Application of Raman Spectroscopy for Detection of Aflatoxins and Fumonisins in Ground Maize Samples

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Mycotoxin detection methods

- Diverse mycotoxin analytical methods available in laboratory and non-laboratory locations

  : **1**) bright greenish-yellow fluorescence (BGYF) test; **2**) thin layer chromatography (TLC); **3**) gas chromatograph (GC); **4**) high performance liquid chromatography (HPLC); **5**) mass spectrometry (MS); **6**) enzyme-linked immunosorbent assay (ELISA); **7**) immune-affinity column assay; and **8**) biosensors

- Reliable, accurate, and precise, but expensive, complex, labor-intensive, and time consuming
- Not allow rapid screening of a large number of samples

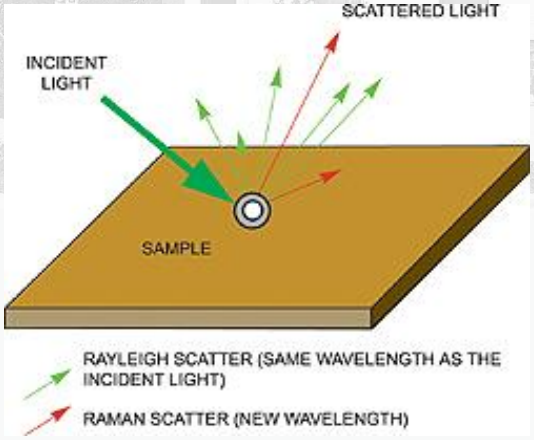
Rapid, sensitive, and accurate methods with minimum effort and cost for early screening of mycotoxin

Spectroscopic techniques

- Spectroscopic techniques such as near-infrared reflectance (NIR), Fourier Transform infrared spectroscopy (FTIR), and Raman spectroscopy are attractive
- Single scan for qualitative and quantitative information pertaining to mycotoxin components and structures.
- Requiring little or no sample preparation and pretreatments
- Each technique uses different physical process ➔ complementary information about mycotoxins
- Applications: limited due to difficult interpretation and spectrum overlapping
- Advent of modern spectral amplification and enhancement techniques : detecting and identifying fungal species and mycotoxins

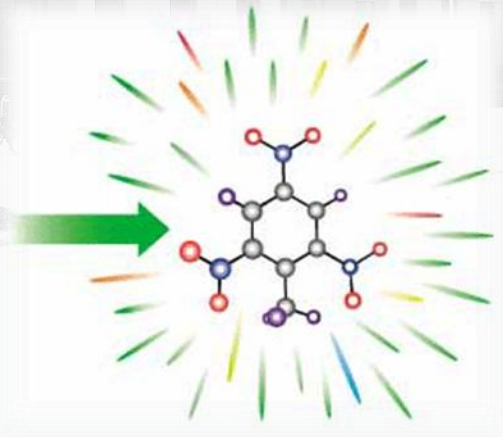
Raman spectroscopy 1

- NIR and FTIR: not well resolved and superimposed with other components and strong HOH bending absorption of water molecules
- Raman spectroscopy: little attention in cereal science and for investigation and detection of mycotoxins in grains and oilseeds.
- Irradiate a substance with monochromatic light and to detect the scattered light with a different frequency to the incident beam
- Raman shifts: differences in the frequencies between the incident and scattered radiation



Raman spectroscopy 2

- Based on the polarity of chemical bonds
- more sensitive to the symmetrical covalent bonds in non-polar group
- Insensitivity to water
- Fewer overlapped bands
- Provide more useful qualitative and quantitative information ➔ molecular level insight into mycotoxin
- Previous studies: showing the promising results for rapid screening of mycotoxin contaminated grains and oilseeds



Objectives

Possibility of Raman spectroscopy technique combined with chemometrics to develop a rapid, inexpensive, and convenient spectroscopic method for classification and quantification of *aflatoxin* and *fumonisin* contaminated maize

a basis and a useful starting point to develop a robust model for real-time monitoring and high-throughput analysis of mycotoxin contaminated samples

Ensure the quality and safety of maize products.

Sample preparation

- Maize samples: OTSC regulatory samples
- Aflatoxin: 132 samples (0.0–1,206.0 μg/kg)
- Fumonisin: 100 samples (0.0–264.0 mg/kg)
- Cover the majority of aflatoxin and fumonisin concentrations found in commercial maize products and routine surveillance samples ➔ appropriate to develop the calibration model for prediction
- Ground to pass a 0.075 mm diameter screen
- Moisture content: kept 15%↓ to ensure stop of fungal growth
- Equilibrated for at least 1 hr at room temperature before use

Raman spectroscopy

- Approximately 5 g directly analyzed by Raman spectroscopy
- Laser power of 160 mW, a 5-mm x 5-mm spot, and exposure times of 2 sec and 5 scans
- A x, y, z-motorized sample holder ➔ automatically align samples to obtain the optimal spectrum

- Spectral data preprocessing
  - Raw spectra of samples ➔ baseline-corrected and normalized
  - Pretreated by a Savitizky-Golay method with smoothing points of 9 ➔ 1st and 2nd derivatives
  - Deconvolution process
  - Eliminate irrelevant chemical information and extract meaningful information ➔ improving classification and predictive accuracy of the models

RamanStation™ 400F

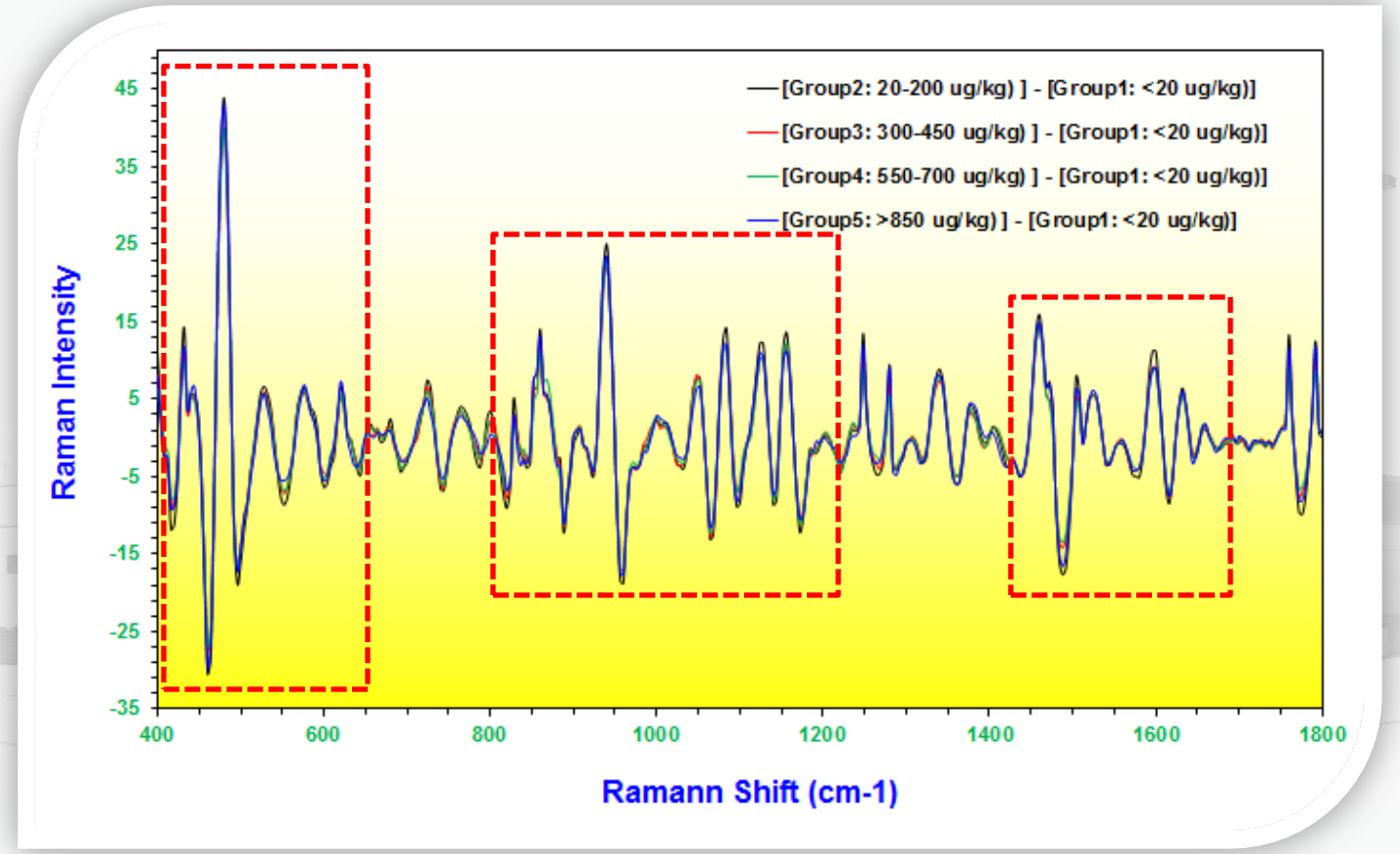
Mycotoxin classification models

- Preprocessed spectra data ➔ converted to ASCII format ➔multivariate statistical techniques: principal component analysis (PCA) & cluster analysis (CA)
- Chemometric models: k-nearest neighbor (KNN), linear discriminant analysis (LDA), principal component discriminant analysis (PCDA), and partial least squares discriminant analysis (PLSDA)
- Aflatoxins: < 20 μg/kg (Group 1, considered as non-contaminated), 20–200 μg/kg (Group 2), 300–450 μg/kg (Group 3), 550–700 μg/kg (Group 4) , & >850 μg/kg(Group 5)
- Fumonisins: < 5 mg/kg (Group 1, considered as non-contaminated), 5–25 mg/kg (Group 2), 25–50 μg/kg (Group 3), and > 50 mg/kg (Group 4).
- Divided into training (75% samples) and validation (25% samples) data sets for developing and testing the classification models
- Performance and accuracy of the models: based on a correct classification rate and a false negative error

Mycotoxin quantification models

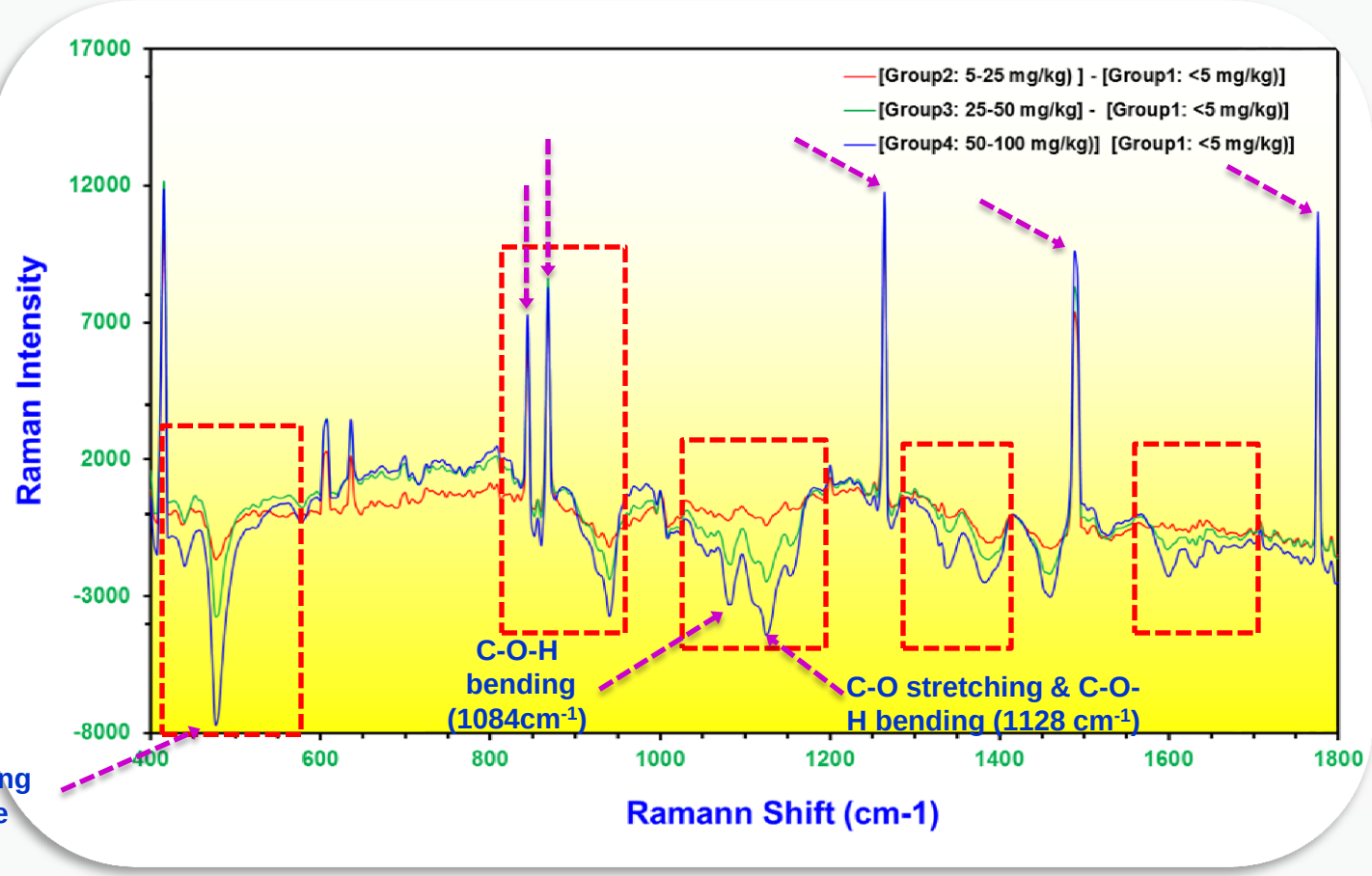
- Chemometric models: multiple linear regression (MLR), principal components regression (PCR), and partial least squares regression (PLSR) algorithms
- Spectra data: divided into 75% training data for calibration model development and 25% validation data for testing the model
- HPLC (for aflatoxins) and LC-MS/MS (for fumonisins) reference measurements: compared and correlated with Raman spectra through the developed models
- Performance of the models: evaluated based on the root mean standard error of prediction, correlation coefficient of determination (r^2), Pearson's correlation coefficients, and residual prediction deviation (RPD) using the external validation data set

Spectra difference (aflatoxins)



Averaged Raman subtractive spectra of **aflatoxin** contaminated samples (Groups 2, 3, 4, and 5) from the averaged spectrum of aflatoxin negative samples (Group1) (2^{nd} derivative)

Spectra difference (fumonisins)



Averaged Raman subtractive spectra of **fumonisins** contaminated samples (Groups 2, 3, 4, and 5) from the averaged spectrum of fumonisin negative samples (Group1) (normalized)

Correct Classification Rates (aflatoxin)[a]

preprocessing method	LDA			PCDA			PLSDA		
	training (%)	validation (%)	false negative error (%) [b]	training (%)	validation (%)	false negative error (%)	training (%)	validation (%)	false negative error (%)
Normalization	97.5	94.3	0.0	90.0	74.3	13.3	92.5	65.7	6.7
1st derivative	96.3	97.1	0.0	90.0	37.1	20.0	97.5	91.4	0.0
2nd derivative	100.0	100.0	0.0	86.3	57.1	6.7	96.3	82.9	0.0
Deconvolution	98.8	100.0	0.0	93.8	54.3	26.7	100.0	74.3	0.0

[a] LDA, linear discriminant analysis; PCDA, principal component discriminant analysis; PLSDA, partial least squares discriminant analysis.

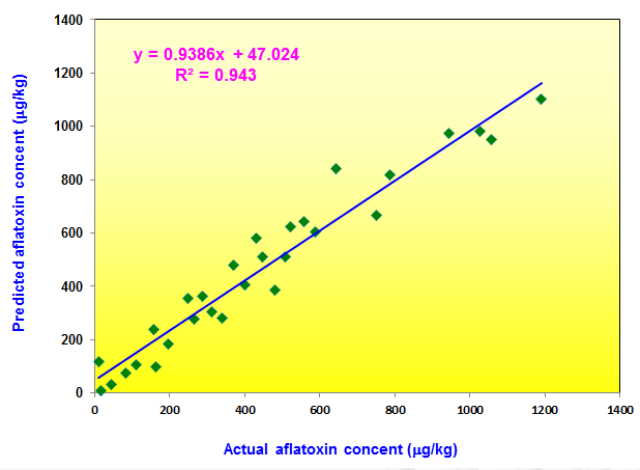
[b] A false negative error (%) was defined as the failure of the method to classify contaminated samples as aflatoxin negative.

Correct Classification Rates (fumonisins)[a]

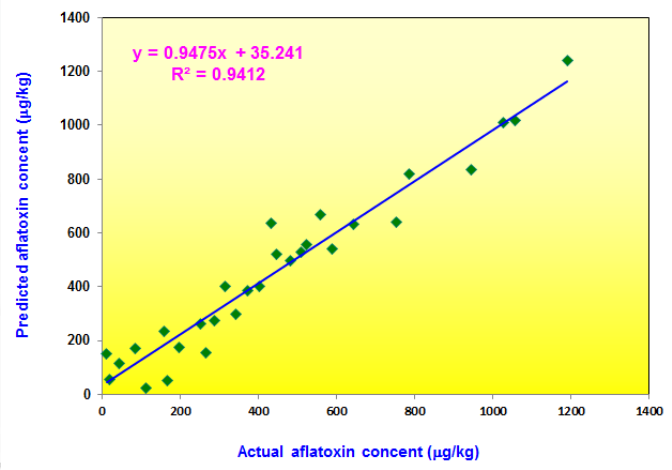
preprocessing method	KNN			LDA			PLSDA		
	training (%)	validation (%)	false negative error (%) [b]	training (%)	validation (%)	false negative error (%)	training (%)	validation (%)	false negative error (%)
Normalization	100.0	100.0	0.0	92.0	100.0	0.0	97.3	92.0	0.0
1st derivative	100.0	96.0	0.0	81.3	96.0	0.0	97.3	92.0	0.0
2nd derivative	100.0	96.0	0.0	92.0	96.0	0.0	100.0	64.0	0.0
Deconvolution	100.0	96.0	0.0	92.0	96.0	0.0	100.0	80.0	0.0

[a] KNN, k-nearest neighbor; LDA, linear discriminant analysis; PLSDA, partial least squares discriminant analysis.
[b] A false negative error (%) was defined as the failure of the method to classify contaminated samples as aflatoxin negative.

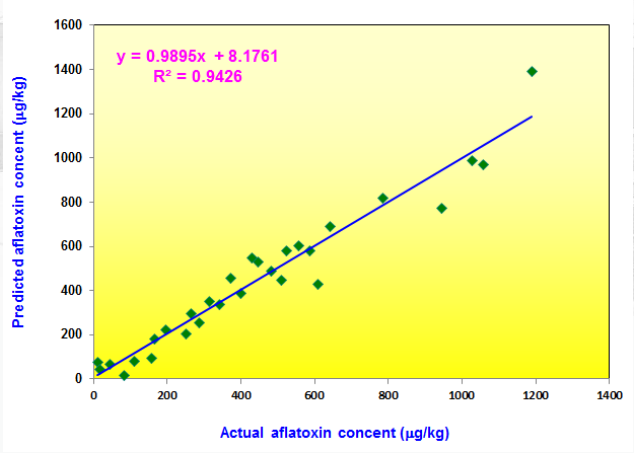
Quantification of aflatoxins in maize samples 1



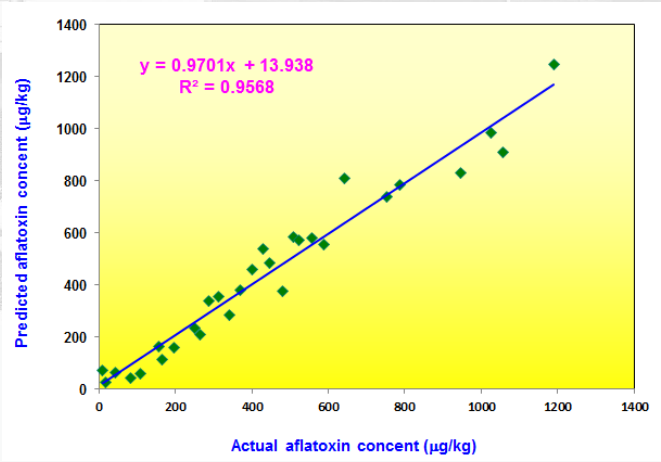
Normalized (PLSR)



1st derivative (PLSR)



2nd derivative (PLSR)



Deconvolution (PLSR)

* *PLSR:* partial least square regression

Quantification of aflatoxins in maize samples 2

chemometrics	preprocessing method	RMSEC (ug/kg)[a]	RMSEP (ug/kg)[b]	slope		R^{2c}	
				Training	Validation	Training	Validation
MLR (multiple linear regression)	Normalization	119	141	0.831	0.781	0.840	0.821
	1st derivative	111	144	0.846	0.648	0.864	0.838
	2nd derivative	82	90	0.923	0.898	0.923	0.930
	Deconvolution	96	97	0.896	0.898	0.896	0.903
PCR (principal component regression)	Normalization	168	184	0.638	0.565	0.640	0.567
	1st derivative	155	179	0.703	0.611	0.713	0.700
	2nd derivative	164	176	0.701	0.555	0.695	0.687
	Deconvolution	144	182	0.763	0.592	0.763	0.668

[a] **RMSEC: root-mean-square error of calibration**
[b] **RMSEP: root-mean-square error of prediction**
[c] **R^2: correlation coefficient of determination**

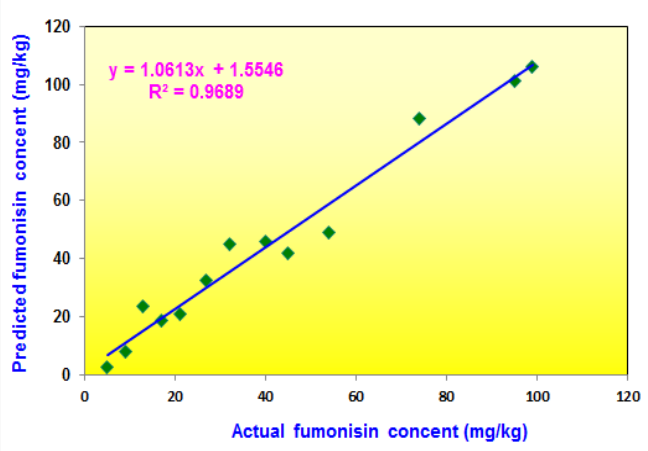
HPLC Ref. vs Predicted Values (aflatoxins)

chemometrics	preprocessing method	paired differences (μg/kg)		r[a]	sig (2-tailed)	RPD[b]
		mean	std error mean			
MLR	Normalization	-48.3	24.7	0.899	0.066	2.248
	1st derivative	-8.3	29.3	0.907	0.940	2.247
	2nd derivative	20.8	16.9	0.955	0.213	3.482
	Deconvolution	-20.2	18.9	0.952	0.585	3.204
PCR	Normalization	-19.0	38.2	0.742	0.770	1.538
	1st derivative	-16.7	34.3	0.862	0.949	1.817
	2nd derivative	1.2	33.8	0.829	0.949	1.750
	Deconvolution	-20.7	35.4	0.873	0.782	1.674
PLSR	Normalization	-9.1	18.1	0.947	0.667	3.205
	1st derivative	-16.9	17.2	0.964	0.444	3.996
	2nd derivative	6.4	15.1	0.966	0.673	3.921
	Deconvolution	-4.1	16.4	0.963	0.911	3.870

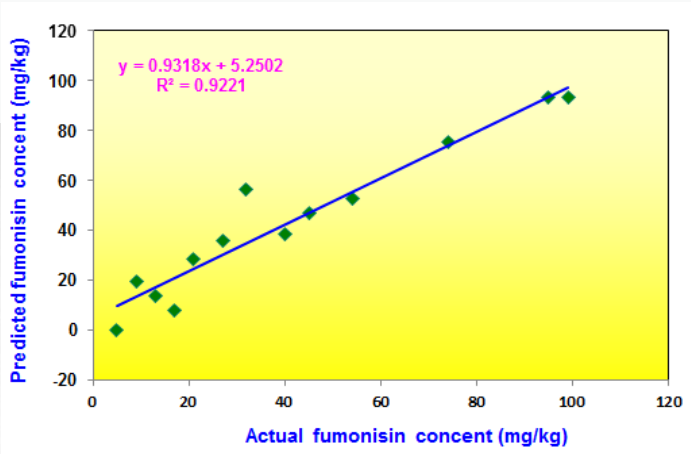
[a] **Pearson correlation coefficient**

[b] **RPD (residual prediction deviation): ratio of standard deviation of reference to root mean square error of cross-validation**

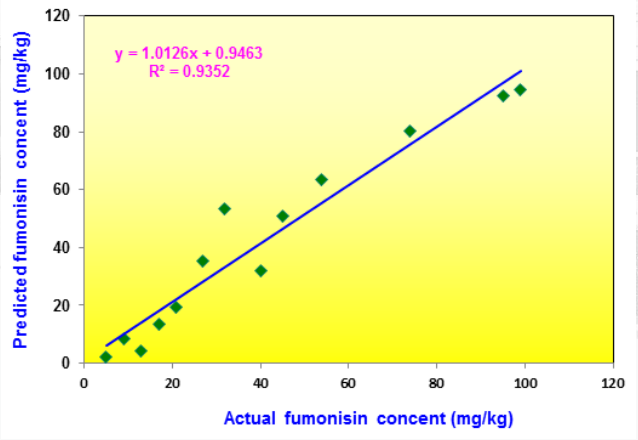
Quantification of fumonisins in maize samples 1



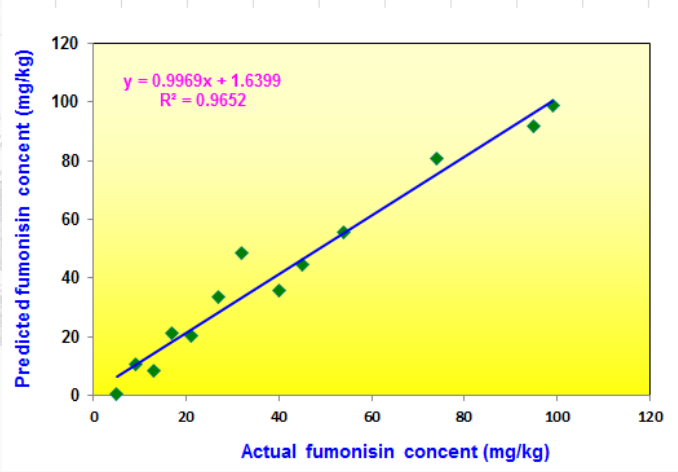
Normalized (MLR)



1st derivative (MLR)



2nd derivative (MLR)



Deconvolution (MLR)

* *MLR*: multiple linear regression

Quantification of fumonisins in maize samples 2

chemometrics	preprocessing method	RMSEC (mg/kg)[a]	RMSEP (mg/kg)[b]	slope		R^{2c}	
				Training	Validation	Training	Validation
PCR (principal component regression)	Normalization	6.895	8.973	0.930	1.050	0.930	0.948
	1st derivative	8.405	8.794	0.896	0.883	0.896	0.917
	2nd derivative	9.016	10.220	0.880	0.775	0.880	0.905
	Deconvolution	9.041	9.669	0.876	0.860	0.880	0.900
PLSR (partial least square regression)	Normalization	5.312	9.585	0.958	1.048	0.958	0.943
	1st derivative	6.692	8.127	0.934	0.929	0.934	0.931
	2nd derivative	8.319	9.615	0.898	0.813	0.898	0.910
	Deconvolution	4.137	7.321	0.975	0.964	0.975	0.946

[a] RMSEC: root-mean-square error of calibration
[b] RMSEP: root-mean-square error of prediction
[c] R^2: correlation coefficient of determination

LC-MS/MS Ref. vs Predicted Values (fumonisins)

chemometrics	preprocessing method	paired differences (mg/kg)		r[a]	sig (2-tailed)	RPD[b]
		mean	std error mean			
MLR	Normalization	-4.06	1.75	0.9843	0.0384	4.324
	1st derivative	-2.47	2.44	0.9603	0.3321	3.579
	2nd derivative	-1.46	2.33	0.9670	0.5423	3.839
	Deconvolution	-1.51	1.65	0.9824	0.3782	5.316
PCR	Normalization	-4.73	2.20	0.9734	0.0529	3.511
	1st derivative	-0.52	2.53	0.9577	0.8423	3.583
	2nd derivative	-0.04	2.95	0.9512	0.9889	3.083
	Deconvolution	0.43	2.79	0.9487	0.8790	3.258
PLSR	Normalization	-5.36	2.29	0.9710	0.0378	3.287
	1st derivative	-1.68	2.30	0.9649	0.4779	3.877
	2nd derivative	0.59	2.77	0.9539	0.8355	3.277
	Deconvolution	-1.93	2.04	0.9726	0.3617	4.303

[a] **Pearson correlation coefficient**

[b] **RPD (residual prediction deviation): ratio of standard deviation of reference to root mean square error of cross-validation**

Conclusions

- Raman spectroscopic method: proved to be successfully applicable as alternative rapid and non-destructive technique
- Classification and quantification models showed a good predictive performance with high accuracy and low error rate
- Ideal for real-time monitoring of critical performance attributes
- Anticipating several difficulties and constraints in using this technique ➔numerous opportunities to improve the accuracy and precision of Raman spectroscopy measurements
- Calibration models would be more stable and practically applicable by continuing to analyze maize samples with diverse genetic and environmental backgrounds and mycotoxin levels
- Raman spectroscopy: easy, rapid, and inexpensive screening system for mycotoxins ➔ a powerful tool for quality control of grains ➔ improve the safety of feed and food products supplied to consumers.

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