Journal of Chromatography A, 1235 (2012) 39–48

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Pareto-optimality study into the comparison of the separation potential of comprehensive two-dimensional liquid chromatography in the column and spatial modes

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ARTICLE INFO

Article history:
Received 18 October 2011
Received in revised form 12 January 2012
Accepted 20 January 2012
Available online 25 January 2012

Keywords:
Two-dimensional liquid chromatography
LC × LC
Spatial chromatography
OPTLC

ABSTRACT

The expected performance of spatial ("flat-bed") two-dimensional liquid chromatography (^{x}LC × ^{x}LC) has been calculated using the Pareto-optimality strategy. This approach allowed different objectives (total peak capacity, total analysis time, and total dilution) to be considered simultaneously and to establish optimal parameters (pressure drop, particle size, bed length, and initial spot size). The performance of spatial two-dimensional chromatographic systems was compared with that of conventional on-line, real-time two-dimensional column-liquid-chromatography systems (^{t}LC × ^{t}LC). The potential gain in peak capacity and/or analysis time of the spatial configuration was confirmed. By restricting the spatial parameters to realistic chromatographic conditions (limiting the stress, as counterbalance for the pressure drop through the sorbent bed, to 2500 kg) it was found that ^{x}LC × ^{x}LC is attractive for very fast analysis of complex samples, rather than for extremely efficient separations. For example, a peak capacity of 780 may be achieved in only 2.7 min using a 100 × 100 mm sorbent bed of a quality currently encountered thin-layer chromatography. Furthermore, if beds can be packed as efficiently as contemporary columns, the predicted peak capacity increases to around 1000, corresponding to a peak-production rate of about 6.3 peaks/s. Possibilities to boost the performance of ^{x}LC × ^{x}LC further are briefly discussed. Unless we can overcome the severe stress requirements of high-performance ^{x}LC × ^{x}LC, conventional ^{t}LC × ^{t}LC may be more amenable to very complex separations, thanks to the very high peak capacities. However, ^{t}LC × ^{t}LC separations will require long analysis times (*e.g.* 10,000 peaks in 37 h, corresponding to 0.075 peaks/s at a pressure drop of 40 MPa). The best trade-off between total peak capacity, total analysis time, and total dilution under restricted (realistic) conditions was obtained using high pressures, small chromatographic beds, small particles, and relatively large sample spots.

1. Introduction

Modern liquid chromatography is all about resolving the highest number of peaks in the shortest possible time. To realize this, high system performances are indispensable. A way to express the separation potential of a chromatographic system is the peak capacity, n_c. This parameter was introduced by Giddings in 1967 as a measure for the number of peaks that can be located – at equal resolution – between the first (unretained) and the last (most retained) peaks in a chromatogram [1]. However, a statistical treatment, assuming the peaks to be distributed randomly, has revealed that the number of single-component peaks cannot exceed about 18% of the peak capacity [2]. Furthermore, the expected total number of peaks p (*i.e.* singlets, doublets, triplets, *etc.*) can be expressed by [2]:

$$p = me^{-m/n_c} \quad (1)$$

where m is the number of components in the sample and n_c the peak capacity.

In the field of systems biology (proteomics, metabolomics, *etc.*) very complex samples are frequently encountered. A typical proteome sample may contain 10,000–50,000 different proteins or – in case these proteins are digested prior to analysis – 100,000–500,000 different peptides [3]. It is clear from Eq. (1) that it is virtually impossible to separate all the components in such a complex sample. However, irrespective of the complexity of the sample, maximizing the number of separated components can only be achieved through maximizing the peak capacity of the chromatographic system (Eq. (1)).

The most straightforward approach to increase the peak capacity in one-dimensional liquid chromatography (1D-LC) is to

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doi:10.1016/j.chroma.2012.01.059

increase the number of plates, *N*. Many theoretical and experimental studies into achieving high peak capacities in one-dimensional column LC (1D-^{t}LC) have been published [4–15]. For example, Shen et al. realized a peak capacity of 1500 for the separation of a tryptic digest of *Shewanella oneidensis* in approximately 33 h with gradient-elution reversed-phase LC (RPLC), using a 2 m × 50 μm I.D. fused-silica column packed with 3.0-μm porous C_{18} particles [14]. Eeltink et al. recently obtained a peak capacity exceeding 1000 in 10 h for the separation of a proteolytic digest of *Escherichia coli* in RPLC, using a monolithic poly(styrene-*co*-divinylbenzene) capillary column of 1 m length [4]. The law of diminishing returns is, however, merciless in 1D-LC. Once n_c is large, even a slight increase will require a very much longer analysis time [16]. For separating the proteomics samples mentioned above 1D-LC clearly falls way short.

Potentially, a much more favourable trade-off can be achieved in comprehensive two-dimensional column LC (commonly LC × LC, but in the context of the present paper denoted as ^{t}LC × ^{t}LC). In this configuration, the theoretical total peak capacity of the two-dimensional set-up $^{2D}n_c$ equals the product of the peak capacities obtained in each dimension separately, whereas the total analysis time $^{2D}t_w$ equals the sum of the analysis times for each dimension [17,18]. The first-dimension separation is usually very slow, but the peak capacity can be increased by an order of magnitude in comparison with 1D-LC, while keeping the analysis time within reasonable limits. However, to make full use of this gain in peak capacity, ^{t}LC × ^{t}LC should make use of two completely different (independent or "orthogonal") separation mechanisms. Any correlation between the retention times in the two dimensions will result in a decrease in the effective peak capacity and the experimentally obtained n_c values will be lower than the product of the peak capacities in the individual dimensions.

Two-dimensional (liquid) chromatographic separations can be divided into two categories, *i.e.* time-based (^{t}LC × ^{t}LC) and spatial (^{x}LC × ^{x}LC) separations. Time-based LC (^{t}LC) is associated with the traditional "column" chromatography, in which solutes are eluted from the separation body to be detected. In the case of spatial chromatography (^{x}LC) the solutes migrate to specific positions in the separation body. In ^{t}LC × ^{t}LC small consecutive fractions from the first dimension are injected in the second dimension. The resulting series of second-dimension chromatograms can be visualized as a two-dimensional color map. In spatial two-dimensional chromatography, the separation is carried out physically in a two-dimensional flat bed, separating the compounds in one direction (first dimension) and then in a perpendicular direction (second-dimension). The compounds may be detected in the two-dimensional plane (^{x}LC × ^{x}LC) or they may be eluted from the bed during the second-dimension separation (^{x}LC × ^{t}LC).

Comprehensive two-dimensional column LC can be operated in either an on-line (^{t}LC × ^{t}LC) or off-line (^{t}LC/×/^{t}LC) mode. In the on-line mode, the second-dimension separations are carried out during the first-dimension elution. In order for a first-dimension peak to be sampled in several second-dimension runs the first dimension should be (very) slow and the second one (very) fast. In the off-line mode, a fraction collector is used to store fractions from the first dimension, carrying out the second-dimension runs in an independent second step. Stop-flow on-line ^{t}LC × ^{t}LC [19] is a compromise in which the times are decoupled, but the sample remains confined within a single instrument. Both on-line and off-line modes have advantages and disadvantages. When the total analysis time is an issue, on-line ^{t}LC × ^{t}LC is usually preferred. However, this approach has a limited separation power due to the short analysis time available in the second dimension. In ^{t}LC × ^{t}LC the second-dimension separation can be viewed as a detector of the first-dimension separation. In order to preserve the separation obtained in the first dimension the eluting peaks should be sampled with sufficient frequency. However, this is not possible because of time limitations. In practice, one accepts a certain decrease in the actual first-dimension peak capacity due to "undersampling" first-dimension peaks and the resulting additional band broadening in the first dimension [20–22]. Furthermore, the second-dimension chromatogram is likely to suffer from injection band broadening, since relatively large volumes of effluent from the first-dimension separation are injected on the second-dimension column. This effect has a negative impact on the second-dimension peak capacity. In the end, the modulation time should be carefully optimized, taking into account all these effects to reach the best possible compromise [23].

Most separations described in literature using the on-line configuration required analysis times of less than 2 h and produced peak capacities between 500 and 1000 [24]. For example, Stoll et al. claimed a peak capacity of 1024 for an on-line ^{t}LC × ^{t}LC separation of plant metabolites in roughly 30 min, corresponding to a peak-production rate of more than 0.5 peaks/s [24,25].

When a high peak capacity is the main objective and the total analysis time is not constrained, off-line ^{t}LC/×/^{t}LC may be preferred. Very long analysis times may be required. For example, Eeltink et al. obtained a peak capacity of 8720 for an off-line 2D-LC separation of an *E. coli* digest in 1560 min, which corresponds to a peak-production rate of about 0.1 peaks/s [26].

A potential way to overcome the limitations of comprehensive two-dimensional column LC is two-dimensional spatial chromatography (^{x}LC × ^{x}LC). Analytes are separated in a porous flat bed to end up at specific locations in the separation medium, rather than specific elution times. Typically, a sample may be injected at (or close to) a corner of the plane and then eluted along the *x*-axis by pumping an appropriate solvent in the direction parallel to that axis. After a certain time the flow of the first dimension solvent is stopped and a second solvent is pumped in the perpendicular direction (along the *y*-axis of the plane) resulting in migration of the analytes in the second dimension. This implies that each separated compound of the sample may ultimately be characterized by a combination of its *x* and *y* coordinates. As before, a high degree of orthogonality between the two separation dimensions is required to make full use of the total two-dimensional peak capacity. This may, for example, be achieved by packing a narrow strip at the edge of the (*x* direction) with a material that is different from that used in the rest of the plane [27,28], by using strongly different mobile-phase conditions for each dimension [28], or by combining electrically driven and pressure-driven separations [29]. No fraction collection or valve switching is required for ^{x}LC × ^{x}LC, but the overriding argument in its favour is that all second-dimension separations are performed simultaneously. This fundamentally implies that shorter analysis times and higher peak capacities can be achieved in comparison with column-based ^{t}LC × ^{t}LC.

Prime examples of spatial two-dimensional separations are two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and two-dimensional thin-layer chromatography (2D-TLC). The former technique is known to have a very high resolving power (peak capacities up to 10,000 have been demonstrated [30]), but it has some important drawbacks. 2D-PAGE is labor-intensive and difficult to automate. It has a limited loading capacity and low efficiency in the analysis of hydrophobic proteins, it is time-consuming, and it cannot be coupled on-line with mass-spectrometric (MS) detection [31–33].

To increase the separation power of conventional TLC, forced-flow (thin) layer chromatographic techniques have been introduced already in the late 1970s. One of these techniques is over-pressured thin-layer chromatography (OPTLC) [34]. The key feature of this approach lies in the fact that the mobile phase does not flow due to capillary action under atmospheric conditions, but is driven by pressure. A closed-bed compartment and a pump are