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Using the hydrophobic subtraction model to choose orthogonal columns for online comprehensive two-dimensional liquid chromatography

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ABSTRACT

A method for choosing orthogonal columns for a specific sample set in on-line comprehensive two-dimensional liquid chromatography (LC × LC) was developed on the basis of the hydrophobic subtraction model. The method takes into account the properties of the sample analytes by estimating new F-weights for the prediction of orthogonality. We compared sets of F-weights and used these F-weights to predict orthogonal column combinations: (1) the standard F-weights determined by Gilroy et al. [1], (2) F-weights determined from the retention of sample analytes, and the same procedure of calculation as described by Gilroy et al. [1], (3) F-weights determined from the retention of sample analytes but using principal component analysis (PCA) for the estimation, and (4) the Gilroy F-weights modified by excluding the C-term in the hydrophobic subtraction model, as suggested by Dolan and Snyder [2]. The retention of 13 neutral and 4 acidic oxygenated polycyclic aromatic compounds (PACs) and 3 nitrogen-containing PAC bases was measured isocratically on 12 columns. The isocratic runs were used to determine the hydrophobic subtraction model analyte parameters, and these were used to estimate new F-weights and predict orthogonal column combinations. LC × LC-DAD analysis was then performed on a test mix using these column sets. We found that the column combination predicted from the new F-weights provide a more orthogonal separation of the PACs than those predicted using the standard F-weights and the F-weights modified by excluding the C-term. This emphasizes the necessity of considering the nature of the sample when choosing orthogonal columns.

1. Introduction

In two-dimensional liquid chromatography two separations are performed on the same sample, either by only collecting the interesting part of the effluent from the first system and running it on the second system (heart-cut), or by transferring the entire first dimension effluent to the second dimension in small samples (comprehensive) [3]. The advantage of comprehensive two-dimensional liquid chromatography (LC × LC) is that the overall peak capacity of the system is – ideally – the product of the peak capacity of the individual systems, while heart-cutting lead to significantly lower peak capacities depending on the number of fractions transferred to the second dimension. Selective LC × LC where only part of the first dimension chromatogram is analyzed in a comprehensive way was recently suggested as a compromise between heart-cut and comprehensive LC × LC [4]. LC × LC has been reported to yield a peak capacity exceeding 1000 in 30 min [5,6], compared to traditional HPLC which has peak capacity in the low hundreds [7]. In online comprehensive LC × LC the full first dimension effluent is sampled into fractions which are analyzed on the second dimension system. Each first dimension fraction is analyzed on the second dimension within the time it takes to collect the next fraction [8]. Online comprehensive LC × LC requires very short second dimension run times, but the overall analysis time is almost the same as if no second dimension was used. Alternatively, in LC × LC the flow on the first dimension column can be stopped while the second dimension chromatogram is made; this is usually referred to as stop-flow LC × LC [9]. Offline comprehensive LC × LC, where the samples of the effluent are stored and analyzed independently of the first dimension, gives the largest peak capacities but at the cost of a very long analysis time.

LC × LC has been used for a wide range of compounds and was first achieved by Bushey and Jorgenson [8] in 1990; they separated proteins with ion-exchange in the first dimension and size-exclusion in the second. Other application range from branched polystyrene [10] and triacylglycerols [11] to organic acid in aerosols [12] and carotenoids in juice [13]. For a more comprehensive

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overview of applications of LC × LC we refer to recent review articles [14–16].

For two-dimensional liquid chromatography it is important that the selectivity in the two chromatographic systems is as different as possible, in order for compounds that are not separated in the first dimension to be separated in the second dimension. If the separation mechanisms in the two dimensions are completely different they are referred to as orthogonal. It is possible to make systems that are close to fully orthogonal, but they suffer from low peak capacity, e.g. size exclusion chromatography coupled with reverse phase chromatography, or mobile phase immiscibility such as when hyphenating normal phase chromatography with reverse phase (RP) chromatography. RP × RP is by far the most common combination, but it can be difficult to achieve a high degree of orthogonality since columns offer rather similar retention mechanisms [14–17]. It is possible to optimize the orthogonality of the chromatographic systems by modifying the mobile phase (e.g. pH, organic modifier) [18], or by careful optimization of the gradients [19], but the choice of column is paramount to the orthogonality and the focus of this article.

Several ways of evaluating the orthogonality in two-dimensional chromatography have been presented in the literature (e.g. [17,20,21]), but predictions of the orthogonality are so far limited. Columns are often chosen based on a priori knowledge, and the selection of the best column combination therefore depends on the research group's experience. A more objective way of finding columns with a high degree of orthogonality is by comparing columns using the hydrophobic subtraction model. The hydrophobic subtraction model was first described by Wilson et al. [22–24] and was later reviewed and extended by Snyder et al. [25]. The model describes the analyte-column interactions present in RP-LC by the following equation:

$$\log \alpha \equiv \log\left(\frac{k}{k_{EB}}\right) = \eta'\mathbf{H} - \sigma'\mathbf{S}^* + \beta'\mathbf{A} + \alpha'\mathbf{B} + \kappa'\mathbf{C} \quad (1)$$

where k is the retention factor of an analyte in a specific chromatographic system and k_{EB} the retention factor of ethylbenzene in the same system. The remaining symbols in Eq. (1) represents either column properties (upper case roman), or properties of the analyte (lower case Greek). Each part of Eq. (1) represent a physical or chemical influence on the retention factor: the hydrophobicity ($\eta'\mathbf{H}$), steric hindrance ($\sigma'\mathbf{S}^*$), acidity/basicity ($\beta'\mathbf{A}+\alpha'\mathbf{B}$) and ion-exchange ($\kappa'\mathbf{C}$) respectively. For an in-depth description of the analyte-column interactions see [25]. The column parameters have been determined for a wide range of columns, and are available from the U.S. Pharmacopeia [26]. The orthogonality of any two columns can be estimated by comparison of their column properties as deduced from the hydrophobic subtraction model [25]. More accurately, this can be done by calculating the F-factor as given by Eq. (2); the higher the F-factor the more different the columns are.

$$F = \sqrt{(a(H_2 - H_1))^2 + (b(S_2^* - S_1^*))^2 + (c(A_2 - A_1))^2 + (d(B_2 - B_1))^2 + (e(C_2 - C_1))^2} \quad (2)$$

The F-weights a–e depend on the importance of that particular interaction in the separation of a specific set of analytes. Gilroy et al. [1] estimated general F-weights values of 12.5, 100, 30, 143 and 83 based on 67 analytes with a wide variety of properties (see Section 2). However, as Gilroy et al. noted [1], this might not be the ideal approach when dealing with a specific class of compounds since the F-weights should reflect the relative importance of each column–analyte interaction, and their importance may vary from one set of analytes to another. For samples containing only neutral compounds is has been suggested to set e to 0, but otherwise keep a–d as stated above [2]. We suggest that instead of using the general F-weights determined by Gilroy et al., F-weights should be determined based on the compounds of interest. In this way the most orthogonal columns for specific sets of analytes can be found, and the separation power of the system maximized.

The aims of this study are therefore to test the validity of the standard F-weights to predict orthogonal column sets for LC × LC separation of a specific set of analytes, to calculate sample-relevant F-weights using analytes of interest instead of the commonly used 67 analytes [1], to compare the predictions of the most orthogonal column combination for the new F-weights and the standard F-weights, and to use these column combination for comprehensive LC × LC separation of a test mixture of 15 neutral and acidic oxygen-containing polycyclic aromatic compounds (PAC15).

As the new F-weights are calculated on the basis of sample compounds or in this case the same compounds as present in the test mix, we expect that the column sets predicted from these new F-weights will provide a more orthogonal separation of the PAC15 that those predicted using the standard F-weights estimated from the retention of 67 analytes with a range of chemical properties. Furthermore, as the PAC15 test set consists of 13 neutral and 2 acidic compounds we expect that removing the C-term from the standard F-weights will improve the prediction for the most orthogonal column set, since the number of acids is low and the pH is at 2.8. To further test the importance of the C-term the PAC15 test set was extended to include three basic nitrogen-containing PACs and additionally two acidic oxygen-containing PACs (PAC20).

Oxygen- and nitrogen containing PACs are formed from oxidation of PACs which are abundant in fossil fuel. Due to the complexity of fossil fuel the formed PACs appear as a complex mixture of compounds requiring a high peak capacity for their complete separation. The oxygen-containing PACs represent a subgroup of PAC degradation products. The degradation products have been found to exhibit equal or greater toxicity compared to their parent PACs [27].

2. Methodology

We use two mathematical approaches to determine the F-weights in Eq. (2): The method described by Gilroy et al. [1] and our novel approach denoted SAmple Dependant Column Orthogonality Determination (SADCOD) which is based on principal component analysis (PCA) [28]. The different approaches are compared using two sets of compounds containing 15 (PAC15) and 20 (PAC20) compounds respectively. Finally, two LC × LC chromatograms of PAC15 are made using relevant column combinations to verify the finding.

The two LC × LC chromatograms was compared by their overall peak capacity calculated with Eq. (3)

$$n_{total} = {}^1n \times {}^2n \times \frac{1}{\langle\beta\rangle} \times f, \quad (3)$$

where ${}^1n$ and ${}^2n$ are the peak capacities in the first dimension and second dimension, which is approximate by dividing the peak width with the length of the chromatographic run, and f is the fraction of the chromatogram which is covered by peaks. $\langle\beta\rangle$ is the correction for undersampling of the first dimension peaks and is given by

$$\langle\beta\rangle = \sqrt{1 + 3.35 \times \left(\frac{t_s}{{}^1w}\right)^2},$$

where t_s is the modulation time and ${}^1w$ is the first dimension peak width [29].