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**Enhancement of Fluorescence Detection in Chromatographic
Methods by Computer Analysis of Second Order Data**

**Progress Report - 8/1/90 - 10/1/93
DOE # DE-FG05-88ER13833**

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This progress report focuses on the results obtained over the last three years with support from the U.S. Department of Energy. This was a renewal of a previous grant, "Compensation for Peak Shifts and Variable Background Responses in Fluorescence Spectroscopy"; publications 1-10 describe the work accomplished during the previous three years of support.

The current proposal was focused on the development of data analysis methods for the evaluation of second-order chromatographic-fluorescence data. Two types of experiments were studied during the course of this project. The first was the liquid chromatographic separation of polyaromatic hydrocarbons (PAHs) followed by detection with full-spectrum fluorescence spectroscopy using an intensified diode array detector. Here, methods such as generalized rank annihilation (GRAM) and adaptive Kalman filtering (AKF), have been developed and evaluated for this purpose. In the second area, we use a thin-layer chromatographic (TLC) or planar electrophoretic separation to effect the separation of the analytes. The analytes are subsequently reacted with a reagent or enzyme substrate, and the resulting reaction is followed by collecting fluorescence intensity information as a function of time and migration distance. Kinetic methods of analysis are then used to quantify the

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component species. Significant advances have been made in both areas; these will be outlined in two sections.

Multiwavelength Fluorescence Detection in Chromatography

During the previous grant period, we were able to finish the construction and initial characterization of an intensified diode array-based, multiwavelength fluorescence detector for liquid chromatography. This system has been applied to the analysis of polyaromatic hydrocarbons (PAHs). During the present grant we have characterized and improved the performance of several data analysis algorithms suited for the analysis of the second-order, bilinear data that are obtained from this instrument. One technique, the Generalized Rank Annihilation Method (GRAM), has been characterized extensively (12,17). This technique requires that the data be in a bilinear matrix format, which is the form of data provided by our liquid chromatographic-fluorescence detection system. A standard sample which contains all the components of interest to be quantified is injected into the chromatograph, and the second-order data matrix comprised of fluorescence intensities as a function of both retention time and emission wavelength is obtained. Similarly, a sample containing the components of interest to be quantified, plus additional, unknown interferant compounds is also injected in the chromatograph, and the corresponding data matrix is collected. The GRAM algorithm permits "target" species present in the sample to be quantified, as long as these species are present in the standard at known concentrations, even if these species suffer from spectral and/or chromatographic overlap with unknown interferences in the sample to be quantified. We have found

that one of the key requirements for successful utilization of this algorithm is that the retention time of the components of interest in the standard and in the sample must be reproducible. We have also determined that the original GRAM algorithm reported by Sanchez and Kowalski (prop. ref. 10) is much less sensitive to these retention time shifts than the newer method proposed by Wilson, Sanchez, and Kowalski (prop. ref. 12) which is based on concatenation of the standard and sample data matrices. Additionally, we have carefully characterized the performance of the algorithm in the presence of heteroscedastic noise, varying degrees of chromatographic overlap, different sampling frequencies, and variable retention times. We find that the algorithm performs well for quantifying perylene when the signal is only partially resolved from that of benzo[a]pyrene. In this case, reliable quantitative results can be obtained for chromatographic resolution values as low as 0.2.

Another technique that is potentially useful for quantifying target species in the presence of unknown interferences is the adaptive Kalman filter (13,17,18). Here data in a bilinear data matrix format are not required for this approach, however, the presence of a spectral or chromatographic region in which the target species are not subject to overlap from unknown interferences is required. In our initial studies in this area, we have focused on spectroscopic data, and have summed all the spectra in the data matrix to describe a single peak envelope. Here, we find that the adaptive filter is effective in quantifying benzo[a]pyrene in the presence of unsuspected perylene, but is subject to errors when attempting to quantify perylene in the presence of a large amount of unsuspected benzo[a]pyrene. Upon inspection of the spectral profiles

for these two compounds, it is clear that the reason for the successful application of the filter in the first case, and the failure in the second case, is because in the former case, there is a significant overlap free region, while in the latter case, the overlap free region is quite small.

A difficulty with the adaptive filter that we have been able to solve during this grant period, is that results of the filter are dependent on the initial guess provided for the concentrations, or alternatively on the order in which the data are processed. By using either a grid search or simplex optimization of the *information yield* from the filter, we have been able to obtain results that do not depend upon the initial guesses or the data processing sequence (18). The combination of the two complementary techniques, GRAM and the adaptive Kalman filter, provides a good approach for the quantitative evaluation of *real*, chromatographic/fluorescence data collected from samples containing PAHs or other fluorescent components.

Kinetic Detection in Thin-Layer Chromatography

Significant progress in the area of kinetic detection subsequent to a planar chromatographic separation step has also been made during the current grant period. The general approach is to separate the analytes, using either thin-layer chromatography (in the case of analytes such as amino acids) or slab gel electrophoresis (in the case of analytes such as enzymes). The separation step is followed by introduction of a reactive reagent (o-phthalaldehyde in the case of amino acids; 4-methyl umbelliferyl phosphate in the case of the alkaline phosphatase isoenzymes), which subsequently reacts to form a fluorescent product. The formation

of this product is followed using a charge-coupled device (CCD) imaging system which collects images of the planar media at regular time intervals. We have completed the construction and programming of this system which allows subsequent images to be captured, stored on a hard disk, corrected for variations in pixel sensitivity and dark current, and intensities for different spots on the media to be integrated. Kinetic fitting programs based on the Kalman filter algorithm have been implemented which allow the kinetics of the reactions as a function of position of the plate or slab gel to be analyzed. The two analytical systems that have been studied to date are the quantification of alkaline phosphatase isozymes, and the quantification of amino acids.

In the case of the alkaline phosphatase isozyme analysis problem (15,16), two forms of the enzyme (arising from bone and liver tissue) are particularly difficult to separate electrophoretically. We have found that we can resolve these species using the differential effect of a denaturant, guanidine hydrochloride, on the two different enzyme forms. We first determine the first-order rate constant for deactivation of samples that are predominately comprised of the bone or liver isozyme. We have found that using repeated linear fits using the Kalman filter with different initial guesses for the rate constants has allowed us to determine the rate constants accurately. In this case the bone isoenzyme deactivates at a faster rate than the liver isoenzyme. Once these rate constants have been determined, we can determine the relative contribution of these two isozymes in a sample mixture. We have also identified important effects on this reaction due to variations of the ionic strength of the reaction medium. The alkaline phosphatase system has allowed us to

demonstrate that this novel analytical approach is feasible, and should extend the applicability of quantitative thin-layer chromatographic and slab gel electrophoresis techniques.

A second analytical problem that has been the focus of the current work is the analysis of amino acids using a combined thin-layer chromatographic/kinetic method for analysis based on the kinetics of formation of the o-phthalaldehyde derivatives of amino acids. In this case, more complex kinetic behavior is observed, as the fluorescent product is unstable, and decays to nonfluorescent products over the time-frame of the experiment. This gives rise to kinetic profiles that may be fit using a consecutive first-order kinetic model. One characteristic of this model is that two distinct sets of kinetic parameters will fit any given kinetic profile equally as well. We have explored this problem and have found that by finding the quantity $k_1 A_0$, where k_1 is the rate constant for the first process, and A_0 is proportional to the initial concentration of analyte (amino acid), we can get consistent calibration curves, no matter which set of kinetic parameters is obtained from the fit. We have explored different means of generating initial guesses for the kinetic parameters to be supplied to the nonlinear regression fitting algorithm (in our case, an extended Kalman filter), and have established the reliability of the method for estimating initial concentrations. Now that we have solved these difficulties, we are ready to apply this approach to the analysis of amino acids subsequent to thin-layer chromatographic separation. This kinetic detection approach is completely general, and should be applicable to a wide variety of analytical separation and quantification problems.

PUBLICATIONS (acknowledging DOE support)

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