Qualitative and quantitative analysis of endophyte alkaloids in perennial ryegrass using Near-Infrared Spectroscopy

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Running title: NIRS to detect Epichloë alkaloids in ryegrass

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ABSTRACT

BACKGROUND: Near-infrared reflectance spectroscopy (NIRS) has been widely used in forage quality control because it is faster, cleaner, and less expensive than conventional chemical procedures. In *Lolium perenne* (perennial ryegrass), one of the most important forage grasses, the infection by asymptomatic *Epichloë* fungal endophytes alters the plant nutritional quality due to the production of alkaloids. In this research, we developed a rapid method based on NIRS to detect and quantify endophyte alkaloids (peramine, lolitrem B and ergovaline) using a heterogeneous set of *L. perenne* plants obtained from wild grasslands and cultivars.

RESULTS: NIR spectra from dried grass samples were recorded and classified according to the absence or presence of the alkaloids, based on reference methods. The best discriminant equations for detection of alkaloids classified correctly 94.4%, 87.5% and 92.9% of plants containing peramine, lolitrem and ergovaline respectively. The quantitative NIR equations obtained by modified partial least squares (MLPS) had coefficients of correlation of 0.93, 0.41, and 0.76 for peramine, lolitrem B and ergovaline respectively.

CONCLUSIONS: NIRS spectroscopy is a suitable tool for qualitative analysis of the endophyte alkaloids in grasses and for the accurate quantification of peramine and ergovaline.

KEYWORDS: ergovaline, *Epichloë* endophytes, grassland, *Lolium perenne*, lolitrem B, peramine

24 **INTRODUCTION**

25

26 Antiquality components are defined as any factor that diminishes the degree to which
27 a forage meets the nutritional requirements of a specific kind of animal.[1] Among the
28 diverse impediments to forage quality there are structural components (*e.g.* lignin)
29 and secondary metabolites (*e.g.* alkaloids).[2] Antiquality components may reduce dry
30 matter intake and digestibility, or cause physiological disorders in herbivores. Such
31 factors represent a high economic cost for the industry, due to losses in potential gain
32 and reproduction of livestock.[1,3]

33 Perennial ryegrass (*Lolium perenne* L.) is one of the most important cool season
34 grasses and the basis of many forage-livestock systems worldwide. Perennial ryegrass
35 has the potential to produce high yields of excellent quality forage and can be used for
36 pasture, hay, silage, turf and conservation purposes.[4] Perennial ryegrass, like several
37 other grass species, is often infected by endophytic fungi of the genus *Epichloë* that
38 confer adaptive advantages to the host grass.[5-7] However, *Epichloë*-endophytes are
39 also responsible for the production of some mycotoxins which function as antiquality
40 factors in forage systems.

41 Lolitrem B, ergovaline and peramine, are the most common alkaloids produced
42 in infected perennial ryegrass plants by *Epichloë* endophytes. These mycotoxins often
43 cause pronounced physiological reactions in herbivores, with negative effects for
44 livestock. Lolitrem B, an indole-diterpene alkaloid, is a tremorgenic compound
45 responsible of ryegrass staggers, a neuromuscular disorder producing ataxia and
46 tremors in mammals.[8-10] The ergot alkaloid ergovaline is a major contributor of fescue
47 toxicosis in livestock, a syndrome that encompass symptoms such as reduced weight

48 gain, hyperthermia, reduced fertility and gangrene of the extremities.[11-13] Peramine is
49 an insect deterrent, with no obvious clinical effect over mammals.[14]

50 The presence of *Epichloë* endophytes in pasture grasses has caused important
51 economic losses in livestock industry due to the toxic effects of the alkaloids lolitrem B
52 and ergovaline.[1,7] As a consequence, current strategies for forage grass improvement
53 focus on the utilization of selected endophytes which maintain insect deterrent
54 properties (peramine) while minimizing the negative impact of alkaloids toxic to
55 livestock (lolitrem B and ergovaline).[16-19] Therefore, one technology in constant need
56 of advancement is a methodology for the detection and quantification of fungal
57 alkaloids in plants.

58 Near-infrared reflectance spectroscopy (NIRS) is a non-destructive technique
59 with a widespread application in food and agricultural research, including the
60 evaluation of forages for quality assessment.[20-24] NIRS is an analytical technique that
61 predicts the chemical composition of materials based on the interaction between the
62 surface of the sample and the incident polychromatic light over a spectral wavelength
63 ranging from 1100 to 2500 nm (near infrared range). NIRS offers several advantages
64 over conventional methods of forage quality analysis: it can evaluate many parameters
65 at the same time using the same spectral signature, is rapid, non-destructive, requires
66 small sample amounts, and no chemical reagents are needed. In the last decade, NIRS
67 has been successfully applied to agricultural commodities for the detection of
68 mycotoxins such as aflatoxins, ochratoxin A, fumonisins or deoxynivalenol.[25-27] NIRS
69 has also been used for the analysis of specific alkaloids in plants for medicinal
70 purposes,[28-29] but there are no reports of the use of this spectroscopic technology to
71 determine lolitrem B or peramine in grasses.

72 Quantitative analysis of alkaloids in plants is based on elaborate procedures of
73 extraction of each alkaloid separately, followed by quantification by high performance
74 liquid chromatography (HPLC). Although these methods are preferred for being exact
75 and precise, in high throughput studies where numerous samples should be screened,
76 there is a need for faster methods. The objective of this work was to evaluate the
77 suitability of NIR spectroscopy for qualitative and quantitative analysis of the alkaloids
78 of fungal origin peramine, lolitrem B and ergovaline in a heterogeneous set of *Lolium*
79 *perenne* plants.

80

81 **MATERIAL AND METHODS**

82 **Plant material**

83 A total of 124 *Epichloë*-infected ryegrass plants were used, 87 were of wild origin and
84 37 belonged to two commercial cultivars. Wild plants were collected at six wild
85 populations of *L. perenne* located in Western Spain.[30] After collection in the field, wild
86 ryegrass plants were transplanted in the experimental farm Muñovela (Salamanca,
87 Spain; 40°54'19" N, 5°46'28" W; 780 masl; annual precipitation 372 mm, and mean
88 annual temperature 12.7 °C). A distance of 50 cm was left between neighboring plants,
89 and they were watered during their establishment but not thereafter. Plants were
90 harvested on May of the next-year at the flowering stage. The set of ryegrass plants
91 from cultivars were obtained by artificial inoculation with known *Epichloë* strains of
92 seedlings of 'Barplus' and 'Romance' cultivars (Barenbrug, NL).[31] These inoculated
93 plants were grown for one year in 2 L pots with a perlite:peat moss (1:1, v/v) potting
94 mix. Pots were maintained outdoors in a randomized arrangement, rotating their

95 position frequently, watering regularly and fertilizing them once a year with a liquid
96 commercial fertilizer. In all cases, ryegrass plants were harvested by cutting all
97 aboveground biomass at approximately 5 cm from the soil surface, and then stored at -
98 80 °C, freeze dried, and ground to 0.5 mm using a hammer mill (Fritsch 15303).

99 **Reference HPLC analysis of alkaloids**

100 Peramine, lolitrem B and ergovaline were analyzed separately by high performance
101 liquid chromatography (HPLC).

102 Peramine was extracted following the technique described by Barker et al.[32]
103 The analysis was performed by HPLC in a Waters module 2695 (Waters Co, MA, USA)
104 with a C18 column 150 x 3.9 mm; 4.0 µm (Waters Nova Pak) using a Photodiode Array
105 detector (Waters 996, MA, USA) set at 230 nm. The mobile phase was isocratic,
106 composed by 15% acetonitrile and 85% of 10 mM guanidine carbonate and 0.16%
107 formic acid buffer, with a flow rate of 0.7 mL min^{-1}. The peramine standard was a gift
108 from G. Lane (AgResearch, New Zealand).

109 Quantification of lolitrem B was based on the method reported by Gallagher et
110 al.[33] The sample peaks were compared with those of lolitrem B from a standard
111 solution (a gift from C. Miller, AgResearch, New Zealand), using a HPLC Waters 2695
112 module (Waters Co, MA, USA), a silica column 250 x 4.6 mm, 5.0 µm (Waters
113 Spherisorb), and a fluorescence detector (Waters 2475, MA, USA) λ_{exc}= 268 nm; λ_{em}=
114 440 nm. The mobile phase was composed of 80% dichloromethane and 20%
115 acetonitrile, with a flow rate of 1.0 mL min^{-1}.

116 The procedure described by Yue et al.[34] was performed to determine the
117 concentration of ergovaline. Its quantification was done by reverse phase HPLC in a

118 Waters 2695 module, a C18 column 150 x 4.6 mm; 2.7 µm (Agilent Poroshell, CA, USA)
119 and a fluorescence detector (Waters 2475, MA, USA) λ_{exc}= 250 nm; λ_{em}= 420 nm. The
120 mobile phase was 35% acetonitrile in 0.01M ammonium acetate with gradient flow to
121 0.8 mL min^{-1}. Ergovaline standard was purchased from F. Smith, Auburn University,
122 USA.

123 **Near-Infrared Spectroscopy**

124 ***Acquisition of infrared spectra***

125 Approximately 2.0 g of each of the 124 ground ryegrass samples were placed on a
126 circular (38 mm diameter and 10 mm thickness) quartz reflectance-sampling cell for
127 their spectrum acquisition. The reflectance spectra between 400 and 2498 nm and
128 acquired at 2 nm wavelength increments were collected using a NIRSystem 6500
129 scanning monochromator (FOSS Analytical, Denmark) fitted with a sample transport
130 module. The spectrum of each grass sample was recorded as log (1/*R*) (*R*= intensity of
131 reflected light at each wavelength) and used for further chemometrical analyses.
132 Instrument control, manipulation of spectral files and chemometric analyses were
133 made with WinISI 4.3 software (FOSS Analytical, Denmark).

134 The collected spectra were randomly divided automatically using WinIsi 4.3
135 software into two subsets, one of them (*ca*. 75% of all the samples) was used for
136 training or calibration of the models and the other samples (*ca*. 25%) were used for an
137 external validation to corroborate the performance of the NIR equations obtained.

138 ***Spectra pretreatment***

139 In both qualitative and quantitative analyses, mathematical pretreatments and
140 principal component analysis (PCA) were applied to spectra of the samples. The

141 mathematical pretreatments applied on spectra were: averaging, characterization of
142 the absorbance (standard normal variate, SNV), correction of the trend (DeTrend, DT),
143 and application of SNV and DT together (SNV+DT).[35] The mathematical pretreatments
144 were combined with smoothing, gaps, and derivative transformations to remove
145 additive baseline effects (first derivative) or a linear baseline (second derivative).[36]
146 Their notation is indicated with four digits (*a*, *b*, *c*, *d*) where *a* is the order of derivative;
147 *b* is the number of points where the derivative is performed; *c* is the number of points
148 where the first smoothing is made; and *d* the number of points where the second
149 smoothing is performed.

150 ***Qualitative NIR analysis***

151 The discriminant model was based on a pattern recognition method, with *a priori*
152 knowledge about the category membership of samples (supervised). A discriminant
153 algorithm known as X Residuals was used, with this method a PCA is performed on
154 each group, then the evaluated spectrum score is multiplied by the PCA loadings for
155 each group, the product is subtracted from the evaluated spectrum and the sample
156 will be classified as belonging to the group resulting with the lowest residual.

157 The NIR spectral information of each sample was used to define the
158 discriminant equations to be developed for detecting presence (+) or absence (−) of
159 each alkaloid in ryegrass samples: peramine (PER− or PER+), lolitrem B (LTM− or LTM+)
160 and ergovaline (ERG− or ERG+). In order to find out optimal NIRS classification
161 equations, it was needed to transform the spectra through the mathematical
162 pretreatments combined with smoothing, gap and derivative transformations
163 providing a total of 40 discriminant equations for each parameter.

164 Once the discriminant models were created, their accuracy was measured as
165 the percentage of samples from the validation set that were correctly classified and
166 with the global percentage of false negatives. Those models with the best classification
167 performance and the lowest percentages of false negatives were selected for
168 identification of the evaluated traits of new ryegrass samples. For this work, a false-
169 positive was defined as a sample without the alkaloid studied but classified by the
170 discriminant model as having the compound; conversely, a false-negative occurs when
171 in a sample the metabolite was present but the models classified the sample as not
172 having the molecule.

173 ***Quantitative NIR analysis***

174 The development of the quantitative models was done through the modified partial
175 least squares method (MPLS),[37] using the spectra and concentrations obtained from
176 the method of reference (HPLC) separately for each alkaloid (peramine, lolitrem B and
177 ergovaline) in the ryegrass from the calibration set. In this procedure, samples in which
178 the alkaloid concentration was zero in the HPLC-analysis were not included.

179 Before the MPLS, a PCA was performed on spectra of the calibration set,
180 generating 20 different files by the combination of the mathematical treatments
181 (spectra averaging, SNV, DT, SNV+DT, smoothing, gaps and derivatives) described
182 above. In this process, the spectral outliers were identified (samples with $H > 3.0$) and
183 discarded. Subsequently, on the 20 files generated by the PCA other 20 pretreatments
184 were applied, generating 400 different equations to be evaluated for the quantification
185 of each alkaloid.

186 When the MPLS was performed, a cross-validation was applied to select the
187 optimal number of factors, and to avoid overfitting.[38] In the cross-validation, the
188 sample set is divided into several groups; each group is then validated using a
189 calibration developed on the other samples. In this process, samples with high
190 residuals are detected and those samples whose *T* statistical, defined as the residual
191 divided by the standard error of cross-validation (SECV), exceeds the value of 2.5 were
192 removed from the calibration set, this procedure was repeated two times to finally
193 obtain the models. The selection of the best NIRS equations for alkaloid quantification
194 was based on the multiple correlation coefficient (RSQ), standard error of calibration
195 (SEC) and SECV.[39-40]

196 The robustness of the NIR models for alkaloid quantification was corroborated
197 through external validations by means of a simple regression between NIRS-predicted
198 values and those obtained by the reference method, to determine the accuracy of the
199 calibration (RSQ, SEP, statistics). The ratio performance deviation (RPD) which is the
200 ratio of standard deviation of the prediction reference data to the standard error of
201 prediction (SEP), was also calculated to evaluate the performance of the calibration.
202 The RPD statistics provides a basis for standardizing the SEP and should be ideally be at
203 least 2. A Student's *t*-test was conducted to verify that the concentrations obtained by
204 both methods (HPLC and NIRS) provided values significantly equal or not (P= 0.05) and
205 the residuals were calculated on alkaloid concentrations.

206

207 **RESULTS**

208 **Chemical measurements**

209 The alkaloid contents of the ryegrass samples by HPLC reference methods are
210 indicated in Table 1. A 61.3% of the *Epichloë*-infected plants contained peramine
211 (PER+) in a concentration ranging from 2.16 to 24.00 mg kg^{-1}; 55.7% had lolitrem B
212 (LTM+) ranging from 0.46 to 6.74 mg kg^{-1}; and 42.7% contained ergovaline (ERG+) with
213 concentrations from 0.02 – 2.11 mg kg^{-1}.
214 Toxic levels of lolitrem B for livestock (>1.80 g kg^{-1}) were detected in approximately
215 27% of the samples in which this alkaloid was present; whereas a two-thirds of the
216 samples with ergovaline have a concentration above the reported safe limit for
217 livestock consumption (0.40 g kg^{-1}).

218 **Qualitative NIR analysis**

219 All the 20 discriminant models obtained for detection of each alkaloid (lolitrem B,
220 ergovaline, and peramine) had good accuracy; the global percentages of good
221 classification in the training set were always higher than 85% (data not shown). In the
222 validation and for all mathematical treatments, it was observed that plants containing
223 any alkaloid (PER+, LTM+ or ERG+) were better recognized than plants without the
224 alkaloid (PER−, LTM− or ERG−).
225 Of the 20 models developed, the one selected for identifying ryegrass samples
226 with or without peramine, was the one with the highest percentage of good
227 identifications in the validation (89.3%), and it was obtained when the spectra were
228 transformed using the s0 treatment, SNV (0,0,1,1), with eight PCs explaining 99.99% of
229 the spectral variability (Table 2). This model misclassified 20% of the PER− plants (8 out
230 of 48); however, it had the lowest percentage of false negatives (1.4%) mistaking only
231 one PER+ plant in the validation set (Figure 2). Wrongly classified PER− plants had

232 different origins and were handled equally during the spectra acquisition. The only
233 PER+ sample classified as PER− had a peramine concentration of 3.88 mg kg^{-1}, which is
234 in the lower limit of concentration found in PER+ plants from the training set (Figure
235 1).

236 The best NIRS discriminant model for lolitrem B detection was obtained using
237 the m1 mathematical treatment, SNV+DT (1,4,4,1), with first derivative transformation
238 of the spectra (Table 2). The selected discriminant model for the detection of lolitrem
239 B misclassified ryegrass samples only in the validation set, six out of 12 LTM− samples
240 (50.0%) where recognized as LTM+, and only two out of the 16 LTM+ samples (12.5%)
241 were not correctly classified (Figure 2). The six LTM− plants, which were not correctly
242 classified were from different origins and LTM+ plants misclassified had individual
243 lolitrem B concentrations of 0.74 mg kg^{-1} and 1.49 mg kg^{-1}.

244 There were two models with the same best parameters for the identification of
245 ERG+ and ERG− plants (Table 2). In cases like that, it is recommendable to choose the
246 model in which the original spectra had been less modified; thus, the model selected
247 was n4, raw spectra without correction of the scattering and transformation using the
248 second derivative (2,4,4,1). All samples in the training set were correctly classified, and
249 in the validation set seven plants from different origins, were incorrectly classified, six
250 out of 16 ERG− samples were identified as having ergovaline and only one out of 13
251 ERG+ samples was classified as ERG− (Figure 3). The concentration of ergovaline in the
252 ERG+ sample classified as ERG− was 0.48 mg kg^{-1}, in the lowest limit of concentration
253 in the training set.

254 **Quantitative NIRS analysis of alkaloids**

255 All the statistical parameters of the best NIRS calibration equations for quantification
256 of peramine, lolitrem B and ergovaline in ryegrass samples are shown in Table 3.

257 ***Quantification of peramine***

258 The most accurate model for quantification of peramine was developed when the
259 spectra of the ryegrass samples were transformed using the mathematical
260 pretreatment s2: standard normal variate (2,4,4,1). Because of the statistical
261 treatments described, the calibration model was obtained with 55 samples; only one
262 spectral outlier was eliminated after application of the *H* criterion (Mahalanobis
263 distance) and no chemical outliers were detected according to the *T* criterion (high
264 residual, $T > 2.5$). The calibration equation obtained had a correlation coefficient (RSQ)
265 of 0.93; a SEC of 1.56 mg kg^{-1} and a SECV of 3.65 mg kg^{-1}.

266 The correlation between the reference values and those predicted by NIRS
267 samples from calibration set is presented in Figure 4. The predictive capability of the
268 model RPD was 3.99, which indicates that the model obtained can be applied to
269 estimate accurately peramine concentration in ryegrass samples with unknown
270 concentration of this alkaloid.

271 The external validation of the NIR equation for quantification of peramine in
272 ryegrass samples was accurate (Table 3). The Student *t*-test indicated that there was
273 no significant difference between the concentration measured by HPLC and the NIR
274 predictions ($P = 0.52$). The mean standard error for quantification of peramine
275 concentration of the NIR equation with respect to the HPLC procedure was 0.25 mg kg^{-1}
276 and the residual errors were 1.95 and 0.25 mg kg^{-1} in the validation samples.

277 ***Quantification of lolitrem B***

278 The best calibrations for lolitrem B quantification by NIRS were obtained using the
279 spectral pretreatment d0 (DT), with the numerals (0,0,1,1) which involves the
280 application of a second-degree polynomial to standardize variations in spectral
281 curvilinearity without transformation by derivatives. No samples were eliminated by
282 the *H* criterion. Similarly to PCA for the MPLS, the best performance for lolitrem B
283 quantification was obtained with the pretreatment d0 and using seven PLS factors. The
284 final calibration set was constituted by 46 samples because two samples were
285 eliminated using the *T* criterion. The NIR model had a RSQ of 0.41 with a SEC and SECV
286 of 0.46 and 0.51 mg kg^{-1} respectively (Table 3).

287 The validation process comparing the concentration of lolitrem B estimated
288 with HPLC with that predicted by a NIRS equation (Figure 4), allowed the calculation of
289 the SEP= 0.44 mg kg^{-1}, and the predictive capability of the NIRS equation (RPD= 1.25).
290 Given the low correlation between the actual and predicted data (RSQ= 0.41), and the
291 low RPD, the results of NIR prediction of lolitrem B concentration should be taken
292 cautiously.

293 The external validation of the NIR equation for quantification of lolitrem B and
294 the HPLC reference method, showed no significant differences (P= 0.39). However,
295 compared with the concentrations of the samples the error of prediction was high
296 (RMSE= 0.39 mg kg^{-1}) also the residuals (0.30 mg kg^{-1}).

297 ***Quantification of ergovaline***

298 The model with the best performance for ergovaline quantification by NIRS was
299 obtained when spectra were transformed by the mathematical treatment s0: SNV,
300 without derivatives (0,0,1,1) in the PCA. In this process, one spectral outlier was

301 detected and eliminated. In the MPLS regression, the mathematical treatment used
302 was d0: correction of trend without application of derivatives (0,0,1,1). The calibration
303 model for quantification of ergovaline had a RSQ of 0.76, a SEC of 0.29 mg kg^{-1} and the
304 SECV was 0.38 mg kg^{-1} (Table 3).

305 When actual ergovaline concentration was compared with the predicted NIR
306 values, the standard error of prediction was 0.26 mg kg^{-1} and this model had a RPD=
307 2.04 (Figure 4). According to this RPD, the NIRS models for quantification of ergovaline
308 can be used to quantify this alkaloid in samples of ryegrass, but the prediction should
309 be taken with caution because the SEP (0.26 mg kg^{-1}) was higher than half of the safety
310 limit for livestock consumption (0.40 mg kg^{-1}).

311 Results of the external validation, in which the performance of the NIR
312 equation for quantification of ergovaline was evaluated, indicated that the
313 concentrations estimated were equal to the data obtained using HPLC (P= 0.56). The
314 RMSE in the calculation of the concentration using NIR was 0.25 mg kg^{-1} and the
315 residuals 0.22 mg kg^{-1} (Table 3).

316

317 DISCUSSION

318 The purpose of this study was to evaluate the suitability of NIRS for qualitative and
319 quantitative analysis of fungal alkaloids in perennial ryegrass plants. The results
320 showed that the spectral information obtained directly from minimally processed grass
321 samples can be used to detect the presence and concentration of the alkaloids
322 peramine, lolitrem B and ergovaline. Qualitative and quantitative NIRS equations
323 fulfilled their purpose independently of the fact that the sample set was composed of a

324 heterogeneous group of ryegrass plants from natural grasslands and cultivars and
325 different growth conditions, which indicates a high robustness of this method.

326 To our knowledge, this is the first report of the use of NIRS for the identification
327 or discrimination of grass samples containing *Epichloë* alkaloids. Our results showed
328 that the accuracy of NIRS discriminant models for the identification of ryegrass
329 samples containing peramine, lolitrem B or ergovaline was acceptable. In general,
330 spectral differences were higher in positive samples (PER+, LTB+, ERG+) than in the
331 negative ones (PER−, LTB−, ERG−), resulting in discriminant NIRS models that identified
332 better those plant samples which had alkaloids than those without. Due to the nature
333 of this study, having a higher number of false negatives might be a greater problem
334 because of the toxic nature of lolitrem B and ergovaline for mammals. However, NIRS
335 discriminant equations for lolitrem B and ergovaline had only 3.1% and 1.9% of false
336 negatives respectively.

337 Studies on the quantitative analysis of *Epichloë* alkaloids by NIRS in forage
338 samples are very scarce; to our knowledge there are only two published reports
339 focusing on in the quantification of ergovaline in tall fescue samples[41-42] and there are
340 no publications of its use for quantitative analysis of peramine or lolitrem B. The NIRS
341 equation developed here for quantification of ergovaline was less accurate (RSQ= 0.76)
342 than the one reported by Roberts et al.[41] (RSQ= 0.93). However, one limitation of the
343 calibration equation of Roberts et al.[41] is that was developed with homogeneous plant
344 samples belonging to a single cultivar, and that equation might not be accurate for
345 other plant samples. In contrast, our calibration equation was developed using a
346 heterogeneous group of perennial ryegrass samples from natural grasslands, plus two
347 commercial cultivars, and this increased the robustness of the models developed. The

348 calibration equation for peramine quantification was very accurate. Therefore, NIRS
349 can be suitable for quantitative analysis of ergovaline and peramine in perennial
350 ryegrass plants.

351 The major methods to determine these alkaloids (peramine, ergovaline and
352 lolitrem B) comprise HPLC with fluorescence or UV detection and liquid
353 chromatography with tandem mass spectrometry.[32-34,43-44] These methods can achieve
354 greater precision and accuracy in quantifying; however, they are based on elaborated
355 procedures of extraction with organic solvents and purification or clean-up, before
356 chromatography. The advantage of NIRS is that no chemical reagents are necessary,
357 what makes it is environmentally friendly, cheaper and less time-consuming, since only
358 ground samples are required.

359 It is possible that NIR is detecting precursors or other fungal metabolites with
360 similar functional groups.[42] For instance, ergovaline is the main ergot alkaloid in
361 *Epichloë*-infected grasses but other compounds of this group like ergonovine have
362 been detected.[45] The biochemical pathway of the lolitrem B synthesis is complex with
363 several intermediate molecules of the indol-diterpene group.[45-47] Thus, other indol
364 diterpenes than lolitrem B, like epoxy-janthitrems, are also produced by *Epichloë*-
365 endophytes. However, that hypothesis should be validated working in the mid-infrared
366 spectral region where it is easier to attribute differences in absorbance to specific
367 chemical bounds and thus to identify possible molecules.

368 Several samples from the commercial cultivars of ryegrass were spectral
369 outliers, what means that their spectra were significantly different from the average
370 spectrum obtained from ryegrass samples from wild origin. Commercial cultivars of

371 ryegrass have been part of continuous breeding programs, improving herbage
372 production, persistence, drought and heat tolerance, and resistance to diseases and
373 pests.[48-50] All these changes may be reflected in the chemical composition of the plants
374 and therefore in their respective spectra. The number of samples from commercial
375 cultivars was lower than that of the plants of wild origin and possibly did not have
376 enough representation in the whole set to be recognized as part of the group.
377 Omission of all spectra from commercial cultivars increased the accuracy of the
378 discriminant models, but at the same time decreased the robustness of the models
379 and consequently their applicability. Therefore, they were kept in the training set.
380

381 **CONCLUSION**

382 This study shows that NIRS can be used for fungal alkaloid detection in
383 perennial ryegrass plants. Considering that *Epichloë*-infected grasses can contain or
384 not these mycotoxins, and the existing variability of alkaloid profiles in endophyte-
385 grass associations, this qualitative technique can be a very helpful tool to discriminate
386 toxic plants, or to select particular endophytes, especially in studies where a high
387 number of samples need to be screened. The NIR quantitative equations generated
388 enabled to estimate accurately the concentration of peramine and ergovaline with
389 similar precision to the HPLC methods, but its accuracy was lower predicting the
390 lolitrem B concentration. The combination of both qualitative and quantitative NIRS
391 models could be a powerful tool for a rapid analysis of toxins in perennial ryegrass
392 plants, and for endophyte research.

393

394

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547
548

549 **Table 1.** Characteristics of the perennial ryegrass samples analyzed by HPLC reference

550 methods and used for the development of NIRS models for detection and

551 quantification of the alkaloids peramine, lolitrem B and ergovaline (mg kg^{-1}).

552

Alkaloid	Plant status	Sample set	n	Range	Mean	SD
Peramine	[a] PER+	Calibration	56	2.16-24.00	6.96	5.83
		Validation	20	2.73-17.82	7.45	6.70
		Average/total	76	2.16-24.00	7.16	5.87
	[b] PER−	Calibration	36	ND		
		Validation	12	ND		
		Average/total	48	ND		
Lolitrem B	[a] LTM+	Calibration	48	0.47-6.74	1.33	1.13
		Validation	16	0.46-2.61	1.27	0.62
		Average/total	64	0.46-6.74	1.32	1.02
	[b] LTM−	Calibration	37	ND		
		Validation	14	ND		
		Average/total	51	ND		
Ergovaline	[a] ERG+	Calibration	39	0.02-2.11	0.74	0.58
		Validation	14	0.19-1.55	0.61	0.39
		Average/total	53	0.02-2.11	0.71	0.54
	[b] ERG−	Calibration	50	ND		
		Validation	16	ND		
		Average/total	71	ND		

553
554
555 [a] Plants with the alkaloid detected. [b] Plants without the alkaloid detected.
556 ND = not detected

557

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560

561 **Table 2.** Results of the best discriminant models for qualitative analysis for peramine,

562 lolitrem B and ergovaline (ALK− = plants free from alkaloid; ALK+= plants with the

563 alkaloid) in the ryegrass samples infected with *Epichloë* endophytes.

564

Alkaloid	Math. treat.[a]	PC	Variability explained (%)	Samples correctly classified (%) [b]						Samples misclassified (%)[b]	
				Training set			Validation set			total	
				ALK−	ALK+	total	ALK−	ALK+	total	ALK−	ALK+
Peramine	s0	8	99.99	80.0	100	92.6	80.0	94.4	89.3	20.0	1.4
Lolitrem B	m1	14	99.86	100	100	100	50.0	87.5	71.4	14.6	3.1
Ergovaline	n4	16	99.82	100	100	100	62.5	92.9	76.7	9.4	1.9
	d4	16	99.82	100	100	100	62.5	92.9	76.7	9.4	1.9

565
566 [a] Transformation of the NIR spectra: n= no scattering; s= standard normal variate
567 (SNV); d= correction of trent (DT); m= SNV+DT. The smoothing, gaps and derivatives
568 are indicated with the number next to letter, for this: 0= (0,0,1,1); 1= (1,4,4,1) and 4=
569 (2,8,6,1).
570 [b] Percentages calculated without spectral outliers.

571

572 **Table 3.** Statistical parameters obtained for the calibration equations developed for
573 quantification of peramine, lolitrem B and ergovaline applying modified partial least
574 squares regression to the NIR spectra of the perennial ryegrass samples, and internal
575 and external validation processes.

	Peramine	Lolitrem b	Ergovaline
Calibration			
Principal component analysis (PCA)			
Pretreatment [a]	s2	d0	s0
Number of principal components (PCs)	11	7	6
Explained variability (%)	99.05	99.93	99.95
Spectral outliers (H> 3.0)	1	0	1
Modified partial least squares (MPLS)			
Pretreatment [a]	s2	d0	d0
Number of samples	55	46	36
Standard deviation (SD) (mg kg^{-1})	5.63	0.47	0.46
Range (mg kg^{-1})	0.83 - 22.64	0.04 - 1.96	0.09 - 1.93
Chemical outliers (T> 2.5)	0	2	2
Multiple correlation coefficient (RSQ)	0.93	0.41	0.70
Standard error of calibration (SEC) (mg kg^{-1})	1.56	0.46	0.29
Standard error of cross validation (SECV) (mg kg^{-1})	3.65	0.51	0.38
Number of PLS factors	11	7	6
Groups in cross-validation	6	6	6
Validation			
Internal validation			
Standard error of prediction (SEP) (mg kg^{-1})	1.46	0.44	0.26
Medium value of the residuals (BIAS) (mg kg^{-1})	0	0.09	0
SEP corrected by the bias (SEPc) (mg kg^{-1})	1.47	0.44	0.26
Multiple correlation coefficient (RSQ)	0.94	0.41	0.76
Ratio performance deviation (RPD)	3.99	1.25	2.04
External validation			
Root mean standard error (RMSE= SEP) (mg kg^{-1})	0.25	0.39	0.25
Average residual (mg kg^{-1})	1.95	0.30	0.22
Student's t-test (P)	0.52	0.33	0.56

576

577 [a] Transformation of the NIR spectra: s= standard normal variate (SNV); d= correction of
578 trend (DT). The smoothing, gaps and derivatives are indicated with the number next to
579 letter as follow; for this: 0= (0,0,1,1); 2= (2,4,4,1).

580

581

FIGURE CAPTIONS

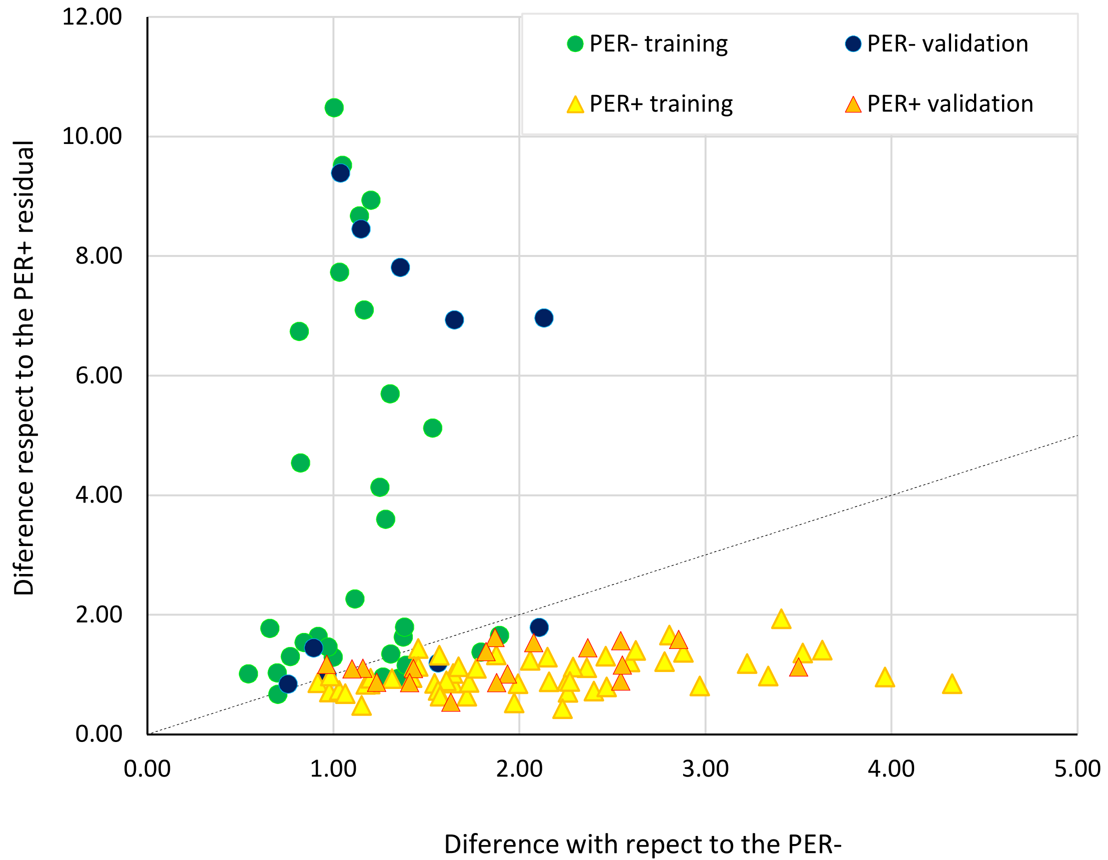
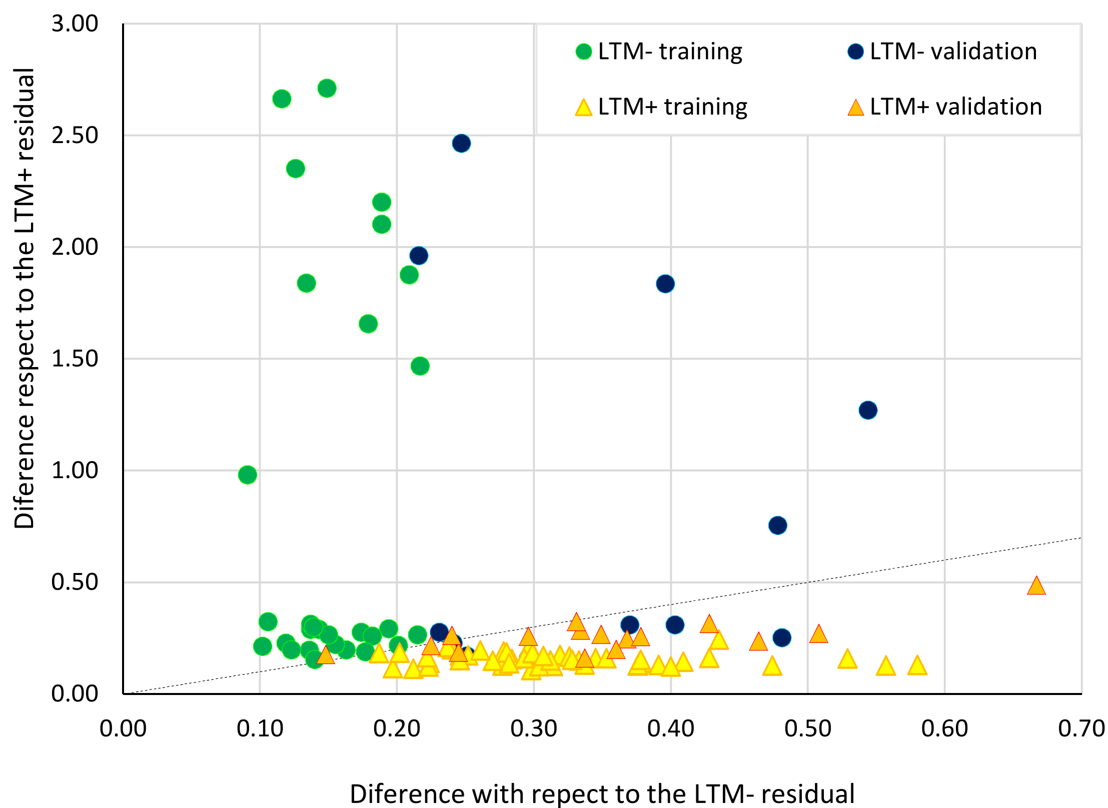


Figure 1. Classification of perennial ryegrass samples according to the presence of peramine (PER−, without peramine; PER+, with peramine) applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment s0: SNV(0,0,1,1).

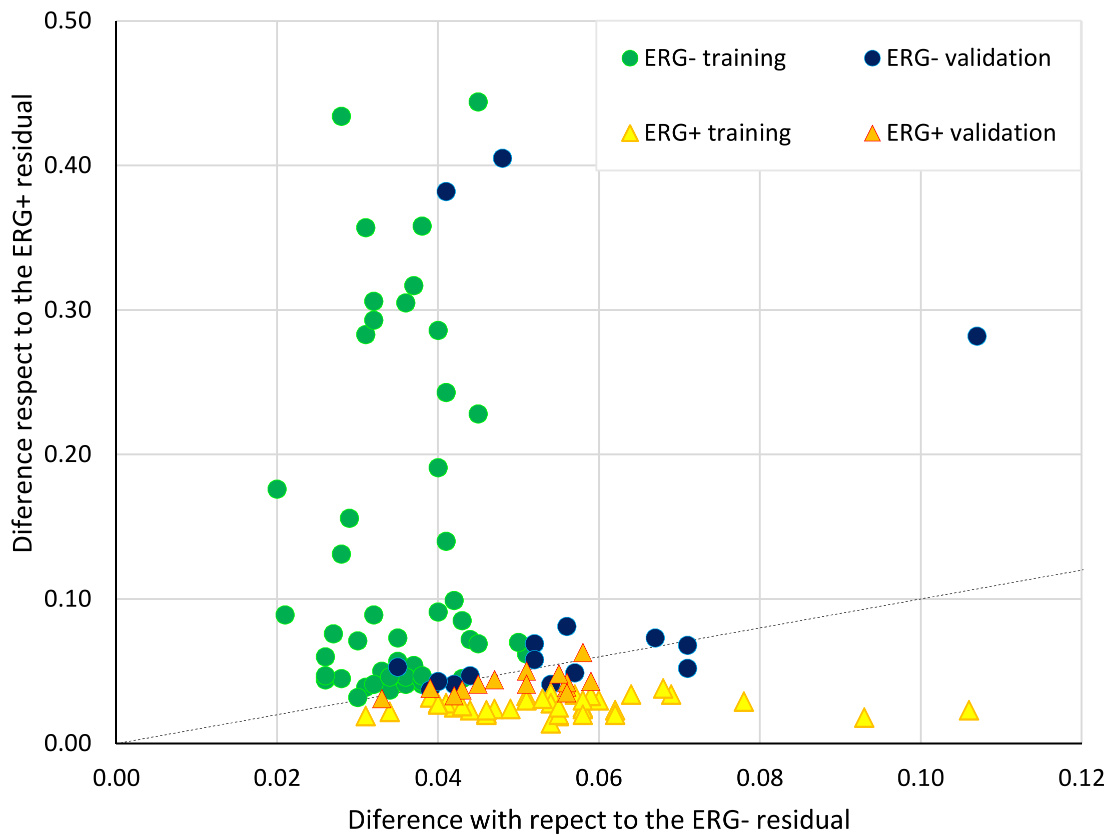
582



583

Figure 2. Classification of perennial ryegrass samples according to the presence of lolitrem B (LTM−, without lolitrem B; LTM+, with lolitrem B) applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment m1: SNV+DT(1,4,4,1).

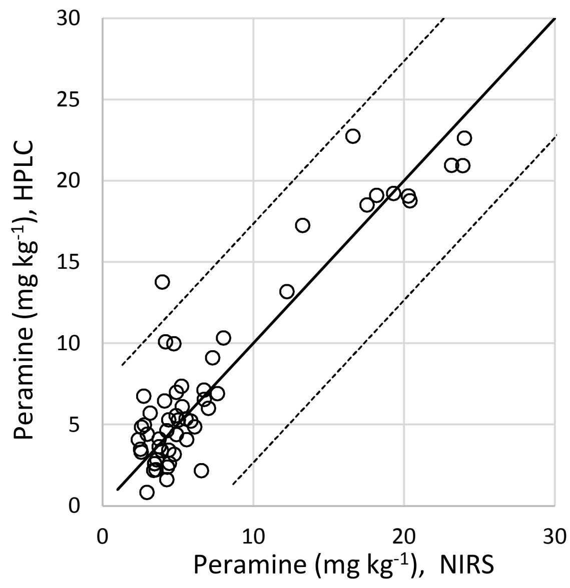
584



585

Figure 3. Classification of perennial ryegrass samples according to the presence of ergovaline (ERG−, without ergovaline; ERG+, with ergovaline) applying the discriminant X Residual algorithm on the NIR transformed spectra with the mathematical treatment n4: raw spectra (2,8,6,1).

586



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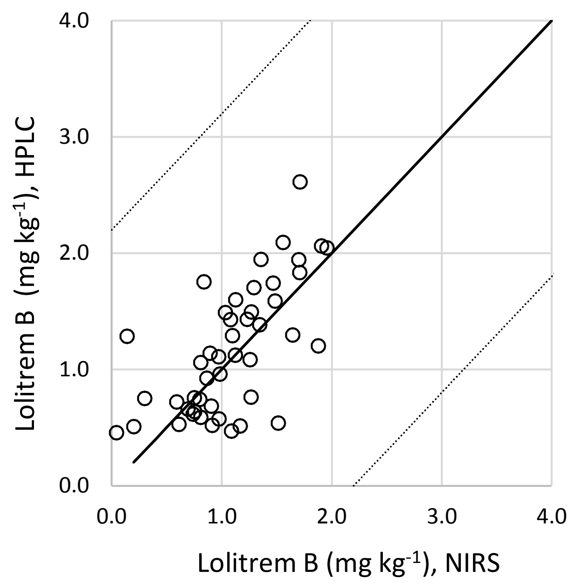

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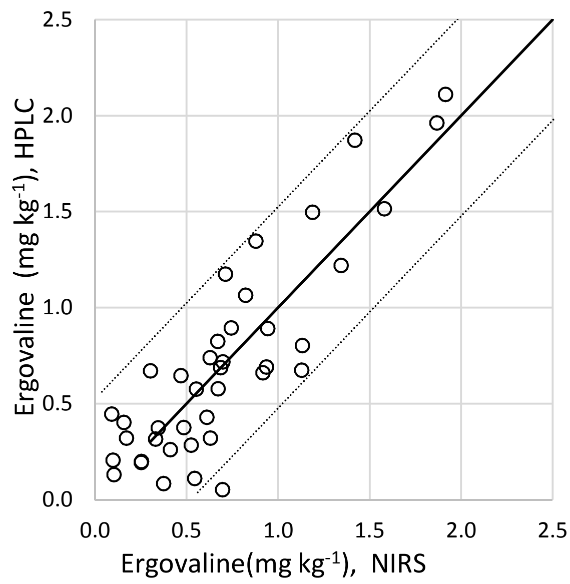

Figure 4. Internal validation comparing values of HPLC reference against the predicted by NIR spectroscopy using the MPLS regression for peramine, lolitrem B and ergovaline concentration in the validation set of perennial ryegrass samples.

590