Journal of Chromatography A, 1218 (2011) 6675–6687

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Effect of first dimension phase selectivity in online comprehensive two dimensional liquid chromatography (LC × LC)

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ARTICLE INFO

Article history:
Received 7 April 2011
Received in revised form 15 July 2011
Accepted 18 July 2011
Available online 24 July 2011

Keywords:
Two dimensional liquid chromatography
Selectivity
Reversed phase
LC × LC method development
Hydrophobic subtraction method
Carbon stationary phases

ABSTRACT

In this study, we examined the effect of first dimension column selectivity in reversed phase (RP) online comprehensive two dimensional liquid chromatography (LC × LC). The second dimension was always a carbon clad metal oxide reversed phase material. The hydrophobic subtraction model (HSM) and the related phase selective triangles were used to guide the selection of six different RP first dimension columns. Various kinds of samples were investigated and thus two different elution conditions were needed to cause full elution from the first dimension columns. We compared LC × LC chromatograms, contours plots, and $f_{coverage}$ plots by measuring peak capacities, peak numbers, relative spatial coverage, correlation values, *etc.* The major finding of this study is that the carbon phase due to its rather different selectivity from other reversed phases is reasonably orthogonal to a variety of common types of bonded reversed phases. Thus quite surprisingly the six different first dimension stationary phases all showed generally similar separation patterns when paired to the second dimension carbon phase. This result greatly simplifies the task of choosing the correct pair of phases for RP × RP.

1. Introduction

Comprehensive two dimensional liquid chromatography (LC × LC) is gaining more and more interest due to its high separating power compared to one dimensional liquid chromatography (1DLC) [1–6]. When dealing with complex samples in metabolomics, proteomics, pharmaceutics, *etc.*, the limited separation power provided by 1DLC can be the bottleneck in achieving successful separations in a reasonable time [3,7–11]. Clearly improvements in technologies with increased separating power are highly desirable. The concept of LC × LC was introduced three decades ago; the enormous improvements since 1990 have confirmed the huge potential of LC × LC for increasing peak capacity; this mainly results from the "multiplicative advantage" of multi-dimensional methods under ideal conditions [12–17]. Recently, Carr and coworkers performed a series of LC × LC studies on metabolomics samples with total analysis times in the range of 15–60 min [18–20] but with the second dimension run on the time scale of 6–40 s by doing the second dimension separations at high temperatures (100–120 °C). The decreased eluent viscosity at higher temperatures makes it possible to use high linear velocities, high flow rates (~3 mL/min) and short cycle times on the second dimension. Stoll et al. compared the peak capacities and numbers of observed peaks of fully optimized 1DLC and practical LC × LC, and showed that LC × LC becomes superior to 1DLC in terms of *Effective* peak capacity within only 5–10 min [19]. LC × LC produced peak capacities of about 1000 within 30 min; this is well beyond what 1DLC could possibly achieve within a reasonable time. Other important issues in LC × LC include theoretical [21–23] and experimental studies of the optimal second dimension time [20], the use of sophisticated second dimension gradients [24–26], the use of parallel second dimension columns [27], and the development of protocols for the optimization of LC × LC [28,29].

To obtain a successful LC × LC separation, a number of criteria must be reasonably satisfied. First, the sampling of the first dimension effluent must be fast enough to limit resolution losses resulting from "under-sampling" effects [30]. Second, the first and second dimension retention times should be minimally correlated [12,31]. This is frequently spoken of as the "orthogonality" requirement. Third, peaks from the actual samples should cover the whole 2D separation space [32]. Under practical conditions the above three criteria are never completely met by real LC × LC systems; therefore, Stoll et al. proposed a new metric called the effective LC × LC peak capacity ($n'_{c,2D}$) which was defined as per Eq. (1) [19]:

$$n'_{c,2D} = {}^1n_c \times {}^2n_c \times f_{coverage} \times \frac{1}{\langle\beta\rangle} \quad (1)$$

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doi:10.1016/j.chroma.2011.07.063

Table 1
Elution conditions.

1DLC or the first dimension in LC × LC		Second dimension in LC × LC	
Elution Condition 1			
A: 1.5 mM H_3PO_4 in water; B: ACN		A: 10 mM H_3PO_4 in water; B: ACN	
Time (min)	B%	Time (min)	%B
0	0	0	0
24	50	0.30	100
24.01	0	0.31	0
Elution Condition 2			
A: 10 mM $HClO_4$ in water; B: ACN		A: 10 mM H_3PO_4 in water; B: ACN	
Time (min)	B%	Time (min)	%B
0	0	0	0
24	50	0.30	100
24.01	0	0.31	0

Here ${}^1n_c$ and ${}^2n_c$ are the first and second dimension peak capacities, respectively; $f_{coverage}$ is the fractional spatial coverage factor corresponding to the peak distribution of the 2D separation based on a modification of a method due to Gilar [32]; and $\langle\beta\rangle$ is the Davis–Stoll–Carr (D–S–C) under-sampling correction factor which can be calculated as per Eq. (2) [33,34]:

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

where t_s is the first dimension sampling time and ${}^1\varpi$ is the average first dimension peak width. In *on-line* LC × LC the sampling time is equal to the second dimension cycle time (${}^2t_c$).

The specific sample under investigation is very important to establishing the effective peak capacity as it can seriously impact $f_{coverage}$. The separation mechanisms used must be orthogonal to ensure the multiplicative behavior of the peak capacities from the two dimensions. Thus, choosing two columns with maximum orthogonality (i.e. different selectivity) for the sample of interest is a key step in LC × LC method development. Schure suggested using *D*, called the dimensionality of chromatographic techniques, to quantitatively measure the multidimensional orthogonality due to its scale-free nature [35]. Janderra and his coworkers used linear free energy relationships (LFER) [36,37] as a tool for the selection of columns to provide low correlation of retention and separation selectivity in LC × LC [24]. They also developed the method of "parallel gradients" in the two dimensions for the separation of phenolic and flavone antioxidants, which increased orthogonality and improved coverage of the LC × LC retention space. Hajek et al. used a PEG coated silica phase in the first dimension and C18 or C8 phases in the second dimension; interestingly they found that the type of alkylsilica phases employed as the second dimension had little effect on the quality of separation when paired with a PEG phase in the first dimension [37]. Huidobro et al. explored separation orthogonality in LC × LC and concluded that the combination CNxC18, gave adequate orthogonality for their sample [38]. When the phases are not sufficiently orthogonal, Bedani et al. described a strategy for optimizing the second dimension gradient conditions to maximize the coverage of the separation space [39].

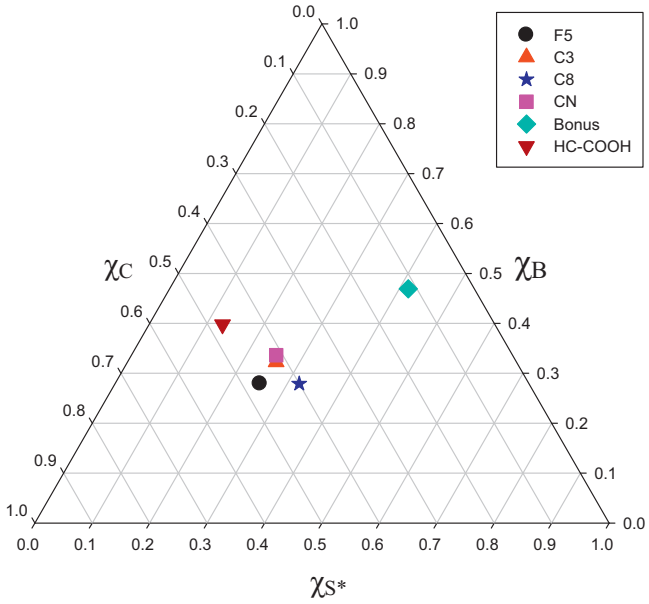

Fig. 1. Selective triangle (S^*–B–C) of the six reversed stationary phases in this study. F5: Discovery HS-F5; C3: Zorbax 300SB-C3; C8: Zorbax 300SB-C8; CN: Zorbax SB-CN; Bonus: Zorbax Bonus-RP; HC–COOH: home-made HC–COOH.

One of the stated purposes of the hydrophobic subtraction model (HSM) for characterizing RP materials [40] is the selection

Table 2
Column selection based on hydrophobic subtraction method.[a]

	C3[b]	CN[c]	C8[d]	HC–COOH[e]	F5[f]	Bonus[g]
C3[b]	0	7	12	26	75	247
CN[c]	7	0	18	32	80	241
C8[d]	12	18	0	23	68	256
HC–COOH[e]	26	32	23	0	54	269
F5[f]	75	80	68	54	0	320
Bonus[g]	247	241	256	269	320	0

[a] This table shows F_s values calculated based on Eq. (3) when pH = 2.8; each F_s value is comparing two stationary phases from the corresponding row and the column.
[b] C3: Zorbax 300SB-C3 (3.5 μ particles).
[c] CN: Zorbax SB-CN (3.5 μ particles).
[d] C8: Zorbax 300SB-C8 (3.5 μ particles).
[e] HC–COOH: home-made HC–COOH (5 μ particles).
[f] F5: Discovery HS-F5 (3 μ particles).
[g] Bonus: Zorbax Bonus-RP (3.5 μ particles).