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The chemometric resolution and quantification of overlapped peaks form comprehensive two-dimensional liquid chromatography

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Abstract

The chemometric resolution and quantification of overlapped peaks from comprehensive two-dimensional (2D) liquid chromatography (LC × LC) data are demonstrated. The LC × LC data is produced from an in-house LC × LC analyzer that couples an anion-exchange column via a multi-port valve with a reversed-phase column connected to a UV absorbance detector. Three test mixtures, each containing a target analyte, are subjected to partial LC × LC separations to simulate likely cases of signal overlap. The resulting unresolved target-analyte signals are then analyzed by the standard-addition method and two chemometric methods. The LC × LC analyses of a test mixture and its corresponding standard-addition mixture results in two data matrices, one for each mixture. The stacking of these two data matrices produces a data structure that can then be analyzed by trilinear chemometric methods. One method, the generalized rank annihilation method (GRAM), uses a non-iterative eigenvalue-based approach to mathematically resolve overlapped trilinear signals. The other method, parallel factor analysis (PARAFAC), uses an iterative approach to resolve trilinear signals by the optimization of initial estimates using alternating least squares and signal constraints. In this paper, GRAM followed by PARAFAC analysis is shown to produce better qualitative and quantitative results than using each method separately. For instance, for all three test mixtures, the GRAM-PARAFAC approach improved quantitative accuracy by at least a factor of 4 and quantitative precision by more than 2 when compared to GRAM alone. This paper also introduces a new means of correcting run-to-run retention time shifts in comprehensive 2D chromatographic data.
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Keywords: Liquid chromatography, two-dimensional; Complex mixtures; GRAM; PARAFAC; Three-way data; Trilinear

1. Introduction

Comprehensive two-dimensional (2D) liquid chromatography (LC × LC) is well suited for the separation and analysis of semi and non-volatile compounds in complex mixtures. Like comprehensive 2D gas chromatography (GC × GC), LC × LC's enhanced peak capacity provides a greater separation space to resolve chemical components. In the 1990s, several papers used the enhanced separation power of LC × LC to successfully analyze complex mixtures that were primarily biological [1–9]. Recent LC × LC papers have expanded the use of LC × LC to other sources of complex mixtures such as food products [10–17]. However, unlike GC × GC the chemometric analysis of LC × LC data has not been heavily pursued, even though it would benefit LC × LC as it has GC × GC. For instance, several papers have successfully applied chemometric methods to GC × GC data to reveal hidden chemical information [18–25]. The goal of the chemometric methods discussed in this paper is to mathematically reveal and quantify overlapped signals, which inevitably occur in very complex mixtures. The generalized rank annihilation method (GRAM) is one chemometric method discussed in this paper. It has been successful at resolving and quantifying severely overlapped GC × GC peaks [18–20]. It has also been applied to overlapped signals from other hyphenated chromatographic methods producing structured 2D data [26–32]. Another chemometric applied to GC × GC data is known as PARAFAC. It has been used to successfully resolve overlapped signals in data from GC × GC—time-of-flight mass spectrometry (GC × GC—TOFMS) [24,25]. Reference [25] demonstrated that coupling PARAFAC with trilinear decomposition (TLD), which is a method similar to GRAM, gave better results than TLD alone. Better signal resolution

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with PARAFAC agrees with the findings of other authors [33,34].

In this paper, chemometric resolution and quantification of unresolved LC × LC data is applied for the first time. The LC × LC data was obtained using an in-house LC × LC analyzer that couples an ion-exchange (IC) column and a reversed phase (RP) column with a single-wavelength UV absorbance detector. The work described in this paper is similar to the first application of GRAM to GC × GC flame-ionization data [18]. However, PARAFAC is used to improve upon the GRAM results. Better signal resolution and quantitative results are gained when GRAM and PARAFAC are coupled as opposed to individually. In addition, a new method for correcting run-to-run retention time shifts in 2D data is introduced.

1.1. Chemometric methods

GRAM is a non-iterative eigenvalue-based method used to resolve and quantify the bilinear signals of compounds that vary in concentration between two data matrices. LC × LC signals for the most part are bilinear. That is, an LC × LC signal for an analyte can be mathematically represented by the product of two vectors, each representing that analyte's signal from one HPLC column. One of the two data matrices subjected to GRAM analysis is called the sample data matrix. It contains the signal for the target analyte from the LC × LC analysis of a test mixture. The other data matrix is called the standard data matrix. It contains the signal for the target analyte from the LC × LC analysis of a standard-addition mixture. That mixture is made by spiking a known amount of target analyte into a portion of the test mixture. Different versions of the GRAM algorithm exist [35–38]. The GRAM algorithm used is based on the one from Wilson et al. [38]. The only input required for GRAM analysis is an estimate of the number of different component signals present in the data matrices. Several methods exist for estimating the number of component signals [39–43]. The data requirements for the GRAM analysis of LC × LC data are identical to those listed for GC × GC [18]. The key requirement for GRAM analysis is that the two data matrices (sample and standard) must be trilinear. In other words, when the data matrices are stacked to make three-way data (i.e., a cube of data), the bilinear signals in common between the data matrices must match perfectly excluding signal intensity. For two stacked LC × LC data matrices, three vectors represent the trilinear signal of an analyte. One vector is the normalized analyte signal for one column and another is the normalized analyte signal for the other column. The third vector represents the relative amount of the analyte in the two data matrices.

PARAFAC is an iterative three-way method that resolves overlapped signals through the optimization of initial estimates using alternating least squares (ALS) and signal constraints. Non-negative and uni-modality are the standard signal constrains. PARAFAC as a chemometric method is well documented in literature [34,44]. The initial estimates used by PARAFAC can come from an eigenvalue-based method (e.g., GRAM), random values, random orthogonalized values, and singular values. PARAFAC, like GRAM, is a trilinear-based method that calculates the three vectors that represent the trilinear signal of an analyte in two stacked LC × LC data matrices. Once each vector is obtained, the resolved signal for that analyte can be reconstructed using the three vectors. In this paper, the target analyte's concentration in a given test mixture is calculated from the vector representing the relative amount of the analyte in the test mixture and its standard-addition mixture. The target analyte's actual concentration in the test mixture is calculated using the known concentration of the spiked analyte in the standard-addition mixture.

1.2. Retention time alignment

Run-to-run retention time shifts are a main cause of non-trilinearity in three-way chromatographic data [30,31,45]. Hence, retention-time shifts need to be corrected prior to chemometric analysis by GRAM or PARAFAC. Rank alignment has been successful at correcting retention-time shifts in three-way data [19,28,31,46]. It is an iterative 2D technique that shifts the bilinear signals in one matrix relative to the other until a minimum in the percent residual variance is reached [47]. At that point, the bilinear signals in common between the two stacked data matrices are aligned. Unfortunately in some cases rank alignment did not correct the run-to-run retention time shifts of the LC × LC data. Therefore, an alternative alignment method was developed. This new retention time alignment method, however, is beyond the scope of this paper and will not be discussed in depth. It involves incrementally applying a time-shift correction to the LC × LC data followed by GRAM and then PARAFAC analysis. The right shift provides the smallest sum of squares or best data fit between the PARAFAC data and the raw data. Simulations have shown that for trilinear methods the fit between raw data and processed data improves as the degree of retention-time shift decreases [45].

2. Experimental

2.1. Test mixtures

Three aqueous test mixtures, A–C, were prepared. The solutes were *p*-chlorobenzoic acid (99% Aldrich Chemical Co., Milwaukee, WI, USA), benzoic acid (99.5% Aldrich), uracil (99+% Acros Organics, Morris Plains, NJ, USA), pyruvic acid (99+% Acros), maleic acid (99% Acros), fumaric acid (99% Aldrich), and phenyl phosphoric acid (98% Aldrich). The water used was purified by a Milli-Q system (Millipore Corp., Milford, MA). Mixture A contained *p*-chlorobenzoic acid (50.0 mg/mL or ppm) and benzoic acid (50.0 ppm). Mixture B contained uracil (5.00 ppm) and pyruvic acid (200.0 ppm). Mixture C contained fumaric acid

(2.50 ppm), maleic acid (25.0 ppm), and phenyl phosphoric acid (100.0 ppm). A standard-addition mixture for each of the three test mixtures was also made by spiking a known amount of the target analyte into an aliquot of each test mixture. The target analytes for mixtures A–C, were *p*-chlorobenzoic acid, uracil, and fumaric acid, respectively. The concentrations for the spiked target analytes in their respective standard-addition mixtures were 4.96 ppm, 50.0 ppm, and 1.24 ppm for *p*-chlorobenzoic acid, uracil, and fumaric acid, respectively. The dilution factor for the target-analyte originally in each mixture was calculated to be 0.9926, 0.9000, and 0.9926 for *p*-chlorobenzoic acid, uracil, and fumaric acid, respectively.

2.2. Instrumentation

Fig. 1 depicts a schematic of the LC × LC analyzer used to generate the data for this paper. The analyzer consists of a GP 40 gradient pump (Dionex Corp., Sunnyvale, CA, USA), which pumps the IC eluent through an injector (Rheodyne, Cotati, CA, USA) with a sample loop and then through a Dionex IonPac AS-11 4 × 250 mm anion-exchange column with 13 μm particles. The IC eluent then flows through a Dionex 4 mm anion self-regenerating suppressor (ASRS). The ASRS is typically used to lower the conductivity of the IC eluent prior to entering a conductivity detector. However, for the purpose of our study, the ASRS was utilized to lower the pH from approximately 12 to 6 to prevent degradation of the RP column. The slightly acidic IC eluent then flows through a 10-port two-position, high-pressure valve (VICI, Valco Instruments Co. Inc., Houston, TX, USA). The 10-port valve was converted into an eight-port valve by connecting two ports via a short segment of stainless steel tubing. The valve is actuated by a microelectric actuator capable of switching between the two valve positions in 70 ms. The valve is fitted with two identical stainless steel sample loops. Each time the valve switches position, one loop is filled with IC eluent while the content of the second loop is pumped to the RP column. That portion of the IC eluent that is not transferred is sent to waste. An isocratic LC-6A pump (Shimadzu America Inc., Columbia, MD, USA) is used to pump the RP eluent. The RP column is either a Platinum 7 × 33 mm EPS C-18 column with 1.5 μm particles and 100A pores (Alltech Associates Inc., Deerfield, IL, USA) or a Synergi 4 × 10 mm Fusion C-18 column with 2 μm particles and 80A pores (Phenomenex, Torrance, CA, USA). Each RP column had a pH operating range of 1–7. The RP eluent is fed into a Shimadzu SPD-6A absorbance detector consisting of an 8 μL flowcell with a 10 mm pathlength. The detector was operated at 220 nm with a cycle time of 20 Hz. As shown in Fig. 1, the majority of the LC × LC components is contained in an oven (Dionex LC 30) set at a constant 27 °C. A personal computer running a program written in LabVIEW 5.0 (National Instruments, Austin, TX, USA) collects the detector's analog signal at a rate of 20 pts/s via a data acquisition (DAQ) board (model AT-MIO-16E-2, National Instruments). The LabVIEW program also controls the valve cycle time via the DAQ board. Both the valve cycle and data collection are initiated when the injector is manually switched from load to inject.

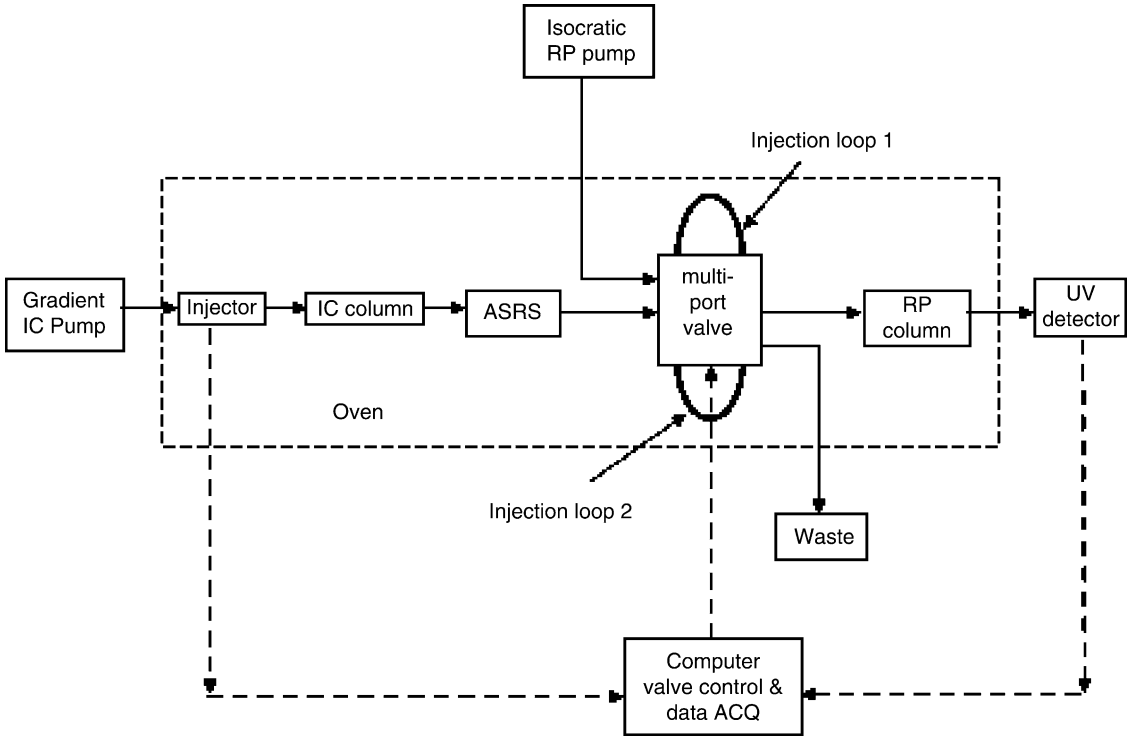

Fig. 1. Schematic of LC × LC instrumentation.

2.3. Instrumental parameters

LC × LC parameters such as mobile phase composition were adjusted to obtain a different degree of signal overlap for each mixture (A–C). The LC × LC parameters was the same between a given mixture and its corresponding standard-addition mixture. For mixture A, the IC eluent was 60 mM aqueous NaOH and was pumped at 1.0 mL/min. A 50-μL injection loop was used for the IC separation. The RP eluent was 65% (v/v) acetonitrile in 5 mM aqueous HCl and was pumped at 3.5 mL/min. The RP column was the Platinum C-18 (see above). The valve cycle time was 6 s and the valve injection loops were 30 μL in volume. Under these conditions, 30% of a sample injected into the IC column was transferred to the RP column. For mixture B, the IC eluent was 20 mM aqueous NaOH and was pumped at 1.0 mL/min. A 150-μL injection loop was used. The RP eluent was 5% (v/v) acetonitrile in 10 mM aqueous HCl and was pumped at 3.0 mL/min. The RP column was the Synergi C-18 (see above). The valve cycle time was 4 s and the valve injection loops were 30 μL in volume. Forty-five percent of the sample volume was transferred to the RP column. For mixture C, the IC eluent was 40 mM aqueous NaOH and was pumped at 1.0 mL/min. All other parameters were the same as those for mixture B. Replicate LC × LC runs were made for each mixture (A–C) and its standard-addition mixture.

2.4. Data analysis

For each of the LC × LC runs the collected data was transferred as a text file to Matlab 6.1 R12 (The Mathworks Inc., Natick, MA). In Matlab, the raw data was first boxcar averaged to 4 pts/s for mixture A and 5 pts/s for mixtures B and C. The data for each run was then converted into a matrix such that each row of the matrix represented a fixed time on the IC column and each column of the matrix represented a fixed time on the RP column. GRAM and PARAFAC analyses were then performed on a given sample and standard data matrix. The Matlab code for the GRAM algorithm came from the PLS Toolbox (Eigenvector Research, Inc., WA, USA). The Matlab code for PARAFAC algorithm came from the N-way Toolbox 2.10 [48]. For the GRAM analysis of mixture A data, two was entered as the expected number of component signals. The rank alignment parameters were two for the number of component signals and 1 data point for the expected maximum retention time shift for both columns. Data-point interpolation was used to determine retention-time shifts less than a data point [47]. PARAFAC parameters were: (1) two for the number of components, (2) non-negativity for the IC and RP dimensions, (3) 1×10^{-6} for the convergence criterion, and (4) 1000 for the maximum number of iterations. For mixture B, the number of component signals entered into both GRAM and PARAFAC was two. Rank alignment was not used because it did not work for some sample-standard combinations. In those cases, the GRAM and PARAFAC results were worse with rank alignment than without. The PARAFAC parameters were identical to those of mixture A, however, both non-negativity and uni-modality were applied to the IC and RP dimensions. For mixture C, the number of components entered was three. Again, rank alignment was not favorable. However, some retention-time alignment was required due to erroneous values (i.e., imaginary numbers) for the concentrations without alignment. Unless specifically stated, each mixture's chemometric parameters were kept constant.

3. Results and discussion

3.1. Chemometric analysis

Fig. 2A–C depict representative sample data matrices for mixtures A–C. They also illustrate one of the problems addressed in this paper. That is, the impossibility of obtaining reliable peak measurements for quantification using standard methods when a target analyte's signal is as badly interfered as those in Fig. 2A–C. Fortunately, unresolved LC × LC signals can be mathematically resolved and quantified by either GRAM or PARAFAC as long as the LC × LC data is sufficiently trilinear. For our study, two LC × LC data matrices make up the LC × LC data inputted into either GRAM or PARAFAC. The first is a sample data matrix (e.g., see Fig. 2A) and the other is a standard data matrix (e.g., see Fig. 3A). Fig. 3A–C depict representative standard data matrices for mixtures A–C. Each standard data matrix is the LC × LC separation of the standard-addition sample produced by spiking a known amount of the applicable target analyte into mixtures A–C. For this paper, GRAM analysis of a sample data matrix with a standard data matrix is always performed first. If the sample and standard data matrices as a whole are trilinear, then GRAM analysis provides an accurate representation of the target analyte's resolved LC × LC signal and its relative concentration in the mixture. Previous GC × GC signals have been accurately resolved and quantified by GRAM [18]. However, for the LC × LC data, we found that not all signals were accurately resolved and quantified by GRAM.

Fig. 4A and B depicts the GRAM-predicted LC × LC signals for *p*-chlorobenzoic acid and benzoic acid in mixture A. It is clear that the LC × LC reserved phase signal for benzoic acid is not accurate because a good portion of its signal dips significantly below the signal baseline. It is well known that chromatographic peaks are non-negative, that is, they do not dip below a baseline having a signal average of zero. Along the anion exchange dimension, the signal profiles (i.e., width and shape) for both acids are typical for LC peaks. More importantly, their signal shapes are non-negative and each has one obvious maximum (i.e., unimodal). Because the signal profile for the *p*-chlorobenzoic acid along the reversed phase column is also non-negative and unimodal, its overall LC × LC signal is probably an accurate representation of the true *p*-chlorobenzoic acid signal. This is good because *p*-chlorobenzoic acid is the target analyte for mix-

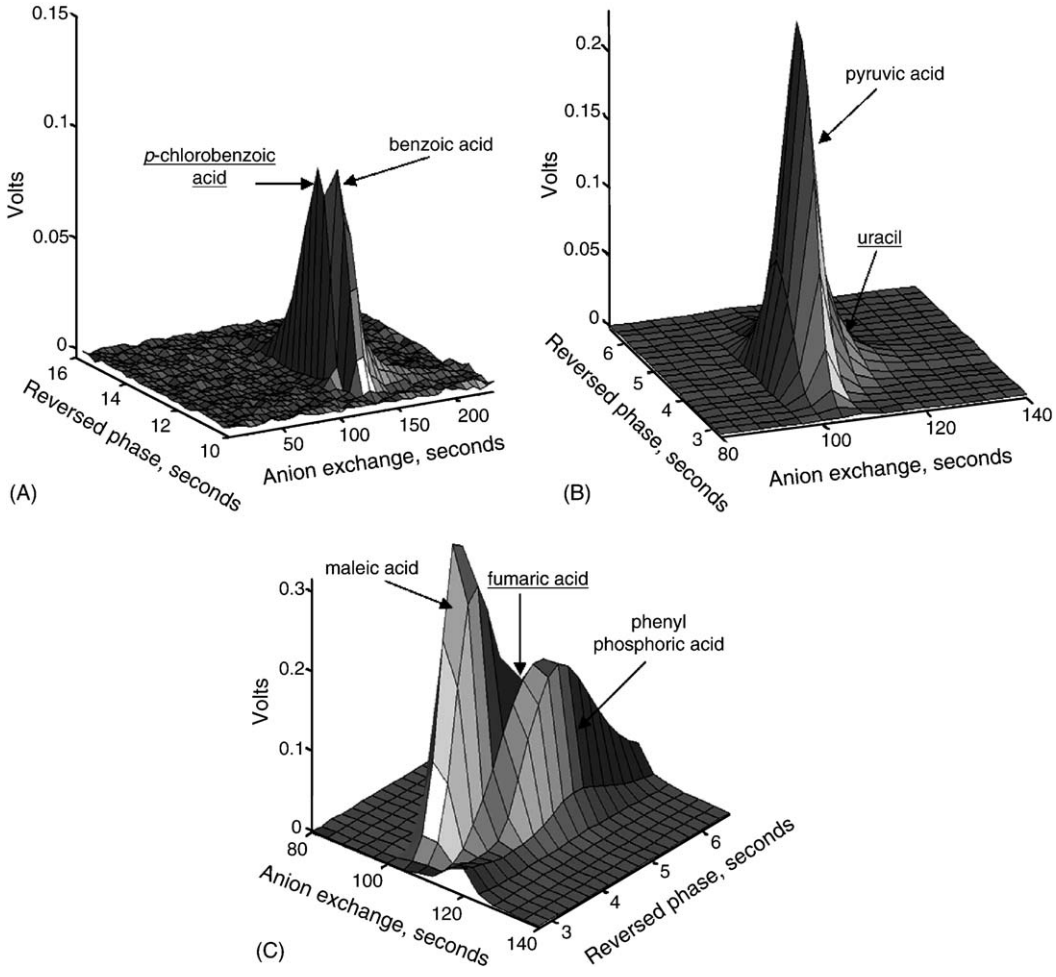

Fig. 2. Three 3D plots each depicting one representative sample data matrix. Each sample data matrix is the LC × LC separation of mixtures A (A), B (B), or C (C). Each sample data matrix contains the unresolved signal of a target analyte (underlined) in the presence of one or more interfering signals.

ture A. However, its signal height cannot be correct because it must compensate for the negative dip of the benzoic acid signal along the reversed phase column. This is because the sum of the GRAM signals for both acids must reconstruct their combined signals in the original sample data matrix. This same problem occurs for the standard data matrix. Therefore, based on the poor signal profile of benzoic acid, one would expect GRAM to give an inaccurate concentration for *p*-chlorobenzoic in mixture A. Indeed, for the GRAM signals shown in Fig. 4, the predicted concentration for the 50.0 ppm *p*-chlorobenzoic is 57.3 ppm. This significant quantitative bias is substantially reduced by PARAFAC analysis initiated by the GRAM data.

Fig. 5A and B depict the PARAFAC-predicted LC × LC signals for *p*-chlorobenzoic acid and benzoic acid in mixture A. The PARAFAC signals are obtained by performing PARAFAC analysis on the sample and standard data matrices as illustrated in Fig. 2A and B. The GRAM signals previously obtained for these two data matrices (see Fig. 4) are used to initiate PARAFAC analysis. Mitchell and Burdick previously used a similar approach when analyzing simulated three-way data [33]. They used the response profiles produced by an eigenanalysis-based method similar to GRAM to initiate PARAFAC analysis. They frequently obtained better signal resolution by coupling the eigenanalysis-based method with PARAFAC than using the eigenanalysis-based method alone. The PARAFAC algorithm can also be initiated using randomly generated profiles or singular value vectors [44,48]. However, for mixture A data, using random profiles produced PARAFAC results that were meaningless while SVD vectors produced results that were better but not satisfactory. The analysis times for these approaches were significantly longer, especially for the random generator profiles. The same results were observed in the data analysis for mixtures B and C.

In Fig. 5A and B, the PARAFAC LC × LC signals for both acids are unimodal and non-negative along both columns. This gives confidence that the mathematical resolution of the overlapped LC × LC peaks is accurate. Indeed, as shown in Fig. 6A and B, the PARAFAC signal for *p*-chlorobenzoic acid matches quite well with the resolved signal of *p*-chlorobenzoic acid, which was obtained by the

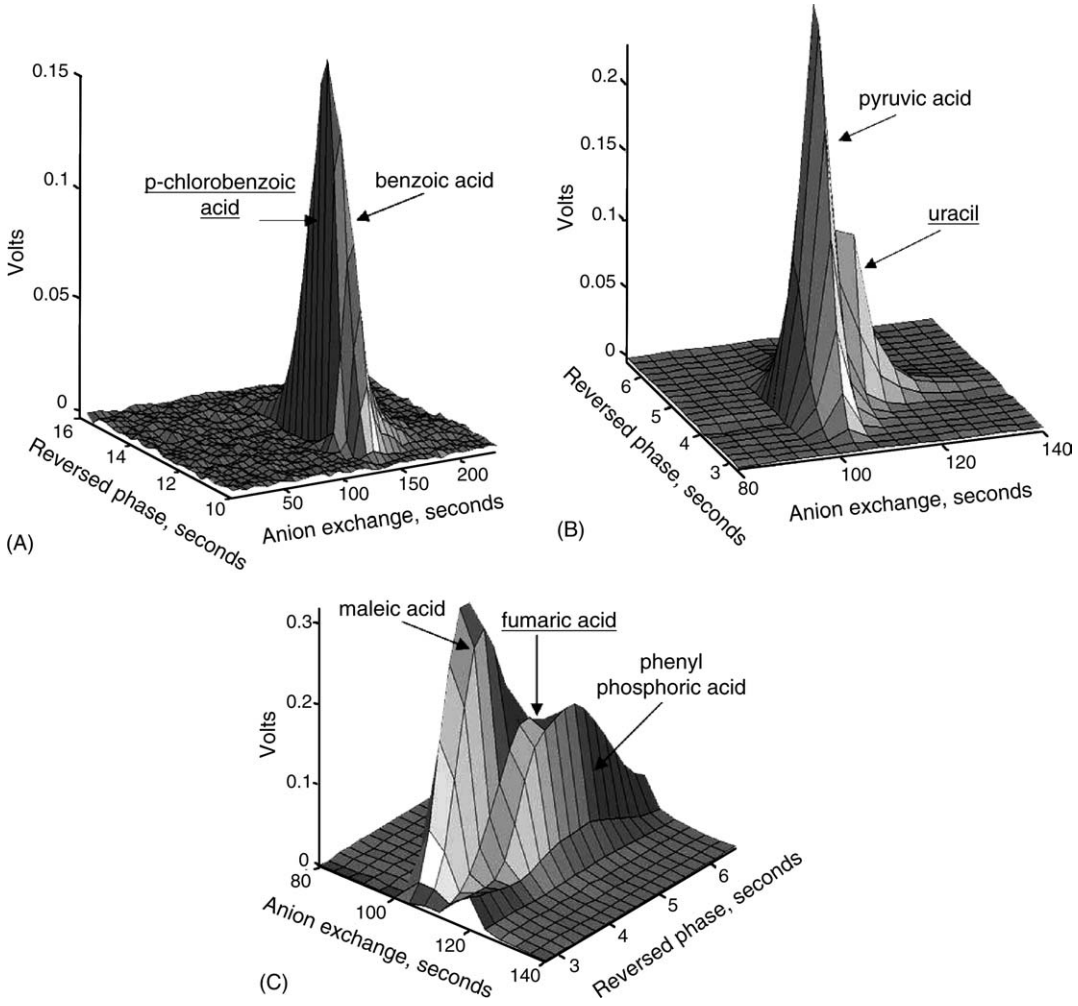

Fig. 3. Three 3D plots each depicting one representative standard data matrix. Parts (A)–(C) are the LC × LC data obtained for the standard addition of the target analyte (underlined) into mixtures A–C, respectively.

LC × LC analysis of a pure 50 ppm *p*-chlorobenzoic acid solution. Therefore, it comes to no surprise that the predicted PARAFAC concentration of 49.7 ppm for *p*-chlorobenzoic acid is very close to the expected value of 50.0 ppm. As shown in Table 1 column 2, the average concentration for *p*-chlorobenzoic acid in mixture A is noticeably more accurate and precise for PARAFAC than GRAM. The mean concentration is based on four chemometric analyses. Each of the four chemometric analyses involved a different sample data matrix and standard data matrix that came from performing two replicate LC × LC runs of both mixture A and its standard-addition mixture. Each unique sample-standard combination was first analyzed by GRAM and followed by PARAFAC analysis initiated by the GRAM results.

Table 1
GRAM and PARAFAC quantitative results for target analytes in mixtures A–C

Chemometric method	Mixture A (chlorobenzoic 50.0 ppm)[a] ($n^b = 4$) predicted conc.[c], bias[d], RSD[e]	Mixture B (uracil 5.00 ppm)[a] $n^b = 9$ predicted conc.[c], bias[d], RSD[e]	Mixture C (fumaric 2.50 ppm)[a] $n^b = 4$ predicted conc.[c], bias[d], RSD[e]
GRAM	57.8 ppm, 16%, 9.9%	11.3 ppm, 130%, 66%	9.14 ppm, 265%, 74%
PARAFAC	51.2 ppm, 2.5%, 4.1%,	5.14 ppm, 2.8%, 21%	4.16 ppm, 66.0%, 12%

[a] Target analyte and its true concentration in mixture.
[b] The number of replicate chemometric analyses performed for each chemometric method. Each analysis was a different combination of one replicate mixture data matrix and one replicate standard data matrix.
[c] Mean of n concentrations for the target analyte as determined by each chemometric method. For mixtures B and C, the concentration means for GRAM and PARAFAC are based on $n - 1$ because of an outlier.
[d] (Predicted conc. − true conc.)/true conc.
[e] Relative standard deviation.

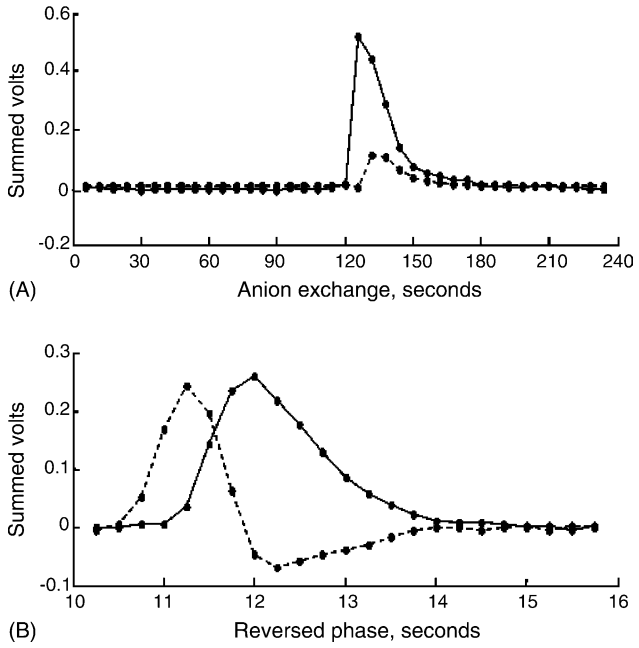

Fig. 4. Overlays of the GRAM-resolved LC × LC signals for benzoic (dashed line) and *p*-chlorobenzoic (solid line) acids in mixture A. Parts (A) and (B) depict the overlaid LC × LC signals after each have been summed onto the anion exchange column and the reversed phase column, respectively. The signals were obtained by the GRAM analysis of the sample data matrix depicted in Fig. 2A and the standard data matrix depicted in Fig. 3A.

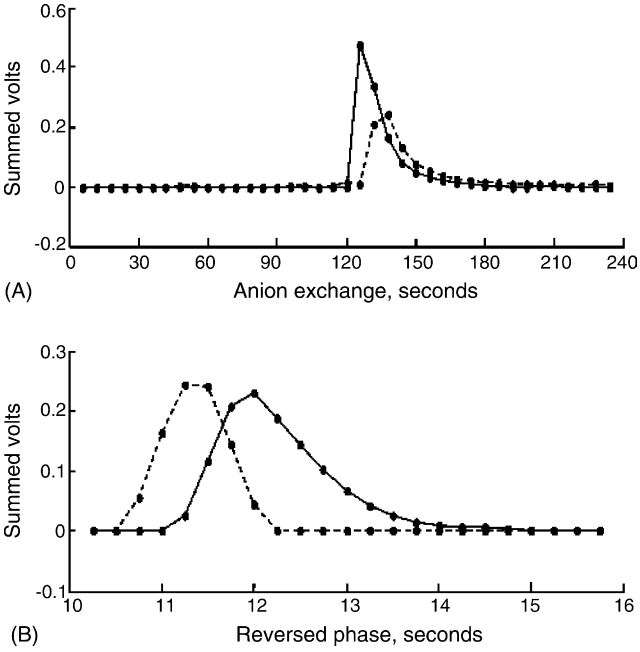

Fig. 5. Overlays of the PARAFAC-resolved LC × LC signals for benzoic (dashed line) and *p*-chlorobenzoic (solid line) acids in mixture A. Parts (A) and (B) depict the overlaid LC × LC signals after each have been summed onto the anion exchange column and the reversed phase column, respectively. The signals were obtained by the PARAFAC analysis of the GRAM signals depicted in Fig. 4.

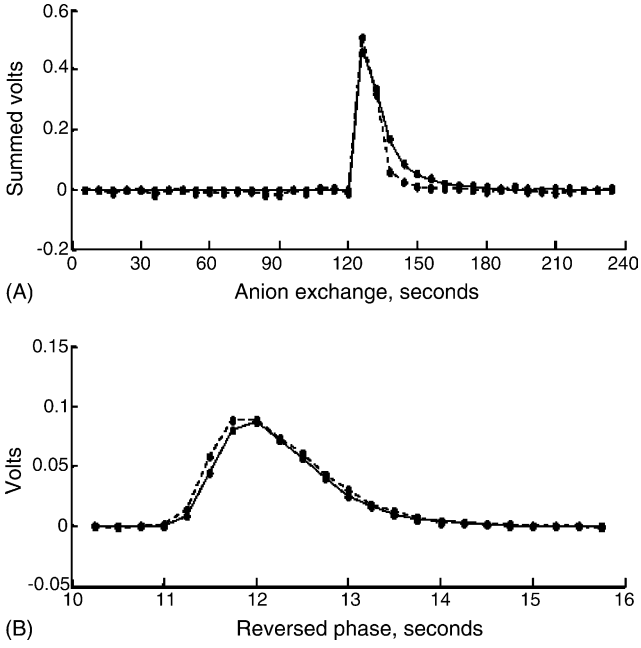

Fig. 6. Overlays of the PARAFAC-resolved LC × LC signal (solid line) and the actual resolved LC × LC signal (dashed line) for *p*-chlorobenzoic acid in mixture A. Part (A) depicts each LC × LC signal after being summed onto the anion exchange column. Part (B) depicts a slice for each LC × LC signal that corresponds to the reversed-phase separation taking place at 126 s.

For mixture A data, the lower accuracy and precision of the GRAM results as compared to the PARAFAC results is likely due to the lack of trilinearity in the LC × LC data. Previous work on GC × GC data has shown that run-to-run retention-time variability is a major cause of non-trilinearity [19]. Therefore, prior to GRAM and PARAFAC analyses, each sample-standard data set from mixture A was subjected to 2D rank alignment. Interestingly, only retention-time shifts along the reversed phase column were detected and hence corrected. However, even after retention-time alignment, the data was not sufficiently trilinear as indicated by mediocre GRAM results. The cause of this non-trilinearity is not obvious and therefore difficult to ascertain. Regardless of the cause, PARAFAC is better able to deal with data that is not fully trilinear because unlike GRAM it takes into account signal constraints. GRAM assumes the original data is perfectly trilinear. Unfortunately, if the real data is not perfectly trilinear, the GRAM representation of the signals can be significantly off the mark. For instance, GRAM may represent the LC × LC signal for the target analyte with a significant negative dip. However, that signal and the GRAM signals for the remaining components are the best trilinear representation of the original data. In contrast, PARAFAC will find trilinear signals that also meet signal constraints such as non-negativity. Therefore, GRAM outputs trilinear signals that fit the original data better than PARAFAC while not necessarily representing the true signal shapes. PARAFAC, on the other hand, outputs trilinear signals that more closely represent the true shapes of the LC × LC signals at the expense of accu-

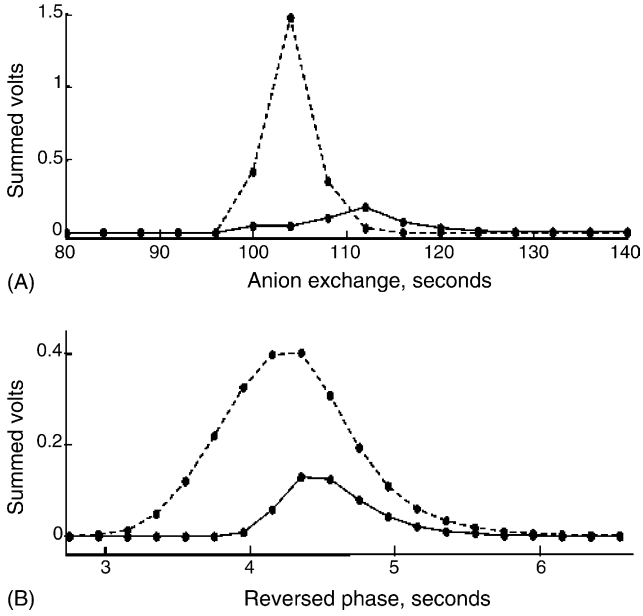

Fig. 7. Overlays of the PARAFAC-resolved LC × LC signals for pyruvic acid (dashed line) and uracil (solid line) in mixture B obtained using the sample and standard data matrices shown in Figs. 2B and 3B. Parts (A) and (B) depict the overlaid LC × LC signals after each have been summed onto the anion exchange column and the reversed phase column, respectively.

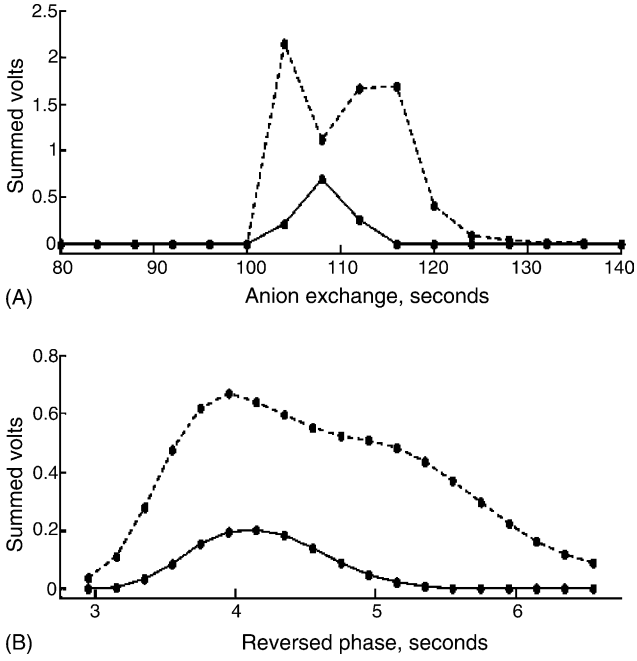

Fig. 8. Overlays of the PARAFAC-resolved LC × LC signal for fumaric acid (solid line) and the combined PARAFAC LC × LC signals for maleic and phenyl phosphoric acids (dashed line) in mixture C. Parts (A) and (B) depict the overlaid LC × LC signals after each have been summed onto the anion exchange column and the reversed phase column, respectively. The signals were obtained using the sample and standard data matrices shown in Figs. 2C and 3C.

rately fitting the original data. Apparently, obtaining a good representation of the true signal shapes is the reason why PARAFAC produces better quantitative results than GRAM (see Table 1, column 2 for mixture A). This statement is also supported by a study discussed in reference [45]. Using simulated three-way data, the study found that a trilinear-based algorithm with signal constraints produces lower quantitative errors than one based on a strictly trilinear approach.

As shown for the LC × LC data from mixture A, coupling GRAM and PARAFAC produces better qualitative and quantitative results than are generally achieved with GRAM alone. In order to validate this statement further, different LC × LC data from mixtures B and C were tested. Depicted in Fig. 7A and B are the LC × LC signals for the uracil and pyruvic acid present in mixture B as determined by the GRAM-PARAFAC approach. While not shown for brevity, the signals for both components match quite well with their true signals determined by performing an LC × LC analysis of each component separately. A total of nine sample-standard combinations were analyzed via the GRAM-PARAFAC approach. The data for each combination originated from performing three replicate LC × LC runs of both mixture B and its standard-addition mixture. Out of the nine sample-standard combinations that were analyzed, PARAFAC analysis improved upon most of the signal profiles provided by GRAM. In particular, PARAFAC produced realistic signal profiles with correct retention times. This again resulted in PARAFAC having better precision and accuracy for the predicted concentration of uracil than GRAM (see Table 1, column 3). Only one of the nine sample-standard combinations resulted in signal profiles for GRAM and PARAFAC that was implausible. The predicted GRAM and PARAFAC concentrations for this particular sample-standard combination were determined to be clear outliers based on Chauvenet's criterion [49].

Fig. 8A and B shows the PARAFAC LC × LC signals for mixture C initiated by GRAM. The PARAFAC signal for the target analyte, fumaric acid, overlaps completely with the combined signals of maleic and phenyl phosphoric acids. For this very difficult case, the GRAM-PARAFAC approach is still able to produce a fairly accurate signal profile for fumaric acid at the correct retention time. This is demonstrated further in Fig. 9A and B. Each figure displays the normalized PARAFAC signal for fumaric acid overlaid with the normalized signal of pure fumaric acid along one of the column axes. The signals were normalized to unit area in order to determine the quality of the PARAFAC signal profiles. This was needed because the PARAFAC predicted signal heights were rather inaccurate. The inaccurate peak heights meant that the predicted mean concentration for fumaric acid were also off the mark as shown in Table 1, column 4. This mean concentration was originally based on four different PARAFAC concentrations obtained from analyzing four different sample-standard combinations. The data for each combination originated from performing two replicate LC × LC runs of both mixture C and its standard-addition mixture. One of the four PARAFAC quantitative results and its corresponding GRAM

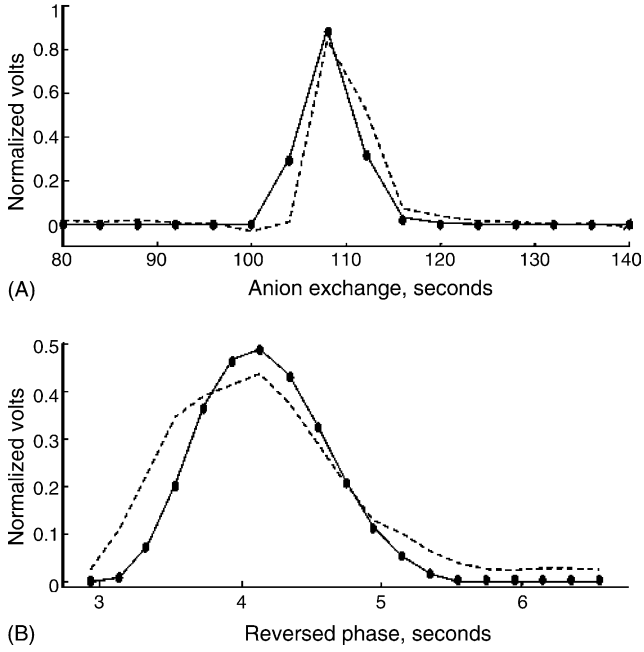

Fig. 9. Overlays of the PARAFAC-resolved signal (solid line) (see Fig. 8) and the actual resolved signal (dashed line) for fumaric acid in mixture C. Parts (A) and (B) depict the overlaid LC × LC signals after each have been summed onto each column axes and then normalized to unit area.

results were removed because of an unrealistic representation of the fumaric acid signal. The fumaric acid signal had a shape that did not resemble an LC × LC peak. The signal profiles for the remaining three PARAFAC analyses looked realistic. To no surprise, all the GRAM signals for fumaric acid were erroneous (e.g., negative dips and bimodal). Although the remaining PARAFAC quantitative results were biased they still had acceptable precision. As shown in Table 1, the GRAM-only quantitative results for mixture C are significantly worse compared to the PARAFAC results. However, for many applications the quantitative accuracy of PARAFAC for fumaric acid in mixture C would be unacceptable. The reason for this poor performance is due to the severity of signal overlap. Indeed, the calculated multivariate selectivity for the fumaric acid signal is near the limit for obtaining acceptable PARAFAC results [24].

4. Conclusions

For the LC × LC data presented in this paper, GRAM alone was generally qualitatively and quantitatively inferior to PARAFAC because it lacked signal constraints. On the other hand, PARAFAC without GRAM significantly increased analysis time and frequently produced unsatisfactory results. The chemometric approach that worked the best used GRAM followed by PARAFAC analysis. It mostly produced fast and reliable signal resolution and quantification of overlapped LC × LC peaks.

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