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Short communication

Comprehensive two-dimensional chromatography with coupling of reversed phase high performance liquid chromatography and supercritical fluid chromatography

Paul G. Stevenson, Abhijit Tarafder, Georges Guiochon*

Department of Chemistry, University of Tennessee, Knoxville, TN, USA

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ABSTRACT

A 2D comprehensive chromatographic separation of blackberry sage fragrant oil was performed by using HPLC in the first dimension and SFC in the second. A C_{18}-bonded silica column eluted with an ACN gradient was used in the HPLC dimension and an amino-bonded silica column eluted with ACN as a modifier in the SFC dimension. This 2D separation was completed in the off-line mode, the fractions from the HPLC column being collected and injected in the SFC column. The retention factors on the two columns have a −0.757 correlation coefficient. The method provides a practical peak capacity of 2400 in 280 min. The first eluted peaks in HPLC are the last ones eluted in SFC and vice versa. The results demonstrate that the coupling of an HPLC and an SFC separation have a great potential for 2D chromatographic separations.

1. Introduction

The first problem in developing a 2D chromatographic separation is to maximize the practical peak capacity that can be achieved by selecting two columns implementing retention mechanisms that are orthogonal or nearly so [1]. The thermodynamics of the retention in reversed phase-HPLC and supercritical fluid chromatography (SFC) are fundamentally most similar but that there are some crucial differences between these two mechanisms in terms of the nature of some of the molecular interactions taking place between the solutes, the mobile and the stationary phases. The opposite polarities of CO_2 and H_2O as well as the profound differences between their respective interactions with the stationary phases are key to the differences between these mechanisms [2]. With polar stationary phases, SFC tends to behave like NP-HPLC [3].

Coupling these two different modes of chromatography for a multidimensional separation should produce a separation that uses a large fraction of the separation space, as confirmed by the few instances reported in the literature of 2D separations combining an SFC and an HPLC system to achieve the analysis of complex samples [4,5].

The aim of this work was to illustrate a LC × SFC separation with LC in the first dimension using a fragrant oil as a complex sample mixture. Performing an off-line separation with HPLC in the first dimension is technically simpler when a fraction collector is available to connect to the HPLC instrument and can be incorporated in the experimental design. The separation was not optimized for any particular parameter, however, peak capacity, separation speed and signal to noise ratio were all considered.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile (ACN), methanol and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pure CO_2 was purchased from Airgas (Knoxville, TN, USA). Blackberry sage fragrant oil from Aztec International INC. (Knoxville, TN) was dissolved in ACN at a concentration of 10 μL/mL. Samples were injected without further dilution.

2.2. Instrumentation

The first dimension separation was performed with an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a mobile-phase degasser, an auto sampler, a binary pump and a column compartment. Samples of 80 μL were injected into the column. The mobile phase composition was increased from 55 to 100% ACN in H_2O over 10 min, then held at this concentration for a further 8 min. The column used in the first dimension was

* Corresponding author.
E-mail address: guiochon@ion.chem.utk.edu (G. Guiochon).

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a Phenomenex Luna C18(2) column (Phenomenex, Torrance, CA) (150 mm × 4.6 mm, 3 μm particle diameter, 100 Å pore size, S/N 380692-5). The first dimension separation was completed with a flow rate of 0.5 mL/min and at a constant temperature of 24 °C, fixed by the laboratory air conditioner.

Fractions from the first dimension were collected from 4 to 18 min every 15 s (125 μL) using a Gilson model 203 fraction collector (Middleton, WI), for a total of 56 fractions. The second dimension analysis was performed immediately upon completion of the first dimension separation.

The second dimension separation was performed on a TharSFC (Waters, Milford, MA) with a PrincetonSFC Amino column (250 mm × 4.6 mm, 10 μm particle diameter, 60 Å pore size, S/N 07-8776) (Princeton Chromatography INC., Cranbury, NJ). Other columns that were tested for the second dimension were a Virdis SFC Silica column (150 mm × 4.6 mm, 5 μm, S/N 0102103071EE 05, Waters, Milford, MA) and a Luna Amino column (150 mm × 4.6 mm, 5 μm, 100 Å pore size, S/N 341534-1, Phenomenex, Torrance, CA). The separations were performed with a sharp concentration gradient whereby the starting mobile phase composition, 90% CO_2:10% ACN, was brought to 40% CO_2:60% ACN within a minute, after what it was kept constant for a further minute. The column was then re-equilibrated to the initial mobile phase composition, for a period of 3 min prior to the next injection. The mobile phase was pumped at 3 mL/min, back pressure of 175 bar and at 27 °C. The injection volume of the second dimension was 50 μL using the partial loop injection method with a 100 μL loop. The chromatograms were collected with a UV detector with the wavelength set at 210 nm.

The one-dimensional separations were conducted according to the corresponding methods with injection volumes of 4 μL on the HPLC dimension and 3 μL on the SFC dimension.

2.3. Data analysis

The retention times for 2D-HPLC peaks were measured according to the method described in Ref. [6]. Calculations for the analysis of the 2D chromatographic peaks and graphics were performed with Wolfram Mathematica 7 (Wolfram Research, Champaign, IL) using algorithms written in-house.

The sample peak capacity was measured according to Eq. (1), where t_g is the gradient time and W is the average width of the peaks measured at 4σ [7,8].

$$n_c \approx \frac{t_g}{W_{4\sigma}} \quad (1)$$

3. Results and discussion

The blackberry sage fragrant oil is an appropriate sample to investigate the possibilities and potential performance of LC × SFC for the analysis of complex mixtures because it contains many components and gives numerous overlapping and co-eluting peaks, as illustrated in Figs. 1 and 2 and most of these compounds absorb in the UV range. This product is also available in abundance.

3.1. SFC method development

Method development for the SFC dimension included first the selection of the proper mobile and stationary phases, then the design of the operating pressure and temperature, and the choice of the gradient conditions. The possibility of using neat CO_2 as the mobile phase was eliminated because we observed an excessive level of baseline noise, which made it difficult to detect all the components in the sample, especially those present in trace amounts. This mainly originates from the compressibility of the mobile phase [9]. ACN was preferred over methanol as the most appropriate modifier of CO_2 because its use reduces the mobile phase mismatch between the SFC mobile phase and the eluate of the HPLC column. Besides, there was no visually significant qualitative difference between the separation performance obtained with these two modifiers.

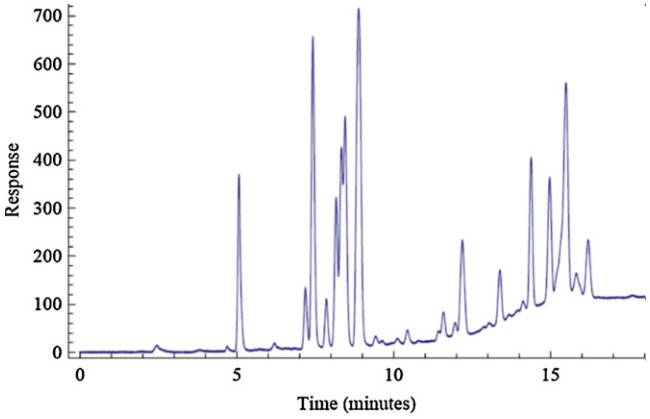

Fig. 1. First dimension separation of fragrant oil completed with HPLC, a C18 column and ACN gradient.

3.2. 2D separation design

The criteria for pairing included a good spread of peaks in both dimensions and some different selectivity (determined by comparing relative peak heights). In the case of the second dimension the 1D separation was performed over a longer time period than shown here, then shortened for the 2D analysis. Several gradient methods were tested, ranging from short to long and from shallow to steep gradients. Eventually, a sharp gradient, the details of which are provided in the experimental section, was selected. While implementing a sharp gradient caused the loss of two peaks, it led to a better peak capacity (peak capacity of 23 peaks per minute, compared to 21 for the shallow gradient), hence a better overall resolution of the sample components, which is a desirable criterion for 2D separations. Also, the final peak eluted 2.3 min earlier with the sharper gradient.

3.3. Multidimensional separation

The first dimension separation is illustrated in Fig. 1, where two clusters of peaks are observed, the first between 7 and 9 min and the second between 14 and 17 min. Due to the nature of the HPLC retention mechanism on a C18-bonded silica column the earlier

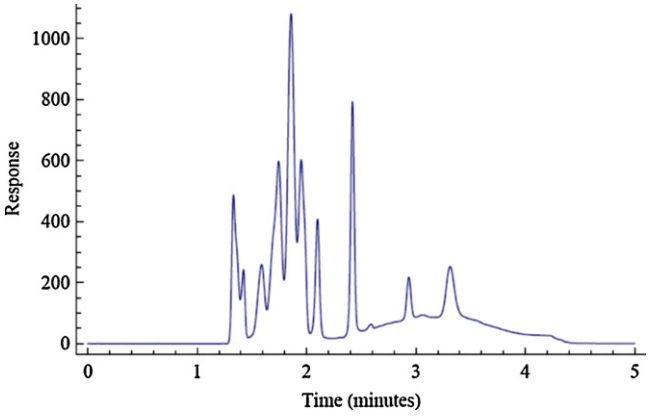

Fig. 2. Second dimension separation of fragrant oil completed with SFC, an amino column and ACN step gradient.

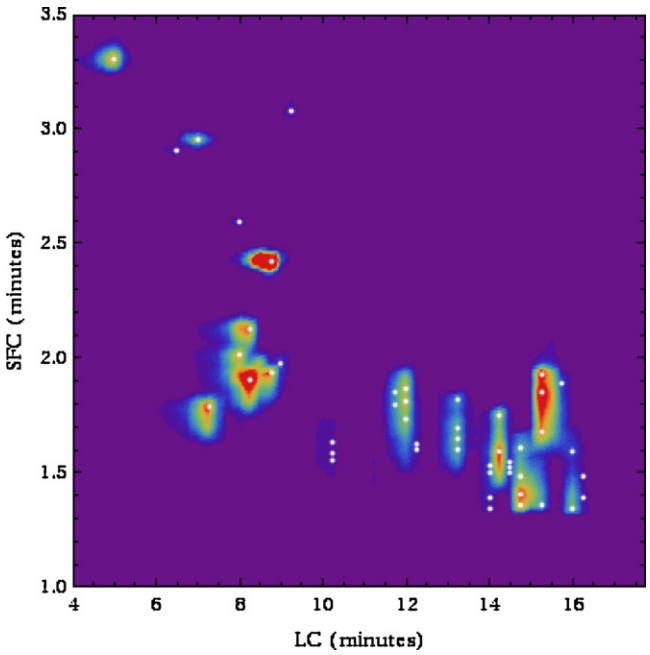

Fig. 3. Multidimensional separation of fragrant oil whereby HPLC was used in the horizontal first dimension and SFC in the vertical second dimension. The red coloring represents a stronger detector response and detected peaks are shown with white points. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

eluting peaks have a higher polarity (thus, a lesser affinity for the non-polar stationary phase) and the later group is more hydrophobic. The first peak elutes at 5.06 min and the last peak at 16.2 min, the average peak width (0.122 min) was calculated by averaging the width at half-height of several singlet peaks. This calculation gave a peak capacity of 92 in the first dimension.

The second dimension separation is illustrated in Fig. 2. A rapid visual comparison of the separations obtained in the two dimensions shows clearly that two different retention mechanisms are involved because there are no discernible similarities between the two chromatograms. In this second separation, the first peak elutes at 1.33 min and the last peak at 3.31 min. The average peak width was measured to be 0.043 min, giving a peak capacity of 46 in the second dimension. When the concentration of the ACN modifier was decreased in order to improve the resolution between the early eluting peaks, the signal to noise ratio significantly decreased to the point that it became difficult to detect most peaks. This is likely caused by fluctuations in the back pressure regulator. Further work needs to be completed to improve the analytical performance of the instrument in the resolution of poorly retained compounds.

The multidimensional separation was completed in the off-line mode of 2D chromatography. The chromatograms of the 56 fractions of eluate of the HPLC column were recorded, exported by the Empower software (Waters, Milford, MA, USA) in text format, and compiled by algorithms built in house with Mathematica 7. The resultant contour plot is illustrated in Fig. 3. The chromatographic peaks were detected and quantified using methods described previously [10,6]. The programs requires that the SFC peaks have a minimum response value greater than 15 mAu. The first derivative threshold used for distinguishing co-eluting peaks was 5×10^{-5}. The second derivative of the peak profile is used to detect closely eluting peaks. However, due to the baseline noise of the chromatograms many false positives were detected. So, we report only the results of the first derivative peak detection method. As a consequence, some shouldering peaks were lost. However, a large number of peaks were detected, sufficiently to provide an accurate representation of the performance of this mode of 2D-chromatographic separations. The trade-off between high resolution and fast analysis is not easily qualified as it depends essentially of the analytical purpose. It can be qualified by the resolution of peaks and the time needed. The shouldering peaks observed could have been resolved with a further optimization of the experimental conditions of the second dimension. However, speed of analysis was preferred to an increase in the peak resolution. To account for the fact that a given compound may elute in successive fractions, a peak region overlap of 90% was required for matching peaks (for more detailed information see [10]). A total of 48 peaks were detected. The apex of their elution peaks are represented by the white dots in Fig. 3.

Fig. 3 reflects the two groups of peaks observed in Fig. 1. The more polar compounds that elute early in HPLC are spread across a large amount of the SFC separation space, utilizing most of the available area. In contrast, the compounds that elute late in the first dimension use a much smaller fraction of the second dimension space. This separation follows a trend that is quite different from that of most LC × LC chromatographic separations in which the peaks are positively correlated, i.e. are grouped in a fan around the first diagonal of the overall separation space. The tip of this fan is close to the point having the hold-up times of both modes as coordinates and the fan widens toward the end of both separations. Instead, there is a negative correlation whereby the peaks scatter around the second diagonal of the separation space. In their analysis of 2D-HPLC data, Mnatsakanyan et al. [11] divided the separation space into four quadrants and analyzed these regions independently. Following the same approach in the case of the fragrant oil separation shows that a very large fraction of the separation space is used in three quadrants while no peak elute in the top right one.

Gray et al. [12] showed that the performance of multidimensional separations can be conveniently measured using a geometric approach to factor analysis (GAFA). GAFA yields information that is visually simple to interpret and for which calculations can be easily automated with minimal programming knowledge. This method is commonly used to examine variations within data sets, in the context of 2D-HPLC. Liu et al. [13] used an alternate, geometric approach to factor analysis to assess the degree of orthogonality of the two retention mechanisms and to estimate the actual peak capacity of the separation. Correlation matrices can be constructed from the scaled retention times of solutes in each dimension. This permits a practical visualization of the peak capacity. Information relating to these calculations can be found in Refs. [12–14].

The GAFA of this data set reveal a spreading angle of 40.8° with a correlation coefficient of −0.757, a theoretical peak capacity of 4232 and a practical peak capacity of 2405. The theoretical peak capacity is a multiplication of the peak capacities of both dimensions thus assumes a spreading angle of 90°, i.e. complete dimension orthogonality. In reality there is some correlation between the two dimensions, and the practical peak capacity is less than the theoretical peak capacity. The negative sign for the correlation coefficient reflects the greater separation of the polar compounds that eluted earlier in the HPLC dimension compared to the later eluting hydrophobic compounds. The total aggregate analysis time (i.e. the sum of the separation time and the column re-equilibration time) for this separation was 280 min. With a practical peak capacity of 2405 this system has the potential of eluting 8.6 peaks per minute.

4. Conclusion

The aim of this manuscript was to illustrate the possibility of coupling LC and SFC to perform a 2D chromatographic separation. This was successfully achieved and our work proves that coupling

these two separations methods provides a good framework for further 2D separations.

The degree of separation space utilization of this analysis shows that when a high theoretical peak capacity is available, a relatively high practical peak capacity can also be achieved provided that the two retention mechanisms have been properly chosen. In the case in point, the practical peak capacity is 57% of the theoretical one, although the degree of orthogonality of the two retention mechanisms provides only a GAFA spreading angle of 40.8° and a correlation coefficient of −0.757.

Acknowledgements

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