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DISCRIMINATION AND QUANTIFICATION BETWEEN ANNUAL RYEGRASS AND PERENNIAL RYEGRASS SEEDS BY NEAR-INFRARED SPECTROSCOPY

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ABSTRACT

Contamination of annual ryegrass with perennial ryegrassby physical seed mixing or gene flow can result in a significant reduction in forage production.Therefore, this study aimed to develop a suitable technique using near-infrared spectroscopy (NIRS) for discriminating and quantifying adulteration of annual ryegrass seeds with perennial ryegrass. A partial least squares regression for discriminating analysis using visible and NIR region was developed using a calibration set(n=120), including 60 samples each of pure annual ryegrass seeds and those contaminated with perennial ryegrass at levels ranging from 10 to 990 g/kg. An independent validation set, consisting of 40 pure samples and 39 adulterated samples, was used to validate the calibration model.In all, 105 samples were used to develop the quantitative analysis model; with each sample subsequently spiked (10–990 g/kg; standard deviation: 294.2 g/kg). A discriminant analysis model developed with mathematic pretreatment 1,4,4,1 in NIR region (1100–2500 nm) successfully discriminated annual ryegrass adulterated with perennial ryegrass seed. A quantitative model developed with mathematic pretreatment 1,4,4,1 in NIR region (1100–2500 nm)also accurately predicted the adulteration with a standard error of cross validation of 76.91 g/kg and a ratio of performance deviationof 3.82. These results demonstrate the usefulness of NIRS combined with chemometrics as a rapid method for discrimination and quantification of perennial ryegrass in adulterated annual ryegrass seedsamples.

Key words: Near-infrared Spectroscopy, Quantitative, Annual Ryegrass, Perennial Ryegrass, Seed.

INTRODUCTION

Two species of ryegrass are grown in South Korea: perennial (*Lolium perenne* L.) and annual (*Lolium multiflorum* Lam.) types, both of which have very similar genomes and are fully interfertile. However, they are morphologically distinct, with annual ryegrass primarily utilized for forage production and perennial ryegrass utilized for either turf or forage purposes. Annual ryegrass is widely grown in southern Korea and has become an important component in winter forage-livestock systems. More than one million hectares of annual ryegrass are annually grown in the rice paddy field after harvesting rice grain.

With the high demand for annual ryegrass seeds, premium varieties and purity of seed are priced higher because better seed variety leads to increased production and economic benefit. Consequently, some illegal seed manufacturers intentionally mislabel poor-quality seed varieties as high-quality seed varieties and sell them to farmers to increase their profit margin. This unethical behavior compromises the interests of the enterprises that develop better quality seed varieties and negatively affects the seed market. Thus, accurately identifying the seed variety and purity has become increasingly critical to the development of the ryegrass seed market and protecting the interests of both farmers and owners of excellent seed varieties.

Several studies have tested ryegrass seed purity by using biochemical assays. Seed certifying agencies in the United States currently use a test called seedling root fluorescence (SRF) to detect contamination between species (Barker and Warnke, 1999). SRF has been used as a phenotypic marker to separate annual ryegrass from perennial ryegrass since its discovery in 1929. Seedling roots of annual ryegrass fluoresce when exposed to ultraviolet light, whereas those of perennial ryegrass do not (Niemyski and Budzynska, 1972; Barker and Warnke, 1999). Inaccuracy of the SRF test (Floyd and Barker, 2002) has led to a search for an alternative or supplemental test that willmore accurately detect annual ryegrass adulterated with perennial ryegrass (Warnke *et al*., 2002). A genetic map-based approach is one method that could be used to expedite the identification of markers for species separation. Several genetic maps of ryegrass have been reported thus far (Jones *et al*., 2002). However, it is a time-consuming method.

Near-infrared spectroscopy (NIRS) is a rapid analytical technique that has been successfully used to detect the quality of agriculture produce, including maize, rice, and wheat (Yan *et al*., 2005; Burns *et al*., 2007; Velasco *et al*., 1999). This method has been used to predict rapeseed attributes (fatty acid, seed weight, oil, protein, and total glucosinolate contents) with partial least

squares regression (PLSR) using a rapid, single-kernel, near-infrared instrument. NIRS has also been successfully applied to identify European wheat varieties with PLSR and principal components analysis (PCA) (Miralbés, 2008; Huang *et al*.,2011), and to analyze hybrid maize seed purity via partial least squares discriminant analysis (PLS-DA).

The objective of this study was to develop NIRS calibration models for qualitative and quantitative determination of perennial ryegrass adulteration in annual ryegrass seed. This model was designed for routine analyses in the identification of seed variety at the forage seed separation stage in order to permit rapid and accurate varietal classification.

MATERIALS AND METHODS

Sample preparation: Five varieties of annual ryegrass ('Tam 90', 'Florida 80', 'Kowinearly', 'Greenfarm', and 'Hwasan 104') and 4 varieties of perennial ryegrass ('Bastion', 'Accent', 'Linn', and 'Boost') were collected from a domestic seed company in South Korea. The pure samples of annual ryegrass and perennial ryegrass were established by mixing different varieties. On the other hand, adulteration of the samples was achieved by combining pre-determined proportions of annual and perennial ryegrass seeds and thoroughly mixing them.

Sample preparation for qualitative analysis: The calibration set consisted of 120 samples, including 60 samples each of pure annual ryegrass seeds and annual ryegrass seeds adulterated with perennial ryegrass seeds. The adulterated samples were prepared in our laboratory by spiking annual ryegrass samples with varying amounts (10–990 g/kg) of the perennial ryegrass samples. The external validation set consisted of 79 samples, including 40 pure annual ryegrass samples and 39 adulterated samples, which were prepared using the same methods as that used for the calibration set.

Sample preparation for quantitative analysis:A total of 105 samples prepared for the quantitative analysis, as described above, were used for qualitative analysis. Each sample was subsequently spiked with varying amounts (10–990 g/kg) of X, with a standard deviation (SD) of 294.2 g/kg.

Near-infrared spectroscopy analysis: A Spectra Star 2500 scanning Monochromator (Unity Scientific, Brookfield, USA), equipped with a spinning module, was used; the sample was placed in a circular cell of anodized aluminum with a quartz window (35 mm diameter; 10 mm depth). The samples were scanned 42 times to create average spectra in the reflectance mode over the range of 680~2500 nm at 1-nm intervals. The sample quantity analyzed was between 3.8 and 4 g. All spectra were recorded as log (1/R), where R is the relative reflectance. Spectra were recorded with the U-CAL software version 2.04 (Unity Scientific).

Development of NIR calibrations: Two calibration strategies were evaluated. The first strategy used a discriminant model to detect if the annual ryegrass variety was adulterated with perennial ryegrass verity. The second strategy used a quantitative model to estimate the content of perennial ryegrass seeds in annual ryegrass sample.

The data were analyzed using mathematical treatments and scatter correction in order to reduce baseline offset arising from particle size and packing density. All multivariate regression equations were obtained using standard normal variate (SNV) and de-trending for scatter correction. SNV scales of each spectrum have a standard deviation of 1.0,which helps reduce particle size effects. The five mathematical treatments used were log (1/R), first derivative, and second derivative. These mathematic treatments were denoted as 0,0,1,1; 1,4,4,1; 1,8,8,1; 2,8,8,1; and 2,16,16,1, respectively. The first number denotes the derivative order. The second number denotes the number of nanometers in the segment used to calculate the derivative. The third and fourth numbers refer to the number of data points used in the first and second smoothing, respectively.

Qualitative strategies: The calibration method applied to this procedure was partial least squares (PLS) discriminant analysis. A score of 2 was used to identify samples adulterated with perennial ryegrass seed and a score of 1 was used for samples of pure annual ryegrass seed. The calibration was conducted by regression of the wavelength information on 1 or 2. Cross-validation was conducted to determine the number of factors to be included in the final equation.The independent validation set was used to validate the capacity of calibration equations. These unknown samples were assigned to either the filename for pure annual ryegrass seed or the filename for the adulterated samples on the basis of which file had the greater score.

The output of discrimination was listed as one of three categories, namely 'Misses', 'Uncertain' and 'Hit'. When the two limits were set in the input screen ('T critical limit' and 'Uncertainty factor') at 2.5, then samples that had a predicted value of more than (2.0+'T critical limit' × SECV) or less than (1.0- 'T critical limit' × SECV) were classified as 'Misses'. Samples that had a predicted value of (1.5±'Uncertainty factor' × SECV/2) were listed as 'Uncertain'. The remaining samples were considered as 'Hits'.

Quantitative strategies: Near infrared calibrations were performed using U-cal software version 2.0 (Unity Scientific). Calibration equations were developed by PLS regression. When developing PLS equations, cross-

validation is recommended to select the optimal number of factors and avoid over-fitting (Shenk and Westerhaus, 1993). Calibration statistics calculated included the standard error of calibration (SEC) and the coefficient of multi-determination in calibration (R^2). The validation results were used to test calibration result. The best calibration equations were selected based on the fact that the lower the standard error of cross-validation (SECV), the higher coefficient of determination in cross-validation (R^2_{cv}), and the best relation between the standard deviation of the reference data of the calibration set and the SECV, known as the ratio of performance deviation (RPD). According to Williams (2001), RPD values of less than 2.4 are equations with low reliability for predictive purposes. With PRD ratios of between 2.4 and 3.0, the predictive quality increases and the equation and can be applied with the aim of approximation or classification in ranges. When the RPD is higher than 3.0, the quality of the equation is acceptable for prediction.

RESULTS AND DISCUSSION

Sample spectra: The second derivative spectra of pure annual and perennial ryegrass and adulterated annual ryegrass seeds are shown in Fig. 1. Differences in size, shape, color, and chemical composition result in differences in the spectra characteristics of annual and perennial ryegrass seed. When corrected for scatter by weighted standard normal variateand de-trending for scatter correction, followed by a second derivative, several absorbance peaks arise corresponding to chemical and morphology characteristics in seed. The 3D principal component analysis graph for the pure annual and perennial ryegrass and adulterated seed is showed in Fig. 2. In this graph, annual and perennial ryegrass seeds were widely separated. However, the adulterated seed cluster, being very diverse in range, was still quite diffuse. Fig. 2 may explain the basis for discrimination and quantitative analysis in annual ryegrass adulterated with perennial ryegrass.

Discriminant analysis: The results of the PLS discriminant performance for perennial ryegrass adulteration in annual ryegrass are presented in Table 1. Three different mathematic pretreatments were tested using the full spectral range (680-2500). Using derivative to transform the data is helpful to develop a calibration equation. Cross validation produced with mathematical treatments 1,8,8,1 and 2,8,8,1, explained a similar percentage (80% and 81%) of the variation existing in the content of perennial ryegrass. However, the mathematic pretreatment 1,8,8,1 was chosen to be the best spectra pretreatment method based on the number of 'Hits' for the independent validation. The same strategy has been successfully tested by Miralbés (2008) to discriminate European wheat varieties with 99.5% accuracy for the calibration set and 94% for the validation set. To compare the effect of spectral range on calibration results, the visible and short-wave NIR (680–1009) and NIR (1100–2500) were taken alone and together. For the data given in Table 1, the full rage (680–2500 nm) gave the greatest number of 'Hits' (74) while the lowest number of 'Hits' (57) were found in 1100-2500nm. Additional information from the visible and short-wave NIR range of the spectrum would appear to offer a useful improvement in discriminant performance.The independent validation score plot for the best discriminant analysis model is shown Fig. 3. Pure annual ryegrass seed clustered around a score 1.0, while those with adulterated perennial ryegrass clustered around 2.0. There were 3 'Uncertain' samples (samples in A zone), 74 'Hits' samples (sample in B zone) and 2 'Misses' samples (samples in C zone). With a breakpoint score of 1.5, all samples were discriminated correctly. The same strategy has been successfully tested by Murray *et al*. (2001) to detect adulterations of fishmeal with meat and bone meal at 30, 60, and 90 g/kg by weight.

Quantitative analysis: The results of quantitative analysis for perennial ryegrass adulteration of annual ryegrass are presented in Table 2. Five different mathematic pretreatments was tested using the full spectral range and for each mathematic pretreatment was compared. The statistics, SECV and R^2_{cv}, confirmed the precision and accuracy of the calibrations for predicting perennial ryegrass content in annual ryegrass. According to SECV, the calibrations developed with full spectral range (680–2500 nm), NIR region (1100–2500 nm) is more stable and accurate than full spectral range. In PLS, the NIR residuals at each wavelength that are obtained after each factor is calculated and are standardized before calculation the next factor. Calibration equations produced with 1,4,4,1 and 2, 8, 8,1 mathematical treatments explained a similar percentage (96%) of the variation existing in the content of perennial ryegrass. However, the SECV was lower when the spectra were treated with 1, 4, 4, 1 (93.13 g/kg) than with 2, 8, 8, 1 (110.97 g/kg) mathematical treatments. The effect of spectral mathematical pretreatments on quantitative calibration results were presented in Table 2, where the full range treated with 1, 4, 41 gave the best calibration result with SECV of 93.13 g/kg and RPD of 3.16. The NIR region alone performed well with SECV of 76.91 g/kg and RPD of 3.82. Additional information from the visible and short-wave NIR region (680–1099) of the spectrum would appear to offer a useful improvement in quantitative performance. A strategy has been successfully used by Li *et al*. (2015) where they investigated the quantitative identification of fishmeal and bone meal via inflorescence spectral imaging.

Conclusion: The calibration and validation results confirm that NIRS could provide the forage seed industry

and inspection adulteration seeds with a rapid, non-destructive, and non-invasive technique for the detection and quantification of perennial ryegrass in annual ryegrass. At present, SRF is the only official method to discriminate between annual ryegrass and perennial ryegrass. However, SRF does not allow a quantitative identification. Furthermore, compared to SRF, NIRS is better for large-scale screening applications. For the detection of perennial ryegrass, the spectroscopy method has a higher accuracy of detection than that of the SRF method, which therefore needs to be combined with confirmatory techniques. Further work is required to enlarge the spectral libraries with authenticated samples in order to increase the robustness and applicability of the qualitative and quantitative NIR models.

Table 1. Calibration and validation results for qualitative analysis of adulteration of perennial ryegrass with annual ryegrass seeds

Spectrum regions (nm)	Mathematic treatments	PLS terms	Calibration set (*n* = 120)					Validation set (*n* = 79)		
			Hits	Uncertain	Misses	R^2_{cv}	SECV	Hits	Uncertain	Misses
680–2500	0,0,1,1	11	102	17	1	0.79	0.231	61	14	4
680–2500	1,4,4,1	12	120	0	0	0.87	0.173	65	13	1
680–2500	1,8,8,1	6	99	20	1	0.80	0.217	64	13	2
680–2500	2,8,8,1	5	111	9	0	0.81	0.210	59	16	4
680–2500	2,16,16,1	4	98	21	1	0.77	0.211	57	16	6
680–1099	0,0,1,1	12	116	4	0	0.76	0.240	58	20	1
1100–2500	1,4,4,1	9	120	0	0	0.91	0.151	74	3	2

Table 2. Calibration and validation results for quantitative analysis of adulteration of perennial ryegrass with annual ryegrass seeds

Spectrum regions (nm)	Mathematic treatments	PLS terms	Calibration					Validation		
			n	Range (g/kg)	S.D.	R^2	SEC (g/kg)	R^2_{cv}	SECV (g/kg)	RPD
680–2500	0,0,1,1	11	105	10-990	294.2	0.94	68.49	0.89	95.19	3.09
680–2500	1,4,4,1	6	105	10-990	294.2	0.96	55.62	0.90	93.13	3.16
680–2500	1,8,8,1	6	105	10-990	294.2	0.94	70.34	0.88	98.92	2.97
680–2500	2,8,8,1	6	105	10-990	294.2	0.96	56.28	0.84	110.97	2.65
680–2500	2,16,16,1	7	105	10-990	294.2	0.95	67.40	0.89	93.28	3.15
680–1099	0,0,1,1	10	105	10-990	294.2	0.95	64.39	0.87	102.18	2.88
1100–2500	1,4,4,1	5	104	10-990	294.1	0.96	59.96	0.93	76.91	3.82

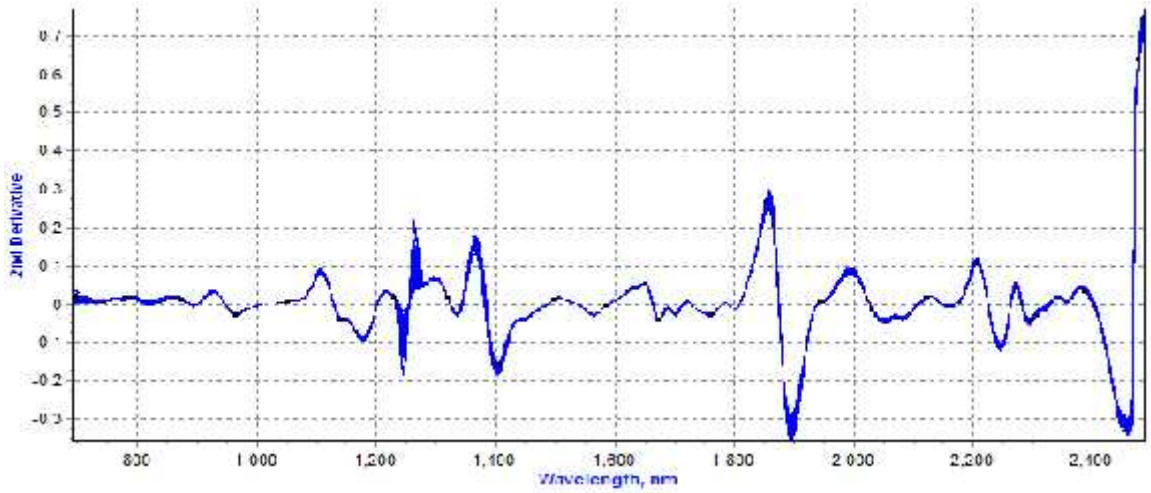

Fig. 1. The second derivative spectra of for the pure annual and perennial ryegrass seeds or annual ryegrass adulterated with perennial ryegrass

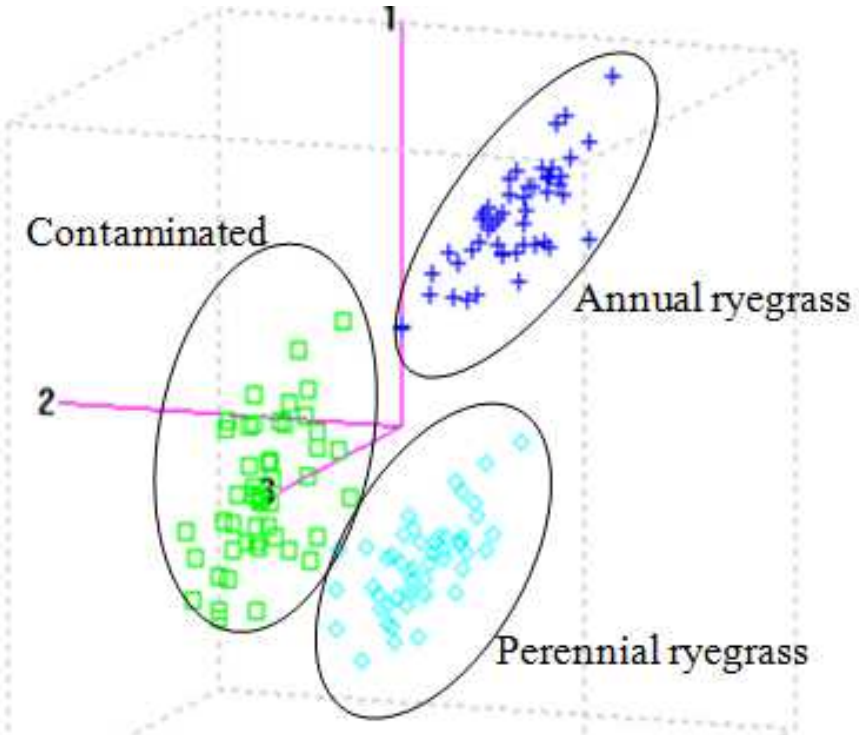

Fig.2. The 3D principal component analysis graph for the pure annual and perennial ryegrass seeds or annual ryegrass seeds contaminated with perennial ryegrass seeds.

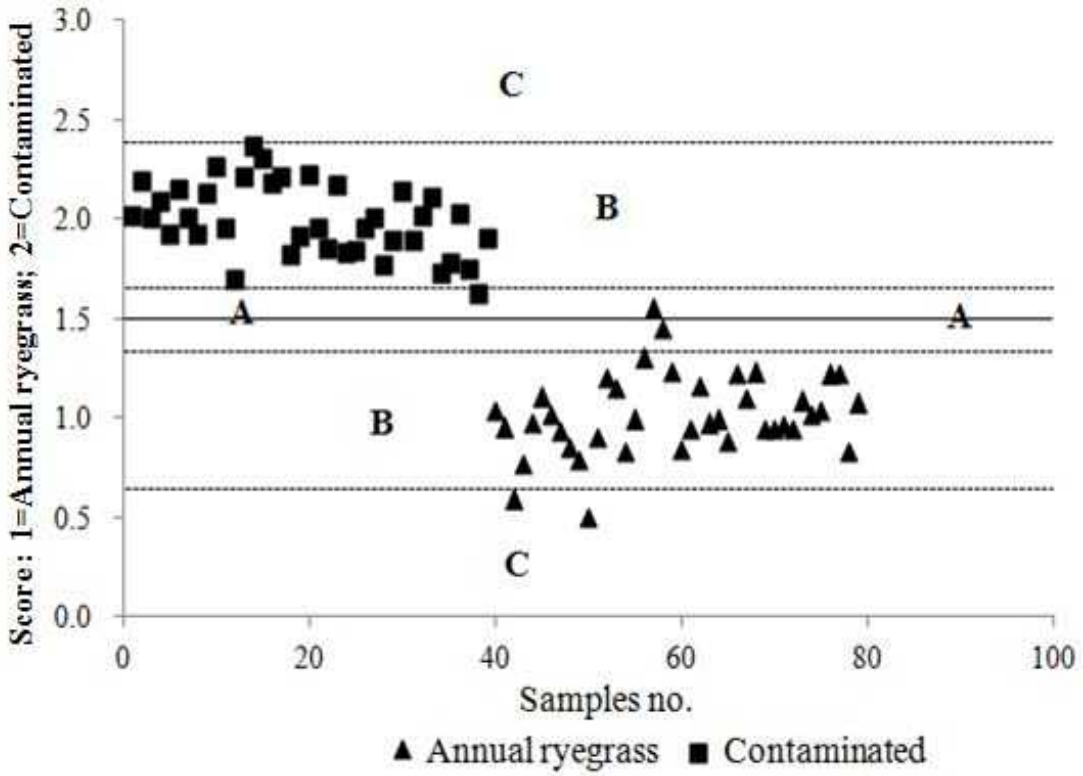

Fig. 3.The results of independent validation for the best PLS discrimination analysis model. Samples in A zone were classified as 'Uncertain'; Samples in B zone were classified as 'Hits'; Samples in C zone were classified as 'Misses.

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