Environmental Science & Technology

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Multidimensional Fluorescence Fingerprinting for Classification of Shrimp by Location and Species

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Supporting Information

ABSTRACT: Parallel factor analysis with soft independent modeling by class analogy (PARAFAC-SIMCA) was used to analyze fluorescence data from shrimp extracts (organic and aqueous phases) to create classification schemes for two species of shrimp from four different countries. Twenty-four shrimp (six from each location: Ecuador, Philippines, Thailand, and United States) were studied; two were classified as statistical outliers. Using PARAFAC scores from the two aqueous fluorescent components and the strongest four components from the organic phase, country of origin was correctly identified at the 95% confidence level for all 22 remaining specimens; three false positives, at lower confidence levels than the true positives, were also indicated. A classification scheme which used all eight fluorescent components reproduced the 22 correct classifications and reduced the number of false positives to one. Finally, a scheme using PARAFAC scores from the two aqueous fluorescent components and the strongest four components from the organic phase, designed to classify according to species, produced 22 correct matches with no false positives. Spectral similarities between known chemical species and the components identified by PARAFAC are suggested for most cases. The results indicate that environmental effects appear in the fluorescence fingerprints of shrimp collected in different locations; therefore, fluorescence measurements on shrimp have the potential to permit geographical classification of shrimp or, conversely, to permit inferences to be made about the animal's environment.

INTRODUCTION

Over the past 15 years, chemometric analysis of multidimensional spectral data has been used extensively to characterize samples including natural waters,[1−3] food products,[4,5] and biological samples.[6,7] The fluorescence signatures of natural waters are often dominated by the contributions of the dissolved organic matter from decay of the indigenous flora and fauna; therefore, they are characteristic of the local ecosystem. In 2005, our group published a study in which the fluorescence fingerprints of natural water samples were used to identify their geographic origins, with success rates of 85−90% in classifying samples taken from four locations within a 10 mile radius.[8] In that study, the N-way partial least squares (NPLS) method was used to classify the spectra, using mathematical factors not directly related to individual chemical components of the samples. In another study involving three ports separated by hundreds of miles, the technique was about 98% successful.[9]

Parallel Factor Analysis (PARAFAC) has emerged as one of the most promising methods for analyzing fluorescence excitation−emission matrixes (EEMs) for characterization purposes. A significant advantage of PARAFAC is that complicated multidimensional spectra can be simplified into a number of component spectra that can be interpreted chemically. Further chemometric analysis of PARAFAC output can allow for the development of a classification method based on chemically interpretable data, in contrast to other classification techniques such as NPLS. In 2007, we demonstrated that soft independent modeling by class analogy (SIMCA) analysis of PARAFAC output may be used for geographical classification of natural water samples.[10] In this work, we extend our range of application to geographical and species classification of marine organisms, whose fluorescence fingerprint might include contributions from the organism itself and from chemicals found in its environment or their metabolites.[7]

The development of a fluorescence based method for geographically classifying shrimp and prawns may prove to be a useful technique for other studies assessing the variety of problems associated with shrimp aquaculture which pertain to ecosystem, animal, and human health. Such problems include habitat destruction, chemical pollution, the spread of viral diseases and parasites, and the reduction of the biodiversity of shrimp species. It is also hypothesized that an increased pollutant load (viruses, heavy metals, PCBs) in shrimp used for

Received: October 22, 2011
Revised: January 25, 2012
Accepted: January 27, 2012
Published: January 27, 2012

food[11,12] may result in a higher prevalence of allergic reactions in humans. Solutions to shrimp aquaculture problems, particularly those pertaining to shrimp and human health, may be found through careful study and monitoring of shrimp.

The International Marine Shrimp Environmental Genomics Initiative (IMSEGI) is an organization with the goals of monitoring ecosystem, animal, and public health.[13,14] The so-called ONE HEALTH project is run by IMSEGI and focuses on studying shrimp and ecosystem health on several different levels. ONE HEALTH is monitoring the following:

- Natural abundances of shrimp species and associated genetic diversity
- Pollutant levels in wild and cultured shrimp including chemicals like heavy metals, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs)
- Correlations between pollutants and the genetic diversity of shrimp populations
- Long-term health effects associated with human consumption of shrimp

IMSEGI is interested in the use of a fluorescence based classification scheme to help address some of the previously mentioned issues by testing shrimp imported to the Unites States.

The work presented in this paper involves the analysis of shrimp collected from four geographically diverse sampling locations including the United States, Ecuador, Philippines, and Thailand. Two different locations for each of two different species were chosen for this study to allow us to assess genetic vs geographic or environmental effects. Subsequent analysis of fluorescence data obtained from the shrimp by PARAFAC-SIMCA resulted in a successful classification scheme, with PARAFAC results that may also potentially provide chemical information about the samples. Additional samples from countries representing the natural range of shrimp-producing/exporting countries are needed to validate this tool.

EXPERIMENTAL SECTION

Sample Collection. The shrimp samples used in this study were collected by one of us (AW) over the period 1996−2004 in the following locations: Hawaii, United States (1998); El Oro, Ecuador (2002); Panay, Philippines (1996); and Samut, Thailand (2004). The GPS and geographic coordinates for the samples, including the name of the town and (province, country) are as follows: Waimanao (Oahu, Hawaii, USA): 21.334, 157.698 [21°19′58″ N and 158°5′33″W]; Jambeli (El Oro, Ecuador): $3.\overline{3}$, 80.067 [3°14′08″ S and 80°04′50″ W]; Roxas City (Panay, Philippines): 122.75, $11.58\overline{3}$ [11°35′ N and 122°45′ E]; Samut Sakhon (Thailand): $13.5\overline{3}$, $100.28\overline{3}$ [13°31′ N and 100°16′ E].

The sample species and time spent in frozen storage for the different locations varied. Shrimp from the United States and Ecuador were of the species *Litopenaeus vannamei* ("Pacific whiteleg shrimp") while those from the remaining two locations were *Penaeus monodon* ("giant tiger prawns"). The United States shrimp were cultured, specific pathogen-free (SPF) *L. vannamei* (Kona Line) from the breeding program of the US Marine Shrimp Farming Program (USMSFP) maintained at the Oceanic Institute in Hawaii.[14,15] Once the samples were received, they were stored at −80 °C and thawed only before extraction.

Sample Preparation. Attempts to measure fluorescence from whole shrimp by mounting them in a fluorometer on a standard front surface accessory did not produce adequate signal. Therefore, an extraction procedure designed to remove PAHs from mussel tissue[16] was adapted for removing fluorescent compounds from shrimp. Approximately 2 g of tissue was removed from the head (with exoskeleton removed) of a shrimp and homogenized in a 2 M solution of KOH in 50:50 EtOH/H_2O. The resulting solution was refluxed for 2 h, allowed to cool, centrifuged, decanted, and filtered using a 45 μm nylon filter (Millipore). A liquid−liquid extraction was performed with *n*-hexane. Both the aqueous and organic extraction phases were kept for analysis. Each organic-phase extract solution was dried with Na_2SO_4 and purged for 15 min with ultrahigh purity nitrogen to prevent oxygen quenching of fluorescence.[17,18] The aqueous-phase extracts were diluted by a factor of 15 to allow the EEMs of both phases to be collected using the same photomultiplier tube settings and because the absorbance values were too high.

Spectroscopy. All EEMs were collected on the same day that a sample was extracted. A Varian Cary Eclipse spectrophotometer was used for all fluorescence measurements. Standard 1 cm quartz cuvettes (Hellma) were used for aqueous sample measurements. For the organic-phase samples, a 1 cm cuvette (Starna Spectrosil far-UV quartz) with a fused 60 mm long Pyrex graded seal neck was used. A size 8 rubber septum was used to cap the cell, allowing stainless steel needles to be inserted and removed easily for the nitrogen purge. Each EEM consisted of measuring emission from 240 to 600 nm in 1 nm intervals for excitation wavelengths from 230 to 600 nm in 5 nm intervals. The PMT voltage was set to 600 for all measurements. The absorption spectra of both the hexane phase and the aqueous phase were measured from 230 to 600 nm in 1 nm intervals using a Cary 300 UV−vis spectrophotometer. A total of 48 EEMs were obtained, one organic-phase and one aqueous-phase EEM for each sample.

Data Preprocessing. Rayleigh scattering signals were removed from each EEM and set to "missing" using a program written in house by Gregory J. Hall and modified by Hao Chen.[8] An EEM of the solvent was subtracted from each data matrix, effectively reducing Raman scatter signals to the level of background noise. For each excitation wavelength, all intensity values at emission wavelengths $\lambda_{em} < \lambda_{ex}$ were set to zero because no appreciable fluorescence signal should be measured at wavelengths shorter than the excitation light. An in house correction program (Hall and Chen) was used to correct for inner filter effects and instrumental bias.[8]

PARAFAC. PARAFAC is a useful tool to elucidate trends in multivariate data, such as stacks of EEMs as in the present case. For a dilute or absorbance-corrected mixture, the fluorescence contributed by each component should be linear in concentration, absorptivity at excitation wavelength, and relative fluorescence probability at emission wavelength.[19] A three way array of fluorescence EEMs of dimensions $I \times J \times K$ can be modeled using PARAFAC by:

$$x_{IJK} = \sum_{r=1}^{R} a_I b_J c_K + e_{IJK} \quad (1)$$

where I is the number of samples, J is the number of emission wavelengths, K is the number of excitation wavelengths, and R is the number of factors or components. The decomposition of the data by PARAFAC results in output excitation and emission spectra (vectors b and c) for each component used to model the data. These output spectra can be interpreted as pure

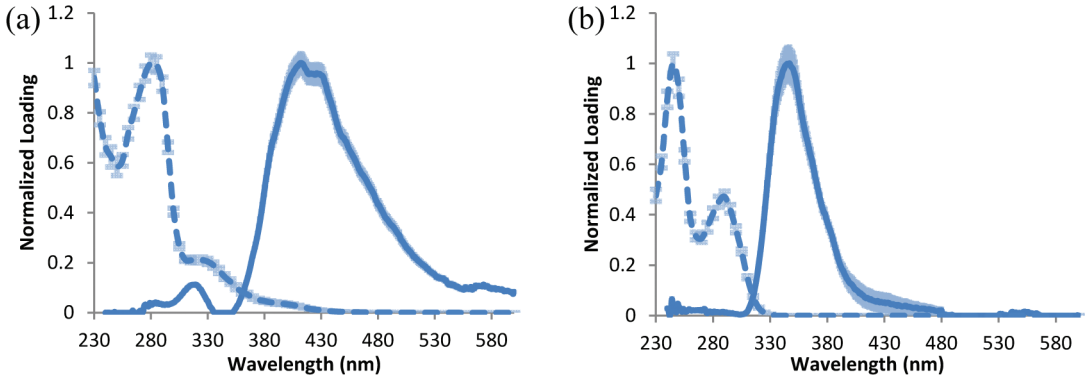

Figure 1. Average excitation (dashed line) and emission loadings (solid line) with standard error of (a) aqueous-phase component 1 and (b) aqueous-phase component 2.

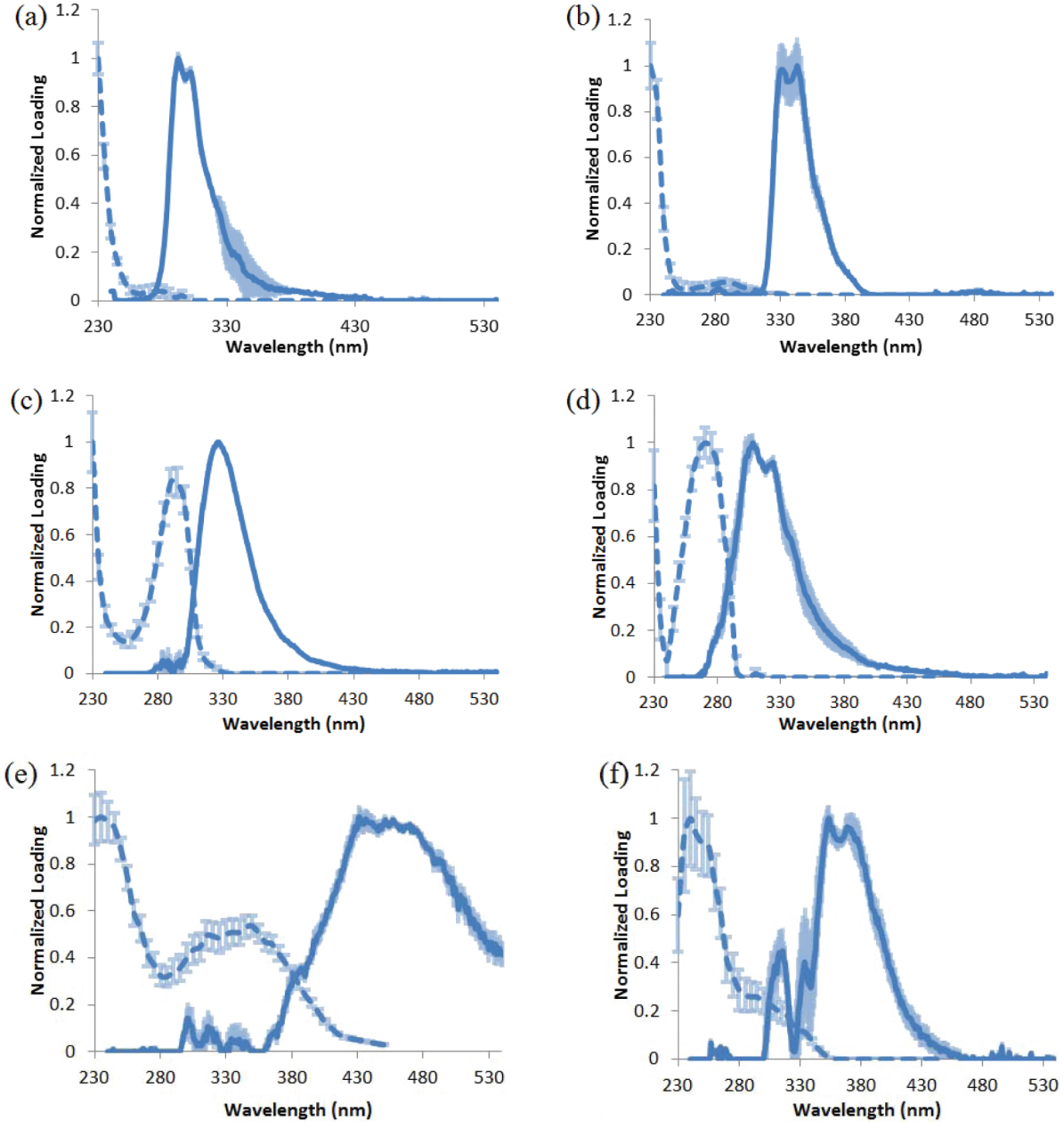

Figure 2. Average excitation (dashed line) and emission loadings (solid line) with standard error of organic phase (a) component 1, (b) component 2, (c) component 3, (d) component 4, (e) component 5, and (f) component 6.

dx.doi.org/10.1021/es2037542 | *Environ. Sci. Technol.* 2012, 46, 2276–2282

component spectra or possibly approximate contributions from a class of similar fluorophores whose concentrations covary. Vector *a* is referred to as the "score" of a component and can be interpreted as a relative contribution of a component to the overall model.

All models were fit using PLS_Toobox 5.2 (Eigenvector Research) in MATLAB 7.8 (Mathworks). Jack-knifing outlier analysis and standard error calculations were performed using the *jkparafac* program by Bro et al.[20] A stop criterion of 1×10^{-6} percent relative change in the residuals was used, and the models were initialized using the best of ten small runs to avoid fitting models to local minima. Nonnegativity constraints on all three modes were used to ensure each model would have a physical interpretation. The EEMs of the two extract phases were modeled by PARAFAC separately. Models were validated by use of split-half analysis, residual analysis, and visual inspection of the excitation and emission loadings.

SIMCA. SIMCA uses principal components analysis (PCA) to define classes of data according to orthogonal vectors, called principal components (PCs), which are chosen to capture the most variance. Samples can be classified according to the defined classes by projecting them onto the model and calculating the distances between each sample and each model using fit statistics. It should be noted that SIMCA is a soft method, and therefore, classes can overlap. For this reason, a sample can be a member of any number of classes including not fitting into any class.

Common fit statistics used in SIMCA models are Hotelling's T^2 statistic and the lack of fit statistic Q.[21] With these values, the total distance, d, of a individual sample can be calculated to a particular model according to the following relationship:[21]

$$d = \sqrt{(Q_r)^2 + (T_r^2)^2} \quad (2)$$

where T_r^2 and Q_r are the reduced Hotelling's T^2 and lack of fit statistic, respectively, normalized to the 95% confidence interval.

RESULTS AND DISCUSSION

PARAFAC Modeling. The aqueous-phase data of 24 EEMs was initially modeled using 1−4 factors. The EEMs of each sample were stacked to form a matrix of dimensions 24 × 361 × 75. One outlier was removed on the basis of having extreme score values. Jack-knifing did not suggest any other samples were outliers. The final model fitted with 23 EEMs was determined to have two components and validated using a split-half analysis. See Supporting Information for key fit parameters (Table S1) and split-half spectra (Figures S1 and S2). The standard errors in the excitation and emission loadings were determined using jack-knifing (Figure 1).

The first aqueous-phase component has not been identified, but the second component has a spectral profile similar to that of the amino acid tryptophan. This component also possibly describes tyrosinate fluorescence resulting from basic conditions such as those used during extraction. Tyrosinate weakly fluoresces at approximately the same wavelengths as tryptophan.[22]

The organic-phase data were initially modeled using 1−8 components. The EEMs of each sample were stacked into a matrix of dimensions 24 × 301 × 55. Jack-knifing yielded two outliers, one of which was the same sample removed as an outlier during the aqueous-phase modeling; both outliers were from the United States. The final PARAFAC model was fitted with 22 samples using six components and validated using a split-half analysis. See Supporting Information for key fit parameters (Table S2) and split-half spectra (Figures S3 and S4). The standard errors of the excitation and emission loadings were calculated using jack-knifed PARAFAC (Figure 2).

The compounds responsible for the organic-phase fluorescence could not be identified with certainty. It is possible that the components model classes of approximately covarying compounds with similar spectral profiles. Four out of the six components have spectral signatures, which resemble aromatic molecules, including fluorene and biphenyl. See Supporting Information (Table S3 and related text) for further discussion of spectral characterization.

SIMCA Classification by Location. Geographical classification of the 22 shrimp samples used to construct the models was performed by combining the scores of both the aqueous- and organic-phase PARAFAC models. Of the organic-phase scores, only those of the first four components were used for SIMCA analysis because the last two components only contribute a small fraction to the overall model (Figure 3). In this way, the most meaningful scores were used for classification purposes.

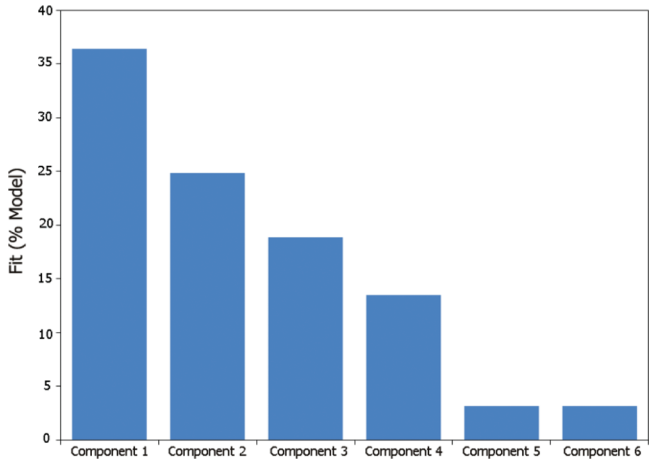

Figure 3. Percent variance explained by each component in six component organic-phase PARAFAC model.

Each model was built using normalized score values from the samples of a single location. The scores of a particular component were normalized as a fraction of the overall sum of scores for that component in order to prevent any one component from having more influence on the model. PCA models explaining between 99 and 100% variance were created for each class using 3 or 4 PCs. The distance of each sample to each class was calculated according to eq 2; those samples with a distance less than 1, the 95% confidence interval, were considered a member of a class (Figures 4, 5, 6, and 7 and Table S-4 in Supporting Information).

Of the 22 samples included in the classification, all were identified as members of the expected classes within the 95% confidence level. Three false positives were observed where samples from Thailand additionally classified into the Philippines class. However, the nearest class of each false positive, determined by shortest distance from a sample to a PCA model, was the correct class for all samples. Recall also that shrimp from these two sites were of the same species.

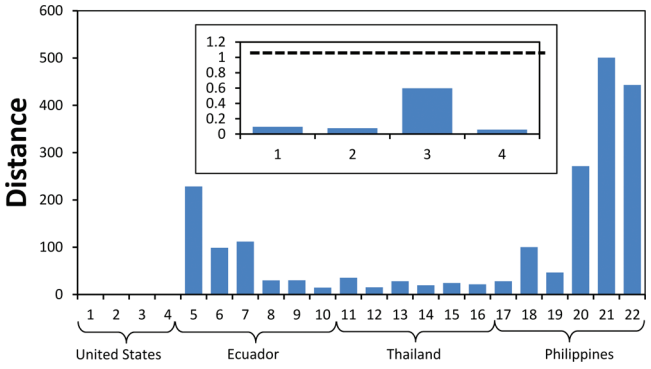

Figure 4. Distance of each sample to the United States model. Distances less than or equal to 1 correspond to 95% probability of assignment to the United States class. In this figure and the three following figures, the inset presents selected samples at an expanded scale.

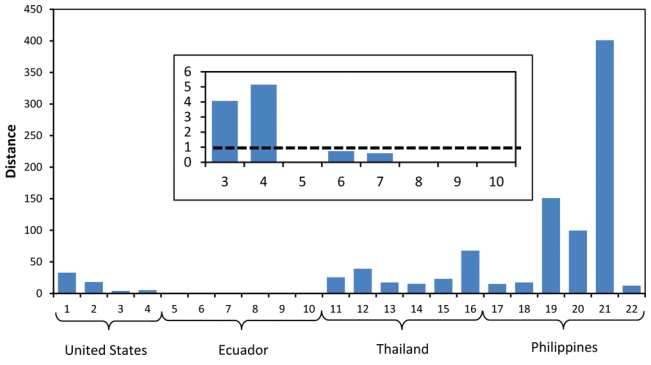

Figure 5. Distance of each sample to the Ecuador model. Distances less than or equal to 1 correspond to 95% probability of assignment to the Ecuador class.

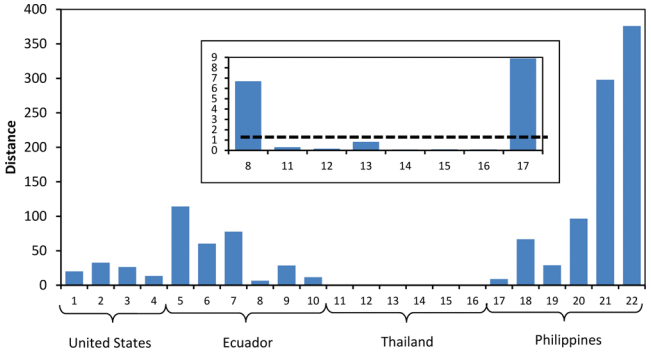

Figure 6. Distance of each sample to the Thailand model. Distances less than or equal to 1 correspond to 95% probability of assignment to the Thailand class.

Constructing a SIMCA model using all six organic-phase components in addition to the aqueous-phase components yielded slightly better results; 21 samples were correctly identified at the 95% confidence interval with one false positive and two outliers. The nearest class to the false positive was the correct class. See Figures S5–S8 in Supporting Information for distance plots.

SIMCA Classification by Species. *Classification using Aqueous-Phase Scores.* Of the 24 shrimp samples used in this study, 12 were of the species *L. vannamei* and the remaining 12 were *P. monodon*. There are significant physiological differences

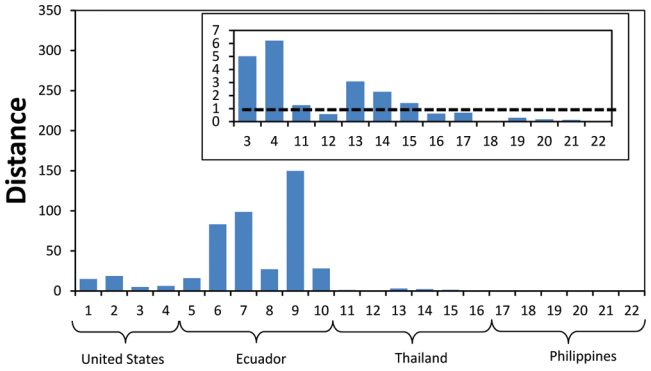

Figure 7. Distance of each sample to the Philippines model. Distances less than or equal to 1 correspond to 95% probability of assignment to the Phillippines class.

between the two species, and the possibility of the use of the aqueous-phase PARAFAC scores to distinguish between the two was explored. Component two was identified as an amino acid contribution and, if both aqueous-phase components model biological compounds, it may be true that the relative amounts of those compounds differ between species. Figure 8

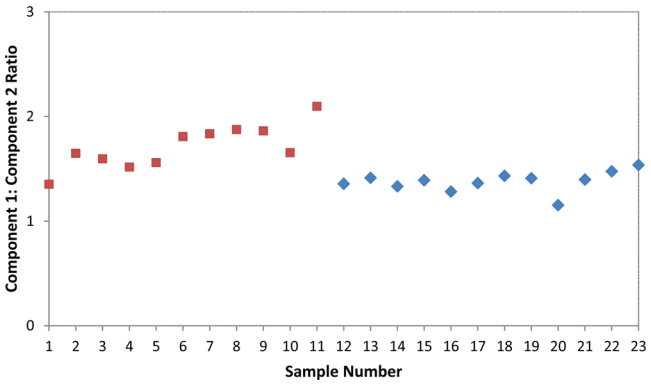

Figure 8. Ratio of scores of the two components in the 23 sample aqueous-phase model.

shows a plot of the scores for each sample, with sample numbers 1–11 corresponding to *L. vannamei* and 12–23 to *P. monodon*. It appeared that for each species the ratio of component scores was similar.

This ratio differed between the two species. The average ratio of component one to component two for the *L. vannamei* shrimp was 1.71 ± 0.21, and for *P. monodon*, the average was 1.38 ± 0.10. Student's *t* test gives $t = 0.0003$, so the difference is significant with greater than 99.9% certainty.

The scores used for this classification were obtained from the 23 sample aqueous-phase PARAFAC model. The score values for each sample were normalized as a fraction of the sum of all scores for a given component to minimize the influence of any one component.[10] Two classes were defined on the basis of species: *L. vannamei* and *P. monodon*. All samples of one species were analyzed using PCA to generate a model, explaining between 99 and 100% variance, for the corresponding class. The PCA models used 2 PCs for the *L. vannamei* class and 1 PC for the *P. monodon* class. A SIMCA model was constructed from the two PCA models, and the scores of all samples were used to calculate the distance of each sample from the PCA

dx.doi.org/10.1021/es2037542 | *Environ. Sci. Technol.* 2012, 46, 2276–2282

class models. Table S6 in the Supporting Information summarizes the results of the classification.

All samples were classified into their designated class. A significant number of samples were classified into both classes. By examining the results in terms of nearest class, only three samples were incorrectly classified. It may be because, with only two components, there were not enough differences between both classes for a more definitive classification. The same classification procedure was attempted by combining the aqueous scores with organic-phase scores.

Classification Using Aqueous- and Organic-Phase Scores. A similar classification by species was attempted using scores from both the organic- and aqueous-phase PARAFAC models. It is possible that the molecules dissolved in the organic-phase extract solutions, such as possible pollutants, are indicative of the natural habitats of the shrimp. For example, a PARAFAC study involving eels in the North Sea showed differences in the types of and concentrations of oil pollutants based on sample collection location.[7] Classification based on species also roughly categorizes the shrimp based on location as the *P. monodon* shrimp used in this study were from Southeast Asia and *L. vannamei* shrimp were from the eastern Pacific.

PCA models were generated using six PCs for the *L. vannamei* class and 5 PCs for the *P. monodon* class. Excluding the outliers, each sample correctly classified into the expected nearest class. More importantly, there was only one false positive (sample D3) where a shrimp was classified into both classes, suggesting that the SIMCA model based on both organic and aqueous-phase scores is better for species classification. Tables S-7 and S-8 and Figures S-9 and S-10 in the Supporting Information summarize the SIMCA results.

SIMCA is a soft method which allows a sample to be a member of any number of classes including none at all. This fact allows the same samples that will be classified to be used to generate the PCA models. It was therefore expected that all samples would be classified into their correct classes since they were used to generate the models. The more significant result is the low number of false positives, which suggests that there are real fluorescence differences between the shrimp samples of different species and geographic locations.

The classification of several samples from Thailand as members of both the Thailand and Philippines classes suggested that the two models overlap to some degree. It is possible that the similarity of the two models can be explained by the geographical proximity to both sampling sites or to genetic factors; shrimp from both locations were the same species. Although separated by thousands of kilometers, both Southeast Asian collection sites were located on the South China Sea. The relative distance between the two locations is significantly less than the distance between any other pair of sites. Originally, we designed the EEM based PARAFAC-SIMCA classification scheme to distinguish between natural water samples based on geographical location and, ideally for country of origin labeling purposes, a PARAFAC-SIMCA based classification scheme would be able to distinguish between samples within the same country or province. Estuarine water samples in the Mystic River Watershed in Massachusetts taken from sites less than 10 miles apart were successfully classified in a past study.[8] Focusing instead on a single type or a small number of types of organism raises the question of whether the fluorescence fingerprints depend on genetics, or environment, or both. Genetic differentiation among and within *P. monodon* of four geographic regions in the Philippines[23] and between *P. monodon* populations from the Andaman Sea and the Gulf of Thailand have been reported.[24] These results may have some bearing on the false positives for some Thailand samples to the Philippines class.

It is possible that the two outliers (from the United States samples) reflect the variation in the origin of the parental stocks used to develop the SPF shrimp, which originated from Sinaloa, Mexico and Esmeraldas, Ecuador. The so-called "SPF" shrimp from the United States have been maintained in culture for many generations.[15,25]

Because the samples were collected in different years, we examined the possibility that sample age could have influenced the classification. PARAFAC scores for the two aqueous components and the six organic-phase components used in the models were plotted versus sample year. Linear correlation coefficients r^2 and significance parameters p were calculated for each of the eight cases. The results are shown in Table 1.

Table 1. Correlation Coefficient r^2, Student's t Value, and Significance Parameter p for Linear Correlations of PARAFAC Scores of Eight Components vs Sample Year

component	r^2	t	p
organic phase 1	0.0018	0.190	0.851
2	0.1953	2.203	0.039
3	0.0798	1.317	0.203
4	0.0071	0.378	0.709
5	0.0351	0.853	0.404
6	0.3892	3.570	0.002
aqueous phase 1	2.00×10^{-5}	0.020	0.984
2	0.0033	0.264	0.795

Correlations are normally considered significant only when $p < 0.05$. It can be seen that in only two cases are the scores correlated with sample year, and even in those cases ($p = 0.039$ and 0.002), the correlations are weak ($r^2 = 0.195$ and 0.389).

The results in Table 1 strengthen the evidence that the environment of the shrimp is a major contributor to the success of classification using multidimensional fluorescence fingerprinting. The results indicate that environmental effects appear in the fluorescence fingerprints of shrimp collected in different locations; therefore, fluorescence measurements on shrimp have the potential to permit geographical classification of shrimp or, conversely, to permit inferences to be made about the animal's environment.

The next step is to validate the PARAFAC/SIMCA technology for commercial application using a larger sample size from each of the major shrimp producing countries and to investigate the correlations between the fluorescence signatures of the animals and available genetic and environmental information. Shrimp samples collected since 1998 through the IMSEGI project in Thailand, Ecuador, Philippines, Peru, and other major shrimp-producing countries are available for further testing and validation of the methodology for traceability purposes.

■ ASSOCIATED CONTENT

Supporting Information

Additional information about PARAFAC and SIMCA modeling and interpretation of spectral loadings. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by a New Directions Grant to J.E.K. from the Tufts Faculty Research Fund. J.K.E. acknowledges support from the Tufts Summer Scholars program as well as a grant from Fundacion para la Conservacion de la Biodiversidad Acuatica y Terrestre de Ecuador (FUCOBI), Guayaquil, Ecuador.

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