Advantages of two-dimensional liquid chromatography in the analysis of complex samples

Pavel Jandera*, Tomáš Hájek, Petr Česla, Veronika Škeříková

Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, CZ-532 10, Czech Republic

* Corresponding author. E-mail: pavel.jandera@upce.cz

Two-dimensional (2D) LC–LC separation techniques can be used to significantly increase the number of compounds separated in a single run in comparison with single-dimension separations and to classify a sample on the basis of structural similarities. In-line “comprehensive” LC × LC has experienced a rapid development over the past years, because it allows automatic performance avoiding tedious and time-consuming off-line fraction transfer between the separation systems.

Combining different LC separation mechanisms, such as reversed-phase (RP), ion-exchange (IEX) and normal-phase (NP) or HILIC chromatography is aimed to accomplish a non-correlated retention with the maximum peak capacity in the time available for the analysis in “orthogonal” two-dimensional LC × LC separation systems. In comprehensive two-dimensional LC × LC, the separation in the second dimension should be accomplished, while the next fraction from the first dimension is collected, usually in less than 1 min. The chemistry of the stationary phase and the composition of the mobile phase in each dimension should match the real-time 2D operation. System incompatibility due to a limited miscibility of the mobile phases and/or too large differences in the elution strengths in the two dimensions should be avoided. Two RP systems with different stationary phases can be combined on-line more easily than an RP system with an NP or HILIC system. Gradient elution significantly increases the number of resolved peaks, both in the first and in the second dimensions. Fast (<1 min) second-dimension gradients with different subsequent time segments improve the overall 2D resolution and separation time.

Key words: two-dimensional LC, comprehensive LC × LC, gradient elution

INTRODUCTION

Ever-increasing demands for the analysis of complex samples containing hundreds or thousands of compounds cannot be successfully met by separation on a single column. This need has triggered the recent development of two-dimensional (2D) separations in order to increase the number of sample compounds separated in a single run. In addition to now almost routine GC × GC applications, various combinations of LC × LC separation modes have recently emerged [1–4].

A separation system can be regarded as multi-dimensional when the mechanism of separation in each dimension is different. Multi-dimensional systems are primarily used to increase the number of separated compounds, which is conveniently characterized in terms of the peak capacity \( n \) defined as the maximum possible number of separated compounds with regularly spaced closely adjacent peaks filling the whole space available in the chromatogram.

The single-dimensional peak capacity \( n \) under isocratic conditions in the range of elution volume between the first \( V_{k,1} \) and the last \( V_{k,Z} \) elution volumes in a single chromatographic run can be calculated as

\[
\frac{n}{4} = \frac{\sqrt{N}}{4} \ln \left( \frac{V_{k,Z}}{V_{k,1}} \right) + 1,
\]

where \( n \) depends primarily on the number of theoretical plates of the column \( N \), but is strongly affected by the separation selectivity of the column which controls the peak
spacing in the chromatogram. As the spacing is more or less irregular, the practical number of peaks that are separated in real samples is always lower than the theoretical peak capacity.

Under isocratic conditions, the width of the peaks increases proportionally to the elution time of solutes, in contrast to gradient elution LC where the bandwidths $w_g$ are significantly narrower and approximately constant during the gradient run due to the continuously increasing elution strength of the mobile phase. Consequently, the gradient peak capacity $n_G$ is higher than in the isocratic mode within the same range of elution times (volumes) from $V_{R,1}$ to $V_{R,Z}$, corresponding to the gradient time $t_G$ (Fig. 1) [1]:

$$n_G = 1 + \frac{\sqrt{N} t_G F}{4 V_m (1 + k)} ,$$

(2)

where $V_m$ is the column hold-up time, $F$ is the flow-rate of the mobile phase, and $k$ is the retention factor at the time of the peak maximum elution, which is approximately independent of the solute elution time under gradient conditions.

Equation (2) applies in linear gradient reversed-phase LC in which the effect of the volume fraction of the organic modifier in the binary aqueous-organic mobile phase on the retention under isocratic conditions is – to the first approximation – described by the equation

$$\ln k = a - m \phi .$$

(3)

In two-dimensional chromatography, where all compounds are subject to separation on two different columns and/or mobile phases, the peak capacity is subject to the multiplication effect, and the number of resolved compounds can be theoretically as high as the product of the peak capacities in the first and second dimensions:

$$n_{2D} = n_1 \times n_2 .$$

(4)

However, the number of peaks resolved in real two-dimensional systems depends on the differences in the separation selectivity for the sample in the first and second dimensions. The highest two-dimensional peak capacity can be achieved in the so-called “orthogonal” systems with completely different retention mechanisms, resulting in a non-correlated retention (selectivity of separation).

**Experimental setup**

2D LC × LC systems employ a combination of two different columns with different separation modes in the first and second dimensions, connected via a ten- or twelve-port switching valve (or several six-port switching valves) and one or more mobile phases (isocratic or gradient elution). The most common two-dimensional LC × LC technique is the fraction heart-cutting, where only the fractions of interest are selected from the effluent of the first-dimension separation system for the separation in the second dimension. In “comprehensive” two-dimensional LC × LC, the entire sample is subjected to two separation mechanisms. The whole effluent from the first dimension is collected in subsequent small-volume fractions in storage loops and then transferred into the second-dimension separation system in multiple repeated alternating cycles controlled by the frequency of the switching valve interface with two sampling loops or enrichment columns (Fig. 2) [2, 3].

Like in single-dimensional separations, the number of peaks that can be really separated in comprehensive LC × LC is always lower than the maximum theoretical peak capacity expected based on Eq. (4). To improve the coverage of the useful 2D retention space available for separation and to increase the peak capacity, efficient columns in “orthogonal” separation systems showing large differences in selectivity for sample compounds should be used. 2D separations should be optimized by matching the column geometry and type. A column with a higher peak capacity should be
Advantages of two-dimensional liquid chromatography in the analysis of complex samples

preferred in the first dimension, as a rule, a long narrow-bore column or a micro-column and a low flow-rate of the mobile phase, if allowed by the detection sensitivity. As the second-dimension separation of the actual fraction should be accomplished during the time of collecting the next fraction, a short and efficient column with the internal diameter equal to or larger than that used in the first dimension and as high flow rates as possible (a column packed with small particles (3 μm or less), a non-porous, superficially porous or monolithic column) should be used at a high flow-rate for fast separation, as the separation time is limited by the switching cycle frequency.

The maximum operation pressure allowed in the instrument in the second dimension determines the time of a comprehensive LC × LC analysis; hence, second-dimension columns packed with small-diameter particles may require UHPLC instrumentation, whereas columns packed with superficially porous particles or monolithic columns show a higher permeability and therefore can be used in conventional HPLC instruments.

The mobile phases and flow rate in the two dimensions, the size of the transferred fractions and the frequency of the fraction transfer cycle should be appropriately adjusted to the performance of the first- and second-dimension systems, taking into account the limited available separation time and the desired resolution in the second dimension. Ideally, every peak from the first dimension should be transferred to the second dimension in at least three or four consecutive fractions.

Because of the consecutive dilution of fractions transferred from the first dimension, detection limits and sensitivity are very important criteria in the development of a 2D separation. In addition to the necessity of using sensitive MS, fluorescence or UV detectors, band broadening connected with sample fraction transfer from the first to the second dimension should be suppressed. Various “modulation” approaches can be applied to suppress the broadening of the chromatographic bands transferred from the first to the second dimension. “Modulation” in GC is usually accomplished by inserting a “cold trap” between the first- and second-dimension columns. This approach cannot be applied in 2D LC × LC in which, however, two small “trapping” columns can be used instead of sampling loops in the 10-port valve as the interface for the fraction transfer between the first and second-dimension columns, so that each fraction from the first dimension is trapped alternately on one of the two trapping columns. At the same time, compounds retained from the previous fraction on the second trapping column are back-flushed onto the analytical column in the second dimension (Fig. 3).

Another possibility is to omit the sampling loops and to connect the first dimension column via a switching valve directly to two identical second-dimension columns operating in a parallel setup in alternating cycles (a fraction from the first-dimension column is trapped alternately on one of the two columns, meanwhile the second column is back-flushed, and the solutes transferred in the preceding fraction from the first-dimension column are separated) (Fig. 4).

Phase system selection and programmed elution in two-dimensional comprehensive LC × LC

In addition to an appropriate choice of the right stationary phase in the first and second dimensions, selection of the mobile phases is equally important, as the whole stationary/mobile phase system controls the retention and the separation selectivity. For this purpose, the mobile phases should be optimized to provide the highest selection differences between the first and the second dimensions. Of course, the mobile phases used in the two dimensions should be compatible. Connecting two reversed-phase systems or an RP and an ion-exchange system usually does not present serious compatibility problems. On the other hand, highly orthogonal
two-dimensional systems including reversed-phase and non-aqueous normal-phase (adsorption) modes with completely different separation mechanisms are much more difficult to couple in-line, because non-aqueous mobile phases are very strong eluents in most reversed-phase systems and often cause a too early elution and a poor separation in the second RP dimension, whereas aqueous-organic mobile phases used in RP systems are not only strong eluents, but also may completely desactivate the adsorption capacity of a polar adsorbent, if used in the second-dimension system. There are two possible solutions of this problem for non-polar or weakly polar compounds, normal-phase separations can be sometimes combined with non-aqueous reversed-phase chromatography, such as in separations of acylglycerols, carotenoids and other lipids. On the other hand, polar compounds can be separated in combined RP–HILIC systems for the separation of carbohydrates, glycosides, carboxylic acids, amino acids, peptides, proteins, metabolites, etc. HILIC (hydrophilic interaction liquid chromatography) is essentially liquid chromatography on polar columns, using aqueous-organic mobile phases. However, even in these systems there may be still some compatibility problems; therefore, two-reversed-phase comprehensive RP × RP systems employing columns with large differences in polarity are very useful for various separation problems [4].

If possible, the mobile phase used in the first dimension should have a low elution strength in the second dimension. If the sample fractions are eluted from the first column with a weaker mobile phase than that used in the second dimension, the transferred fraction is concentrated (focused) in a narrow zone on the top of the second-dimension column before the elution step, which leads to narrow second-dimension bandwidths.

Because of a higher peak capacity, gradient elution is generally preferred to isocratic LC for separations in the first dimension (Fig. 1). Temperature programming offers another possibility for increasing the peak capacity, especially in the separation of macromolecular or oligomer compounds. In the second dimension, the time necessary for the re-equilibration of the column before the transfer of the next fraction decreases the time available for the separation if repeated gradients are run with each transferred fraction. This problem can be in some cases avoided by using parallel gradients in the two dimensions over the full 2D separation time. If the second-dimension gradient ramp over the whole 2D separation time is shallow enough, the separation conditions are
Advantages of two-dimensional liquid chromatography in the analysis of complex samples

Fig. 5. Two-dimensional comprehensive LC × LC separation of phenolic acids and flavones with simultaneous gradients of acetonitrile in buffered aqueous organic mobile phase (0.01 mol/L CH₃COONH₄, pH 3.1). First dimension: a PEG column (5 µm, 150 × 2.1 mm i. d.), 65 µL/min; second dimension: a Kinetex C18 column (2.6 µm, 50 × 3.0 mm i. d.), 2.5 × mL/min. a – fast 2nd D gradient with a broad acetonitrile concentration range; b – fast 2nd D gradients with two segments of acetonitrile concentration range; c – segmented 2nd D gradient parallel to the first-dimension gradient with a shallow acetonitrile concentration range. Acetonitrile concentration profiles are shown in the insets (broken line for 1st D, full line for 2nd D) [6]
Pavel Jandera, Tomáš Hájek, Petr Česla, Veronika Škeříková

quasi-isocratic and do not cause problems in the evaluation of the 2D data [5]. Synchronized gradients run simultaneously in the first and in the second dimensions and, using elevated temperatures (if permitted by the sample stability and column type), can significantly improve the regularity of the coverage of the available retention space and reduce the time of analysis. The selectivity of separation and the number of resolved peaks can be further increased by using serially connected columns with a different chemistry of the stationary phases in the first dimension and a fast and efficient column in the second dimension. Such setups show some features of three-dimensional separation systems.

Two-dimensional LC × LC separations have become increasingly used for numerous practical applications, including separations of peptides and proteins, pharmaceuticals, industrial products and natural compounds in plant extracts and food samples. Figure 5 shows a comprehensive LC × LC separation of natural antioxidants in 30 min in an orthogonal reversed-phase 2D system on a polyethylene glycol column in D1 and a fused-core C18 silica column in D2 with parallel gradients and a fast separation at a high pressure and a flow-rate in D2 [6].

CONCLUSIONS

As compared with uni-dimensional separations, 2D separations provide a significantly higher number of resolved peaks (peak capacity) and improved possibilities for separation into classes with common structural features. Comprehensive LC × LC offers an automated method for the analysis of the whole sample in two dimensions.

The equipment can be easily assembled from a gradient liquid chromatograph (preferably with a micro-flow rate possibility), an additional gradient pump (preferably enabling a high-pressure operation) and a ten-port two-way switching valve interface. 2D data evaluation is possible with slightly adapted standard software for 3D data processing (e.g., ORIGIN); special software for LC × LC is becoming available.

Selection of non-correlated “orthogonal” 2D systems, sample transfer modulation, optimization of column dimensions and flow rate, employing gradient elution in D1, D2, high temperature operation and monolithic, sub-2 um, or fused-core silica 2nd D columns enable to increase peak capacity and to speed up 2D separations.

ACKNOWLEDGEMENTS

The support of this research by the Ministry of Education, Youth and Sports of the Czech Republic under the project MSM 0021627502 and by the Grant Agency of the Czech Republic under the project 203/07/0641 is acknowledged.