Modes of operation and parameter selection in on-line comprehensive two-dimensional liquid chromatography
Bedani, F.

Citation for published version (APA):
Bedani, F. (2012). Modes of operation and parameter selection in on-line comprehensive two-dimensional liquid chromatography

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 1

Introduction

Abstract

The increasing popularity of two-dimensional liquid chromatography (2D-LC) techniques is largely due to the fact that they promise to resolve samples that current one-dimensional liquid chromatography (1D-LC) methods cannot adequately deal with. 2D-LC operation is referred to as on- or off-line depending on whether the transfer of first-dimension fractions to the second-dimension column is or is not automated. This difference strongly influences 2D-LC performance. As much as for any other LC methods, in order to make full use of 2D-LC resolving power, careful optimization is essential. However, optimizing 2D-LC experiments is not a trivial matter, and particularly so when 2D-LC analyses are performed on-line. In the first part of the chapter, the most relevant current limitations in performing on-line 2D-LC separations are introduced. In the second part, strategies to improve these issues are outlined. Their detailed treating is given in Chapters 2-5. Main aim of this thesis is to discuss novel modes of operation and strategies for parameter selection in on-line comprehensive 2D-LC.
1.1 Two-dimensional liquid chromatography (2D-LC) techniques

The tremendous improvement in performance experienced by liquid chromatography (LC) along its history has mostly been driven by the ambition to extend the technique’s applicability to the separation of samples of ever increasing complexity. The quest for higher LC “resolving power” has triggered considerable research efforts, which have resulted in significant technological advances. Successful approaches include the use of monolithic columns [1], high temperatures [2], and the implementation of so called ultra-high pressure liquid chromatography (UHPLC) [3]. UHPLC allows the optimum operation of columns packed with very small (sub-2 μm) totally non-porous [4] and superficially porous particles [5]. Faster and more efficient separations at higher linear velocities can then be achieved. Despite all progress made, it is now evident that even after these improvements one-dimensional liquid chromatography (1D-LC) techniques cannot deal with the complexity of samples from many fields (e.g., food, environmental analysis, and life sciences) [6]. More “powerful” LC methods are hence essential. Promising pioneering achievements [7, 8] and theoretical considerations [9] have drawn scientists attention towards two-dimensional (2D) LC techniques.

2D-LC consists in subjecting the whole sample, or just the part of interest, to two distinctly different consecutive separations. In the first case, the 2D-LC run is said to be “comprehensive”; in the second one, the term heart-cutting 2D-LC is used. Following the nomenclature of Schoenmakers et al. [10], “comprehensive 2D-LC” is normally referred to as LC × LC and “heart-cutting 2D-LC” as LC – LC. Both LC × LC and LC – LC can be performed in two different ways [11]: so called off-line operation requires the eluate from the first-dimension (1D) to be collected as narrow fractions and stored. Fractions of
Chapter 1

interest are subsequently reinjected onto the second-dimension (2D) column. “On-line” operation means that transfer of effluent fractions from the first- to the second-dimension is executed within the confinement of the 2D-LC system. Then, a rapid separation is performed such that the 2D column is ready to receive the next fraction and perform a new separation. Clearly, in order to perform on-line 2D-LC operation, an automated set-up is required. This is achieved by using an interface between the two dimensions. In most current implementations of on-line 2D-LC, two-position 8- or 10-port valves equipped with loops matching the volume of the fractions to be transferred are used [12]. Despite offering a convenient way to “store” 1D fractions prior to transfer to the 2D column, several aspects of these interfaces need to be improved. Their most serious limitations are: they do not allow the use of non-miscible solvents in the two dimensions; they lead to an increase in 1D peaks band broadening; they make the focussing of 1D fractions as narrow bands on top of 2D columns complex; finally, some classes of analytes (e.g., non-polar peptides) are very susceptible to adsorption to their surfaces.

The most notable “asset” of LC × LC techniques is that their resolving power is ideally given by the product of the resolving power of the two underlying 1D separations [13, 14]. However, the LC × LC resolving power estimated in this way can only be utilized if two conditions are met: the two phase systems (stationary and mobile phase combinations) are “fully” orthogonal and the resolving power generated independently by the two columns can be maintained. Two separations are said to be orthogonal if there is no relationship between the retention data of the different components of the sample in the two separations [11]. As explained later in this thesis, meeting these two requirements simultaneously is a very complex matter.

LC × LC techniques have been employed in the analysis of several classes of analytes. Successful applications include – but are not limited to – the separation of proteins and peptides from proteomics samples [15, 16], of essential [17] and vegetable [18] oil extracts from food matrices, of pharmaceuticals and metabolites [19] and of synthetic polymers [20]. More and more often, LC × LC is also employed to reduce sample complexity prior to performing tandem mass spectrometry (MS/MS) analysis [21, 22]. Clearly, given the wide difference in the properties of the compounds analyzed, various phase systems have been employed in the two dimensions of 2D-LC systems. Examples
Introduction

of orthogonal systems include the combination of the following stationary phases: normal-phase (NP) and reversed-phase (RP), ion-exchange (IEX) and RP, and RP with size exclusion chromatography (SEC). 2D-LC implementations employing RP stationary phases in both dimensions – previously thought not to provide sufficient orthogonality – have been experiencing a steep rise in popularity. However – and once more – it is important to stress that, in terms of optimizing LC × LC experiments, the combination of the two phase systems is what really matters. In other words, the choice of the mobile phases is as important as that of the stationary phases.

As recent publications show [11, 19], on-line LC × LC is currently the most popular 2D-LC technique and the one to which most research strides are dedicated. However, despite the considerable attention that this mode of operation has been drawing, improvements in both theory and instrumentation are strongly needed. They should lead to better optimization strategies and higher robustness, respectively. Moreover, further efforts should be made to provide analysts with commercially available, easy-to-use software for analyzing 2D separation data. Their lack is a significant impediment to wider adoption of 2D-LC techniques by non-experts. In the next Section, the most important “current limitations” associated with on-line LC × LC operation will be identified. In Section 1.3, strategies to overcome these limitations are introduced. These strategies constitute the main topic of this PhD thesis and get thoroughly discussed in Chapters 2-5.

1.2 Current limitations in on-line LC × LC

Both on- and off-line LC × LC techniques can provide analysts with a valid tool for achieving the resolving power needed to separate a complex mixture whenever 1D-LC techniques fail doing so. As mentioned before, the key instrumental difference between on- and off-line LC × LC techniques concerns the way 1D fractions are transferred to the 2D column: in on-line LC × LC, this step is performed automatically by means of an interface. No operator involvement is thus required; in off-line LC × LC, fractions eluting from the 1D column are manually collected in vials and then stored [23]. Following storage, (re)injection in the 2D column and subsequent analysis are performed. As a result of this set-up difference, important consequences arise, which affect the analysis time and the resolving power achievable in both on- and off-line LC × LC. In this Section, these
consequences are identified and concisely discussed with the final aim to shed light on “current limitations” in on-line LC × LC.

On- vs off-line LC × LC operation. The automation of on-line LC × LC operation leads to clear advantages over off-line LC × LC. First of all, the chance of “spoiling” 1D collected fractions is dramatically reduced. Secondly, the total analysis time is likely to be lower. Thirdly, high-throughput operation is possible. However, automation also implies a severe time constraint on the performing of 2D analyses. In other words, on-line LC × LC operation is much less “flexible” than off-line LC × LC one. In off-line LC × LC, not only these “rigidities” do not apply but also it is not even required to analyze all the collected fractions. Further, samples can be concentrated and even dissolved in different solvents, thus allowing the use of immiscible mobile phases in the two dimensions. Another serious consequence of the above mentioned on-line LC × LC “lack of flexibility” is that method development is more challenging here than for off-line LC × LC experiments. This aspect is discussed below.

Method development in LC × LC. In any LC implementation, the optimization of the separation conditions is a central issue. Main aim here is to predict the optimum settings to achieve the highest resolving power in the shortest possible analysis time, or to estimate the resolving power needed to separate the target compounds in a given analysis time. On-line LC × LC optimization is more difficult than optimization in off-line combination. This is the result of three main reasons: (1) as the first- and the second-dimension are coupled, changes in one parameter in one dimension have consequences for several others in both dimensions [19]. As a result, LC × LC should be optimized as a complete system [24]; (2) detection issues are much more serious in LC × LC than in off-line 2D-LC [25]. Hence, not just resolving power and analysis time but also the dilution factor should be taken into account when developing optimization strategies; (3) in LC × LC, given the impracticability of altering the sample solvent composition during transfer, the mobile phase at the beginning of the 2D column should be very carefully chosen. When this is not properly done, reinjection onto the 2D column may result in undesirable phenomena, such as band broadening, peak distortion, peak splitting or breakthrough [26, 27]. Despite all challenges that the developing of rigorous optimization strategies poses, important progress in on-line LC × LC optimization has recently been made [24].
An important question needs still to be answered: how does the previously described lack of flexibility in on-line LC × LC relate to the generated resolving power? Some insight about this issue is given below.

Resolving power in on-line LC × LC. In order to find the best compromise between analysis time and resolving power, each 1D peak should be sampled between about 2 and 4 times \([28, 29]\). As a result, 2D analyses have to be run fast, which strongly limits the choice of column formats to be used in the 2D and finally prevents achieving high 2D resolving powers. Successful strategies here employ monoliths \([30]\) or columns packed with small particles \([31]\) in combination with high temperatures \([32]\) or high pressures \([33]\). In off-line LC × LC, given the fact that first- and second-dimension are completely decoupled, analysts can employ longer and more efficient columns than those typically used in on-line LC × LC. Higher resolving powers can thus be expected. However, if that is done analysis time will be even longer.

In most on-line LC × LC applications the analysis time is lower than in off-line LC × LC ones. This is relevant because in the wide majority of “real” analytical applications, minimizing analysis time (not to mention operator involvement!) is of paramount importance. However, when compared to 1D-LC applications, run time in on-line LC × LC is still rather high. Efforts should hence be devoted to reduce it. The current trend in on-line LC × LC is to use gradient elution in the 2D. When this is done, in order to achieve “full” orthogonality, the gradient span has to be very wide. However, wide spans are likely to result in long analysis times. As will be evident from the above, the optimization and use of on-line LC × LC is not trivial. New instruments and strategies are thus needed to make the technique perform to its full potential. Recent research advances aimed to overcome some of the remaining issues are the main focus of this Thesis.

1.3 Outline of the Thesis

In the previous Section, the most serious on-line LC × LC limitations which either prevent maximizing the resolving power or determine a not-optimal use of analysis time have been identified and discussed. In this Section, an overview of on-line LC × LC strategies that can help improving these limitations is presented. As said before, the detailed description of these strategies is given in Chapters 2-5.
Chapter 1

Strategies to optimize on-line LC × LC experiments. Given the number of parameters involved, the optimization of LC × LC separations is a complex matter. Because of that, the choice of on-line LC × LC experimental conditions is still mostly based on analyst experience rather than on rigorous theory-supported strategies. Recognizing this limitation, several research groups have developed theories to help analysts in the complex task of on-line LC × LC optimization. In Chapter 2, state-of-the-art strategies that support on-line LC × LC method development through a rigorous choice of chromatographic parameters are critically reviewed. Final aim of this chapter is to provide practitioners with a clear understanding of which aspects can be optimized using current strategies (and which ones cannot).

Maximizing on-line LC × LC resolving power. In Chapter 3, a strategy to improve resolving power in on-line LC × LC is presented. In on-line LC × LC, the 1D column can be operated in two different ways: the most common and widespread used one is continuous low-flow operation [34]; the other option is that of alternating high flow and zero flow operation, i.e. using stop-flow. When operating on-line LC × LC in stop-flow mode, the 1D pump is switched off for the time of the 2D run and it is switched on again when transferring the next fraction. Stop-flow operation improves the “limitation” of the time constraint in the 2D by basically decoupling first- and second-dimension operation. As a result, longer 2D analysis times can be used. The consequences of stop-flow operation in terms of band broadening are studied from a theoretical perspective and system performance in stop-flow is compared to operation at a continuous (low) flow.

Minimizing on-line LC × LC analysis time. One of the most serious disadvantages of on-line LC × LC experiments is that the total analysis time is usually quite high. One of the reasons for that is that, in order to boost their resolving power, 2D separations are predominantly carried out in gradient mode. This has mostly to do with the facts that (a) wide organic modifier spans are often necessary to elute the most retained compounds and that (b) reconditioning times cannot be avoided. In Chapter 4, a strategy is developed which relates optimum gradient operation in the 2D to the degree of orthogonality. This can result in lowering the total analysis time compared to conventional approaches. Alternatively, this novel strategy can be used to improve the separation power at constant time.
Introduction

*Importance of sample solvent strength in on-line LC × LC.* In Chapter 5, a novel approach to estimating the effect of sample solvent strength on band width is discussed. In the proposed strategy, the effect on total resolving power of the difference in eluting strength between the solvent in which 1D fractions are transferred and the mobile phase at the beginning of the 2D column is accounted for. Further, this novel approach also allows analysts to predict if breakthrough phenomena are likely to arise. The designed approach is tested both on a one-dimensional RPLC system operated in gradient elution mode and on an on-line LC × LC system using gradient elution RPLC in the 2D.
1.4 References