment, MA, U.S.A.) at 4000 rpm at 0°C for 4 min. The supernatant was decanted into a 25-ml measuring cylinder and capped with aluminium foil, and the volume recorded. An aliquot of 0.5 ml (if high-toxin grass was used; otherwise 1.0 ml for low-toxin grass) was taken and evaporated to dryness under a stream of dry nitrogen in a 3-ml glass vial. The dried extract was then ready for the Sep-Pak clean-up procedure.

The grass pellet from the centrifuge tube was returned to the extraction flask using 15 ml of fresh extraction solvent for the transfer. The extraction and centrifugation steps were repeated a further five times.

Lolitrem B spike recovery experiment

The quantitative recovery of lolitrem B from solvent extraction of grass samples was conducted using the analytical procedure above, but prior to solvent addition to the grass, the grass was spiked with a known quantity of lolitrem B.

Lolitrem B extraction versus shaking time experiment

In this experiment the effect of shaking time on the orbital shaker *versus* lolitrem B level was examined. Following the analytical procedure, equal portions (1.0 g) of high-toxin grass were weighed out into eight flasks; solvent was then added to each flask and the flasks placed on the orbital shaker. Duplicate flasks were removed from the shaker at 0.5 h and, after the short settling period, extract aliquots (0.5 ml) were taken from the two flasks and cleaned-up, followed by lolitrem B analysis on the HPLC, column. Further duplicate flasks were successively removed from the orbital shaker at 1.0, 2.0 and 4.0 h, and sampling and lolitrem B analysis were carried out as above.

RESULTS AND DISCUSSION

Perennial ryegrass samples were obtained from several local sites in Hamilton, and two samples selected as representative high-toxin and low-toxin reference grass samples. These reference samples, together with lolitrem B standard solutions, were used to develop the HPLC analysis method. This included optimisation of the chromatography conditions, as well as sample extraction and clean-up procedures.

HPLC analysis

The discovery of the strong fluorescence properties of the lolitrems in solution[14] was an important breakthrough in our investigations aimed at developing a rapid and sensitive method of analysis for lolitrem B. It offered considerable scope for circumventing interference problems as experienced in HPLC with UV detection[13]. Also, fluorescence offered greater specificity and enhanced sensitivity of detection, and a more rapid analytical protocol due to reduced demand for time-consuming clean-up procedures.

The chromatographic conditions finally chosen (as described in the Experimental section), were found to be optimal for consistent resolution of the lolitrem B peak from earlier eluting fluorescent peaks, while at the same time allowing short turn-around times between sample injections.

A rapid extract clean-up via silica Sep-Pak treatment was found to be highly advantageous for the HPLC analysis. First, absorption of polar impurities on the Sep-Pak silica protected and prolonged the life of the HPLC column: the same analytical column has been used repeatedly throughout our investigations on lolitrem B, and after hundreds of analyses shows little deterioration in performance. Second, the HPLC analyses were made simpler with fewer peaks being present. Third, a shorter HPLC sample turn-around time was possible, since all extract compounds eluted off the HPLC column in a short time (*ca.* 6 min). Prior to application of the sample aliquot to the Sep-Pak, it was found necessary to dry the extract in a stream of nitrogen, to eliminate the methanol. The presence of methanol, a polar solvent, caused inefficient operation of the Sep-Pak in the purification procedure, and it also caused an interfering fluorescent peak to elute immediately following the lolitrem B peak in the HPLC analysis. The recovery of lolitrem B from the silica Sep-Pak was found to be virtually quantitative with the solvent systems and procedure adopted.

Typical chromatograms of high-toxin and low-toxin grass samples with fluorescence detection are shown in Fig. 1. Also shown in Fig. 1 is a chromatogram of purified lolitrem B as used for the external standard. The retention time for lolitrem B was 4.0 min and the chromatogram run time of 6 min allowed a high rate of sample throughput: 10 samples per hour; 80 samples per 8-h working day.

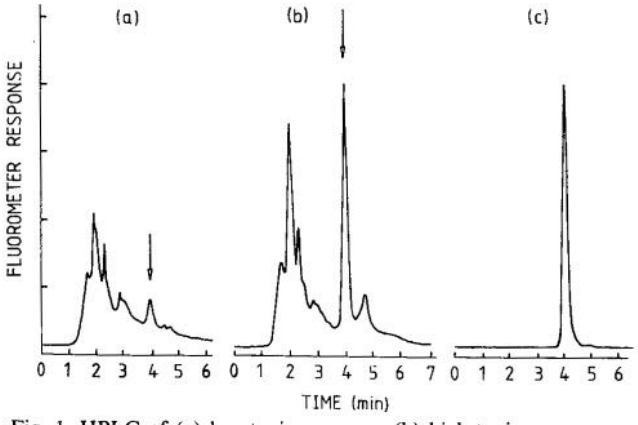


Fig. 1. HPLC of (a) low-toxin ryegrass, (b) high-toxin ryegrass, and (c) lolitrem B standard, showing lolitrem B peak at 4.0 min.

Fluorescence detection

The fluorescence excitation and emission spectra for lolitrem B in the HPLC mobile phase of dichloromethane–acetonitrile (80:20) were determined manually by repeated injections of lolitrem B standard solutions, and manual adjustment of the monochromator settings between injections. Well-defined, sharp excitation and emission maxima were found to occur at 268 nm and 440 nm, respectively. The excitation maximum at 268 nm corresponded to the major UV absorption maximum of lolitrem B[13,14]. Subsequent to these determinations, all HPLC with fluorescence detection was conducted at these excitation and emission maxima wavelengths.

The fluorometer response to lolitrem B (Fig. 2) showed a linear relationship

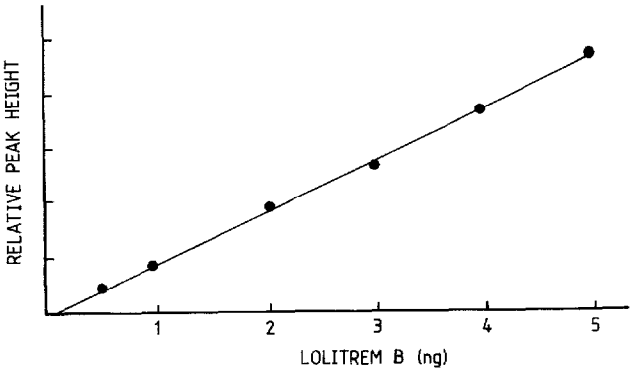


Fig. 2. HPLC fluorometer response to lolitrem B.

between peak height and amount of lolitrem B injected over the range of interest, 0.5–5.0 ng, in the HPLC mobile phase.

The improved sensitivity with fluorescence detection compared to UV detection was demonstrated by the intense full-scale fluorescence signal observed for an injection of 3 ng of lolitrem B. By comparison, a barely detectable signal could just be observed for this amount of lolitrem B on the most sensitive UV detector setting at 268 nm[13].

The use of both a UV detector and a fluorescence detector coupled to a dual-pen chart recorder, in the HPLC system, is highly advantageous. The arrangement is of diagnostic value, in that it allows differentiation of lolitrem B from other extraneous peaks that might occur in the same chromatographic window region. The presence of compounds more weakly fluorescent than lolitrem B would be indicated by a much higher UV detector signal:fluorescence detector signal ratio than in the case of lolitrem B. Further, setting the UV detector wavelength at a wavelength region other than 268 nm where the absorption of lolitrem B is maximal[13], whilst leaving the fluorescence detector monochromators unchanged, would assist in revealing the presence of other molecules.

Lolitrem B content of reference ryegrass samples

The amount of lolitrem B in the high-toxin and low-toxin ryegrass reference samples was determined using the analytical procedure. The results, and the statistical analysis, are shown in Table I. As shown in the table, sixteen replicate determinations

TABLE I

ANALYSIS OF LOLITREM B IN REPLICATES OF HIGH-TOXIN AND LOW-TOXIN RYEGRASS SAMPLES

Sample	*Number of replicates*	*Lolitrem B content** $\bar{x}$	*S.D.*	*S.E.*	*C.V. (%)*
Low-toxin grass	16	0.234	0.009	0.002	3.8
High-toxin grass	14	1.68	0.068	0.018	4.1

* μg lolitrem B/g grass.

on the low-toxin grass gave a mean lolitrem B content of 0.23 μg/g of grass. The estimated standard error in the determination and the % C.V. were very low. A similar determination on fourteen replicates of the high-toxin grass gave a mean lolitrem B content of 1.68 μg/g of grass, again with a low standard error and % C.V.

Efficiency of extraction procedure

Following the above determinations, the lolitrem B contents of the high-toxin and low-toxin ryegrass reference samples were determined by exhaustive solvent extraction. The results are recorded in Table II. After six extractions of the high-toxin grass, a negligible amount of lolitrem B appeared in the extract; in the case of the low-toxin grass, exhaustive extraction was terminated after five extractions.

TABLE II

TOTAL LOLITREM B CONTENT OF HIGH-TOXIN AND LOW-TOXIN RYEGRASS AS DETERMINED BY EXHAUSTIVE EXTRACTION

Extraction	*Lolitrem B content (ng lol. B/g grass)*							
	High-toxin grass replicate				*Low-toxin grass replicate*			
	A	*B*	*C*	$\bar{X}$	*A*	*B*	*C*	$\bar{X}$
1	1132	1277	1170		159	164	151	
2	372	385	365		57	57	54	
3	113	98	99		22	26	18	
4	26	35	36		12	8	8	
5	31	24	21		8	3	7	
6	0	4	4		–	–	–	
Total lolitrem B content	1675	1823	1695	1731	259	258	238	251

Calculation of the recovery estimates of the analytical procedure on the basis of the above exhaustive extraction experiments are tabulated in Table III; the recoveries were 93% for the low-toxin grass and 97% for the high-toxin grass.

A further check on the validity of the analytical procedure was carried out with a spike experiment. Lolitrem B was added at two levels, 0.30 ppm and 1.80 ppm, to samples of low-toxin ryegrass. Both spike levels were tested in three replicate samples each. The results are tabulated in Table IV. At both spike levels, the mean recovery of lolitrem B was greater than 90%.

TABLE III

RECOVERY ESTIMATE OF ANALYTICAL PROCEDURE

Sample	$\bar{x}$ *(μg lol. B/g grass) (analytical procedure)*	$\bar{X}$ *(μg lol. B/g grass) (exhaustive extraction)*	*Recovery (%)*
Low-toxin grass	0.234*	0.251	93
High-toxin grass	1.68	1.73	97

* Values of $\bar{x}$ and $\bar{X}$ obtained from Table I and Table II.

TABLE IV

RECOVERY OF LOLITREM B SPIKE ADDED TO LOW-TOXIN RYEGRASS

Replicate	*Lolitrem B content (ng/100-μl injection)*		
	Grass only	*Grass plus 0.30 ppm lol. B spike*	*Grass plus 1.80 ppm lol. B spike*
1	0.33	0.60	2.27
2	0.30	0.59	2.07
3	0.32	0.60	2.17
x	0.32	0.60	2.17
% Recovery*	–	90.3	98.7

$$* \text{ \% Recovery} = \frac{\text{actual recovery in ng/100-}\mu\text{l injection}}{\text{calculated spike level in ng/100-}\mu\text{l injection}} \times 100\%.$$

Optimisation of the shaking time of the ryegrass with the extraction solvent was achieved by determination of the lolitrem B recovery *versus* shaking time for replicates of high-toxin ryegrass. The results are shown in Table V. Whereas a 0.5-h shaking time gave incomplete extraction, a 1.0-h shaking time gave a recovery in close agreement with that obtained by exhaustive extraction. Further shaking time did not improve the recovery. Thus a 1-h shaking time was adopted routinely for the analytical method.

TABLE V

LOLITREM B EXTRACTION FROM HIGH-TOXIN RYEGRASS *VERSUS* SHAKING TIME

Extraction time (h)	*Duplicate no.*	*Lolitrem B content (μg lol. B/g grass)*	$\bar{x}$
0.5	1	1.46	1.45
	2	1.44	
1.0	1	1.67	1.66
	2	1.64	
2.0	1	1.68	1.68
	2	1.68	
4.0	1	1.62	1.64
	2	1.66	

Application of method

The method outlined in this paper has been applied successfully in our ryegrass staggers research programme in New Zealand, to the routine analysis of ryegrass plants and seed for lolitrem B content. An interesting application has been to determine the component(s) of the ryegrass plant in which the lolitrem B neurotoxin is most concentrated. Thus, in some preliminary studies, we dissected ryegrass plants into leaf blade, "basal leaf sheath/stem", and "upper leaf sheath/stem" components. The basal leaf sheath/stem component was defined as the first inch of the plant (less

any leaf blades) immediately above the root crown; all leaf sheath/stem material above this height was collectively categorised as upper leaf sheath/stem component. No attempts were made in this preliminary work to further subdivide these components into strictly defined anatomical components. Table VI shows some lolitrem B analysis results for some high-toxin ryegrass plants sampled on several consecutive weeks from a trial site in Hamilton during late-winter, early-spring. There is a marked concentration of the lolitrem B neurotoxin in the basal leaf sheath/stem region of the plants, compared with the very low levels in the leaf blade component. These results are clearly important in relationship to the ingestion of lolitrem neurotoxins by grazing livestock, and further studies are underway to observe the changes and increases of neurotoxin levels that will occur over the summer and autumn seasons, when ryegrass staggers most frequently occurs[3–5,7,9,10].

TABLE VI

ANALYSIS OF LOLITREM B IN DISSECTED RYEGRASS PLANTS

Sample date	*Replicate**	*Lolitrem B content (μg lol. B/g grass) in dissected plant component***		
		A	*B*	*C*
25.9.84	1	1.94	1.13	0.23
	2	3.06	1.81	0.37
	3	3.30	1.86	0.49
	4	1.28	1.03	0.24
	$\bar{x}$	2.40	1.46	0.33
2.10.84	1	2.88	1.32	0.12
	2	3.40	1.54	0.18
	3	2.40	1.34	0.18
	4	2.10	1.24	0.20
	5	2.80	1.10	0.24
	6	1.82	1.34	0.32
	7	3.02	1.64	0.26
	8	2.86	1.32	0.18
	$\bar{x}$	2.66	1.36	0.21

* Each replicate was from a different sub-plot at the same plot site.
** A = Basal leaf sheath/stem; B = upper leaf sheath/stem; C = leaf blade.

The method has also been used to examine ryegrass seed cleanings which had been fed to horses, which subsequently developed a clinical syndrome resembling ryegrass staggers. The seed cleanings were found to contain 5.3 ppm lolitrem B[15].

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. G. Keogh, *N.Z. J. Exp. Agric.*, 1 (1973) 55.

4 R. T. Gallagher, R. G. Keogh, G. C. M. Latch and C. S. W. Reid, *N.Z. J. Agric. Res.*, 20 (1977) 431.
5 P. H. Mortimer, in R. F. van Kampen and L. F. James (Editors), *Effects of Poisonous Plants on Livestock*, Academic Press, New York, 1978, pp. 353–361.
6 C. G. Mackintosh, M. B. Orr, R. T. Gallagher and I. C. Harvey, *N.Z. Vet. J.*, 30 (1982) 106.
7 R. T. Gallagher, in M. Baxter (Editor), *Proceedings VIII ISHAM Congress 1982*, Massey University, Palmerston North, New Zealand, 1983, pp. 469–472.
8 R. T. Gallagher, A. D. Hawkes, P. S. Steyn and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, (1984) 614.
9 A. J. Aasen, C. C. J. Culvenor, E. P. Finnie, A. W. Kellock and L. W. Smith, *Aust. J. Agric. Res.*, 20 (1969) 71.
10 B. J. Shreeve, D. S. P. Patterson, B. A. Roberts, S. M. MacDonald and E. N. Wood, *Vet. Rec.*, 103 (1978) 209.
11 G. W. Lanigan, A. L. Payne and P. A. Cockrum, *Aust. J. Exp. Biol. Med. Sci.*, 57 (1979) 31.
12 R. T. Gallagher and A. D. Hawkes, *N.Z. J. Agric. Res.*, in press.
13 R. T. Gallagher and A. D. Hawkes, *J. Chromatogr.*, 322 (1985) 159.
14 R. T. Gallagher and A. D. Hawkes, in preparation.
15 B. L. Munday, I. M. Monkhouse and R. T. Gallagher, *Aust. Vet. J.*, in press.