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Evaluation of the Identification Power of RPLC Analyses in the Screening for Drug Compounds

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Abstract

The identification of drugs of abuse is an important issue in forensic science. The main goal is to trace and identify as many drugs as possible in the shortest possible time preferably with a simple analysis method. One possibility is to screen samples using a Liquid Chromatography – Diode Array Detection (LC-DAD) system. However, when simultaneously performing another analysis on a chromatographic column exhibiting selectivity differences from the first one, i.e., orthogonal or dissimilar columns, a greater number of drugs can be possibly identified without investing a lot of extra time or money. The primary difficulty is then selecting the most appropriate columns. In this paper, it is demonstrated that selecting the most dissimilar columns based on measures such as correlation or Snyder's F_s value is not optimal, because these measures do not take into account the identification power of the individual systems. This implies that a large number of drugs may not necessarily be identified on the systems selected using these criteria. Therefore, three other measures are tested to evaluate the identification power obtained by parallel screening on two columns or by comprehensive two-dimensional LC (LC×LC). The simplest approach is counting the number of compounds separable with a difference in retention time greater than a predefined critical value. However, this measure does not reflect the co-elution pattern of the unidentified drugs nor the separation degree of all compounds. The second tested measure, information, enables differentiation between systems identifying the same number of compounds, but resulting in a different co-elution pattern. Multivariate selectivity, the third tested parameter, takes into account the degree of separation of all compounds and has the advantage that it reflects the gain in identification power achieved by introducing DAD data. All three proposed measures also enable evaluation of whether the corresponding LC×LC method will result in a greater identification power.

Keywords

information theory; two-dimensional separations; Snyder F_s; correlation; multivariate selectivity

In forensic laboratories, powder samples, liquids or biological fluids often need to be screened for a large number of drugs in a short time. For this purpose, the most simple

analysis method with a high identification power is desired. The analyst should thus find a compromise between simplicity and efficiency. One-dimensional (1D) reversed phase liquid chromatography (LC) coupled to diode-array-detection (DAD) is one possible choice of several possible screening procedures.[1,2] Although LC coupled with mass spectrometry (MS) offers more identification power than DAD detection, the cost of MS as a screening tool means that LC-DAD analyses are still quite useful. In ref. (3) it is even stated that the identification power of LC-DAD is similar to that of low resolution LC-MS-MS. Another means of improving the identification power is to carry out simultaneous screening on two dissimilar columns, i.e., columns showing significant selectivity differences. Simultaneous LC analyses, can improve the identification power of the analysis with only a little increase in analysis time and only a moderate increase in cost.[4] If this approach does not result in a sufficient separation, one can consider applying more complex two-dimensional liquid chromatography (LC×LC) methods that can provide higher peak capacities than one dimensional separations[5]. If the number of studied drugs is large, it can be difficult for the analyst to decide whether the more complex analysis will imply a gain in information about the drugs. The primary issue, however, in using either a parallel 1D-LC approach or an LC×LC method, is the selection of the two columns that will give maximal identification power. The term "identification power is used frequently in the field of toxicological analysis[6-10]. Here, the term identification power is used to indicate a measure of the number of compounds, which can be identified unambiguously; in the best case, this metric will also reflect the degree of separation of the unidentified compounds. In this paper, several approaches are discussed to assess the identification power of (1) a single 1D-LC separation, (2) two parallel 1D-LC separations and (3) of the corresponding LC×LC method in a quantitative way based on the measured retention times of a set of drugs of abuse. The most common way to find a combination of two columns, that will provide maximal information, is to choose the two most dissimilar columns (i.e., columns showing significant selectivity differences), which can be evaluated based on the correlation between the retention times of the drug compounds[11] or based on Snyder's F_s values[12]. In addition, other research groups have developed tools to select (dis)similar columns. Hoogmartens et al.[13] for example, introduced an F value based on four test parameters characterizing the column. The group of Vander Heyden applied chemometric techniques such as hierarchical clustering[14], auto-associative multivariate regression trees [15] or an orthogonal projection approach[16] on the retention times of a representative set of pharmaceutical compounds in order to select the most dissimilar chromatographic systems. Jandera has described the degree of column orthogonality in terms of the differences in functional group selectivities[17,18]. However, the fact that two columns are very dissimilar does not imply that the separations obtained on both columns exhibit high efficiency and/or selectivity. Moreover, it is also not guaranteed that all compounds are retained for a reasonable time. Therefore, it would be preferable to use measures that accurately assess the identification power of the individual separations, as well as the dissimilarity between the two separation systems. A very simple, straightforward approach is to count the number of the compounds of interest (N) that can be separated on one column by a difference in retention time greater than a predefined critical value. When two parallel 1D separations are considered, one can first consider the first column and then check whether the initially unresolved peaks are separated on the second column with a difference in retention time higher than a predefined value. For LC×LC, a critical resolution, i.e., the minimal resolution between two peaks still enabling their identification, must be defined for both separation dimensions. It should be pointed out that this approach is distinctly different from simply reporting the peak capacity, which deals only with hypothetical, equally spaced peaks, and not real compounds with measured retention times. Two more advanced tools, i.e., information theory[19] and multivariate selectivity,[20-22] can also be used to determine the quality of a given separation that uses either one or two columns. Multivariate selectivity has the advantage that the

increase in identification power resulting from the use of spectral information (for instance by the use of DAD instead of single wavelength detection) can be evaluated.

In this paper some well established approaches, i.e., F_s and R^2, will be compared with more innovative approaches (number of compounds separated (N), information theory and multivariate selectivity) regarding column selection in screening for unknown drugs using LC-DAD.

THEORY

Correlation Coefficient (R^2) or Snyder's F_S Value

The dissimilarity between two chromatographic columns can be assessed by calculating the Pearson's correlation[23] (R^2) between the retention times of a set of compounds measured on the considered columns.[11] If the retention times for the same compounds on the two columns are very different (i.e., they show a low correlation), it implies that the columns exhibit selectivity differences, and the columns are concluded to be dissimilar. The chance is then very high that the second column delivers complementary information resulting in an improved identification power.

Another method to assess the selectivity differences between two columns is the use of the well-established column selectivity function (F_s) of Snyder.[12] It is based on Snyder's hydrophobic subtraction model, which involves five column properties that define the column selectivity and that will determine the retention (more specifically the selectivity factor) of a solute. These parameters are hydrophobicity (H), steric interaction (S*), hydrogen-bond acidity (A), hydrogen-bond basicity (B) and the cation-exchange activity (C).[24] The F_s value between two columns can then be calculated as follows (eq 1):

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

Each term in this equation is weighted according to its relative importance to the overall selectivity.[12] The greater the obtained F_s value, the more dissimilar the columns. The weighting factors in Eq. 1 are based on Snyder's solutes, and it might be questioned if these are also applicable for gradient analysis of drugs. However, the appropriate experimental information to calculate new, reliable weighting factors was not available to us. Fan *et al.* have shown that the weighing factors in eq 1 are appropriate for the evaluation of column similarities for gradient elution separations of these drug compounds.[25]

Number of Separated Compounds

A very simple and logical measure to assess the separation power of an analytical method is to count the number of compounds that are separated by more than a preset critical difference in retention time (Δt_{crit}). In the present work, if two peaks are separated with a resolution (Rs) of 0.7, they are considered as distinguishable from each other, and the drugs corresponding to these two peaks can be distinguished. The Δt_{crit} is then calculated as follows (eq 2):

$$\Delta t_{crit} = Rs \times w = 0.7 \times w \tag{2}$$

where *w* is the average base width of the peaks, defined as 4σ.

When two 1D separations are performed in parallel, one can first determine which compounds are not separated on the first column with a difference in retention time greater

than Δt_{crit}. Then it can be determined whether these compounds are separated on the second column. For LC×LC, two Δt_{crit} values must be considered, one for each dimension of the LC×LC method. If either of the Δt_{crit} values is exceeded, then the corresponding compounds are deemed to be separated.

Information Theory

When an unknown compound is considered to be possibly one of a number of different compounds (in our case, the compound is one of 25 drugs), additional information is required to narrow the list of possible candidate compounds.[16] This information can be produced by an analytical separation technique that can identify all or a number of the possible compounds. Information theory allows for calculation of the reduction of uncertainty achieved by an analytical procedure and thus makes it possible to evaluate qualitative analyses in a quantitative way.

Before this technique can be applied, the retention times are normalized according to eq 3:

$$t_n = \frac{t_R - t_{min}}{t_{max} - t_{min}}$$

(3)

where t_R is the measured retention time, t_{min} is the retention time of the earliest eluting compound and t_{max} the maximal retention time obtained on the considered column. This results in normalized retention times with values between zero and one. This normalization effectively takes into account that when a method is developed, the mobile phase gradient will be optimized such that the retention times of the compounds will be spread across the gradient time.[26]

The information content of a 1D analysis method, I[10] can be defined as

$$I = \sum_k - p_k \log_2 p_k = \sum_k - \frac{n_k}{N} \log_2 \frac{n_k}{N}$$

(4)

where p_k is the probability of the incidence of a single possible result, k, out of N possible results. In this case n_k is the number of compounds eluting during the kth retention time class, and N is the number of compounds. The size of the retention time classes is based on the Δt_{crit} calculated using eq 2.

The information content of the combination of two chromatographic systems in a comprehensive 2D format, I(1,2), can also be calculated using eq 4.[27,28] In the case of LC×LC, the retention time classes are two-dimensional squares defined by two ranges of retention times, one range for the first dimension separation and one range for the second dimension separation..The information can again be calculated with eq 4 and now the probability is determined by counting the number of drugs located in each square. The mutual information of two systems, I (1;2) can be determined as follows (eq 5):

$$I(1;2) = I(1) + I(2) - I(1,2)$$

(5)

where I(1) and I(2) indicate the information obtained from the two systems independent of one another, and I(1,2) represents the information obtained from the LC×LC technique. The mutual information reflects the fact that some of the information obtained by the two separations is redundant.

The similarity coefficient, R(1,2), is another measure of the qualitative information yield. When its value approaches one, it means that the two combined systems do not yield any more information then the better of the two systems individually. It can be calculated using the formulas below (eqs 6 and 7):[19,28]

$$d(1,2) = 1 - \frac{I(1;2)}{I(1,2)} \tag{6}$$

$$R(1,2) = \sqrt{\left[1 - (d)^2\right]} \tag{7}$$

In the case of two parallel 1D separations, several simplified procedures were used to attempt to calculate I(1,2). The most successful approach was to assume that all non-separated compounds are co-eluting with only one compound. This resulted in realistic values for the information yield of the method, but does have a risk of overestimating the information yield of the parallel separations.

Multivariate selectivity

The evaluation of the identification power of chromatographic systems can also be based on a multivariate selectivity parameter (SEL)[21,22,29,30] This parameter quantifies the precision of quantification for a compound present in a mixture, as compared to the precision of quantification when analyzed as a pure component. This implies that a compound with a high selectivity can be unambiguously identified. The average of the multivariate selectivities of all compounds has been used as an estimator for the identification power of the analysis.[21] Another option is to count the number of compounds with a multivariate selectivity greater than 0.98, because it can be shown that if a compound is separated from all others by at least a resolution 0.7, that this corresponds to a multivariate selectivity of 0.98. An advantage of the multivariate selectivity measure is that spectral information can also be included, and consequently, the gain in identification power obtained by adding spectral information will be reflected in the SEL values.

For the case, where the identification is based on the retention times measured on one chromatographic system, SEL according to Messick, Kalivas and Lang (MKL) is given by eq 8[20,29]

$$SEL_i = \left[\left(\mathbf{A}_1^T \mathbf{A}_1\right)^{-1}\right]_{ii}^{-1/2} \tag{8}$$

where $\mathbf{A}_1$ is a matrix containing the pure component chromatographic profiles in the columns of the matrix. When the chromatographic profiles on one system are combined with the spectra during identification (i.e., LC-DAD analysis) this equation is adapted as follows (eq 9)[20,29]:

$$SEL_i = \left[\left(\mathbf{A}_1^T \mathbf{A}_1\right)^{-1} \cdot \left(\mathbf{A}_3^T \mathbf{A}_3\right)\right]_{i,i}^{-1/2} \tag{9}$$

where $\mathbf{A}_3$ is a matrix containing the spectra of the individual components of the candidate compounds, and the inner dot represents the element-wise Hadamard product. The latter equation can also be used when LC×LC is used, and no spectra are used for identification. In

that case, $\mathbf{A}_2$, a matrix containing the pure component chromatographic profiles on the second dimension column, is substituted in place of $\mathbf{A}_3$. A last possibility is that LC×LC is combined with DAD detection. The multivariate selectivity is then calculated as follows (eq 10)[20,29]:

$$SEL_i = \left[\left(\mathbf{A}_1^T \mathbf{A}_1\right)^{-1} \cdot \left(\mathbf{A}_2^T \mathbf{A}_2\right) \cdot \left(\mathbf{A}_3^T \mathbf{A}_3\right) \right]_{i,i}^{-1/2}$$

(10)

The chromatographic profiles used to calculate the multivariate selectivity are Gaussian peaks simulated with Matlab based on the measured retention times and peak widths (4σ). The retention times are first normalized to values between zero and one and the σ values are adjusted correspondingly. In LC×LC the σ values for the first and second dimension peaks can be derived from the observed peak capacity values.[31] In the first dimension, the interval between the simulated points in the profile also must be increased, to reflect the low sampling rate in this dimension.

EXPERIMENTAL SECTION

The retention times of 25 drug compounds (methapyrilene, zolpidem, aminoflunitrazepam, pyrilamine, chlorpheniramine, chlodiazepoxide, bromazepam, desipramine, oxazepam, nitrazepam, amitriptyline, estazolam, perphenazine, clonazepam, desalkylflurazepam, nordazepam, triazolam, flunitrazepam, temazepam, clobazam, lormetazepam, diazepam, halazepam, prazepam and buclizine) were determined on 13 different columns.[25] Four different groups can be distinguished among the tested columns.[25] The first group contains Type B silica-based C_{18} columns (Discovery C_{18}, StableBond C_{18}, ACE C_{18} and Alltima C_{18}), which show very similar selectivity. The silica-based phenyl columns (StableBond Phenyl, Betabasic Phenyl and Prontosil Phenyl) form a second group. They are similar to each other, but differ from the C_{18} columns. The third group consists of two polar embedded silica-based columns: the Prism RP and the Bonus RP. A variety of dissimilar columns are included in the last group: two cyano columns (Novapak CN and Inertsil CN) and two in-house prepared columns (HC-OH and HC-SO$_3$).[32,33] For all columns, a gradient of 30-70% ACN over 10 minutes was applied, except for the HC-SO$_3$ column, for which a 20-70% ACN gradient was performed. For the StableBond C_{18} columns, both gradients were tested. This resulted in 14 different chromatographic systems.

All calculations were performed using Matlab, v. 7.5 (Mathworks, Inc., Natick, MA).

RESULTS AND DISCUSSION

Data Pretreatment

Before using the proposed methods to determine the identification power of a considered RPLC system, all retention times measured on each stationary phase are normalized according to eq 3, resulting in values ranging from zero to one. The normalization is applied to correct for the fact that the same gradient was applied on almost all columns. A consequence of the latter practice is that on some columns the peaks cover a large portion of the available separation area, such as the SB C_{18} column, while on other columns, such as the Novapak CN column, all compounds elute during the last three minutes of the analysis. When performing this normalization, the peaks will cover the whole separation space, which would also be achieved when optimizing the gradient for each stationary phase.

Parameters for Characterization of the Identification Power of Separation Methods

The parameters used for the calculations of the identification power of the methods for drug analysis are summarized in Table 1. For the correlation calculations, no specific parameters besides the observed retention times are required. The relationship between retention on two different columns is calculated, and is independent of the scale or spread of the retention times. For the F_s calculations, the results depend solely on the values obtained from the Snyder database of column selectivity parameters.[12] For determining the number of compounds separated, as well as for the information calculations, we normalize retention times between zero and one, but we also need a Δt_{crit} value to determine at what degree of separation a pair of peaks will be deemed to be separated. The average σ for the experimental data was found to be 0.032 min, and was consistent across the different columns. From this σ value, we obtain a Δt_{crit} value of 0.091 min using eq 2. The normalized σ value is 0.0048, and then, using eq 2, the Δt_{crit} is found as 0.0135 (both dimensionless quantities). We use these σ and Δt_{crit} values for the normalized data for all columns when evaluating the single and parallel column separations. This gives rise to a peak capacity of 52 for each column.

For the determination of the appropriate σ and Δt_{crit} values for LC×LC separations, we use the reported peak capacities for a fast LC×LC separation as 50 and 17.4 for the first and second dimension separations, respectively.[31] The first dimension peak capacity is very similar to the value found above. However, the reported peak capacity on the first dimension column does not take into the peak broadening caused by undersampling. Davis et al.[34] have given the following expression for the effective peak width, $^1\sigma_{eff}$, as

$$^1\sigma_{eff} = \sqrt{^1\sigma^2 + 0.21 t_s^2} \tag{11}$$

where $^1\sigma$ is for the peak as it elutes from the first dimension column and t_s is the second dimension cycle time. The observed peak capacity on the first dimension column was found using a first dimension gradient time of 23 min and using a second dimension cycle time (sampling time) of 0.35 min. We scale the sampling time to obtain a dimensionless t_s value of $0.35/23 = 0.0152$. Then we calculate an appropriate dimensionless $^1\sigma$ value for our data based on the observed peak capacity[19] of 50 over the first dimension gradient ($^1\sigma = 1/(50 \times 4) = 0.0050$). When introducing the calculated values of $^1\sigma$ and t_s in equation 11, a dimensionless $^1\sigma_{eff}$ value of 0.0086 is obtained. For the second dimension separation, the peak capacity of 17.4 was achieved over a 17.4 s gradient. Therefore, an appropriate dimensionless $^2\sigma$ value is $1/(17.4 \times 4) = 0.0144$. The corresponding (dimensionless) Δt_{crit} values (calculated using eq 2) then become 0.024 and 0.040, for the first and second dimension columns, respectively.

The bin size applied in information theory was based on the Δt_{crit} values calculated above. Practically, this means that for 1D and parallel 1D-separations the bin size is set to 0.0135 grid units. In LC×LC, the grid size is 0.024 in the first dimension and 0.040 in the second dimension. Different grid sizes are required, because the peak capacity and peak widths are different under the different conditions used for the first and second dimension separations. This also implies that a change in the order of the columns in the LC×LC system will result in a different information yield.

For the multivariate selectivity calculations, we also elected to use normalized retention time values. Gaussian peak simulations were carried out for an x-axis range from 0-4σ to 1+4σ, so that the peaks eluting at normalized retention times of zero and one were simulated with a full range of data points describing a Gaussian peak. For the single and parallel column

separations, the dimensionless sampling interval was $\sigma/10 = 0.00048$. For the LC×LC calculations, the first dimension sampling interval (t_s, determined as described above) was 0.0152. The second dimension sampling interval was $^2\sigma/10 = 0.00144$.

Correlation Coefficient (R^2) and Snyder's F_s Value

From the squared correlation coefficients calculated between the retention times of the 25 drugs on the 14 chromatographic systems shown in Table 2, it can be observed that the HC-SO_3 column is the most dissimilar to all other columns, because it exhibits low squared correlation coefficients with all other columns. The most dissimilar combinations according to this metric ($R^2 = 0.01$, shown in bold on Table 2) are the HC-SO_3 column and the Discovery C_{18}, the BetaBasic Phenyl, the HC-OH or the StableBond C_{18} (SB C_{18}* with the 20-70% gradient). In Figures 1A-C examples are shown for two highly correlated columns, two columns with an intermediate correlation and two very dissimilar columns, respectively.

Snyder's F_s values, shown in Table 3, do not totally agree with the results indicated by the correlation coefficients. The HC-SO_3 column is considered dissimilar to the others (high F_s values) as concluded from the correlation coefficients; however, the RP columns and the Novapak CN exhibit similarly high F_s values with respect to the other columns, while they exhibit relatively higher correlation coefficients. According to the F_s values, the most dissimilar columns are the HC-SO_3 column and the Bonus RP column ($F_s = 469$, shown in bold in Table 3). Also the HC-SO_3 column and the Novapak CN or the Prism RP columns exhibit similar high F_s values. Both of those column combinations showed low correlation coefficients, though they were not considered the most dissimilar based on correlation.

Number of Separated Compounds

In Table 4 it can be seen that in a single 1D-separation, the highest number of drugs (N = 15) could be identified using the NovaPak CN column. When combining the NovaPak CN column with the HC-SO_3 column or with the Discovery C_{18} column in parallel 1D-analyses, this number was significantly increased (N = 23), and only two compounds remained indistinguishable. The first column combination also exhibits low correlation and a large F_s value. However, this is not the case for the Novapak CN column and the Discovery C_{18} column. The selection of this pair can be explained by the fact that the latter columns exhibit large individual N values. A large number of compounds (N = 20) could also be separated when the poorly correlated Discovery C_{18} and HC-SO_3 columns were combined. However, this was not the case for the other three combinations indicated by the correlation coefficients: the HC-SO_3 column paired with the BetaBasic Phenyl column, the StableBond C_{18} column (with the 20-70% ACN gradient) or the HC-OH column. This observation can be explained by the fact that relatively poor separations were obtained (seven, eight and eight compounds resolved by at least 0.7, respectively). This remark is also applicable to the column combinations indicated by the F_s values to be the most dissimilar. This confirms the consideration in the introduction: the most dissimilar columns as identified by the R^2 or F_s values will not always result in the highest identification power. This result is because the correlation and F_s calculations do not reflect the quality of the individual separations, only the differences between the two separations. This is consistent with the conclusions of Gilar et al.,[35] who emphasized that the percent utilization of the available separation space was key in providing the best indicator of "orthogonality" of separations. From Table 4 it can also be concluded that the analysis of the drugs on two different columns in parallel (instead of only one column) results in significantly better identification power.

When considering comprehensive LC×LC, the highest number of drugs (19) can be separated, when the Discovery C_{18} is placed in the first dimension and the Novapak CN in the second dimension (Table S-1). These findings are also not consistent with the

intermediate R^2 and F_s values calculated for this column combination. At first glance, it is also surprising to note that the LC×LC separations almost invariably lead to fewer compounds being separated than even the single 1D separations. This observation arises from the Δt_{crit} value in the first dimension being increased from 0.0135 to 0.024 and the Δt_{crit} value in the second dimension being increased from 0.0135 to 0.040. These changes in Δt_{crit} indicate that there is a considerable decrease in resolving power (and therefore peak capacity) upon going from a 1D-LC method to a LC×LC method, specifically for the case of fast LC×LC. In the first dimension, this loss of resolution is due to undersampling, while in the second dimension, the loss of resolution is due to the requirements of the very fast gradient. These results underscore the importance of carefully considering whether a LC×LC method will really improve the resolving power of an analysis. However, a slow LC×LC method would require different Δt_{crit} values, which could result in a higher resolving power.

Information Theory

Considering a 1D-LC analysis, the Novapak CN column exhibits the largest information value (4.40), as shown in Table S-2, and thus the most informative separation can be obtained on this column. The same column was selected based on the number of compounds separable with a difference in retention time larger than the Δt_{crit} (Table 4). When relating the N values to the information value, this means that an information content of 15/25 $\log_2 1/25 = 2.79$ is contributed by the 15 separated drugs and $4.40 - 2.79 = 1.61$ is the information yield for the non-separated drugs. This demonstrates that in information theory not only the number of totally separated compounds is important, but also the degree of co-elution of the non-separated compounds has a considerable influence on this metric.

When performing the information calculations for the analysis on two stationary phases in parallel as described previously, it is confirmed that combining two independent 1D-separations increases the information content and thus also the identification power (Table S-2). Information theory indicates one column combination that yields the maximal information ($I = 4.64$): the Discovery C_{18} combined with the Novapak CN column. This column pair is also indicated as having maximal identification power based on the number of separated compounds (N). The HC-SO$_3$ column and Novapak CN, resulting in the same number of identifiable compounds, exhibit a slightly lower I(1,2) value (4.48). This is caused by the fact that in a situation of maximal information yield two compounds can be located in different bins, although they are not separated with a difference in retention time larger than Δt_{crit} or *vice versa*. The number of separated compounds, N, is moderately correlated with I(1,2) ($r^2 = 0.523$, Figure S-1). However, one N value corresponds with many different information values, which is caused by the fact that for the same number of identifiable drugs different I(1,2) values can be obtained due to different co-elution patterns.

The most dissimilar systems according to information theory (lowest R(1,2) values in Table S-2) are the Discovery C_{18} and the BetaBasic Phenyl. This is not consistent with the results obtained with the correlation coefficients or the F_s values. The Pearson's correlation coefficient is unrelated to the similarity values ($r^2 = 0.0042$, Figure S-2). Secondly, it is noticeable that the similarity (R(1,2)) between all columns is very high. This is unexpected, since columns with different substituents were included, which should provide some selectivity differences. A possible explanation for the high R(1,2) values is that many compounds can be separated on one column, and thus, there is not much room for improvement by adding a second separation.

When applying information theory for comprehensive LC×LC analyses (Table S-3), in some cases the information is lower than when a 1D separation was performed. This is caused by the fact that in LC×LC the size of the bins was larger and thus two compounds will need a

greater difference in retention time to be considered separated. The highest information could, amongst others, be obtained with the column combination also selected based on the N value: the Discovery C_{18} column in the first dimension and the Novapak CN in the second dimension. In addition, several other combinations of columns, denoted in bold in Table S-3, yielded maximal information. According to the information results for LC×LC, the highest dissimilarity occurs between the HC-SO_3 column and the following two stationary phases: StableBond C_{18} and Prism C_{18}. This confirms the earlier findings (correlation coefficients and F_s values) that HC-SO_3 is most dissimilar to the other columns. Although the HC-SO_3 column provides a rather low information yield when used in a single 1D separation, used in the first dimension of a 2D separation the broader peaks do not seem to degrade the separation as much as for the better performing columns.

Multivariate Selectivity

In case of single 1D-LC separations, the addition of spectra resulted in a higher average multivariate selectivity for all columns (Table 5). However, the number of identifiable compounds (number of drugs with SEL > 0.98) could not be increased for most columns. The highest average selectivity based on the retention times without spectral information was obtained on the StableBond phenyl column, but the highest number of compounds could be separated on the Novapak CN column. When adding the spectral information, the same columns are indicated as being most useful for identification. However, based on the average selectivity the Novapak CN was also selected. The above results are consistent with those based on the critical difference in retention time or based on information theory, since these techniques also indicated the Novapak CN as the column on which the highest number of compounds can be identified. When adding spectral information, the average selectivity on the Novapak CN column increased modestly (0.85 vs. 0.99), but no increase in the number of compounds was observed (17 compounds identified either with or without spectral information considered). When comparing the number of separable compounds determined based on Δt_{crit} (Table 4) and based on SEL without spectral information (Table 5), the values are similar for all columns. This is not surprising as we based our selectivity threshold on a target resolution of 0.7 that was used in deriving the Δt_{crit} values. It can also be seen that adding spectral information benefits those separations with lower initial average selectivity more than those separations with already high average selectivity. This can be seen by comparing the HC-SO_3 column, where the average selectivity increases by 27 % (from 0.54 to 0.68) to the StableBond phenyl column, where the average selectivity increases by only 4.7 % (from 0.86 to 0.90).

When performing independent 1D-LC separations on two columns in parallel (Table S-4), higher average selectivity values are obtained than with simple 1D-LC, and the number of identifiable compounds increases substantially. The maximal average SEL (0.99) without including spectral information was obtained with the Novapak CN column combined with either the Discovery C_{18} or the Alltima C_{18} columns. The highest number of drugs (22) could be separated using these same combinations. Information theory and the Δt_{crit} approach also identified the Discovery C_{18}/Novapak CN combination as a top choice for the maximum identification power. The addition of the spectral information resulted, as expected, in greater average multivariate selectivities, but not a greater number of identifiable drugs in most cases. Whether or not the spectral information is included, the combinations of the Novapak CN column with either the Discovery C_{18} or the Alltima C_{18} columns are selected as providing the best identification power for parallel 1D separations.

The average multivariate selectivity values, obtained with LC×LC (Table S-5), are greater than those obtained for 1D-separations and, in most cases greater than those obtained for two parallel 1D-analyses. The reason that the LC×LC results are not always better than the parallel 1D-analyses is due to the need to use a larger Δt_{crit} for both the first and second

dimension separations. In the first dimension, this is due to the undersampling inherent in the LC×LC method, and in the second dimension, this is due to the decrease in peak capacity for a very fast gradient run. An average selectivity of one was obtained for analyses on several column combinations indicated in boldface on Table S-5 and given in Table 6. However, this does not imply that all drugs are identifiable when using these columns. The maximal number of drugs (25) could only be identified when placing the StableBond C_{18} in the first dimension and the Novapak CN in the second dimension, and, when using spectral information, the combination of the Discovery C_{18} with the Novapak CN. The other column combinations exhibiting high average multivariate selectivity result in 21 to 23 identifiable drugs. The latter column combinations also exhibited maximal information. However, with information theory the number of column combinations giving maximal information was larger. This is possibly caused by the fact that the scale of information values is not continuous and similar separations will result in the same value of I(1,2). For most column combinations the addition of spectral information resulted in higher average selectivities and a higher number of compounds with a multivariate selectivity higher than 0.98. However, this was not always the case when the selectivity was already high without spectral information.

Comparison of Information Theory and Multivariate Selectivity—The measures to evaluate the identification power of the separation of the 25 drugs with information theory and with multivariate selectivity (SEL) should be correlated if both approaches are evaluating the same situation (in this case the same combination of parallel columns) well. The information measure I(1,2) is moderately correlated with both the average SEL (R^2=0.47, Figure S-3) as well as the number of compounds with SEL>0.98 (R^2=0.60, Figure S-4). Correlations for the I(1,2) with the selectivity measures for LC×LC methods show somewhat better correlations, with I(1,2) vs. the average selectivity giving an $R^2 = 0.715$ (Figure S-5), and I(1,2) vs. the number of compounds with selectivity >0.98 giving an $R^2 = 0.708$ (Figure S-6). The reason for this may be that when estimating the information yield for two chromatographic systems applied in parallel we always assume that a non-identifiable drug is only co-eluting with one other drug. However, in reality this is not always the case. Consequently, the obtained information value is only an approximation and not as well correlated with the multivariate selectivity, which takes into account the degree of co-elution correctly.

CONCLUSIONS

The column choices with the most identification power indicated by each of the studied methods are summarized in Table 6. We have demonstrated that (dis)similarity measures such as F_s and R^2 do not always indicate the two dissimilar chromatographic systems with the highest identification power. In addition, these measures do not allow distinction between parallel 1D separations and comprehensive 2D separations. An advantage of the F_s metric, however, is that columns can be selected without carrying out actual separations of the target compounds. This tool allows us to choose which columns to select for experimental measurements, which allow for the calculation of the more informative metrics. The measures proposed in this paper enable the comparison between (1) a single 1D-LC method, (2) two 1D-LC separations performed in parallel and (3) the corresponding comprehensive LC×LC method. The number of identifiable compounds (N) can be determined by counting the compounds with a difference in retention time greater than a critical value, which depends on the efficiency of the LC analysis (the peak width). However, N does not take into account the degree of overlap. Information theory enables distinction between two separations with the same number of identifiable compounds, but with a difference in co-elution pattern. Two pairs of two co-eluting compounds, for instance, will yield more information than four co-eluting compounds. The only measure capable of

quantifying the degree of co-elution as well as the degree of separation is multivariate selectivity. The latter can evaluate the number of compounds additionally identified by applying multivariate curve resolution methods when incorporating spectral information obtained with a DAD. Consequently, multivariate selectivity takes thus into account the degree of resolution between nominally unresolved peaks. We feel that this metric offers the most potential for comparing the performance of different analytical methods. It should be mentioned that only a specific set of compounds was considered, and that the results only can be generalized to the extent that these drug solutes serve as general probes of the separation characteristics of the system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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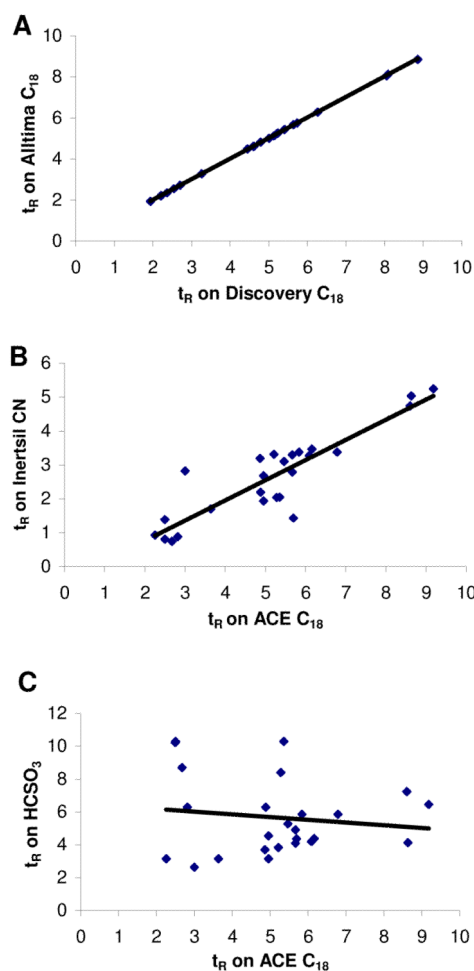
Figure 1.
t_R of 25 drugs on (A) Alltima C_{18} plotted vs t_R of the drugs on Discovery C_{18} (very highly correlated); y = 1.0028(±0.0020)x + 0.004(±0.010); s_E = 0.0184 ; R^2 = 0.9999; N = 25, (B) Intertsil CN plotted vs t_R of the drugs on ACE C_{18} (moderately correlated);y = 0.595(±0.006)x − 0.43(±0.34); s_E = 0.586 ; R^2 = 0.7968; N = 25, (C) HC-SO$_3$ plotted vs. t_R of the drugs on the ACE C_{18} (poorly correlated); y = −0.17(±0.25)x + 6.5 (±1.4); s_E = 2.38; R^2 = −0.0246; N = 25

Table 1

Parameters for calculations of the identification power

Separation System	σ (min)	σ^a (norm)	$\Delta t_{crit}{}^a$ (norm)	Sampling Interval (norm)
1D separation	0.032^b	0.0048	0.0135	0.00048^f
Parallel 1D separations	0.032^b	0.0048	0.0135	0.00048^f
2D separation				
1st dimension		0.0050^c /0.0086^d	0.024	0.0152^g
2nd dimension		0.0144^e	0.040	0.00144^f

[a] The normalized σ and Δt_{crit} values are obtained by normalizing the retention range from zero to one. The Δt_{crit} values are calculated using eq 2.

[b] The value of σ for the 1D separations was estimated from the average peak width of peaks in the experimental data.25

[c] The first dimension σ value, $^1\sigma$, was estimated from an observed peak capacity in LC×LC separations of 50,31 such that $\sigma = 1/(50\times4) = 0.0050$. This value was used in the multivariate selectivity calculations.

[d] The effective first dimension σ value, $^1\sigma_{eff}$, was calculated from $^1\sigma$ using eq 11. This value was used to calculate Δt_{crit}.

[e] The second dimension σ value was estimated from an observed peak capacity in LC×LC separations of 17.4,31 such that $\sigma = 1/(17.4\times4) = 0.0144$.

[f] The sampling interval was calculated as $\sigma/10$.

[g] The sampling interval for the first dimension separation was determined from the typical sampling rate of 0.048 Hz used in actual LC×LC experiments.31

Table 2

Squared Correlation Coefficients Calculated Between the Retention Times of 25 Drugs Measured on 14 Chromatographic Systems.

R^2	SB C$_{18}$	ACE C$_{18}$	Disc C$_{18}$	Allt C$_{18}$	SB Ph	BB Ph	Pront Ph	Prism RP	Bonus RP	Nova CN	Inertsil CN	HC-OH	SB C$_{18}$*	HC-SO$_3$*
SB C$_{18}$	1.00													
ACE C$_{18}$	0.99	1.00												
Disc C$_{18}$	0.99	1.00	1.00											
Allt C$_{18}$	0.99	1.00	1.00	1.00										
SB Ph	0.96	0.95	0.94	0.94	1.00									
BB Ph	0.97	0.97	0.98	0.98	0.96	1.00								
Pront Ph	0.97	0.96	0.95	0.95	0.98	0.96	1.00							
Prism RP	0.83	0.80	0.77	0.78	0.83	0.74	0.86	1.00						
Bonus RP	0.90	0.87	0.85	0.85	0.89	0.82	0.92	0.99	1.00					
Nova CN	0.47	0.50	0.51	0.51	0.43	0.51	0.40	0.26	0.32	1.00				
Inertsil CN	0.80	0.80	0.79	0.79	0.77	0.76	0.78	0.71	0.76	0.29	1.00			
HC-OH	0.94	0.94	0.94	0.94	0.97	0.96	0.95	0.77	0.83	0.49	0.74	1.00		
SB C$_{18}$*	0.93	0.92	0.92	0.91	0.90	0.91	0.92	0.72	0.80	0.45	0.73	0.88	1.00	
HC-SO$_3$*	**0.03**	**0.02**	**0.01**	**0.02**	**0.03**	**0.01**	**0.04**	**0.19**	**0.13**	**0.06**	**0.07**	**0.01**	**0.01**	1.00

Calculated from retention times measured using a 30-70% ACN gradient, except for the columns denoted by the *, which were measured using a 20-70% ACN gradient.[25] The least correlated columns are indicated in bold.

Table 3

Fs Values Calculated Between the Columns Determined Using Snyder's H, S, A, B, C values.

Fs	SB C$_{18}$	ACE C$_{18}$	Disc C$_{18}$	Allt C$_{18}$	SB Ph	BB Ph	Pront Ph	Prism RP	Bonus RP	Nova CN	Inertsil CN	HC-OH	SB C$_{18}$*	HC-SO$_3$*
SB C$_{18}$	0													
ACE C$_{18}$	12	0												
Disc C$_{18}$	14	3	0											
Allt C$_{18}$	8	7	10	0										
SB Ph	18	23	24	18	0									
BB Ph	26	23	23	22	16	0								
Pront Ph	29	32	30	32	32	30	0							
Prism RP	250	250	252	246	241	246	273	0						
Bonus RP	267	266	268	262	257	261	289	24	0					
Nova CN	231	230	232	226	222	226	253	38	52	0				
Inertsil CN	49	50	48	53	55	52	24	296	312	276	0			
HC-OH	30	25	24	29	41	41	47	253	269	235	57	0		
SB C$_{18}$*	0	12	14	8	18	26	29	250	267	231	49	30	0	
HC-SO$_3$*	207	208	205	211	214	209	182	454	**469**	435	160	208	207	0

Calculated from retention times measured using a 30-70% ACN gradient, except for the columns denoted by the *, which were measured using a 20-70% ACN gradient.25 The most dissimilar column pair is indicated in bold.

Table 4

Number of Drugs Separated with a Difference in Normalized Retention Times Greater Than 0.0135 (Rs > 0.7).

N separated	SB C$_{18}$	ACE C$_{18}$	Disc C$_{18}$	Allt C$_{18}$	SB Ph	BB Ph	Pront Ph	Prism RP	Bonus RP	Nova CN	Inertsil CN	HC-OH	SB C$_{18}$*	HC-SO$_3$*
SB C$_{18}$	*10*													
ACE C$_{18}$	15	*9*												
Disc C$_{18}$	17	14	*13*											
Allt C$_{18}$	15	13	13	*11*										
SB Ph	21	16	22	20	*11*									
BB Ph	15	13	17	15	17	*7*								
Pront Ph	18	15	19	17	14	15	*9*							
Prism RP	17	15	19	18	15	13	14	*9*						
Bonus RP	18	17	20	19	17	14	14	12	*8*					
Nova CN	18	21	**23**	21	17	20	19	17	17	*15*				
Inertsil CN	15	17	22	20	18	14	17	16	16	19	*10*			
HC-OH	12	16	18	14	16	13	18	17	17	17	15	*8*		
SB C$_{18}$*	12	15	18	16	18	11	15	16	15	19	13	11	*8*	
HC-SO$_3$*	18	17	20	17	18	15	18	18	18	**23**	17	12	17	*10*

Calculated for the 14 individual chromatographic systems (*italics*) and on all their possible combinations in simultaneous 1D-separations from retention times measured using a 30-70% ACN gradient, except for the columns denoted by the *, which were measured using a 20-70% ACN gradient[25]

Table 5

Average Multivariate Selectivity and Number of Compounds with a Multivariate Selectivity Greater Than 0.98.

	Without spectra		With spectra	
	Av. SEL	**SEL>0.98**	**Av. SEL**	**SEL>0.98**
SB C$_{18}$	0.65	10	0.74	10
ACE C$_{18}$	0.65	9	0.76	9
Disc C$_{18}$	0.63	13	0.74	13
Allt C18	0.64	13	0.75	13
SB Ph	**0.86**	11	**0.90**	11
BB Ph	0.57	9	0.73	10
Pront Ph	0.69	9	0.77	9
Prism RP	0.55	9	0.67	9
Bonus RP	0.73	10	0.80	10
Nova CN	0.85	**17**	**0.90**	**17**
Inertsil CN	0.77	12	0.83	12
HC-OH	0.57	8	0.71	9
SB C$_{18}$[*]	0.56	8	0.71	8
HC-SO$_3$[*]	0.54	10	0.68	10

Calculated from 1D-LC retention times measured using a 30-70% ACN gradient, except for the columns denoted by the [*], which were measured using a 20-70% ACN gradient.25 Values in bold indicate the maximal values.

Table 6

Summary of Results for Identification Power of Separations.

	R^2[a]	Fs[b]	N[c]	Information[d]	Similarity[e]	Average Selectivity[f]	N (SEL>0.98)[g]
Single 1D-LC	NA[h]	NA[h]	N=15 Nova CN	I=4.40 Nova CN	NA[h]	SEL=0.86 SB Phenyl	N=17 Nova CN
Single 1D-LC w/DAD	NA[h]	NA[h]	NA[h]	NA[h]	NA[h]	SEL=0.90 SB Phenyl Novapak CN	N=17 Nova CN
Parallel 1D-LC	R^2 = 0.01 HC-SO_3*&Disc C_{18} or BB Phenyl or HC-OH or SB C_{18}*	Fs=469 HC-SO_3*&Bonus RP	N=23 HC-SO_3*&Novapak CN Disc C_{18}&Nova CN	I(1,2)=4.64 Disc C_{18}&Nova CN	R(1,2)=0.971 Disc. C_{18}&BB Phenyl	SEL=0.99 Disc C_{18}&Nova CN Allt C_{18}&Nova CN	N=22 Disc C_{18}&Nova CN Allt C_{18}&Nova CN
Parallel 1D-LC w/DAD	NA[h]	NA[h]	NA[h]	NA[h]	NA[h]	SEL=0.99 Disc C_{18}&SB Phenyl Allt C_{18}&SB Phenyl Disc C_{18}&Nova CN Allt C_{18}&Nova CN	N=22 Disc C_{18}&Nova CN Allt C_{18}&Nova CN
Comprehensive LC×LC	NA[h]	NA[h]	N=19 Disc C_{18}&Novapak CN	I(1,2)=4.64 SB C_{18}&Nova CN ACE C_{18}&Nova CN Disc C_{18}&Nova CN or Inertsil CN Allt C_{18}&Nova CN or Inertsil CN or HC-SO_3* Pront Ph&ACE C_{18} Nova CN&SB C_{18} or ACE C_{18} or Disc C_{18} or Allt C_{18} Inertsil CN&ACE C_{18} HC-SO_3*&Disc C_{18} or Allt C_{18}	R(1,2)=0.969 SB C_{18}& HC-SO_3 Prism C_{18}& HC-SO_3 HC-SO_3& SB C_{18}	SEL=1.00 SB C_{18}&Nova CN ACE C_{18}&Nova CN or Inertsil CN Disc C_{18}&Nova CN or Inertsil CN Allt C_{18}&Nova CN or Inertsil CN Nova CN&SB C_{18} or ACE C_{18} or Disc C_{18} or Allt C_{18}	N=25 SB C_{18}&Nova CN
Comprehensive LC×LC w/DAD	NA[h]	NA[h]	NA[h]	NA[h]	NA[h]	SEL=1.00 SB C_{18}&Nova CN ACE C_{18}&Nova CN or Inertsil CN Disc C_{18}&Nova CN or Inertsil CN Allt C_{18}&Nova CN or Inertsil CN Nova CN&SB C_{18} or ACE C_{18} or Disc C_{18} or Allt C_{18}	N=25 SB C_{18}& Nova CN Disc C_{18}& Nova CN