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Impact of reversed phase column pairs in comprehensive two-dimensional liquid chromatography

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ABSTRACT

A major issue in optimizing the resolving power of two-dimensional chromatographic separations is the choice of the two phases so as to maximize the distribution of the analytes over the separation space. In this work, we studied the choice of appropriate reversed phases to use in on-line comprehensive two-dimensional liquid chromatography ($LC \times LC$). A set of four chemically different conventional bonded reversed phases was used in the first dimension. The second dimension column was either a conventional bonded C18 phase or a carbon-clad phase (CCP). The $LC \times LC$ chromatograms and contour plots were all rather similar indicating that the selectivities of the two phases were also similar regardless of the reverse phase column used in the first dimension. Further, the spatial coverage seen with all four first dimension stationary phases when paired with a second dimension C18 phase were low and the retention times were strongly correlated. However, when the C18 column was replaced with the CCP column much improved separations were observed with higher spatial coverages, greater orthogonalities and significant increases in the number of observed peaks.

1. Introduction

One of the main challenges in $LC \times LC$ *method development* is selecting appropriate pairs of stationary phases. This is evident in the definition of the effective 2D peak capacity given in Eq. (1) [1]:

$$n'_{c,2D} = \frac{{}^1n_c \cdot {}^2n_c \cdot f_{cov}}{\langle\beta\rangle} \quad (1)$$

here ${}^1n_c$ and ${}^2n_c$ are the first and second dimension peak capacities respectively, f_{cov} is the fractional coverage of the 2D space occupied by the sample peaks and $\langle\beta\rangle$ is the first dimension undersampling correction factor. Our chief concern in this paper is the effect of different reversed phases on the resolution and how they influence f_{cov}.

Rutan et al. evaluated several approaches to determine f_{cov} [2]. Two of the more common approaches are the Gilar approach [3] and the Gilar–Stoll approach [4]. Both approaches plot the normalized retention data onto a grid that has been fitted over the 2D separation space. The grid is divided into a set of rectangular bins. The f_{cov} is calculated by dividing the number of occupied bins by the total number of bins in the grid. The Gilar approach includes only bins that are occupied by retention data points [3]. The Gilar–Stoll approach includes empty bins between retention data points [4]. The Gilar–Stoll approach was used to calculate f_{cov} because it was used previously in a similar work [5].

A fundamental requirement for choosing a pair of columns is that their selectivities should be quite different [6,7]. Such columns are often called "orthogonal" meaning statistically unrelated. A number of approaches for quantifying the relative selectivity of a pair of columns have been discussed in the literature. Three common, but fundamentally different metrics, used to gauge the effectiveness of the choice of conditions are the Pearson correlation coefficients (R^2) using plots of the retention times on the two phases t_R vs. t_R [8,9], the fractal dimensionality of the retention times in the 2D space occupied [10,11] and the F_s metric of the hydrophobic subtraction model (HSM) [12–14].

In this work we were concerned with methods that can be used to choose column pairs in an a priori fashion. One of the simplest methods for selecting a pair of stationary phases in $LC \times LC$ is to choose those phases that minimize the Pearson correlation coefficient of the measured retention times determined by doing 1D separations of a limited set of analytes chosen to *represent* the actual sample on a variety of stationary phases. An appropriate pair of stationary phases for 2D work would show a very weak correlation

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between each pair of retention times [4]. Clearly, this approach attempts to maximize the orthogonality of two phases, but it can fail to maximize the fractional coverage. High orthogonality is a necessary, but insufficient condition for maximizing the effective peak capacity according to Eq. (1). D'Attoma et al. proposed that this method be used to select appropriate pairs of stationary phases for $LC \times LC$ work [9]. However, this method can be tedious and often requires using chromatographic conditions that are not conducive to good $LC \times LC$. Further, this method is performed using a specific set of compounds that might not accurately represent the sample of interest.

A second method that can be used for column selection is the dimensionality of a pair of columns. Giddings developed the idea of sample dimensionality in multidimensional separations [10]. Schure used the concept of 'box-counting' dimensionality by using the fractal dimension to characterize the 2D space occupied by the actual sample [11]. In contrast to the Pearson correlation coefficient this is not an a priori method for column selection in that it requires the actual sample to be run. Additionally, the fractal dimension is not simply related to f_{cov}. The two aforementioned methods for column selection are totally empirical approaches, that is, experimental data is required for either to be used.

By contrast to the above methods, the hydrophobic subtraction model (HSM) is an a priori approach to column selection which does not require any new data to be used. As previously suggested by Snyder et al. HSM should be useful for screening and selection of pairs of reversed phases for use in 2D-LC [12]. Wilson et al. showed that majority of all RP phases involve only five types of selectivity controlling processes between the solutes and the stationary phase [13]. These are the hydrophobic ($\eta' H$), steric ($\sigma' S^*$), hydrogen bond donating ($\alpha' B$), hydrogen bond accepting ($\beta' A$), and cation exchange ($\kappa' C$). Eq. (2) shows the empirically founded relationship between the retention factor (k') and these interactions:

$$\log k' = \log k'_{EB} + \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C \quad (2)$$

The Greek letters in Eq. (2) refer to the chemical properties of the analytes and the Roman letters refer to the corresponding complimentary chemical properties of the stationary phase. The term $\log k'_{EB}$ refers to the retention factor of ethylbenzene which is used as the reference analyte [12,14]. Snyder et al. further proposed that the HSM phase parameters could be used to determine the difference in selectivity between two RP phases by using Eq. (3) [12,14]:

$$F_s = \{[12.5(H_2 - H_1)]^2 + [100(S_2^* - S_1^*]^2 + [30(A_2 - A_1)]^2 + [143(B_2 - B_1)]^2 + [83(C_2 - C_1)]^2\}^{1/2} \quad (3)$$

The F_s value calculated from Eq. (3) indicates the degree of difference between the two stationary phases. The numerical weighting factors in Eq. (2) were chosen to represent a "typical" set of solutes [12,13]. As an example, if an actual data set did not contain any solutes that were ionic then there could be no contribution to selectivity from coulombic interactions and the corresponding weighting factor for that term should be zero. From the perspective of $LC \times LC$, an F_s value greater than 100 indicates that the selectivity of the two phases should be very different from each other [12,14].

Zhang and Carr developed "triangle plots" to graphical represent visualize and compare the differential selectivities of a large number of phase in a single plot [15]. Based on the theoretical study of Zhao and Carr which proved that column selectivity is fundamentally related not to the absolute value of the phase parameters but only to their ratio, Zhang's work used the ratios S^*/H, A/H, B/H and C/H to show hundreds of phases in a set of four triangle plots [15]. Eq. (3) shows that the S^*, B, and C terms provide the greatest contribution to the F_s value and should incorporate the largest difference in selectivity between any two phases. Accordingly the values of the three terms are normalized relative to the H term as per the selectivity concept of Zhao and Carr [16]. The distance between the two stationary phases provides a measure of the degree of difference in their selectivity.

Despite the fact that HSM captures the major contributions to phase selectivity for directing the choice of columns for $LC \times LC$, it has some drawbacks [17,18]. Marchand et al. discussed some of the drawbacks related to the C-term and the pH of the mobile phase [17]. They showed that when the mobile phase pH is less than 3, a majority of the free silanols on the stationary phase surface are protonated resulting in less tailing for ionized basic compounds. Further, the paper also showed that the contribution of the C-term to the selectivity of the stationary phase will be reduced when very acidic mobile phases are used. Snyder and Dolan previously proposed that the C-term be dropped under these mobile phase conditions [19]. Thus, eliminating the C-term should allow for a more pertinent F_s value and a better comparison of the selectivity difference between two reverse phases under low pH conditions or as described above when the sample does not contain any charged analytes. Græsbøll et al. proposed that use of the F_s parameter with the C-term dropped could be used as a metric to select a pair of reverse phase columns for $LC \times LC$ [20]. The major limitation to this approach is that the nature of the compounds in the sample of interest must be known so as to guide the inclusion or exclusion of the C-term for comparing the phases. Clearly, dropping the C-term would only be effective for samples containing only uncharged analytes or when the pH is so low that C is effectively negligible.

Our recent work in $LC \times LC$ has focused on using CCPs as the second dimension [5,21]. In addition to the general hydrophobic interaction, the primary types of interactions that take place between a CCP material and the analyte are electrostatic and π–π interactions [22]. These unique and strong interactions give CCP materials quite different selectivities and considerably enhance solute retention compared to bonded type RP phases [21–23]. Further, CCPs are also chemically very stable and allow the use of higher temperatures to achieve higher speeds in LC [24–26]. Therefore, use of such phases has enabled both fast and unique separations of complex samples by $LC \times LC$.

In a previous study, we compared $LC \times LC$ with six chemically different reverse phases in the first dimension combined with a CCP column as the second dimension [5]. The results showed that use of CCPs in the second dimension produced respectably high and virtually the same fractional coverages independent of the type of RP used in the first dimension.

In this work, we studied the use of four chemically different reverse phase materials as the first dimension of $LC \times LC$. A conventional C18 stationary phase was used in the second dimension. Despite the differences in the selectivity between each first dimension phase relative to the second dimension phase as measured by the HSM F_s parameter, the 2D chromatograms showed strong correlations and low fractional coverage of the two dimensional separation space. In contradistinction, when the second dimension C18 phase was replaced with a CCP type phase, the chromatograms showed greater coverage of the separation space and an increase in the number of observed peaks.

2. Experimental

2.1. Chemicals and reagents

HPLC grade water and acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO). HPLC grade methanol was purchased from Thermo Fisher Scientific (Waltham, MA). Phosphoric