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Kinetic Detection of Overlapped Amino Acids in Thin-Layer Chromatography with a Direct Trilinear Decomposition Method

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Kinetic fluorescence detection (KFD) was employed to determine the concentrations of two overlapped components after a thin-layer chromatographic (TLC) separation. Two amino acids, glycine and glutamine, were used as model analytes. These species exhibited very similar retardation factors (R_f) under our experimental conditions. A reaction that produced a fluorescent product was performed subsequent to the separation. The reaction can be described by the following scheme: amino acid + OPA → fluorescent product → nonfluorescent product. Here OPA stands for *o*-phthalaldehyde. The kinetic profile of the reaction was monitored with a charge-coupled device camera by taking sequential images of the separation medium after the reaction starts. A direct trilinear decomposition (TLD) method was used to analyze the resulting third-order data that consist of fluorescence intensities as a function of elution distance, reaction time, and sample number. This approach was used to determine the initial concentrations of the two overlapped components based on the different kinetics and retention exhibited by these species. This paper discusses the kinetic approach and the applicability and limitations of the direct trilinear decomposition (TLD) method using both synthetic and experimental data. Efforts to optimize the experimental conditions are also reported. The major focus of this work is to explore the application of this novel kinetic fluorescence detection method for TLC separations (TLC-KFD) combined with the TLD data analysis method.

Hyphenated instruments have seen increasing applications in analytical chemistry laboratories in the past few years. These instruments, such as liquid chromatography-UV diode array detection (LC-DAD), gas chromatography/mass spectrometry (GC/MS), and excitation/emission fluorescence have been used for many important analyses. A two-dimensional matrix can be obtained for each sample from these hyphenated or second-order techniques. Based on the two-dimensional data, calibration in the presence of unknown interferents can be performed, which is impossible for zero- or first-order methods. This is the so-called "second-order advantage".[1] Chemometric methods, such as the generalized rank annihilation method (GRAM) and the direct trilinear decomposition (TLD) method,[2] have been developed to extract useful information from the data obtained from second-order instruments. These chemometric methods allow taking the second-order advantage, and they have been utilized to solve several practical problems.[3-8] The present work was dedicated to the evaluation of a novel second-order technique combined with a chemometric method and the application to a chemical problem, in which uncharacterized interferents or background components contribute to the measured response.

Kinetic fluorescence detection subsequent to thin-layer chromatographic separation (TLC-KFD) provides a second-order analytical method.[9] A two-dimensional data array, which is represented as fluorescence intensities as a function of elution distance and reaction time, is obtained for each sample. Combination of the data matrices from several samples constitutes a third-order data array. The TLD method can be used to analyze the third-order data and provide estimates for the real physical factors that underlie the data. In the case of TLC-KFD, the real physical factors are the retention, kinetics, and composition for each component. This approach allows the determination of overlapped species after TLC separation in the presence of unknown interferents.

To test the feasibility of this general analytical approach, two amino acids, glycine and glutamine, were chosen as model compounds. These two species demonstrate similar retardation factors in TLC under our separation conditions. After TLC separation, the species on the separation medium undergo an in situ reaction with *o*-phthalaldehyde (OPA). The reaction scheme is

amino acid + OPA → fluorescent product → nonfluorescent product

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The mechanism of this reaction as it occurs in solution has been investigated by several workers[10,11] and can be approximately described by a consecutive first-order reaction model. The formation and decay of the intermediate fluorescent product (λ_{ex} = 366 nm, λ_{em} = 440 nm) was monitored with a charged-coupled device (CCD) camera by taking sequential images of the TLC plate. A third-order data array for this experiment was obtained by stacking the sequential chromatograms of all the samples along the reaction time coordinate. Therefore, the three-way data consisted of the fluorescence intensities as a function of elution distance, reaction time, and sample number.

Both GRAM and TLD methods are useful for analyzing data matrices obtained from hyphenated instrumentation. However, GRAM is restricted to the use of only one standard and one mixture sample at a time. TLD can be used to process a third-order data array which consists of two-dimensional data matrices for several samples (standards and unknowns) simultaneously. Therefore, more precise solutions can be obtained by the signal averaging of multiple calibration standards that occurs when TLD is used. In order to use TLD, the three-way data should obey the trilinear model described in the equation[2]

$$R_{ijk} = \sum_{r=1}^{N} X_{ir} Y_{jr} Z_{kr} + E_{ijk} \quad (1)$$

where R_{ijk} represent the elements of a third-order data array, which are the fluorescence intensities as a function of retention, reaction time, and sample number, and E_{ijk} indicates the error contribution. In our case, the **X**, **Y**, and **Z** are matrices describing the retention, kinetic, and relative sample composition behavior for each pure component, and each represents a real physical or chemical factor. It has been found that a unique factor analysis decomposition, i.e., a unique solution to **X**, **Y**, and **Z**, can be achieved, given the array R, when the following assumptions are valid: the physical or chemical factors are linearly independent in at least two orders (e.g., **X** and **Y**) and not identical in the third order.[2]

EXPERIMENTAL SECTION

Nine 500 nL samples containing the amino acids glycine and glutamine (Sigma Chemical Co., St. Louis, MO) in the concentration range of 2.5−10 mM were applied to a nonfluorescent, scored, silica gel TLC plate (Catalog No. 4805-411; Whatman International Ltd., Clifton, NJ) using a 2.0 μL syringe (Hamilton Co., Reno, NV) and a TLC spotter Model PS01 (Desaga, Heidelberg, Germany). The plate was dried at 100 °C in an oven, and was subsequently developed using a mobile phase of 1-butanol (Sigma)/acetone (Sigma)/acetic acid (J. T. Baker Inc., Phillipsburg, NJ)/deionized water (7:7:2:4). The development was carried out in a "sandwich"-type chamber. After development for 15 min, the plate was air-dried in the hood for 5 min, followed by oven drying at 100 °C for 10 min. The plate was then dipped in a 0.05% (w/v) solution of *o*-phthalaldehyde (Calbiochem Co., La Jolla, CA) in methanol (EM Science, Gibbstown, NJ), which contained 0.2% (v/v) 2-mercaptoethanol (J. T. Baker Inc.) and 0.09% (w/v) Brij-35 (Eastman Kodak Co., Rochester, NY),[12] and the plate was immediately removed and placed on the imaging stand (Camag, Muttenz, Switzerland) and illuminated with 366 nm light. The CCD camera was a Star I camera system (Photometrics, Tucson, AZ). Images were obtained at 2 min intervals, with integration performed for 30 s. The pixels spanning the complete width of each lane (usually 38 pixels/lane) were summed, and the data were represented as the total fluorescence intensities as a function of elution distance. Hampton and Rutan described this experimental setup in more detail, as it applies to the detection of fluorescent products produced by enzymatic action, after separation of enzymes via gel electrophoresis.[13] Data were obtained for 9 different sample lanes, and for 40 time intervals during the reaction, providing an 87 (pixels, elution distance) × 40 (images, reaction time) × 9 (sample number) third-order array of intensity values. These original experimental data were corrected to eliminate the effects of dark current, uneven illumination, and variation in sensitivities among the pixels, using a dark image and a flat field image. The flat field image was generated by imaging a TLC plate doped with a green fluorophor and illuminated at 254 nm. This procedure has been explained in detail by Hampton and Rutan.[13]

The corrected data array (R) was input into a program written in MATLAB (The MathWorks Inc., Natick, MA), based on the TLD method described by Kowalski.[2] The resulting **X**, **Y**, and **Z** data matrices describe the retention and kinetic behavior, as well as the relative composition, in each sample for each individual pure component.

In the background study, reflectance spectra were obtained using a UV/visible spectrophotometer (Shimadzu Model UV 265).

RESULTS AND DISCUSSION

In the preliminary experiments, the images were taken without a bandpass filter in front of the CCD camera. It was found that the background varied with time during imaging and that the measured signals were dominated by the background. This phenomenon has been observed with TLC plates from different sources. Therefore, the kinetic differences between the two amino acids were overwhelmed by the large, varying background signals and could not be adequately resolved from the experimental data. This led to a careful examination of the background factors, which led to the development of the optimal detection conditions.

Background Study. The purpose of this study was to evaluate the different parameters controlling the background contribution to the overall signal. Factors considered included the use of a bandpass filter (centered at 450 nm with fwhm = 25 nm, S25-450-R-H840; Corion, Holliston, MA), the effects of the wetting degree of the TLC plates, interferences due to the diffuse reflection of the silica surface and the glass stage underneath the TLC plate, and fluorescence due to the glass substrate supporting the silica gel layer. The major contribution comes from the diffuse reflection due to the silica gel. A TLC plate was divided into six regions, as illustrated in Figure 1. Black paper was placed underneath the right half of the TLC plate, and the bottom half of the TLC plate was dipped into methanol before imaging. Regions 3 and 4 were the bare glass substrate without silica gel. Forty images were taken of the TLC plate with an integration time of 17 s, and at an interval of 2 min. The experiment was done

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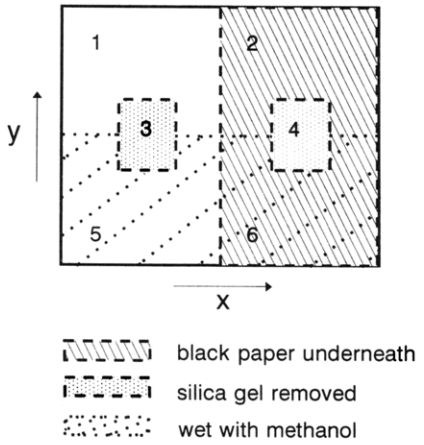

Figure 1. Six regions of the TLC plate evaluated in the background study.

Table 1. Signals from Different Regions in the Background Study

	mean signal	
region no.[a]	without filter	with filter
1	1.04×10^5	108
2_b	1.01×10^5	29
3_{ns}	6.18×10^4	267
$4_{b,ns}$	1.79×10^4	26
5_w	1.07×10^5	130
$6_{b,w}$	9.40×10^4	70

[a] b, black paper underneath this region; w, wet with methanol before imaging; ns, no silica.

twice, one with the bandpass filter in front of the CCD camera, and one without it. All the images were corrected in the same manner as the actual experimental data using a dark image and a flat field image. The digital signals were summed across 38 pixels over the width of a sample spot and then a mean value and a standard deviation were calculated for each region using 30 pixels along the *y* direction.

The mean signals of the background from the different regions at the end of the imaging process are summarized in Table 1. It is observed that the bandpass filter reduces the detected signals by more than 99.9% in all cases. This value is consistent with the transmittance of the bandpass filter at 366 nm. On the other hand, the signals listed in Table 1 were obtained when the wet regions were almost dry. Therefore, it is concluded that the detected signal is mainly from the diffuse reflection of the incident illumination (366 nm) from the silica gel and that the bandpass filter suppresses this reflected light significantly. The diffuse reflection from the silica gel dominates the detected signal when no bandpass filter is placed in front of the CCD camera.

Regions 3 and 4 had no silica gel on the glass substrate. Therefore, the difference between the signals from regions 3 and 4 should be due to the effect of the glass stage on which the TLC plate stays during imaging. It is seen that region 4 (where black paper prevents the excitation light from reaching the glass stage) showed significantly lower intensities than region 3 (70% reduction in signal intensity without the filter; 90% reduction in signal intensity with the filter). That means that the glass stage contributes significantly to the background intensity, and the black paper underneath the TLC plate eliminates this factor effectively. However, the signal intensity of region 3 relative to that of region 4 for with filter is higher than that without the filter. The possible explanation is that the glass stage has two effects: reflection of the exciting radiation at 366 nm and fluorescence at a higher wavelength that is closer to 450 nm. Comparing regions 1 and 2 and 5 and 6 leads to the same conclusion. Under conditions when the filter is not present, the intensities from regions 2 and 6 are just slightly lower than those of regions 1 and 5, respectively. The reason is that, without the filter, the diffuse reflection due to the silica gel dominates the detected signals. The effects of the glass stage were not detected. However, with the filter, the fluorescence contribution from the glass stage becomes significant, since most of the reflected light contribution is effectively removed. This can be seen by comparing regions 1 and 2, and 5 and 6 with the filter present.

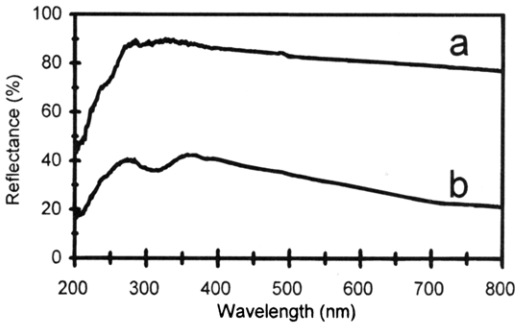

Figure 2. Diffuse reflectance spectra of the TLC plate under (a) dry and (b) wet conditions.

The effects of wetting the silica gel with methanol (the solvent used to introduce the OPA reagent) can be observed by comparing regions 2 and 6 under both filter conditions. Regions 2 and 6 had black paper underneath, and the contribution of the glass stage to the background signal was removed. When the filter was not used, region 6 gave lower intensities than region 2. This is because diffuse reflection is the major background contributor in this case, and wetting the silica gel decreases the diffuse reflection. However, in the case with the filter, region 6 showed a significantly higher signal than region 2. A plausible explanation is that the wet region 6 fluoresces at a wavelength close to 450 nm. Therefore, it is concluded that wetting the TLC plate has two effects. First, the diffuse reflection due to the silica gel is reduced, and hence, the transmittance of the incident light is increased (366 nm). This explanation was confirmed by measuring the diffuse reflection due to the silica gel under dry and wet conditions. Figure 2 shows the reflectance vs wavelength for a TLC plate under dry and wet conditions. It can be seen that the reflectance is lower under wet conditions as compared with dry conditions overall. Second, the transmitted light induces fluorescence of the glass, and the wet silica gel also allows this fluorescence emission to be transmitted and detected by the CCD camera. The TLD results shown in the subsequent section support this interpretation.

One of the goals of this study was to optimize the detection system in order to minimize the background contribution. Based on the above discussion, the black paper underneath the TLC plate and the bandpass filter successfully eliminated the effects of the glass stage and minimized the diffuse reflection due to the silica gel. In fact, regions 2 and 6 represent the signals obtained under the optimal conditions. Note that the intensities listed in Table 1 represent the sum of intensities from 38 pixels. Therefore, under

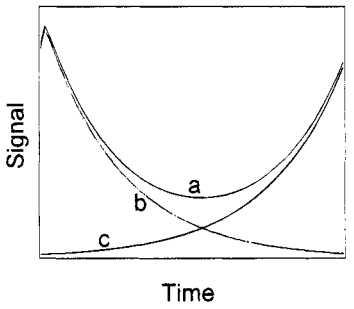

Figure 3. Background factors: (a) total background signal; (b) glass fluorescence signal; and (c) diffuse reflection due to the silica gel.

optimal conditions, the mean background signal detected by each pixel is only a few counts.

This study was also aimed at understanding the time-dependent background variations observed in the preliminary experiments. Curve a in Figure 3 represents the kinetic curve for a wet region under optimal conditions (black paper on the glass stage, and bandpass filter). Note that the extent of wetting of the silica gel changes with time due to evaporation. It appears that this kinetic curve can be decomposed into two factors, represented by curves b and c. A plausible model is that curve b is caused by changes in detected fluorescence due to evaporation of methanol, and curve c is the diffuse reflection due to the silica gel. The diffuse reflection increases as the wetting degree of a TLC plate decreases. During imaging, the wetting degree decreases because of evaporation. Therefore, curve c is assumed to represent this changing factor. As discussed above, the fluorescence from the glass substrate can not be removed since this background fluorescence wavelength is close to the emission maximum (440 nm) of the products monitored during the reaction between amino acids and OPA. Moreover, this fluorescence intensity varies with the degree of wetting. When the TLC plate becomes completely dry, no fluorescence is seen. Therefore, curve b represents this factor. This fluorescence is therefore not a serious interference. Although other fluorogenic derivatization reagents could be used to shift the detected signal away from this interference, the OPA derivatives used here provide us with the best chance of observing the different reaction rates required for kinetic resolution of overlapped zones.[10-12] Note that the variations in both background factors discussed above are related to wetting. They cannot be removed since the reaction between amino acids and OPA has to be carried out under wet conditions on the TLC plates. This requirement has been confirmed experimentally. On the other hand, evaporation during imaging is unavoidable. The evaporation process is hard to control and may vary from one experiment to another. Therefore, the background kinetics may exhibit slightly different profiles from Figure 3, but the overall kinetic trend for the background is always similar to curve a in Figure 3. However, this variable background does not prevent us from applying the TLC-KFD method to this specific system, since the chemometric method, TLD, has the capability of resolving the varying background factors from the analyte signal as long as the background factors are relatively consistent in behavior in all the sample lanes for one experiment. Mathematically, the TLD algorithm treats the changing background factors as additional components. An understanding of the background factors is very helpful in interpreting the output from the TLD algorithm.

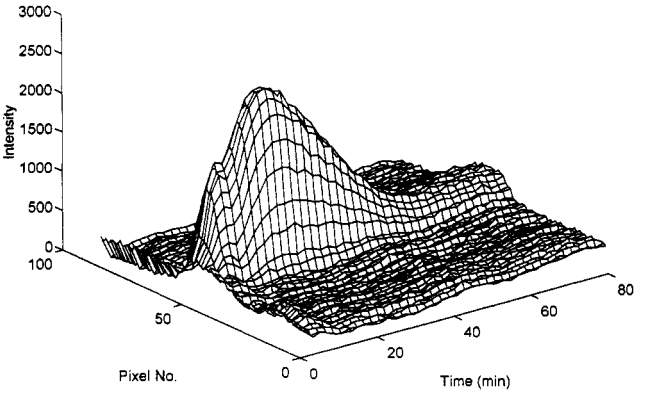

Figure 4. Experimental data for a representative mixture sample.

Experiments with Amino Acids. The experimental procedures were as described in the Experimental Section, but the detection system was optimized as described in the previous section with the bandpass filter in front of the CCD camera and the glass stage covered with black paper. Figure 4 shows a representative data matrix from a TLC-KFD experiment obtained for a single sample, expressed as fluorescence intensity as a function of elution distance (pixel number) and reaction time (min). For each experiment, nine samples were applied to the TLC plate. Therefore, a three-way data array can be constructed from these nine data matrices. Analysis of the three-way data array using the TLD algorithm yields three two-dimensional matrices, **X**, **Y**, and **Z**, as shown in Figure 5. These profiles describe the chromatographic behavior, kinetics, and relative contribution of each physical component, respectively. For our experimental data, it was found that the best results (i.e., the most chemically reasonable) were obtained when four components were assumed to be present. These components were identified as being due to the two background components, and the products of the glycine–OPA, and glutamine–OPA reactions, respectively. This interpretation was based on our understanding of the background factors and observation of the kinetic and elution behavior of the pure samples. Both glycine and glutamine were observed to react with OPA to form an intermediate fluorescent product, followed by a decay process. Glycine demonstrated faster reaction kinetics than glutamine. On the other hand, glycine was retained more than glutamine on the TLC plate under our separation conditions. The retardation factors (R_f) for glycine and glutamine are 0.33 and 0.35, respectively.

Calibration was performed using the two-dimensional matrix depicted in Figure 5C. Both the actual (standard) concentration and predicted concentration (from a linear least squares fit) for each sample are listed in Table 2. Small concentrations for glutamine in samples 1–3, and glycine in samples 4–6 were "predicted", when actually their concentrations should be zero. That is attributed to the experimental noise and the similarity of the kinetic and elution behaviors between glycine and glutamine. A summary of the calibration results from three different experiments is shown in Table 3. Experiments 1a and 1b were performed on the same day, and experiment 2 was done on a different day. It can be seen that the reproducibility of this experiment is adequate, but the accuracy and precision of the concentration prediction are only moderate. The reason may be that the two amino acids have relatively similar kinetic profiles and their elution distances are extremely close. In fact, their elution distances differ by only 5 pixels (0.7 mm on the TLC plate).

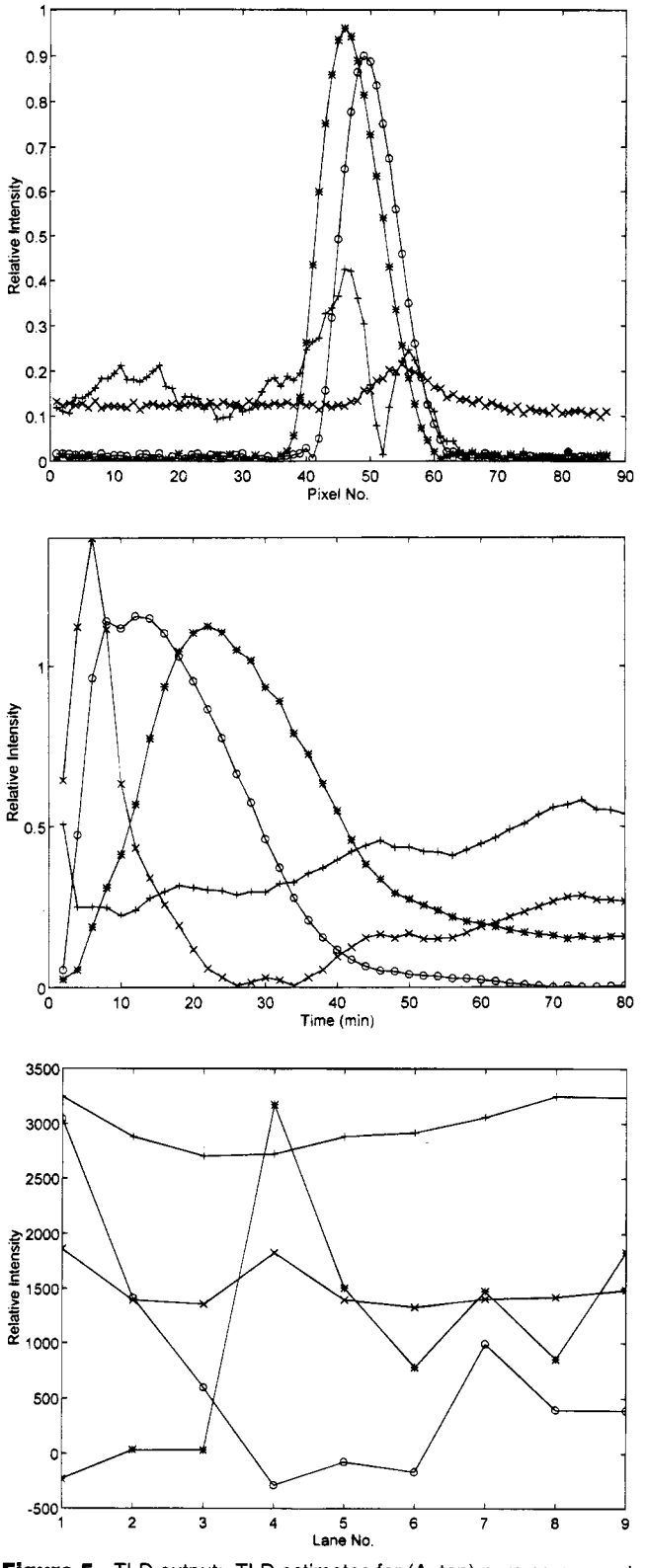

Figure 5. TLD output: TLD estimates for (A, top) pure component chromatograms, (B, center) pure component kinetic profiles, and (C, bottom) relative contributions from each component. (○): glycine; (*) glutamine; (+) and (×) background factors.

The resolution of the TLC chromatographic separation can be seen by comparing the chromatograms shown in Figure 6A–C. Generally, the prediction for the glutamine concentration is more accurate than for glycine. This may be due to the kinetic similarity of glycine with one of the background factors. Another reason may be that the reaction kinetics for glutamine are slower than those for glycine. Therefore, the kinetic data for glutamine span a longer time period and provide more information than the kinetic data for glycine.

Table 2. Calibration of Glycine and Glutamine Concentrations Using TLD

	glycine (mm)		glutamine (mm)	
sample no.	actual	predicted	actual	predicted
1	10.0	11.9	0	−0.8
2	5.0	5.4	0	0.1
3	2.5	2.3	0	0.0
4	0	1.1	10.0	10.2
5	0	0.3	5.0	4.8
6	0	0.6	2.5	2.5
7	5.0	3.8	5.0	4.8
8	2.5	1.5	2.5	2.7
9	4.0	1.5	6.0	5.9

Table 3. Relative Standard Deviation (RSD) of the Slopes of the Calibration Curves for Glycine and Glutamine

	% RSD	
expt no.	glycine	glutamine
1a	9.7	2.2
1b	11	12.8
2	11	7.1

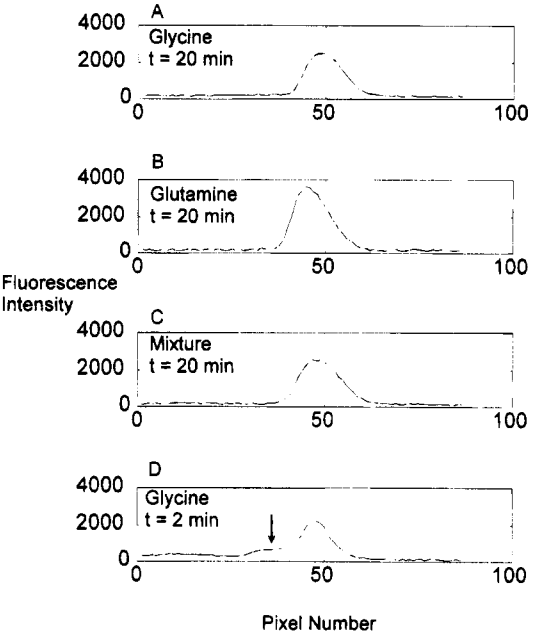

Figure 6. Chromatograms of (A) glycine at $t = 20$ min, (B) glutamine at $t = 20$ min, (C) glycine/glutamine mixture at $t = 20$ min, and (D) glycine at $t = 2$ min, where the arrow indicates the impurity.

Another problem with these experiments was that each sample spot had an extra spot appearing on the plate directly above the main spot. These extra spots exhibit relatively low fluorescence intensities and disappear within 10 min after the reaction starts. The chromatogram shown in Figure 6D is a chromatogram of glycine after only 2 min of reaction time, where the arrow points out the impurity. This may be another reason for poor accuracy in concentration prediction. Efforts have been put into identifying the source of these extra spots. Originally, these extra peaks were thought to be caused by the dipping process. Dipping was

performed along the mobile phase elution direction. This may cause migration of the sample spots on the TLC plate during the dipping process via a mechanism similar to the development of a TLC plate. Therefore, dipping the plates perpendicular to the mobile phase direction and dipping horizontally were also tried. The extra peaks showed up as before in these two experiments, so that the dipping method does not account for these spots. Other potential causes, such as the impurity of reagents, the presence of the preabsorbent band on TLC plates, and the type of development vessel have also been evaluated. Freshly purchased amino acids from Lancaster, as well as recrystallized samples, were tested. Application of the samples above the preabsorbent band, and the use of a full-sized development tank instead of the "sandwich"-type development chamber (which was used in our experiments generally), have also been tried. It was found that the extra bands showed up in all of the above experiments. The source of these bands remains an unresolved problem.

The TLC-KFD experiment was also performed with one pure sample of glycine and glutamine, and seven mixtures, as well as two pure samples of glycine and glutamine, plus five mixtures. It was found that when only one pure sample of each kind was present, the TLD results were very poor, but when two pure samples of each were applied, the TLD results were comparable to the results obtained for the experiment with three pure samples of each analyte (shown in Table 2). Since the kinetic and chromatographic behaviors of the two analytes, glycine and glutamine, are very similar, one pure sample of each analyte may not provide enough information for the TLD algorithm to resolve the two different species.

Computer Simulations. Computer simulations were performed in order to better understand the reasons for the results discussed above. First, we wanted to see whether the number of pure samples of each analyte included in the experimental design affected the capability of the TLD algorithm. The plots in Figure 5A,B and the concentration profiles for the two background components in Figure 5C were used to generate the synthetic data. Note that panels A–C of Figure 5 were obtained from the output of the TLD algorithm for real experimental data. The concentration profiles for the two analytes were varied to mimic different experimental designs for the calibration standards. It was found that when no noise was added to the simulated data, the TLD algorithm could reconstruct these data even for the case when no pure samples were present, as long as the concentration profiles for the two analytes were linearly independent. However, when Gaussian-distributed noise with a standard deviation of 80 (the standard deviation for experimental data) was added to the synthetic data (S/N = 51), the output from the TLD program was not consistent with the profiles used to construct the data. Therefore, it was concluded that noise is one of the factors that affects the capability of TLD program. However, at the same noise level, the TLD algorithm reconstructed the synthetic data with one pure sample of each analyte very well. As mentioned before, the TLD program did not provide good calibration curves for the real experimental data that had only one pure sample of each component. The reason may be that, in the actual experiments, migration and spreading of the sample spots during imaging might occur, and the interaction between the two analytes might affect the kinetic and chromatographic behavior of each analyte. These factors were not included in the simulated experiment. This is probably also the reason that the calibration curves for the experimental data with three pure samples were not significantly better than the results obtained when two pure analyte spots were present. On the basis of the above computer simulations and evaluation of the experimental data, it is recommended that improving the S/N ratio, minimizing migration and spreading of sample spots during imaging, and application of as many pure samples of each analyte as possible on each TLC plate would improve the quality of the calibration obtained using the TLD algorithm.

The TLD method requires that physical factors be linearly independent in at least two of the three directions. These two directions are considered to be more informative than the third one (referred to as the *z* direction). We would like to know how this requirement affects our experimental design. Our TLD program was designed to allow us to assign the least informative direction for our experimental data. Therefore, there are three ways to analyze our experimental data using TLD, since any one of the three directions can be assigned as the least informative direction. In the following computer simulations, the synthetic data sets were analyzed using all the three methods. For this TLC-KFD approach, the real physical factors are the kinetic, chromatographic, and concentration variations of glycine, glutamine, and the background factors. For this next set of computer simulations, the data were generated the same way as described above, including noise, unless stated differently. Through our computer simulations, we found that the TLD could not reconstruct the profiles used to generate the data for the following three cases: (1) when the concentration profiles for the two analytes used to synthesize the data were linearly dependent on each other, but the kinetic and chromatographic curves for the two analytes were different, (2) and (3) when either the kinetic or the chromatographic profiles for the two analytes were the same, but the concentration profiles were linearly independent. When the chromatographic and kinetic matrices resulting from the TLD algorithm match the original curves used to generate the synthetic data, and a linear calibration curve is obtained, the TLD is considered to reconstruct the original data. From cases 2 and 3, it is seen that the concentration composition direction is generally the least informative under our experimental conditions. This is due to the fact that, along the concentration composition direction, there are the fewest data points (only nine data points). For the glycine and glutamine experiment, the kinetic and chromatographic behaviors of the two analytes are not the same. Therefore, the restriction imposed by the TLD algorithm on our experiment design is that the concentrations of glycine and glutamine should not be linearly dependent with one another in the nine samples. This can be avoided in our experimental design, at least for known calibration samples.

CONCLUSIONS

It has been demonstrated that the TLC-KFD method combined with the TLD algorithm is suitable for the determination of two overlapped amino acids, glycine and glutamine. The capabilities and limitations of the TLD algorithm in this application have been analyzed with computer simulations. The glycine/glutamine two-component model mixture represents the case of highly overlapped chromatographic peaks and high similarity in kinetic behavior. On the basis of our experimental results for glycine/glutamine mixtures, and the computer simulations, it can be concluded that, for other unresolved amino acid–OPA derivative combinations, if the differences in chromatographic separation and

reaction kinetics are enhanced compared with our test mixture system, and the S/N ratio could be maintained, the reliability of the established method should be improved upon. Our computer simulation results provide guidelines not only for the glycine/glutamine model system but also for separation of other classes of unresolved analytes using a similar approach.

It is worth mentioning that quantitative analysis with the TLD method can be achieved without making a priori assumptions about the specific characteristics of the resulting kinetic and chromatographic profiles for each individual component. This is a great advantage in the determination of two overlapped species using TLC-KFD approach, since in these experiments the background exhibits kinetics and the kinetic behavior of the background and the analytes may not be reproduced exactly from one experiment to another. Establishing a kinetic model for the different components mathematically or experimentally is almost impossible. Considering these difficulties with the TLC-KFD method, the TLD algorithm is nearly a perfect data analysis method for our experiment. Compared with "hard modeling" approaches, the TLD method imposes many fewer restrictions on experimental conditions, but requires data from multiple sample lanes for analysis. Calibration can be achieved through evaluation of the output from the TLD program directly. Compared with hard modeling methods, TLD takes much less time for data analysis.[14] It takes about 2 min for TLD to analyze one set of experimental data of nine samples vs about 45 min for a hard modeling method.[14] Generally, this TLD method is applicable to any multidimensional chromatographic technique where the signals combine in a linear fashion.

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