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Two dimensional assisted liquid chromatography – a chemometric approach to improve accuracy and precision of quantitation in liquid chromatography using 2D separation, dual detectors, and multivariate curve resolution

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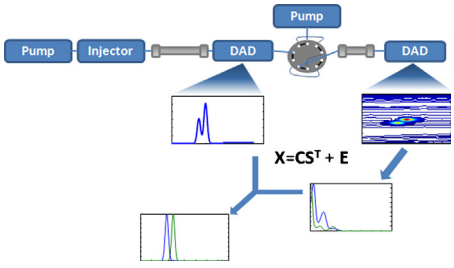
HIGHLIGHTS

- A novel method for quantitation in LC × LC is proposed based on use of dual detectors.
- Curve resolution was carried out on data from both detectors.
- The combined use of data from both detectors lead to improved quantitative results.
- In some cases, use of only first dimension data results in improved quantification.

GRAPHICAL ABSTRACT



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ABSTRACT

Comprehensive two-dimensional liquid chromatography (LC × LC) is rapidly evolving as the preferred method for the analysis of complex biological samples owing to its much greater resolving power compared to conventional one-dimensional (1D-LC). While its enhanced resolving power makes this method appealing, it has been shown that the precision of quantitation in LC × LC is generally not as good as that obtained with 1D-LC. The poorer quantitative performance of LC × LC is due to several factors including but not limited to the undersampling of the first dimension and the dilution of analytes during transit from the first dimension (^{1}D) column to the second dimension (^{2}D) column, and the larger relative background signals. A new strategy, 2D assisted liquid chromatography (2DALC), is presented here. 2DALC makes use of a diode array detector placed at the end of each column, producing both multivariate ^{1}D and two-dimensional (2D) chromatograms. The increased resolution of the analytes provided by the addition of a second dimension of separation enables the determination of analyte absorbance spectra from the ^{2}D detector signal that are relatively pure and can be used to initiate the treatment of data from the first dimension detector using multivariate curve resolution–alternating least squares (MCR–ALS). In this way, the approach leverages the strengths of both separation methods in a single analysis: the ^{2}D detector data is used to provide relatively pure analyte spectra to the MCR–ALS algorithm, and the final quantitative results are obtained from the resolved ^{1}D chromatograms, which has a much higher sampling rate and lower background signal than obtained in conventional single detector LC × LC, to obtain accurate and precise quantitative results. It is shown that 2DALC is superior to both single detector selective or comprehensive LC × LC and 1D-LC for quantitation of compounds that appear as severely

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overlapped peaks in the ^{1}D chromatogram – this is especially true in the case of untargeted analyses. We also anticipate that 2DALC will provide superior quantitation in targeted analyses in which unknown interfering compounds overlap with the targeted compound(s). When peaks are significantly overlapped in the first dimension, 2DALC can decrease the error of quantitation (i.e., improve the accuracy by up to 14-fold compared to 1D-LC and up to 3.8-fold compared to LC × LC with a single multivariate detector). The degree of improvement in performance varies depending upon the degree of peak overlap in each dimension and the selectivities of the spectra with respect to one another and the background, as well as the extent of analyte dilution prior to the ^{2}D column.

1. Introduction

The increasing need for the analysis of complex samples necessitates the development of new analytical techniques and data analysis strategies. Particularly in "omic" type applications, these samples can contain several hundred to several thousands of compounds [1]. Chromatography is often the analytical method of choice because of its ability to separate complex mixtures; however, traditional 1D chromatography is being pushed to its limits in regards to peak capacity, particularly when analysis time is limited. Many of these applications require analyte quantitation not merely identification. To do this, the analytes must be adequately resolved in order to accurately determine how much is present [2].

Comprehensive two-dimensional liquid chromatography (LC × LC) can provide a significant advantage over one-dimensional liquid chromatography (1D-LC) in terms of resolving power. Ideally, the theoretical peak capacity for a two-dimensional (2D) separation is equal to the product of the peak capacities of each separation dimension. In practice, the effective peak capacity is lower for several reasons, including the difficulty in choosing a pair of columns which maximize the use of the 2D separation space [3], partially due to the lack of orthogonality between commercially available columns [4], and the undersampling of the first dimension separation [5]. Despite these limitations Stoll, Wang, and Carr determined through both theoretical and experimental studies that when separation times are greater than 10 min and the ^{2}D separation is conducted sufficiently rapidly, LC × LC has superior effective peak capacity as compared to 1D-LC [5–8]. While multi-dimensional chromatography has definite advantages in terms of peak capacity and peak capacity per unit time, the precision and accuracy of these methods compared to 1D chromatography often are not considered. Indeed, multi-dimensional liquid chromatography methods often suffer in terms of quantitative performance in comparison to their traditional 1D counterparts [2]. Stoll et al. recently reported percent relative standard deviations (%RSD) for 2D peak areas ranging from 0.7% to upwards of 15% with most falling between 1.5% and 7% when manual peak integration was employed [9]. In another paper, Stoll et al. compared the precision of 1D-LC and LC × LC and found that the 2D peak areas were on average *seven-fold less precise* based on the %RSD [5]. Kivilompolo et al. reported %RSDs for peak volumes ranging from 3% to 13% for antioxidants in wines and juices [10]. Dugo et al. were able to quantify more polyphenols in red wines due to the increased resolving power of LC × LC; however, for the compounds detected in both LC × LC and 1D-LC, the %RSD in LC × LC was 12-fold higher than in 1D-LC [11].

Factors that can contribute to this poorer precision include the use of multiple sample delivery valves and loops which require precise control, the undersampling of the first dimension, and lower signal-to-background ratios at the ^{2}D detector. These high background signals result from the use of fast second dimension gradients and the dilution of the ^{1}D effluent as it is transferred to the second chromatographic dimension. When short overall analysis times are required and a gradient is used in the second dimension, the speed of the gradient causes a substantial increase in the ^{2}D baseline due to dynamic refractive index effects in the UV–visible detector cell [12]. Dilution of the analytes occurs in two ways. First, the nature of LC × LC causes the analytes to be diluted when being delivered to the second dimension [13]. One strategy to counteract the dilution issue is to increase the volume of ^{1}D effluent injected onto the ^{2}D column, thereby increasing the number of moles of analyte delivered to the second dimension; however, this can lead to volume overload of the ^{2}D column. This problem can be somewhat ameliorated by on-column focusing in the second dimension, that is, the narrowing of the analyte zone caused by the high retention of analytes dissolved in very weakly eluting solvents [9,13,14]. This on-column focusing can be enhanced by intentionally diluting the ^{1}D effluent with a weak solvent before delivery to the second dimension. This is useful for avoiding injection broadening on the ^{2}D column caused by the delivery of the ^{1}D effluent in a stronger solvent [9,15]. The amount of dilution needed is dependent on the analytes and should be optimized to avoid unnecessary loss of the signal-to-background caused by the loss of signal intensity.

Even given the superior peak capacity of LC × LC, many analytes may still be poorly resolved in complex samples, making quantification difficult. Another approach for improving peak resolution is to use a curve resolution technique, such as multivariate curve resolution (MCR), which allows for the resolution of overlapped peaks during the data analysis process. Using MCR, Bailey et al. were able to detect 18 peaks in a separation space which had a calculated chromatographic peak capacity of only 5 using a diode array detector (DAD) [2]. Curve resolution can essentially improve resolution without increasing the complexity of instrumentation, while maintaining the quantitative advantages of 1D chromatography. While curve resolution methods work well for moderately overlapped peaks, they can fail when peaks are severely or completely overlapped.

Here, we present a new approach for improving quantitation in LC × LC. We term this method 2D assisted liquid chromatography (2DALC). This strategy is outlined graphically in Fig. 1. In this approach the higher resolving power of LC × LC is combined with the superior precision available from 1D chromatography by using a DAD at the end of both the first and second dimension columns. This strategy partially overcomes the inevitable resolution loss caused by the undersampling of the first dimension chromatogram, as well as the decrease in the signal to background ratio resulting from dilution encountered in LC × LC. This is accomplished by first using MCR with alternating least squares (ALS) to get an improved estimate of the pure component spectra from the ^{2}D DAD data and then using the resolved spectra to initiate MCR–ALS analysis of the ^{1}D DAD data. Calibration and quantification can then be performed using the resolved ^{1}D chromatograms.

2. Strategy

Fig. 1 shows the overall strategy developed here. First, the 2D chromatogram is analyzed with MCR–ALS to obtain resolved spectra of the real chemical components. Then, these spectra are