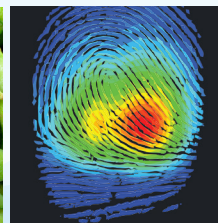# HORIBA
Scientific

A-TEEM Fluorescence for Identification and Quantification of Fulvic Acid Adulteration in Commercial Humic Products

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Introduction

Humic substances (HS) are being increasingly used as bio-stimulants to aid agricultural crop growth and productivity. These materials are obtained primarily from soft coals including lignites, sub bituminous coals and oxidized sources of both coal types (i.e. leonardites and humates, respectively). Other sources include peats, compost (e.g. vermicompost) and sapropels. Humic substances result from humification of dead plant tissues in soils and composts. Humic substances in peats and coals have been further transformed by peatification/humification, in peats, and coalification of peatified materials, in coals. Regardless of source, they are operationally classified into three fractions. The fractions are humic acids (HA), fulvic acids (FA) and humin. These are the alkaline soluble-acid insoluble, alkaline soluble-acid soluble and alkaline insoluble-acid insoluble fractions, respectively. For agricultural purposes the focus is primarily on HA and FA. There is a published standardized method for quantification of HA and FA in source materials and in products that contain them (1), and this method has been adopted, with minor modifications, by the ISO as the international standard method (2). Humic substances are typically extracted from source materials using an alkaline extract (e.g. KOH) and separated from non-soluble products with centrifugation or settling. The HA and FA fractions can then be separated by decreasing the pH (e.g. with conc. HCl) to pH 1 which results in precipitation of the HA. The resulting supernatant is referred to as the fulvic fraction (FF) and it contains hydrophobic FA (i.e. the material that binds to a DAX 8 resin) and the hydrophilic fraction, which contains material not bound by the resin (1). The FF is referred to for commercial applications as FA. HA and FA are applied in both solid and liquid formulations. The methods to quantify FA only quantify the hydrophobic FA fraction. In addition, while HS are readily available, various adulterants like lignosulfonates, molasses, corn steep liquor and organic acids (e.g. acetic acid) are sold as FA.

Hence, there is a need for a quick, accurate and precise method to identify and quantify the FF and adulterants in products sold as FA. This app note demonstrates that the A-TEEM molecular fingerprinting technique can easily distinguish between different components in mixtures that contain FF and other ingredients considered to be adulterants, including lignosulphonates, organic acids, molasses, and provides a quantitative evaluation of mixtures.

Material and Method

Bio Huma Netics, Inc. (BHN) provided pure FF extract, labeled BHN-1, and defined mixtures, labeled BHN-2, BHN-3, BHN-4, and BHN-5, which contained BHN-1 and various ingredients that are known FF adulterants. In addition, four other pure adulterant ingredients, FA-20, molasses, lignin-based product, and acetic acid were provided. Sample IDs and their compositions by weight of the mixtures and pure materials are listed in Table 1. Total carbon analysis and results were provided by (BHN) per AOAC method 972.43.

Sample ID	Composition (wt.%)	Total Carbon (%)
BHN-1	100% Fulvic Fraction (FF)	0.032
BHN-2	50% FF + 50% FA-20	4.133
BHN-3	75% FF + 25% molasses	9.151
BHN-4	80% FF + 20% acetic acid	6.458
BHN-5	75% FF + 25% lignin-based	4.641
FA-20	100% FA-20	8.297
Molasses	100% molasses	32.308
Lignin-based	100% lignin-based (Lignin)	15.034
Acetic Acid	100% acetic acid (AA)	-

Table 1: BHN Sample Information

A-TEEM Data Acquisition

Samples were diluted 50, 5000, or 15,000 times with DI water in order to stay within the linear range for correct fluorescence measurement (Beer Lambert's Law). Measurements were conducted using the Aqualog. Blank used was sealed pure water standard (Starna). Each sample was measured four times in 1 cm quartz cuvettes. Integration time used ranged from 0.1 to 1 second, depending on the signal strength to avoid saturating the CCD detector. The excitation wavelength range was set

from 240 to 600 nm with an increment interval of 3 nm, and emission wavelength range was the default 250 to 800 nm with 8 pixel binning, and CCD gain was set to medium. The saturation mask width was 10 nm. Each A-TEEM fingerprint was collected in 40 seconds to 3 minutes (Figure 1). Each contour plot was post-processed with EEM interpolation, IFE correction, 1st and 2nd order Rayleigh masking, and normalized to its maximum fluorescence intensity value.
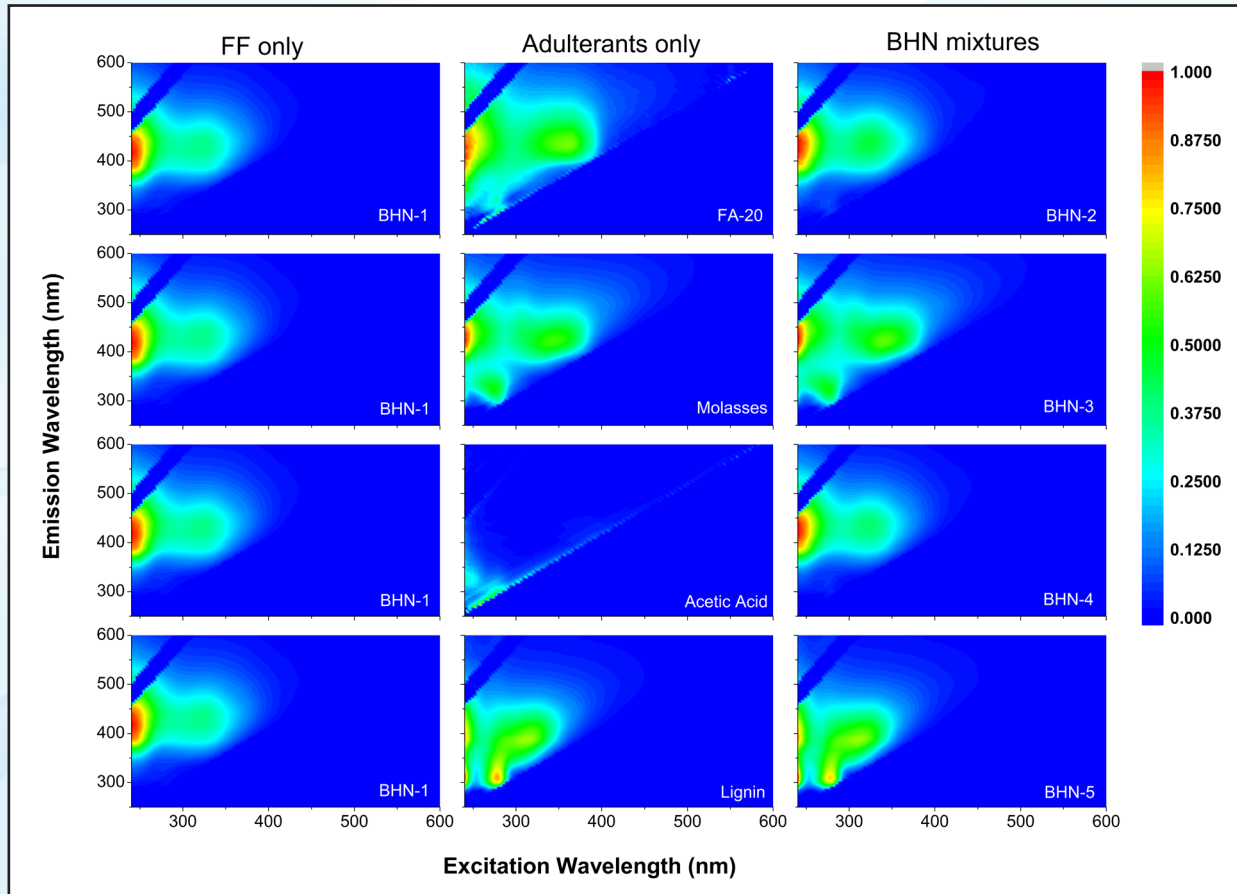

Figure 1: A-TEEM fluorescence fingerprints of BHN-1 (FF), ingredients that are known FF adulterants and BHN-1/adulterant mixtures.

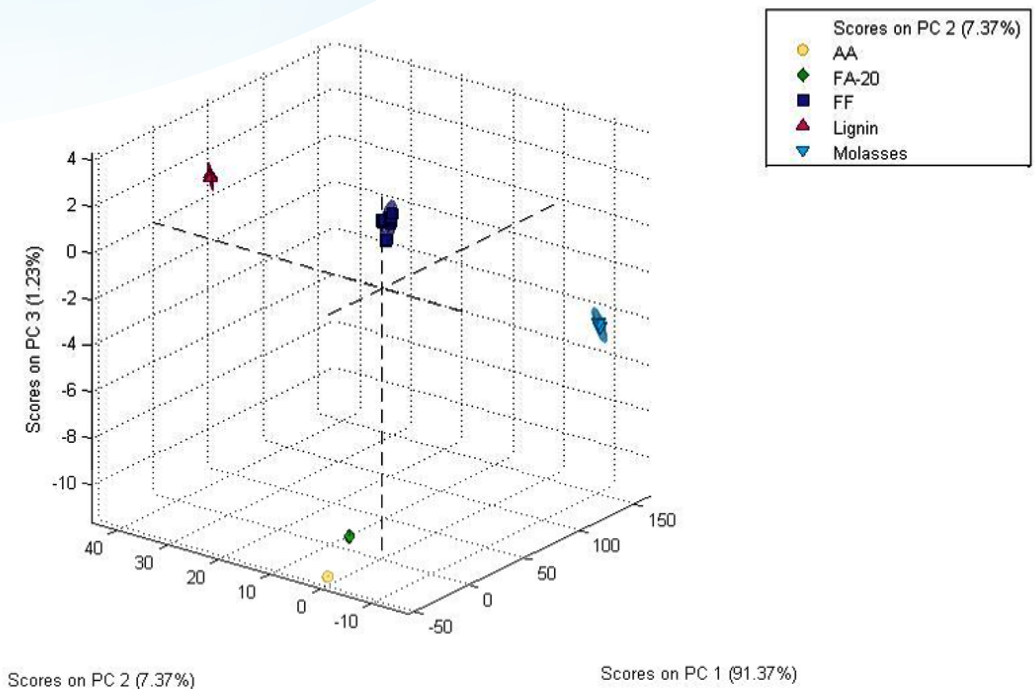## Multivariate Analysis
A-TEEM Classification

Figure 2: 3-D score plot of parallel factor analysis (PARAFAC) of different ingredients in the prescribed mixtures.
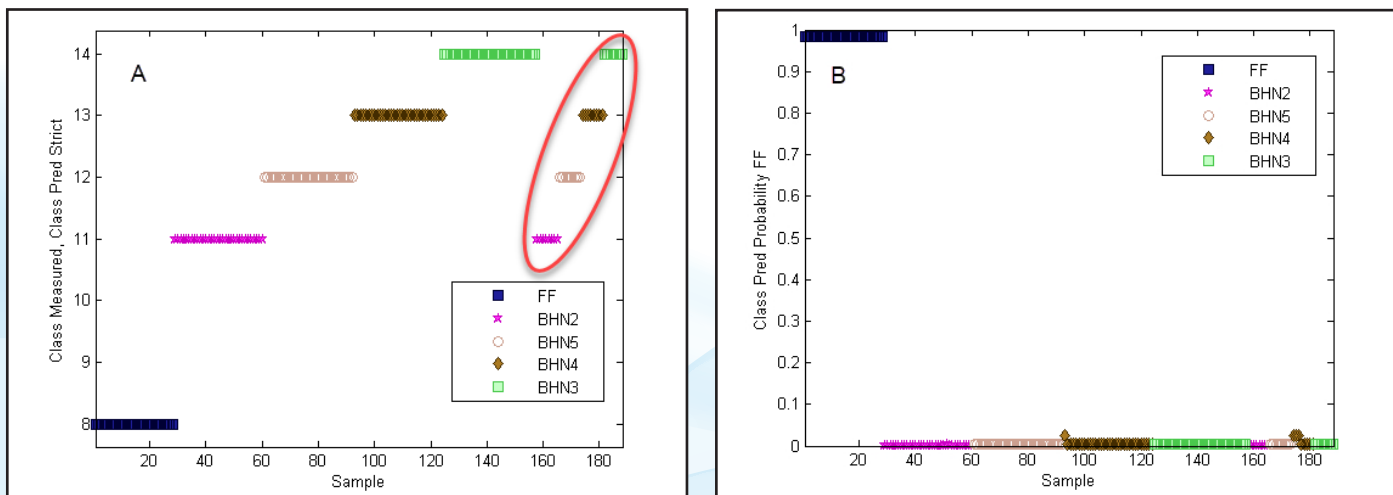
Figure 3. Gradient-boosted tree discriminant analysis (XGBDA) classification prediction results for the unadulterated fulvic fraction BHN-1 (FF) (n =28) and adulterated mixtures (BHN2-5) samples (n = 32). Panel (A) shows the class prediction for the Pred Strict rule evaluations as reported in the confusion matrix (not shown) with 32 validation samples highlighted in a circle. Panel (B) shows the class prediction probability for the FF samples.

3-D PARAFAC analysis can classify these products based on their A-TEEM molecular fingerprints acquired with the Aqualog. Figure 2 demonstrated the ellipses border with 95% confidence interval. Moreover, Gradient Boosted Tree Discriminant Analysis (XGBDA) can also distinguish FF from different adulterated mixtures for 157 calibration samples and 32 validation samples. Figure 3A implies both classes, FF vs. adulterated mixtures (BHN2-5), were resolved without any misclassification, whereas Figure 3B shows the probability for FF identification was significantly greater than 0.5 which exceeded the strict rule requirement of $p > 0.5$ for a positive identification. Both FF and adulterated mixtures were correctly classified.

A-TEEM Regression
Four regression models using Partial Least Squares analysis (PLS) were established for each adulterant, BHN-2, BHN-3, BHN-4, and BHN-5, respectively. Each model contained 60 calibration samples which were prepared with FF mixed with the respective adulterant in a progressive percentage of 0, 15, 25, 40, 50, 60, 80 and 100% by weight. Eight validation samples used for each model were the prescribed BHN mixtures respectively. The PLS regression models correctly predicted the prescribed mixtures BHN-2, BHN-3, BHN-4, and BHN-5 to contain

50% FA-20, 25% molasses, 21% acetic acid, and 28% lignin, respectively, all with the R2 values of calibration and cross validation being both higher than 0.996 (Table 2).

Conclusion
A-TEEM molecular fingerprints acquired with the Aqualog, and coupled with multivariate analysis, demonstrated successful classification and quantification of fulvic acids and other ingredients considered to be adulterants. Therefore, the A-TEEM molecular fingerprinting technology has the potential to be applied for routine regulatory and industrial use to verify contents of commercial humic products.

References
1. Lamar, R., Olk, D.C., Mayhew, L., Bloom, P.R. 2014. A New Standardized Method for Quantification of Humic and Fulvic Acids in Humic Ores and Commercial Products, Journal of AOAC International, Volume 97, Issue 3 (1), p.721- 730.

2. International Standards Organization. 2018. Fertilizers and soil vonditioners-Determination of humic and hydrophobic fulvic acids concentrations in fertilizer materials. ISO19822:2018.

Mixture Name	Prescribed Adulterant Content	PLS Model Predicted Adulterant Content	PLS Regression Model Goodness-of-Fit				
			RMSEC	RMSECV	RMSEP	R2 Calibration	R2 Validation
BHN-2	50% FA-2	50% FA-20	1.479	1.550	1.405	0.998	0.998
BHN-3	25% molasses	25% molasses	0.905	1.614	1.585	0.999	0.997
BHN-4	20% acetic acid	21% acetic acid	1.904	1.951	2.048	0.996	0.996
BHN-5	25% lignin	28% lignin	1.706	1.976	3.536	0.997	0.996

Table 2: Partial Least Squares (PLS) Regression Models Prediction Results

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