

Développement de méthodes bidimensionnelles en ligne LCxLC-UV/MS et LCxSFC-UV pour l'analyse de composés pharmaceutiques

Marion Iguiniz

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Développement de méthodes bidimensionnelles en lignes LCxLC-UV/MS et LCxSFC-UV pour l'analyse de composés pharmaceutiques

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TITRE en Français : Développement de méthodes bidimensionnelles en ligne LCxLC-UV/MS et LCxSFC-UV pour l'analyse de composés pharmaceutiques

RESUME en Français : La chromatographie en phase liquide bidimensionnelle est une technique à fort potentiel, qui offre un grand pouvoir de séparation. Après avoir démontré son intérêt dans l'industrie pharmaceutique et présenté les enjeux liés à l'analyse quantitative, une attention particulière est portée sur le développement de méthodes. Dans l'idée de développer une stratégie d'analyse générique, la première étape est de sélectionner un set de trois systèmes 2D par le biais d'une approche développée au laboratoire. La deuxième étape est d'évaluer le potentiel de ces systèmes pour l'analyse quantitative. Ces deux étapes ont conduit à la proposition d'une stratégie d'analyse qui pourrait être appliquée à l'analyse pharmaceutique dans un contexte industriel. Enfin le potentiel du couplage RPLCxSFC est envisagé dans deux cas de figure différents. Premièrement, dans le but de comparer ce couplage aux séparations RPLCxRPLC développées dans le cadre d'une stratégie analytique générique, en termes de pouvoir de séparation. Deuxièmement, dans le cadre de l'analyse de composés chiraux, en développant un couplage sRPLCxSFC, en mode « sélective compréhensive » permettant une analyse achirale/chirale simultanée. Les avantages d'une telle approche ont été mis en avant en la comparant aux approches conventionnelles.

TITRE en Anglais : Development of on-line two-dimensional LCxLC-UV/MS and LCxSFC-UV methods for the analysis of pharmaceutical samples

RESUME en Anglais : Two-dimensional liquid chromatography (2D-LC) is a powerful technique considering its high separation power. After showing the advantage of 2D-LC in the pharmaceutical area and presenting the challenges related to quantitative analysis, special attention was paid to method development. With the aim of developing a generic analytical strategy for pharmaceuticals, the first step of our approach consisted in selecting a set of three 2D-systems with the help of a methodology previously developed. In a second step, the potential of these 2D-systems was evaluated for the purpose of quantitative analysis. An analytical strategy able to be applied to pharmaceutical analysis in an industrial context was proposed. Finally, the potential of RPLCxSFC was investigated in two different cases. Firstly, for comparing this on-line two dimensional technique to on-line RPLCxRPLC with respect of the separation power. Secondly, for chiral compounds by developing a selective RPLCxSFC method for simultaneous achiral-chiral analysis. The advantage of such method was highlighted by comparing to conventional approaches.

Discipline : Chimie analytique

MOTS-CLES : Chromatographie bidimensionnelle en ligne, RPLC, SFC, composés pharmaceutiques, analyse chirale

ADRESSE DU LABORATOIRE : Institut des Sciences Analytiques (ISA), 5 rue de la Doua, 69100 Villeurbanne (France)

PRINCIPALES ABREVIATIONS

| ¹ D | Première dimension |
|---------------------|---|
| ² D | Deuxième dimension |
| 1D-LC | Chromatographie en phase liquide unidimensionnelle |
| 2D-LC | Chromatographie en phase liquide bidimensionnelle |
| AA | Acétate d'ammonium |
| ACN | Acétonitrile |
| AMS | Acide méthanesulfonique |
| BPR / ABPR | Régulation de la contre pression |
| Ce | Composition du gradient en solvant organique à l'élution |
| CO ₂ | Dioxide de carbone |
| DAD | Détecteur à barrettes de diodes |
| di | Diamètre interne de la colonne |
| d _p | Diamètre de particules |
| ESI | Electrospary |
| F | Débit |
| FA | Acide formique |
| γ | Taux d'occupation de l'espace de séparation 2D |
| H ₂ O | Eau |
| HILIC | Chromatographie en phase liquide à interaction hydrophile |
| ICH | Conférence internationale sur l'harmonisation des critères d'homologation |
| | des produits pharmaceutiques à l'usage de l'homme |
| L | Longueur de la colonne |
| LC | Chromatographie en phase liquide |
| LCxLC | Chromatographie bidimensionnelle en mode « compréhensive » |
| LC-LC | Chromatographie bidimensionnelle en mode « heart-cutting » |
| LOD | Limite de détection |
| LOQ | Limite de quantification |
| MeOH | Méthanol |
| MHC | "Multiple heart-cutting" |
| MS | Spectrométrie de masse |
| n _{2D} * | Capacité de pics pratique |
| n _{2D,eff} | Capacité de pics effective |
| NH ₄ OH | Hydroxyde d'ammonium |
| Od | Degré d'orthogonalité |
| PA (API) | Principe actif (Active pharmaceutical ingredient) |
| RPLC | Chromatographie en phase liquide à polarité des phases inversées |
| σ^2_{col} | Dipersion de la colonne |
| σ^2_{ext} | Dispersion extra-colonne |

| SFC | Chromatographie en fluide supercritique |
|------------------|--|
| sLCxLC | Chromatographie bidimensionnelle en mode « sélective compréhensive » |
| t ₀ | Temps mort de la colonne |
| t _D | Temps de délai |
| t _G | Temps de gradient |
| t _{iso} | Temps de palier isocratique |
| ts | Temps d'échantillonnage |
| UHPLC | Système chromatographique à très hautes pressions |
| UV | Détecteur ultraviolet |
| V _{inj} | Volume injecté |
| Z | Rapport de split |

Les exposants 1 et 2 sont utilisés pour préciser s'il s'agit d'une grandeur de première ou seconde dimension.

SOMMAIRE

| Introdu | ction générale | 1 |
|----------------|--|----|
| Chapitr | e 1 - Contexte | 3 |
| Introd | luction | 5 |
| A. lı pharm | ntérêts et applications de la chromatographie bidimensionnelle pour l'analyse de composés naceutiques | 6 |
| В. Т | raitement des données et analyse quantitative en chromatographie bidimensionnelle | 29 |
| 1. | Prétraitement des Chromatogrammes2 | 29 |
| 2. | Analyse quantitative en LCxLC | 31 |
| Conclu | usions | 33 |
| Référe | ences | 34 |
| Chapitr | e 2 - Conditions expérimentales | 39 |
| Introd | luction | 11 |
| A. lı | nstrumentation | 12 |
| 1. | Systèmes chromatographiques | 12 |
| 2. | Interfaces | 14 |
| 3. | Spectromètre de masse QDa (Waters) | 16 |
| 4. | Caractéristiques des systèmes | 17 |
| B. P | hases stationnaires | 51 |
| С. Р | hases mobiles | 52 |
| 1. | Chromatographie en phase liquide | 52 |
| 2. | Chromatographie en fluide supercritique | 52 |
| D. E | chantillons5 | 53 |
| 1. | Projet S56922 | 53 |
| 2. | Projet S68502 | 53 |
| 3. | Echantillon Chiral | 53 |
| 4. | Autres échantillons | 54 |
| Ε. Τ | raitement des données et calculs | 55 |
| 1. | Optimisation de méthodes LCxLC | 55 |
| 2. | Données 1D | 55 |
| 3. | Données 2D | 55 |
| Référe | ences | 57 |

| Chapitre 3 - Mise en place d'une stratégie 2D-LC générique. Partie 1 : Sélection de syst bidimensionnels intéressants | è mes 59 |
|---|-----------------------|
| Introduction | 61 |
| A. Stratégie de selection de conditions intéressantes | 62 |
| Conclusions | 82 |
| Références | 83 |
| Chapitre 4 - Mise en place d'une stratégie 2D-LC générique. Partie 2 : Aspects quantitat éléments de validation | t ifs et 85 |
| Introduction | 87 |
| A. Sensibilité et aspects quantitatifs en lcxlc | 89 |
| B. Proposition d'une stratégie d'analyse | 99 |
| C. Transfert de méthode en 2D-LC | 102 |
| Conclusions | 106 |
| Références | 107 |
| Chapitre 5 - Intérêt et développement du couplage entre la LC et la SFC pour l'an d'échantillons pharmaceutiques | alyse 109 |
| Introduction | 111 |
| A. LCxSFC pour l'analyse de composés pharmaceutiques et comparaison avec la lcxLC | 112 |
| 1. Recherche de conditions intéressantes | 112 |
| 2. Mise en place du couplage RPLCxSFC | 115 |
| B. sLCxSFC pour l'analyse achirale-chirale de composés pharmaceutiques | 119 |
| Conclusions | 131 |
| Références | 132 |
| Conclusions générales et persepectives | 135 |
| ANNEXES | 139 |

La synthèse d'un principe actif est un procédé long en plusieurs étapes, dans lequel la présence d'impuretés résiduelles semble inévitable (intermédiaires de synthèse, sousproduits, etc.). Dans ce contexte, le département de « Développement Analytique » d'ORIL Industrie, filiale du groupe pharmaceutique Servier, a une double mission : (1) assurer un appui aux chimistes organiciens du groupe, afin de leur permettre une meilleure compréhension des réactions de synthèse et en améliorer les performances, étape par étape ; (2) garantir la qualité du principe actif, par l'identification et la quantification des impuretés, dont le contrôle est régulé par les directives ICH (Conférence internationale sur l'harmonisation des critères d'homologation des produits pharmaceutiques à usage humain). Ce travail nécessite la mise au point de méthodes séparatives performantes, capables de séparer à la fois des composés possédant des polarités et des acido-basicités très diverses, et des composés très proches en structure du principe actif.

La chromatographie en phase liquide (LC) est une technique de choix pour ce type d'analyses dans l'industrie pharmaceutique. Cependant, l'utilisation d'une seule méthode d'analyse peut être insuffisante pour séparer tous les composés d'un échantillon, du fait de la diversité des impuretés potentielles présentes. La stratégie actuelle consiste à utiliser deux (ou plus) méthodes unidimensionnelles différentes, dans le but d'atteindre un niveau de spécificité adéquat. Le cas le plus parlant est le cas des principes actifs chiraux puisque leur caractérisation nécessite l'utilisation d'au moins deux méthodes de séparation: une séparation achirale permettant de déterminer la pureté chimique du principe actif et une séparation chirale pour évaluer la pureté énantiomérique du principe actif. L'inconvénient de ce type d'approche est que la caractérisation d'un principe actif peut devenir un processus laborieux, consommateur de temps et nécessitant plusieurs préparations d'échantillon différentes en amont des analyses.

Actuellement, l'un des défis majeurs de l'industrie pharmaceutique est de réduire la durée d'émergence des nouveaux traitements, notamment en réduisant les délais d'analyse dans les phases précoces de développement. Dans cette optique, toute technique innovante est à prendre en considération. L'objectif de ces travaux de thèse a donc été de développer des méthodes d'analyses bidimensionnelles en ligne permettant de résoudre différents challenges rencontrés dans l'analyse pharmaceutique.

Le premier chapitre présente un état de l'art des applications de la 2D-LC pour l'analyse de composés pharmaceutiques, ainsi que des différentes approches de traitement des données, notamment pour l'analyse quantitative. Le chapitre 2 décrit les conditions expérimentales (appareils, colonnes, phases mobiles et échantillons) utilisées dans les différentes études. Les Chapitres 3 et 4 sont dédiés à la mise en place d'une stratégie 2D générique pour l'analyse d'échantillons pharmaceutiques. Le Chapitre 3 présente la sélection de systèmes RPLC pour la mise en place d'analyses RPLC-UV/MS génériques. Cette sélection est basée sur une méthodologie développée au laboratoire, qui consiste à évaluer le potentiel d'un grand nombre de combinaisons LC/LC. Les différents systèmes 2D-LC possibles sont comparés par le biais du calcul du degré d'orthogonalité et de la capacité de pics pratique. Le Chapitre 4 traite de l'aspect quantitatif des méthodes, et de certain critères de validation

permettent de vérifier les performances quantitatives des analyses LCxLC, et de proposer une stratégie de sélection en deux étapes pour la mise en place d'analyses 2D. Enfin, le Chapitre 5 est dédié à l'évaluation du potentiel du couplage entre la RPLC et la SFC. En effet, depuis un certain nombre d'années la SFC a connu un regain d'intérêt, en particulier dans l'industrie pharmaceutique, que ce soit pour la séparation de composés chiraux ou l'analyse achirale d'échantillons pharmaceutiques. Cette technique, qui se positionne comme complémentaire et orthogonale à la RPLC, présente donc un grand intérêt pour une seconde dimension. Ainsi, deux couplages différents sont étudiés : un couplage RPLCxSFC pour la mise en place d'analyses 2D génériques et un couplage sRPLCxSFC pour l'analyse achirale simultanée de composés pharmaceutiques.

Quatre études sont présentées sous forme d'articles :

Article 1 (chapitre 1)

"Two-dimensional liquid chromatography in pharmaceutical analysis. Instrumental aspects, trends and applications"

M. Iguiniz, S. Heinisch, Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482–503. http://dx.doi.org/10.1016/j.jpba.2017.07.009.

Article 2 (chapitre 3)

"Comprehensive two dimensional liquid chromatography as analytical strategy for pharmaceutical analysis"

M. Iguiniz, F. Rouvière, E. Corbel, N. Roques, S. Heinisch, Journal of Chromatography A, 1536 (2018) 195–204. https://doi.org/10.1016/j.chroma.2017.08.070.

Article 3 (chapitre 4)

"Quantitative aspects in comprehensive on-line two dimensional liquid chromatography for pharmaceutical applications"

M. Iguiniz, E. Corbel, N. Roques, S. Heinisch, Talanta 195 (2019) 272–280. https://doi.org/10.1016/j.talanta.2018.11.030.

Article 4 (chapitre 5)

"On-line coupling of achiral Reversed Phase Liquid Chromatography and chiral Supercritical Fluid Chromatography for the analysis of pharmaceutical compounds"

M. Iguiniz, E. Corbel, N. Roques, S. Heinisch, Journal of Pharmaceutical and Biomedical Analysis 159 (2018) 237–244. https://doi.org/10.1016/j.jpba.2018.06.058

CHAPITRE 1

Contexte

Ce chapitre a fait l'objet d'une publication :

Article 1

"Two-dimensional liquid chromatography in pharmaceutical analysis. Instrumental aspects, trends and applications"

M. Iguiniz, S. Heinisch, Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482–503. http://dx.doi.org/10.1016/j.jpba.2017.07.009.

L'analyse d'échantillons pharmaceutiques peut être un véritable challenge en chimie analytique. En effet, ces échantillons contiennent un certain nombre de composés, qui peuvent avoir des propriétés acido-basiques et des polarités très variées. De plus, les impuretés peuvent avoir des structures chimiques proches du celle du principe actif, ou totalement différentes. En LC, des composés aussi variés au sein d'un même échantillon peuvent rendre la mise au point de conditions chromatographiques performantes plus difficile, voire impossible en utilisant qu'une seule méthode.

La chromatographie en phase liquide bidimensionnelle est une technique à très fort potentiel, tant en termes de sélectivité que de pouvoir de séparation. Que ce soit en mode heart-cutting, sélective compréhensive, ou compréhensive totale, cette approche permet de résoudre un grand nombre de challenges analytiques tels que la caractérisation d'échantillons complexes en un temps record, la résolution de co-élutions dans la séparation de première dimension, ou encore la réduction d'effets matrices dans le cas d'un couplage avec la spectrométrie de masse.

Ce chapitre 1 établit, dans une première partie, un état de l'art des applications de la 2D-LC pour l'analyse de composés pharmaceutiques. Une attention particulière est portée sur les différents montages utilisés, les modes chromatographiques appliqués dans chaque dimension, et les différents objectifs des méthodes bidimensionnelles développées.

Une deuxième partie est dédiée au traitement des données en LCxLC, plus particulièrement dans le cas d'analyses quantitatives.

A. INTERETS ET APPLICATIONS DE LA CHROMATOGRAPHIE BIDIMENSIONNELLE POUR L'ANALYSE DE COMPOSES PHARMACEUTIQUES

Article 1

"Two-dimensional liquid chromatography in pharmaceutical analysis. Instrumental aspects, trends and applications"

M. Iguiniz, S. Heinisch, Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482–503. http://dx.doi.org/10.1016/j.jpba.2017.07.009.

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Two-dimensional liquid chromatography in pharmaceutical analysis. Instrumental aspects, trends and applications





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ABSTRACT

The interest in two-dimensional liquid chromatography (2D-LC) has been growing up since the last decades. This promising technique appears as a relevant solution for various analytical challenges encountered in pharmaceutical analysis.

The objective of this review is to give an overview of past, current and emerging trends in 2D-LC techniques applied to pharmaceutical compounds. The referenced studies cover the late 1980s to the present. Information regarding the different aspects of this analytical technique, including chromatographic conditions, instrumental setup and compounds of interest, was compiled and summarized into a synoptic table. Particular attention is paid to key features including (i) the interfaces used for coupling the two dimensions, (ii) the application fields, and (iii) the chromatographic modes that can be combined together. Finally an attempt is made to predict future advances in two-dimensional separation techniqes for pharmaceutical analysis.

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Contents

| Introd | luction | . 483 |
|--------|--|---|
| Gener | ral aspects of two-dimensional liquid chromatography | . 484 |
| Instru | imental aspects for on-line 2D-LC. | . 484 |
| 3.1. | Direct transfer | |
| 3.2. | Loop storage | .493 |
| 3.3. | Trap columns. | |
| 3.4. | Additional set-ups | . 494 |
| | 3.4.1. Parallel columns in the second dimension | . 494 |
| | 3.4.2 Make-up flow technique | .494 |
| Applie | cations in pharmaceutical area | .494 |
| | Introd Gener Instru 3.1. 3.2. 3.3. 3.4. Appli | Introduction General aspects of two-dimensional liquid chromatography Instrumental aspects for on-line 2D-LC 3.1. Direct transfer 3.2. Loop storage 3.3. Trap columns 3.4. Additional set-ups 3.4.1. Parallel columns in the second dimension 3.4.2. Make-up flow technique Applications in pharmaceutical area |

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Abbreviations: API, active pharmaceutical ingredient; LC, liquid chromatography; IEX, ion exchange chromatography; HILC, hydrophilic interaction chromatography; IPC, ion-pair chromatography; micel, micellar chromatography; NPLC, normal phase chromatography; RPLC, reversed phase chromatography; SEC, size-exclusion chromatography; BMC, biopartitioning micellar chromatography; DAD, diode array detector; EC, electrochemical detection; Flu, fluorescence detection; MS, mass spectrometry detection; post-Flu, fluorescence detection after precolumn derivatization; UV, UV absorbance detection; ELSD, evaporating light scattering detection; CAD, charged aerosol detection; Pre-Flu, fluorescence detection after precolumn derivatization; MS/MS, tandem mass spectrometry detection; NMR, nuclear magnetic resonance; C18, octadecyl silica; OS, octadecyl silane; PFPP, pentafluorophenylpropyl; SCX, strong cation exchange; CSP, chiral stationnary phase; RAM, restricted acces media; BSA, bovine serum albumine; AGP, immobilized a1-acid glycoprotein; T, temperature; F, flow rate; ACN, acetonitrile; APM, ammonium phosphate monobasic; b-CD, b-cyclodextrines; DCM, dichloromethane; DMNA, dimethylonitrosamine; EDTA, dissodium ethylene diamine tetraacetate; E13N, triethylamine; Et2NH, diethylamine; EtA, ethanolamine; EtOH, ethanol; FA, formic acid; H3PO4, phosphoric acid; KH2PO4, potassium dihydrogen phosphate; MeOH, methanol; NaCl, sodium chloride; NaOH, sodium hydroxyde; NN-DMOA, *NN*-dimethyloctan-1-amine; NS, sodium nonyl sulfate; OSA, octane sulfonate; SBS, sodium butane sulfonate; SDS, sodium hexpl sulfate; SLS, sodium lauryl sulfate; SLS, sodium nonane sulfonate; SOS, sodium octane sulfonate; TEA, triethylammonium; TFA, trifluoroacetic acid; THF, tetrahydrofuran; brij 35, polyoxyethylene (23) lauryl ether.

| | 4.1. | Interest of 2D-LC for pharmaceutical analysis | 496 |
|----|--------|---|-----|
| | | 4.1.1. Trace analysis | 496 |
| | | 4.1.2. Chiral analysis | 496 |
| | | 4.1.3. Sample profiling | 496 |
| | | 4.1.4. Use of a second dimension for MS detection | 498 |
| | 4.2. | Preparative 2D-LC | 498 |
| 5. | LC tec | nniques for 2D-LC. | 498 |
| | 5.1. | Coupling IEX or IPC with RPLC. | 498 |
| | 5.2. | HILL or NPLC in one or both dimensions | 498 |
| | 5.3. | RPLC in both dimensions | 498 |
| | 5.4. | Other couplings | 490 |
| 6. | Conclu | isions and perspectives in the field | 490 |
| | Refere | | 500 |
| | | | 000 |

1. Introduction

In the pharmaceutical area, "safeness and efficacy" are of prime importance for drug development. The safeness of a drug product is dependent not only on the toxicological properties of the active substance itself, but also on the impurities and degradation products that it contains. Thus, according to International Conference on Harmonization (ICH) guidelines, it is advisable to consider 0.05% as reporting limit, 0.1% as identification limit and 0.15% as quantification limit for impurity control of both drug substance and drug products [1,2]. In the cases where a chiral drug substance is developed as a single enantiomer, the control of the second enantiomer has to be considered in the same manner as for other impurities. In term of "specificity", identification methods should be able to discriminate between compounds of closely related structures which are likely to be present. As a result, the use of either two different chromatographic techniques offering different retention mechanisms [3] or a combination of analytical techniques such as chromatography and mass spectrometry [4] into a single analytical procedure is highly recommended by ICH guidances [5].

High-Performance Liquid Chromatography (HPLC) hyphenated to different detection techniques (DAD, MS, ...) has been proved to be an analytical technique of choice for forced degradation and impurity profiling [6,7], as well as for pharmacokinetics and metabolism studies [8,9]. The continuous introduction of new RPLC columns with more stable packing materials allows larger applicable pH and temperature ranges and the possible use of fully aqueous mobile phases. For these reasons, RPLC has become the most widely used separation technique in the pharmaceutical area [10-13]. However, a unique separation is often insufficient to identify impurities whose number can be unknown and whose structures are usually similar to that of the active substance. In this regard, additional data obtained from different orthogonal separations can be very useful to enhance the whole separation power although this is done at the expense of substantial additional time and costs. Furthermore, quantifying impurities usually present in very small amounts can be challenging. In this case, the need for preconcentration and/or sample treatment is required in order to improve the limit of detection and to reduce matrix effects (e.g. ion suppression in LC-MS).

Two-dimensional liquid chromatography (2D-LC) has been used for several years now in pharmaceutical analysis to overcome the above issues and also to increase the separation speed compared to 1D-LC separation [14,15]. According to Giddings [16], the term « multi-dimensional separation» (including 2D-LC) refers to a technique in which: (i) components are subjected to two or more largely independent separative displacements and (ii) components adequately resolved in one displacement step, remain resolved throughout the separation process. This second requirement excludes tandem arrangements in which the mobile phase



Fig. 1. Year-wise publications dealing with 2D-LC separations of pharmaceutical compounds based on Scopus (from 1980 to April 2017).

emerging from one column is sent directly to another one. The present review focuses on 2D-LC as defined by Giddings. Consequently, both on-line sample treatment and in series-coupled columns (e.g. [17–21]) were not considered here. Our objective was to give an overview of 2D-LC applications in the pharmaceutical field, with a critical regard on both the current trends in this field and the main issues involved by such separation techniques. Some theoretical considerations on 2D-LC are proposed at the beginning of this review. However, for more extensive information, we refer the interested reader to recent relevant reviews and published books [15,22–28].

The graph shown in Fig. 1 shows the number of articles, for each decade, dealing exclusively with 2D-LC for pharmaceuticals. For this purpose, literature research via Scopus was carried out from 1980 to April 2017, with "two dimensional liquid chromatography" and "pharmaceutical analysis" or "pharmaceutical compounds" or "pharmaceuticals" as keywords. Resulting data were cross tabulated with those of different websites (Scifinder; Wiley; Direct Science...). It should be underlined that this review exclusively concerns the field of pharmaceuticals. As a result; the numerous studies that have been reported in the field of Chinese medicine (e.g. [29-32]) are not discussed here; considering that the related application fields are mainly natural products rather than pharmaceuticals. Furthermore only small molecules were considered; biopharmaceuticals being out of scope in this paper. Fig. 1 underlines the growing interest in 2D-LC for pharmaceuticals since the early 1980s. In the 1980s the number of publications was still small but in the 1990s the total number reached up to 30. The decreasing number of publications in the 2000s could be explained by the context of this period which saw a huge development of LC-MS instruments; LC-MS technique having evolved during that time from an experimental to a mature state. At the same time; ultra high-performance liquid chromatography (UHPLC) emerged as an alternative technique to reach very high separation power in LC. Considering the potential of these new techniques; the analysts started to work with them at the expense of 2D-LC methods; much more difficult to set up. However; since the 2010's; a renewed interest for 2D-LC can be observed; probably due to advances in both hardware and software resulting in efficient commercially available instrumentation.

The first section of this paper delivers a short reminder of some general aspects related to two-dimensional liquid chromatography in order to make the rest of this review clearer. In a second section, different instrumental setups and interface designs are presented. The different application fields for 2D-LC in pharmaceutical analysis are discussed in the next section and the different chromatographic modes used for the two dimensions are presented. Finally current trends of 2D-LC in pharmaceutical areas are summarized and an attempt to predict the future ones is made. Articles dealing with the 2D-LC separations of pharmaceuticals have been extensively reviewed since the early 1980's until today. For sake of clarity, the whole discussion is supported by a synoptic table (Table 1). Compounds of interest, chromatographic conditions in each dimension, interface designs and detection techniques were considered to build Table 1. The cited references were first sorted by analytical purposes and applications (see Section 4) and then by chromatographic modes used in both dimensions. Trends and statistics given in this paper were drawn from this table.

2. General aspects of two-dimensional liquid chromatography

2D-LC separations can be carried out either in off-line or in online mode. Off-line 2D-LC can be performed with a conventional HPLC system. Fractions from the first column are collected and reinjected into a second dimension at a later time. The same instrument can be used for both dimensions by only changing stationary phase and/or mobile phase. The off-line mode is convenient with no time constraint in the second dimension. Moreover a fraction treatment is technically possible between the first and the second dimension. However, this mode is time consuming, not suitable for automation and little reproducible. Furthermore, there is a high risk of sample loss and/or carry-over. In contrast, on-line 2D-LC is faster, easy to automate and, in any case, more reproducible. Fractions from the first dimension are continuously transferred via a specific interface to the second dimension in order to be further separated. The peak capacity in on-line 2D-LC is usually lower than in off-line 2D-LC. However it is far much higher than in 1D-LC. The main drawback of this approach is its more complex method development. In addition, unlike in off-line 2D-LC, solvent compatibility issues can be encountered depending on the conditions in both dimensions. An intermediate way was proposed [33]. It consists in stopping the first dimension flow-rate (stop-flow mode) while analyzing the transferred fraction. This is usually done without sampling loops by directly sending the first dimension fraction to the head of the second column. On-line mode is maintained thereby avoiding sample loss and carry-over. The main advantage of this approach is its peak capacity which can be increased since the analysis time of the second dimension is not limited. However, it is more time consuming and problems of solvent compatibility may still exist.

2D-LC separations can be heart-cutting (LC-LC) or comprehensive (LCxLC) in approach [16]. The selective comprehensive mode (sLCxLC) introduced by Groskreutz et al. [34] is a trade-off between both approaches. Fig. 2 shows an illustration of these 2D-LC techniques. In LC-LC, only a limited number of ¹D-fractions (most often a single fraction) are transferred to the second dimension. This approach is generally used when only a few compounds of the sample are of interest. In LCxLC, the entire sample is subjected to separations in both dimensions. In addition, the separation obtained in the first dimension has to be essentially maintained in the second dimension [35]. Successive small fractions from the first dimension are stored and analyzed turn by turn in the second dimension. In this approach, the whole LCxLC analysis time being that of the first dimension, very fast ²D- separations have to be performed. In sLCxLC, selected regions of the first dimension separation are sampled according to a comprehensive two-dimensional approach.

3. Instrumental aspects for on-line 2D-LC

In on-line 2D-LC, two LC systems are connected via an appropriate interface. This latter consists in one or several two-position switching valves with either 6, 8, 10 or 12 ports. A 6-port switching valve can be only used in LC-LC. Other valves have been used in on-line LCxLC as can be seen in Fig. 3a showing the relative distribution of interfaces used in on-line 2D-LC for pharmaceutical analysis. Dual 6-port and even more rarely, dual 10-port switching valves are barely used in comprehensive 2D-LC. It appears in Fig. 3a that about 50% of 2D-LC systems are configured with one single 2-position 6-port switching valve. This can be explained by the fact that, the analysts started to develop 2D-LC methods with their available instrumentation and most instruments were already configured with such valves for on-line sample enrichment or sample treatment (heart-cutting). 8- port, 10-port and even dual 6-port switching valves were used from the beginning of the 90's.

Three different methods can be used to transer the fractions from the first to the second dimension [36]: (i) direct transfer, (ii) temporary storage in sample loops and (iii) temporary storage in trapping columns. Fig. 3b shows the relative distribution of these three on-line approaches. It is noteworthy that just a few applications (9%) make use of off-line 2D-LC, suggesting that on-line 2D-LC (with either comprehensive or heart- cutting approach) is more suitable and hence more attractive than off-line 2D-LC for pharmaceutical analysis. This is probably due to the fact that despite some advantages previously discussed, off-line 2D-LC is very time consuming and overall cannot be automated. As can be observed in Fig. 3b, temporary loop storage is the most widely used approach (40% of the 2D-LC separations reported in the pharmaceutical area). This is likely due to the great convenience of this transfer mode compared to the two other ones. Actually column focusing becomes less mandatory with loop storage, than with direct transfer making method development less complex since in the latter case, the use of trapping columns requires an evaluation of their trapping efficiency.

3.1. Direct transfer

Direct transfer is exclusively used in LC-LC, generally with a 2position 6- port switching valve (Fig. 4). By switching the valve from position 1 to position 2, a fraction of ¹D-eluent is transferred and focused onto ²D-column. After switching the valve back to its initial position (position 1), the compounds are eluted by ²D eluent. Direct transfer was widely used until the 2000's with about 40% of 2D-LC set-ups [37–53]. Actually, at that time more than 90% of 2D-LC separations were developed in the heart-cutting mode under isocratic conditions in at least one dimension which probably explains the extensive use of direct transfer. Indeed, finding conditions that favor on-column focusing can be challenging, but still easier under isocratic conditions. Furthermore, the optimization of 2D-conditions is more straightforward compared to alternative transfer modes (sample loops, trapping columns) for which sample loop volumes or focusing power have to be considered. Nowadays, the widespread use of gradient elution in both dimensions led to a decreased use of direct transfer. Yet it is still applied to specific sep-

| Table 1 2D-LC analysis in ph | armaceutical area (Up | o to April 2017). | | | | | | | |
|---|--------------------------------------|--|--|---|------------|-----------------------------------|--------------------|-----------|------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| Trace analysis (de IPC – RPLC | termination of drug t. Ampicillin | races into biological Human plasma/urine | matrices) Perkin-Elmer 3 × 3TM Isocratic elution phosphate buffer pH7.4 + F: 1 mL/min F: 1 mL/min | Microspher C18 Isocratic elution phosphate buffer pH74/MeOH F: 1 mL/min | Heart-cut | 6-port switching valve | Loop transfer | Post-Flu | [56] |
| IPC – RPLC | Basic drugs | Human serum | YMC ODS Isocratic elution KH2PO4 + SOS + phosphoric acid pH 3.5 in water/ACN | YMC ODS Isocratic elution KH2PO4 in water/ACN | Heart-cut | 6-port switching valve | Direct transfer | ΛŊ | [37] |
| IPC – IPC – RPLC | lbuprofen | Human serum | P: I mu/mun - 1: 25°C YMC ODS (1)(2) Isocratic elution (1) Phosphate buffer pH3.5 + TBAB/ACN (2) Phosphate buffer PH7 + TBAB/ACN PH7 + TBAB/ACN | 0P: 1 mL/min - 1: 25°C 0P: 80 TM Isocratic elution Phosphate buffer pH7/ACN F: 1 mL/min - T: 40°C | Heart-cut | 6-port switching valve (X2) | Direct transfer | ٨n | [38] |
| IPC – RPLC | Manidipine & metabolite | Human serum | Develosil ODS 3K Isocratic elution Phosphate buffer pH3 + SNS/ACN F · 0 3 ml/min – T · 40 °C | Develosil ODS 5K Isocratic elution Phosphate buffer pH3/ACN F· 0 3 m1/min – T· 40 °C | Heart-cut | 6-port switching valve | Direct transfer | UV | [39] |
| IPC – RPLC | Phenyl- propanololamine | Human plasma/urine | <i>YMC ODS</i> Isocratic elution phosphate buffer pH3.5 + SBS/ACN F: 1 ml/min - T: 40°C | YMC ODS Isocratic elution phosphate buffer pH3.5/ACN F: 1 m1/min - T: 40°C | Heart-cut | 6-port switching valve | Direct transfer | UV | [40] |
| IPC – RPLC | Propanolol | Human plasma | YMC ODS Isocratic elution KH2PO4 in water + SOS/ACN F-1 m//min - T- 40°C | YMC ODS Isocratic elution KH2PO4 in water/ACN F-1 m1/min - T-40°C | Heart- cut | 6-port switching valve | Direct transfer | UV | [41] |
| IEX – RPLC | Ro 23-7637 | Dog plasma | Zorbax 300 SCX Zorbax 300 SCX Isocratic elution sodium formate pH3 in water/ACN | Zorbax Rx-C18 Zorbax Rx-C18 Isocratic elution ammonium acetate Bitfer pH4/ACN F: 1 m//min T: A7.00 | Heart-cut | 6-port switching valve | Loop transfer | ΛΛ | [63] |
| IEX – RPLC | Ro 24- 0238 | Human plasma | P-SCX P-SCX P-SCX Isocratic elution potassium formate buffer pH3/ACN P-0.4 ml/min - T-46°C | Zorbax Rx-C8 Zorbax Rx-C8 Isocratic elution ammonium acetate buffer $pH4/ACN$ F. 0.2 m1 /min $-T$. 46°C | Heart-cut | 6-port switching valve | Direct transfer | ٨Ŋ | [42] |
| RPLC – IPC | Probenicid | Rat plasma | Inertial ODS-3 Isocratic elution phosphate buffer pH7/ACN F: 1 mL/min – T: 40°C | Inertsil ODS- 2 Isocratic elution phosphate buffer pH7 + TBAB/ACN F: 1 mL/min – T: 40 °C | Heart-cut | 6-port switching valve | Direct transfer | ۸ | [43] |
| RPLC – IPC | Remoxipride metabolite | Human plasma | Supelcosil LCCN Isocratic elution | YMC ODS-A Isocratic elution | Heart-cut | 6-port switching valve | Loop transfer | EC | [62] |

M. Iguiniz, S. Heinisch / Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482–503

| Table 1 (Continued) | | | | | | | | | |
|-----------------------|--------------------------------|-----------------------|--|---|------------|--------------------------------|-------------------------|-----------|------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| RPLC – IPC | buspirone | plasma | phosphate buffer pH2 + DMNA + EDTA/ACN F: 0.8 mL/min - T: 28 °C Spherisorb 0DS2 Isocratic elution Water + KH2PO4 + 0.1% | phosphate buffer pH2 + DMNA+ NS+ EDTA/ACN F: 1.3 mL/min – T: 28 °C Spherisorb 0DS2 Isocratic elution Water + KH2PO4 + 0.1% | Heart-cut | 6-port switching valve | Direct transfer | 3 | [44] |
| RPLC – IPC | Zidovudine-b-p- glucuronide | Rat plasma | Et3N pH2.5/ACN F: 1.2 mL/min Inertsil ODS-3 Isocratic elution phosphate buffer | Et3N pH2.5 + SLS/ACN F: 1.2 mL/min Inertsil ODS-3 Isocratic elution phosphate buffer pH7 + | Heart-cut | 6-port switching valve | Direct transfer | ٨n | [43] |
| RPLC – IEX | Bupivacaine | Human plasma | pH //ALN F: 1 mL/min – T: 40 °C Superspher RP-select B Isocratic elution phosphate buffer | i BAB/ACN F: 1 mL/min – T: 40°C <i>Nucleosil 5 SA</i> Isocratic elution Ammonium phosphate | Heart-cut | 6-port switching valve | Loop transfer | ΠΛ | [57] |
| RPLC – IEX | Ropivacaine | Human plasma | pH3/ACN F: 1 mL/min – T: 27°C <i>RP-select B</i> Isocratic elution phosphate buffer | buffer pH2.6/ACN F: 1 mL/min – T: 27°C Nucleosil 5 SA Isocratic elution ammonium phosphate | Heart-cut | 6-port switching valve | Loop transfer | UV | [57] |
| RPLC – IEX | Sameridine | Human plasma | pH3/ACN F: 1 mL/min – T: 27°C Kromasil C18 Isocratic elution phosphate buffer | buffer pH2.6/ACN F: 1 mL/min – T: 27°C Spherisorb 5SCX Isocratic elution ammonium phosphate | Heart-cut | 6-port switching valve | Trap column transfer | UV | [88] |
| IPC – IPC | Efletirizine | Human plasma/urine | pH3/ACN F: 1 mL/min – T: 27°C Primesphere C18 Isocratic elution Potassium phosphate | buffer pH2.6/ACN F: 0.4 mL/min – T: 27 °C Spherisorb ODS2 Isocratic elution Potassium phosphate | Heart-cut | 10-port switching valve | Direct transfer | UV | [45] |
| IPC – IEX | Vanillylmandelic acid | Human urine | pH3+ OSA/ACN F: 1 mL/min – T: 40°C Nucleosil C18 Isocratic elution EDTA in water PH3.5+ | pH3 + OSA/ACN F: 1 mL/min – T: 40 °C Nucleosil 5 SB Isocratic elution EDTA in water | Heart-cut | 6-port switching valve (X2) | Direct transfer | EC | [46] |
| RPLC – RPLC | Chloramphenicol | Pig tissue | TBP F: 1 mL/min – T: 27°C Chromsep C-18 Isocratic elution ammoniacal buffer pH9.5/ACN | pH3.5/MeOH F: 1 mL/min – T: 26°C PRP-1 Isocratic elution ammoniacal buffer pH9.5/ACN | Heart-cut | 6-port switching valve (X2) | Direct transfer | 3 | [47] |
| RPLC – RPLC – RPLC | Mefenamic acid | Human serum | F: 1 mL/min – T: ambiant YMC ODS Isocratic elution Phosphate buffer pH5/ACN F: 1 mL/min – T: 40°C | F: 0.5 mL/min – T: ambiant YMC ODS (1) – ODS 80 TM (2) Isocratic elution (1) Phosphate buffer PH3.5/ACN | Heart-cut | 6-port switching valve (X2) | Direct transfer | Ŋ | [38] |
| | | | | pH6/ACN F: 1 mL/min – T: 40°C | | | | | |

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| Table 1 (Continued) | | | | | | | | | |
|------------------------------------|---|--|--|---|---------------|---|-------------------------|-----------|-------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| RPLC – RPLC | Methandrostenoloi & metabolites | neEquine plasma/urine | Spherisorb Phenyl Isocratic elution Water/MeOH F: 1 mL/min | Spherisorb Octyl Isocratic elution Water/MeOH F: 1 mL/min | Heart-cut | 6-port switching valve | Trap column transfer | NV + MSMS | [87] |
| RPLC – RPLC | Ramelteon | Human serum | Eclipse Plus C18 Isocratic elution ortho-phosphoric acid in water/ACN F: 0.5 mL/min - T: 30 °C | Restek Ultra PFPP Isocratic elution ortho-phosphoric acid in water/MeOH F: 1 mL/min – T: 30°C | Heart-cut | 6- port switching valve | Loop transfer | ۸n | [61] |
| RPLC – RPLC | TCV-116 and metabolites | Human serum/urine | Inertsil ODS-2 Isocratic elution phosphate buffer pH4/ACN F: 1 mL/min – T: 40°C | Inertsil ODS- 2 Isocratic elution phosphate buffer pH6/ACN F: 1 mL/min – T: 40°C | Heart-cut | 6-port switching valve | Direct transfer | Flu | [48] |
| RPLC – RPLC | Vancomycin | Standard mixture/Human plasma | RP ASTON C18 Isocratic elution Water/ACN + ammonium acetate pH3.8 F: 1 mL/min | ACR C18 Isocratic elution Water/ACN+ ammonium acetate pH5,2 F: 1.2 mL/min | Heart-cut | 10-port switching valve | Trap column transfer | ۸n | [101] |
| NPLC – NPLC | Dexamethasone | Bovine tissue | Spherisorb phenyl Isocratic elution Water/acetic acid/2-propanol/hexane F: 1.5 mL/min | Spherisorb CN Isocratic elution Water/acetic acid/2- propanol/ hexane F: 1.5 mL/min | Heart-cut | 6-port switching valve | Trap column transfer | ۸n | [88] |
| NPLC – NPLC Trace analysis (see | Melengestrol acetate baration and detection | Bovine tissue a of low level impuriti | Spherisorb phenyl Isocratic elution DCM/MeOH/water/ hexane F: 1.5 mL/min – T: 30 °C es) | Silica column Isocratic elution DCM/MeOH/water/ hexane F: 1.5 mL/min – T: 30°C | Heart-cut | 6-port switching valve | Trap column transfer | Ŋ | [06] |
| RPLC × RPLC | API & degradants | Standard mixture | Zorbax Extended C18 Gradient elution Water/ACN + 0.1% FA F: 0.15 mL/min T: ambiant | Ascentis Express C18 Gradient elution Water/ACN+ Acetate buffer pH8.6 F: 1.5 mL/min – T: ambiant | Comprehensive | 12-port switching valve | Loop transfer | ΛΛ | [80] |
| RPLC – RPLC | API & synthetic intermediates | synthesis environment | CAPCELL PAK ACR C18 Isocratic elution Water/ACN + ammonium acetate F: 3 mL/min - T: 50°C | CAPCEIL PAK MG-II Gradient elution Water/ACN+ ammonium acetate F: 0.45 mL/min - T: 50°C | Heart-cut | 6-port switching valve (x2) | Trap column transfer | An an | [94] |
| RPLC – RPLC | API & impurities | standard mixtures | Waters XTerra RP18 Gradient elution Potassium phosphate pH2.6 in water/ACN F: 1 mL/min | Waters Symmetry C18 Gradient elution Water + 0,03% FA/AGN F: 1 mL/min – T: 30°C | Heart-cut | 6-port switching valve (X2) | Loop transfer | ۸n | [66] |
| RPLC – RPLC | API & impurities | standard mixtures | Eclipse Plus C18 Gradient elution Water/ACN + 0.1% FA F: 0.2 mL/min – T: 25 °C | Eclispe Plus Phenyl-Hexyl Gradient elution Water/ACN+ 0.1% FA F: 3 mL/min – T: 60°C | Heart-cut | 8-port switching valve (dual 4-port) | Loop transfer | A) | [67] |

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| Table 1 (Continued) | | | | | | | | | |
|---------------------------------------|---|-------------------------------------|---|---|-------------------------------------|---|---------------------------------|-------------------------|-------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| RPLC – RPLC | API & impurities | resarch compounds synthesized | Symmetry Shield RP18 gradient elution Water+ 0.1% ortho-phosphoric acid/ACN | ACE Phenyl column Gradient elution Water + 0.1% ortho- phosphoric acid/MeOH | Heart-cut | Off-line | automated fraction collector | 'n | [109] |
| RPLC × RPLC | API & impurities | resarch compounds synthesized | F: 0.8 mL/min – T: 22°C Zorbax SB-CN Isocratic elution Water/ACN + 0,05% phosphoric acid F: programmed – T: 26 of | F: 1 mL/min – T: 10°C Zorbax SB-CN Isocratic elution Water/ACN + 0,05% phosphoric acid F: 1,7 mL/min – T: 35°C | Comprehensive (selective/pseudo) | 12-port switching valve | Loop transfer | Δ | [81] |
| RPLC – RPLC | APIs & impurities | resarch compounds synthesized | 202 C Zorbax Bonus-RP Gradient elution Water + MeOH + TFA TFA/ACN + MeOH + TFA F·0.3 ml/min – T·40°C | Zorbax Extend-C18 Gradient elution Ammonium acetate buffer pH9.2/ACN F. 0.6ml /m:nT-40°C | Heart-cut | 8-port switching valve (dual 4-port) | Loop transfer | ٨n | [68] |
| RPLC – RPLC | API & related isomer | resarch compounds synthesized | Eclipse XDB-C18 gradient elution Water + 0.1% FA/ACN F: 1 mL/min - T: 25°C | XSelect CSH Phenyl-Hexyl gradient elution Water/ACN + 0.1% FA F: 1 mL/min - T: 25°C | Heart-cut | 8-port switching valve (dual 4-port) | Loop transfer | UV + MS | [71] |
| RPLC – RPLC | API & metabolites | Rats excreta | XTerra TM RP18 Gradient elution Water 0.1% FA/ACN | Discovery C18 Gradient elution Water + 10 mM ammonium acetate/ACN – T: 40 °C | Heart-cut | Off-line | automated fraction collector | UV/radiomonitor + MS | [110] |
| RPLC – RPLC | BVT2938, Tolterodine, Amperozide & metabolites | Biological matrices | F: 1 mL/min – T: 40°C Amide column Gradient elution Ammonium formate F: 0 2 ml/min | PFPP column Gradient elution Ammonium formate PH3.6/ACN | Heart-cut | 6-port switching valve | Trap column transfer | SM | [91] |
| RPLC – HILIC | API & impurities | standard mixtures | Waters XTerra RP 18 Gradient elution Water + 0.05% FA/ACN F- 1 m1/min | Acclaim Trinity P1 Gradient elution armonium formate pH4 in water/ACN F. 0.5 ml/min_Tr. 35.07 | Heart-cut | 6-port switching valve (X2) | Loop transfer | UV+ CAD | [66] |
| RPLC x Mixed mode | API & impurities | resarch compounds synthesized | Ace C18 Isocratic elution Water/ACN+ 0.2% phosphoric acid F: programmed – T: 35 °C | Primesep B Isocratic elution Water/ACN + 0.2% phosphoric acid F: 1 mL/min – T: 35 °C | Comprehensive (selective/pseudo) | 12-port switching valve | Loop transfer | A | [81] |
| Chiral analysis IEX – RPLC* | Dihydropyridine Calcium Antagonist | dog plasma | Nucleosil SA Isocratic elution Water+ KH2P04/MeOH F. 0.8 mL/min – T: Ambiant | Ultron ES-OVM lsocratic elution Water + KH2PO4/MeOH F. 0.8 mL/min – T: F. 0.ambiant | Heart-cut | 6-port switching valve (X2) | Direct transfer | EC | [49] |
| IEX – RPLC* | Propafenone | Human Plasma | Partisil SCX Gradient elution Water + Perchloric and phosphoric acids pH2.4/ACN F: 0.7 mL/min | Chiraleel ODR Isocratic elution Water + Perchloric and phosphoric acids PH2.4/ACN F: 1.3 mL/min – T: 15 °C | Heart-cut | 6-port switching valve | Direct transfer | Δ | [50] |

| Table 1 (Continued) | | | | | | | | | |
|---------------------|--------------------------------------|--------------------------------|--|---|------------|--------------------------------|---------------------------|-----------|-------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| RPLC – RPLC* | Carvedilol | Pharmaceutical formulations | Chromolith RP18 Isocratic elution Water + sodium acetate PH6/ACN F: 2 mL/min - T: 35 °C | Chiralcel OD-RH Isocratic elution Water + sodium acetate pH6/ACN F: 1 mL/min - T: 35 °C | Heart-cut | 6-port switching valve (X2) | Loop transfer | Flu | [51] |
| RPLC – RPLC* | Chlortalidone, terbutaline | Human blood/plasma | Nucleosil CN (blood)/Phenyl (plasma) Isocratic elution ammonium acetate buffer/MeOH | Nucleosil Phenyl (blood)/C18 (plasma) Isocratic elution b-CD + ammonium acetate buffer/MeOH | Heart-cut | 6-port switching valve | Loop transfer | UV/EC | [58] |
| RPLC – RPLC* | Diperodon | Rabbit blood serum | For the second s | Chirobiotic T Chirobiotic T Socratic elution MeOH/ACN + acetic e- 0.5 ml /min | Heart-cut | a switching valve | Direct transfer | ΛN | [52] |
| RPLC – RPLC* | hydroxychloroquine & metabolites | Plasma | Utremex CN-bonded Isocratic elution N.N-DMOA phosphate – ammonium acetate in water pH4.5 F: 0.6 mL/min – T: ambiant | CH 201 May man Isocratic elution sodium phosphate buffer pH7/EtOH/ACN F: 0,9 mL/min – T: ambiant | Heart-cut | Off-line | Collection of fraction | Â | [112] |
| RPLC – RPLC* | ibutilide, artilide | Human/animal plasma | Ultremex 3 C8 Isocratic elution 0.1% TEA + 0.1% TEA + F - 1 m1/min | Pirkle dinitrophenyl-D- phenylgtycine Isocratic elution MEOH + 0.3% TFA + 0.3% TEA F 1 m1/min | Heart-cut | 6-port switching valve | Trap column transfer | pre-Flu | [92] |
| RPLC – RPLC* | Ketoprofen | Human Plasma | Inertsil ODS-2 Isocratic elution Water/ACN 0,1% Perchloric acid F. 1 m1/min | Ultron ES-OVM Isocratic elution Water + KH2PO4/THF F- 1 ml /min | Heart-cut | 6-port switching valve (X2) | Trap column transfer | ΛN | [96] |
| RPLC – RPLC* | Ketorolac, paracetamol | Human Plasma | Discovery C18 Gradient elution Water + 0.1% FA/ACN F: 0.2 mL/min – T: | ChiralPak AD-RH Isocratic elution Water + 0.1% FA/ACN F: 0.15 mL/min – T: Ambiant | Heart-cut | 6-port switching valve | Direct transfer | WS | [53] |
| RPLC – RPLC* | Leucovorine | human plasma | μBondapak Phenyl gradient elution Phosphate buffer pH5/MeOH F: 2 mL/min - T: ambiant | Resolvosil BSA Isocratic elution Phosphate buffer pH5 F: 0.5 mL/min – T: 40°C | Heart-cut | 6-port switching valve | Loop transfer | ΛN | [60] |
| RPLC – RPLC* | Methylphenobarbital phenobarbital | , Human Plasma | LiChrospher RP18 ADS Isocratic elution Phosphate buffer pH5/MeOH F: 0.8 mL/min – T: 27 °C | Chirateel OJ-R Isocratic elution Phosphate buffer pH8/ACN F: 0.6 mL/min | Heart-cut | 6-port switching valve | Direct transfer | ΛN | [113] |
| RPLC – RPLC* | Phenprocoumon & metabolites | Human Plasma | Grom Sil ODS-3 CP C18 Gradient elution Ammonium acetate buffer pH3.9/MeOH F: 0.15 ml/min | Chira Grom 2 Isocratic elution Water/ACN + FA F: 0.2 mL/min | Heart- cut | 6-port switching valve (X2) | Trap column transfer | SM/SM | [27] |
| RPLC – RPLC* | p-Hydroxyphenyl phenylhydantoin | rat liver microsomes | LiChrospher RP-18 Isocratic elution | LiChrospher RP-18 Isocratic elution | Heart-cut | 6-port switching valve | Direct transfer | UV | [114] |

M. Iguiniz, S. Heinisch / Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482–503

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| Table 1 (Continued) | | | | | | | | | |
|---------------------|---|-------------------------------------|--|--|-------------------------------------|---|-------------------------|-----------|---------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| RPLC – RPLC* | Stereoisomers (from anti-HCV therapeutic) | resarch compounds synthesized | APM in water/ACN F: 1.2 mL/min – T: 35 °C Correcs C18 Gradient elution Water + 0.1% | Water + octyl-L-prolinamide + ammonium and nickel(II) actate pH9/MeOH F: 1.2 mL/min – T: 35°C Teicoplanin Isocratic elution | Heart- cut | 8-port switching valve (dual 4-port) | Loop transfer | Â | [72] |
| RPLC* x RPLC* | Synthetic intermediates | resarch compounds synthesized | Phosponoric acid/ACN/MeOH acid/ACN/MeOH F. 0,22 mL/min – T: 40°C Critrateel 0J-3R Gradient elution Water + 0,1% Phospohoric | acıd/ACN F: 1 mL/min – T: ambiant <i>Chiralce(OD-3R</i> Isocratic elution Water + 0,1% Phosphoric acid/ACN | Comprehensive | 8-port switching valve (dual 4-port) | Loop transfer | Â | [72] |
| RPLC* x RPLC | Synthetic intermediates | resarch compounds synthesized | acid/ACN/MeOH F: 0,05 mL/min – T: 40 °C Chiraleel OJ- 3R Gradient elution Water + 0,1% Phospohoric Phospohoric | F: 3 mL/min – T: ambiant <i>Chiraleel OD-3R</i> Isocratic elution Water + 0,1% Phosphoric acid/ACN | Comprehensive (selective/pseudo) | 8-port switching valve (dual 4-port) | Loop transfer | Â | [72] |
| RPLC – RPLC* | Terbutaline | Human plasma | acuptory/meon B: 0.2 ml/min – T: 40 °C B: 0.2 ml/min – T: 40 °C Nucleosil-Phenyl Isocratic elution ammonium acetate pH4.6 | F: 2 mL/min – T: ambiant b-Cyclodextrin Isocratic elution ammonium acetate pH4.6/MeOH | Heart- cut | 6-port switching valve | Loop transfer | EC/MS | [64] |
| RPLC – RPLC* | Verapamil & metabolites | Human plasma | F: 0.2 mL/min Inertsil ODS-2 gradient elution Water/ACN 0.1%TFA | F: 0.7 mL/min ES-OVM column Isocratic elution Water + KH2PO4/EtOH | Heart-cut | 6-port switching valve (X2) | Loop transfer | ΛŊ | [69,70] |
| RPLC – RPLC* | Verapamil & metabolites | serum | F: 1 ml/min - T: ambiant Hisep HPLC column Isocratic elution Phosphate buffer pH53/ACN | F: 1 mJ/min – T: ambiant CHIRAL AGP-CSP Isocratic elution Phosphate buffer pH5.3/ACN | Heart-cut | 6-port switching valve | Loop transfer | Flu | [65] |
| RPLC-RPLC* | Warfarin & Hydroxywarfains | Standard mixtures | F: 1 mL/min - T: ambiant Eclipse plus C18 Gradient elution Water + 0.1% phosphoric acid/ACN F: 0.4 mL/min - T: 36 °C | P: 1 mJ/min – T: ambiant Vancomycin column Variet e lution Water + 0.1% phosphoric acid/ACN F: 2.5 mL/min – T: | Heart-cut | 8-port switching valve (dual 4-port) | Loop transfer | ۸n | [72] |
| RPLC* – RPLC | Bupivacaine | Human Plasma | AGP column Isocratic elution Phosphate buffer PH7/2-Propanol | amplant Nucleosil C8 Isocratic elution Ph3/ACN | Heart-cut | 10-port switching valve | Trap column transfer | ٨N | [86] |
| RPLC* – RPLC | Leucovorin | dog plasma | F: U.S mL/min Resolvosil BSA-7 Isocratic elution Sodium phosphate in water pH5.1 | F: 1 mL/mm LiChrocart RP-18 gradient elution Sodium phosphate in water ph5/MeOH F: 1 mL/min _ T: amhiant | Heart-cut | 6-port + 10-port switching valves | Direct transfer | ٦V | [100] |
| RPLC* – RPLC | Metoprolol | Human Plasma | <i>AGP column</i> Isocratic elution Phosphate buffer PH7/2-propanol F: 0.8 mL/min | Nucleosil C18 Isocratic elution Phosphate buffer pH3/ACN F: 1 mL/min | Heart-cut | 10-port switching valve | Trap column transfer | Flu | [86] |

M. Iguiniz, S. Heinisch / Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482–503

| Table 1 (Continued) | | | | | | | | | |
|---|---|-------------------------------------|---|--|------------|---------------------------------|---------------------------------|------------|-------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| RPLC* – RPLC | Oxazepam | Human Plasma | BSA column Isocratic elution Phosphate buffer pH6.8/1-propanol F: 1 m//min | Nucleosil C18 Isocratic elution KH2PO4 in water/ACN/MeOH F: 1.5 ml/min | Heart-cut | 10-port switching valve | Trap column transfer | ΛΛ | [98] |
| RPLC* – RPLC | Terbutaline | Human Plasma | AGP column Isocratic elution Phosphate buffer pH7,5 F- 0.8 m1 /min | Nucleosil C18 Isocratic elution Ammonium acetate buffer pH6/MeOH F-1 m1/min | Heart-cut | 10-port switching valve | Trap column transfer | EC | [98] |
| RPLC – NPLC* | API & impurities | resarch compounds synthesized | Symmetry Shield RP18 gradient elution Water + 0.1% ortho-phosphoric acid/ACN | Chiratpat AD-H Isocratic elution hexane/isopropanol | Heart-cut | Off-line | automated fraction collector | ٨Ŋ | [109] |
| NPLC – RPLC* | Disopyramide | Plasma | F: 0.0111/Junu – 1: 22 °C Li <i>Chrosorb Si 60</i> Isocratic elution MeOH+ 0,6% perchloric acid/JPCM F: 1 mL/min | F. O.S.III./JIIII – 1. 40 -C. EnantioPac a1-AGP Isocratic elution plosphate buffer pH7 + NaCl/2-propanol F. O,3 mL/min – T: 23 °C | Heart-cut | Off- line | Collection of fraction | ΛΛ | [111] |
| NPLC – NPLC* | 2 -phenyl-2,3- dihydro-4- quinolone | reaction mixture | NP NH2 column Isocratic elution n-hexane/isopropanol F: 1 mL/min | OD-H column Isocratic elution n-hexane/isopropanol F: 1 mL/min | Heart-cut | 10-port switching valve (X2) | Loop transfer | ΛN | [73] |
| NPLC – NPLC* | lfosfamide, cyclophos- phamide, trofosfamide | Human Plasma | D,L-naphthylalanine Isocratic elution hexane/2-propanol F: 1 mL/min – T: ambiant | Chiraleel OD Isocratic elution hexane/isopropanol or 2- methyl-1-propanol/ACN F: 1.1 mL/min – T: F: 1.1 mL/min – T: | Heart- cut | 6-port switching valve | Loop transfer | Λ Λ | [59] |
| NPLC – NPLC* | Mefloquine | Human Plasma/whole blood | Adsorbosphere CN Isocratic elution hexane/2- propanol/MeOH + 0.005% F: 2 mL/min – T: ambiant | Supelco LC- (S) -naphthylurea Isocratic elution hexane/2- propanol/MeOH + 0.005% F: 1 mL/min - T: ambiant | Heart-cut | 6- port switching valve | Trap column transfer | λn | [93] |
| NPLC – NPLC* | Pimobendan & metabolites | Human plasma | Spheri-5 silica Isocratic elution A: n-hexane/EtOH + 0.1% Et2NH F: 1 mL/min – T: 35°C | Chiraleel OD Isocratic elution A: <i>n</i> -hexane/EtOH + 0.1% Et2NH F: 1 mL/min – T: 35 °C | Heart-cut | Off-line | automated fraction collector | ΛΛ | [115] |
| HILIC – HILIC ⁴ MS detection (despi | Salbutamol, Salmeterol, Atenol ite non compatible ¹ D cc | Urine samples onditions) | Kinetex HILIC Isocratic elution Acetate buffer PH6/MeOH/ACN F: 0.4 mL/min | Chirobiotic V Isocratic elution Acetate buffer pH4/MeOH F: 0.4 mL/min | Heart-cut | 6-port switching valve | Direct transfer | ΛΛ | [54] |
| RPLC – RPLC | API & impurities | standard mixtures | Zorbax Eclipse XDB C18 Gradient elution potassium phosphate PH6.5 in water/ACN F: 1.2 mL/min | Zorbax Eclipse XDB C18 Gradient elution Water + 0.05% FA/ACN F: 1.2 mL/min | Heart-cut | 6-port switching valve (X2) | Loop transfer | SM | [66] |

M. Iguiniz, S. Heinisch / Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482-503

491

| Table 1 (Continued) | | | | | | | | | |
|---|---|---|--|--|---------------|---|---------------------------------------|-----------|-------|
| Coupling | Analytes | Matrix | First dimension | Second dimension | 2D-LC mode | Interface | Interface | Detection | Ref |
| RPLC – RPLC | Duloxetine & impurities | Standard mixture | Zorbax Eclipse Plus C18 Isocratic elution Phosphate buffer/ACN/MeOH F: 1 mL/min | Zorbax Eclipse Plus C18 Isocratic elution Water/ACN+ 0.1% FA F: programmed | Heart-cut | 6-port switching valve | Direct transfer | MS | [55] |
| RPLC x RPLC | Aprazolam & excipients | Degraded aprazolam tablet | Zorbax SB-CN Gradient elution ammonium acetate buffer pH4/ACN F: 0.25 mH/min – T: 40°C | Acquity UPLC BEH- C18 Gradient elution ammonium acetate buffer pH4/ACN E: 1 mL/min – T: 65°C | Comprehensive | Off-line | Manual collection of the fractions | A | [117] |
| RPLC × RPLC | Drugs & barbiturates | Standards mixtures | X-Terra MS C18 Gradient elution Water 0.1%TFA/ACN 0,07%TFA | Zorbax SB-Phenyl Gradient elution Water 0.1%TFA/ACN 0,07%TFA | Comprehensive | 12-port switching valve | Trap column transfer | UV + MS | [66] |
| RPLC x RPLC | Naproxen & degradants | Standard mixture | V. Coster II. 2010 Gradient elution Water + perchloric erid/ACN F. 0.25 ml/min - T. 40°C | Poroshell 120 SB-C18 Poroshell 120 SB-C18 Gradient elution Water + perchloric F: 3 ml /min - T: 60 °C | Comprehensive | 8-port switching valve (dual 4-port) | Loop transfer | N | [78] |
| RPLC x RPLC | Steroids | standard mixtures | Zorbax SB-CN Zorbax SB-CN Gradient elution Water/ACN F: 0.1 mL/min | Zorbax Sp | Comprehensive | 10-port switching valve (X2) | Loop transfer | ٨Ŋ | [82] |
| RPLC x RPLC | Sulfonamid drugs | standard mixtures | Zorbax SB-CN Gradient elution Water + 0.1% FA/ACN F: 0.1 m1/min | Zorbax SB- C18 Gradient elution Water + 0.1% FA/ACN F: 4 mL/min | Comprehensive | 10-port switching valve (X2) | Loop transfer | Ŋ | [82] |
| RPLC × RPLC | Taxanes | standard mixtures/extract samples | Eclipse Plus C18 Gradient elution Water/MeOH F: 0.06 mL/min T: 30 °C | Eclispe Plus Phemyl-Hexyl Gradient elution Water/ACN+ FA F: 4 mL/min T: 40 °C | Comprehensive | 8-port switching valve (dual 4-port) | Loop transfer | UV + MS | [29] |
| NPLC × RPLC | Drug mixture | Standard mixture | Betasil Diol Betasil Diol Isocratic elution n-hexane/butanol + 0.2% ETA F:40 µL/min T: 30 °C | Chromolith Performance RP18 Gradient elution Water/ACN F: 5 mL/min T: 30°C | Comprehensive | 10-port switching valve | Loop transfer | A | [76] |
| RPLC × BMC | Ibuprofen & impurities, other drugs | standard mixtures | Dionex C18 Gradient elution Water+ 0,1% acetic acid/MeOH F: 0.7 ml/min | C18 based on perfusive silica matrix Isocratic elution pH7,4 + NaCl + brij 35 in water F: 0.8 mL/min - T: 37 °C | Comprehensive | 10-port switching valve | Loop transfer | Λſ | [77] |
| Preparative and s , RPLC – RPLC | emi-preparative 2D-L Buspirone, Propanolol | .c Standard mixture | SunFire C18 (semi-prep) Isocratic elution Water/ACN + 0,05% TFA E: 5 mL/min | SunFire C18 Gradient elution Water/ACN+ 0,05% TFA E: 1 mL/min | Heart-cut | multi-position switching valves | Loop transfer | UV + NMR | [74] |
| RPLC – RPLC | Mixture of API | resarch compounds synthesized | XBridge CT8 OBD (prep) Gradient elution Water/ACN + 0,05%TFA (pH1) F: 50 mL/min | XBridge C18 0BD (prep) Gradient elution 10 mM ammonium hydrogen carbonate (pH9.5)in Water/ACN F: 50 mL/min | Heart-cut | multi-position switching valves | Loop transfer | UV + MS | [75] |



Fig. 2. Schematic representation of (a) Heart-cutting, (b) Comprehensive 2DL-LC and (c) Selective comprehensive 2D-LC.



Fig. 3. Relative use (a) of two-position switching valves and (b) of transfer methods, in on-line 2D-LC publications dealing with the separation of pharmaceuticals based on Scopus (from 1980 to April 2017).

arations [54,55] such as chiral separations, generally carried under isocratic conditions. The increasing interest for multi heart-cutting and comprehensive analyses might also explain the declining use of the direct transfer mode.

3.2. Loop storage

Unlike direct transfer, loop transfer can be used both in isocratic and gradient elution mode. In this setup, the valve is equipped with one (in LC-LC) or several (in LC \times LC) loop(s), thereby allowing to collect the fractions from the first dimension and to store them before injection in the second dimension. Interfaces based on a 6port switching valve equipped with a single loop are well adapted to LC-LC analyses where only one fraction of the first dimension has to be transferred to the second dimension. Such equipments have been used for trace analysis or for chiral applications [56–65] for which, only one first dimension peak is usually of interest. Interfaces designed with 8-port, 10-port and two 6-port switching valves have been also used. The main advantage compared to a single 6- port valve is that two loops can equip the interface, thereby providing numerous possible set-ups for 2D-LC analysis. Indeed, such interfaces can be used for heart-cutting and multi heart-cutting 2D-LC [51,66–75] as well as for comprehensive and selective comprehensive 2D-LC [72,76–82].

It should be highlighted that sample loops are less suitable when two chromatographic systems with non miscible mobile phases M. Iguiniz, S. Heinisch / Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482-503



Fig. 4. Schematic representation of the two positions of a 6-port switching valve (direct transfer). Adapted from [36].

(typically NPLC and RPLC) are combined, or when there is a significant difference in eluent strengths between ¹D and ²D (for example HILIC and RPLC). In this case, system peaks, artifacts and/or peak broadening can occur in the second dimension which may seriously affect the separation power of the 2D-LC system [83].

Fig. 5 shows two 2D-interfaces with two different 8-port valves, both designed for comprehensive on-line 2D-LC in the loop transfer mode. Nowadays, the conventional 8-port valve shown in Fig. 5a has become rarely used due to its asymmetric configuration, one loop being emptied in the forward-flush mode and the other one in the back-flush mode. As pointed out [84,85], this asymmetry results in slight differences in retention times, which may complicate data treatment. Recently, a 8-port valve (Fig. 5b) was designed as a dual 4-port valve which allows working in symmetrical configuration [86].

3.3. Trap columns

Sample loops can be replaced by trap columns as illustrated in Fig. 6 with a 10-port valve. The choice of the adsorbent depends on both the analyte properties and the solvents used in both dimensions. Compared to sample loops, trap columns are expected to enhance sensitivity (by sample enrichment) and to reduce injection issues. However, method development is less straightforward because a good trade-off between trapping efficiency and rapid compound desorption has to be found [28].

Some examples of 2D-LC separations using trap columns were reported, either with one single 6-port switching valve [87-93], one 8-port, one 10-port or two 6-port switching valves [94-99]. This approach has been generally used to enhance the sensitivity of 2D-LC methods. The compounds eluted from ¹D-column are focused on the trap column before being backflushed to ²D column. Kammerer et al. [97] went further on the use of this transfer mode by developing the so-called "peak parking system" based on chiral cartridges connected to multiposition switching valves in addition to the two 6-port switching valves of the interface. Such a system was able to perform multi heart-cutting achiral-chiral analysis in a single step. Each compound separated on ¹D column was individually stored on a chiral cartridge and then analyzed on ²D chiral column. Another attractive application was proposed by Li et al. [95]. They developed a 2D-LC set-up for the analysis of Vancomycin. This analyte has several pKa values, making its retention sensitive to mobile phase pH variation in RPLC. A first RPLC separation was therefore performed at a low pH value in order to separate protonated Vancomycin and its impurities. The fraction containing Vancomycin was trapped on IEX column where nonionic and anionic coeluted impurities were removed. Finally, a second RPLC separation was carried out at a higher pH value thereby achieving quantitation of Vancomycin. This 2D-LC system was found to drastically reduce matrix interference.

3.4. Additional set-ups

3.4.1. Parallel columns in the second dimension

Parallel columns in the second dimension offer the possibility of performing analysis of consecutive fractions in parallel. This setup is generally achieved by using either two 10-port or one 12port switching valves as shown in Fig. 7a and b respectively. The main advantage of this configuration is that the separation time in the second dimension can be twice as long, allowing significant increase in effective peak capacity. The use of two parallel columns was reported for pharmaceutical compounds [80–82,99,100]. The main limitation of this approach is that the two columns must be strictly identical in terms of both retention and efficiency. Moreover, the use of two parallel columns in the second dimension enhances the complexity of 2D-instrumentation with a need for both additional gradient system and additional detector, as highlighted in Fig. 7.

3.4.2. Make-up flow technique

Make-up flow (also called Assistant flow) technique consists in adding a post-column flow after the first separation in order to reduce the eluent strength of ¹D mobile phase before sending the fractions in ²D, thereby limiting injection issues by better focusing the sample onto the column. Fig. 8 illustrates the principle of this technique. This approach was used with sample loops and direct transfer to promote peak focusing at the head of ²D column [55,69,70,78,96]. The make-up flow technique was also used with trap columns [94,97,101] to enhance trapping efficiency of trap columns thanks to better focusing of the compounds at the column inlet. However, it should be noted that an additional pump is required and needs to be controlled and synchronized with other instruments.

4. Applications in pharmaceutical area

As mentioned above, Table 1 gives an extensive overview of 2D-LC applications found in the literature for pharmaceutical compounds.


Fig. 5. Schematic representation of two different two position switching valves equipped with two identical loops for on line comprehensive 2D-LC (loop transfer): (a) conventional 8-port valve and (b) dual 4-port valve. Adapted from [86].



Fig. 6. Schematic representation of a two position 10-port switching valve equipped with two identical trap columns for on line comprehensive 2D-LC (trap column transfer). Adapted from [85].



Fig. 7. Possible setups for parallel columns in the second dimension with (a) two 10-port switching valves and (b) one 12-port switching valve. Adapted from [82] and [99], respectively.

M. Iguiniz, S. Heinisch / Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 482-503



Fig. 8. Scheme of make-up flow for 2D-LC. Adapted from [78].

4.1. Interest of 2D-LC for pharmaceutical analysis

The interest of 2D-LC for pharmaceutical analysis has significantly grown during this last decade. Whereas these techniques have been used for a long time in chiral analysis as well as for trace analysis, new trends have emerged since 2000, including sample profiling and micro preparative separation. In addition to the foregoing, the growing need for mass spectrometry detection can make a suitable second dimension very useful as soon as a given separation is not achieved in MS compatible conditions. Fig. 9 shows the relative distribution of 2D-LC applications in the pharmaceutical area and the importance of them over the years. Trace analysis and chiral analysis are historically the most common fields for utilizing 2D-LC techniques in pharmaceutical analysis. This can be explained by the fact that the heart-cutting mode is usually sufficient for such applications and of course much easier to set up and to develop than the comprehensive mode. Advances in both theoretical concepts [102–108] and instrumentation have been made during the 2000's. As a result, LC \times LC which is essential for sample profiling, began to be used at this time. However since $LC \times LC$ remains much more difficult to develop than LC-LC, the number of applications increases slowly. Along with the need for identifying and characterizing impurities at increasingly low levels, new demands have appeared that required 2D-LC techniques. Those include the need for micro preparative or semi-preparative separations before structural analysis and the more recent growing need for MS detection. In this latter case, the use of MS-compatible mobile phases in ²D allows MS coupling while developing non compatible-MS conditions in ¹D.

4.1.1. Trace analysis

In pharmaceutical analysis, peak co-elution is a major concern since impurities can co-elute with API or with other components. A major application of 2D-LC is directed towards the separation of peaks which co-elute in conventional 1D-LC methods. This is of prime importance, for example, for peak purity assessment. LC-LC with a heart cut of the fraction containing API and its co-eluted impurities can address this problem. Two different approaches were found. Some authors used LC-LC for the assay of trace level of drugs in biological matrices [37-48,56,57,61-63,87-90,101], reaching detection limit as low as 50 ng/L with UV detection. This first approach allowed the development of sensitive methods in the context, for example, of pharmacokinetics or therapeutic drug monitoring (TDM) studies. Others applied 2D-LC techniques for separation, detection and sometimes identification of low level impurities [66-68,71,80,81,91,94,109,110] during the early stages of drug development.

4.1.2. Chiral analysis

For separating enantiomers, heart-cutting (or multi heartcutting) can be useful. Fig. 10 illustrates an example of achiral-chiral 2D-LC analysis. In this case, although the compounds were well separated on the chiral column, a first C18-dimension with only a small sample fraction sent to ²D, allowed to significantly extend the lifetime of the chiral column. The first dimension is generally used to separate the compounds of interest from other components. Targeted chiral compounds are either collected and stored into loops or cartridge before transfer or directly transferred to an enantioselective second dimension column that can separate their enantiomers [49-54,58-60,64,65,69,70,72,73,92,93,96,97,100,109,111-115]. The main benefit of this method is that the chiral separation can be enhanced by excluding interfering substances from the second column. For example, with the 2D-LC system proposed by Liu et al. [73], the enantiomeric excess was directly obtained from crude reaction mixtures in 30 min only, without carrying out laborious prepurification. A reversal set-up was also used to enhance the sensitivity of the chiral separation for low concentrations of enantiomers, thanks to on-column focusing in the second dimension [98,100]. Finally, an elegant approach in LC-SFC was recently developed by Venkatramini et al. [116]. Although SFC (supercritical fluid chromatography) is not strictly speaking a LC technique, it should be mentionned. This approach consisted in separating diastereosisomers in ¹D RPLC while separating their enantiomers in ²D-chiral by multi-heartcutting. Such a coupling can be of prime interest in the future as it combines the high separation power of RPLC for non enantioselective species with the high separation power of SFC for enantiomers.

4.1.3. Sample profiling

Operated in the comprehensive mode, 2D-LC can provide a powerful information with compounds distributed over a large two-dimensional separation space. 2D-LC separations must therefore be taken into consideration for impurity profiling during pharmaceutical degradation studies as well as for the analysis of drug mixtures. Some related applications can be found in the literature [76–79,82,99,117]. For example, Huidobro et al. [117] developed an off-line RPLCxRPLC system for the analysis of impurities during stress and stability studies on Alprazolam tablets. More recently, an on-line RPLCxRPLC-UV/MS approach was proposed for the analysis of API and related impurities [118]. A preliminary study was carried out to search for two suitable generic LCxLC systems among a panel of 20 different LC systems (around 200 possible 2D-LC systems). As can be seen in Fig. 11 good separation of API, synthetic intermediates and other related impurities was obtained. Furthermore, a practical peak capacity of 1000 was achieved in less than 50 min with a number of impurity detected in RPLCxRPLC-UV/MS, 40% higher than in conventional 1D-LC-UV/MS.



Fig. 9. Year-wise reported studies in the five main application areas and their relative distribution. This graph only relates to 2D-LC for pharmaceuticals (see Table 1).



Second dimension





Fig. 11. On-line LCxLC separation of an API and its related impurities, (a) being the MS 2D-Contour plot and (b) the MS 3D-Chromatogram. Adapted from [118].

4.1.4. Use of a second dimension for MS detection

When an efficient LC method with UV detection has been developed with non volatile buffers, compound identification is not possible without investing huge additional time to develop an equivalent LC method with MS compatible mobile phases. Using a 2D-LC approach to transfer compounds from the original ¹D separation to MS-compatible ²D separation is an attractive option which permits to keep the original method for routine analysis [55,66]. Changing the mobile phase additive in the second dimension while keeping the same elution order for impurities as in the first dimension can be convenient. An additional benefit of 2D-LC is that the second separation can enhance MS sensitivity for low level impurities by reducing ionization suppression due to their proximity with major components [119].

4.2. Preparative 2D-LC

Micro preparative or semi-preparative 2D-LC can be useful to isolate and eventually concentrate compounds of interest with a view to perform structural analysis. Only two different approaches were proposed so far. The first one consists in coupling together preparative and analytical techniques [74]. The preparative ¹D separation is used to isolate the compounds of interest from a complex matrix, thereby allowing the ²D column to separate a few components from a simpler matrix. Same conditions were used in both dimensions since the final goal was not to improve the separation in the second dimension but to structurally characterize low level impurity by NMR.

The second approach consists in using a preparative separation in each dimension for the purification of target compounds in complex mixtures. This was done for the purification of standard pharmaceutical compounds by changing the mobile phase pH between both dimension to ensure orthogonality [75]. High levels of purity (>95%) were obtained in 23 min only using a heart-cutting technique without intermediate treatment.

5. LC techniques for 2D-LC

From a theoretical point of view, all chromatographic techniques may be used in 2D-LC, the main objective being to find the highest degree of orthogonality between both dimensions. The selection of first and second dimension systems can therefore be critical. Fig. 12 shows the relative distribution of the different chromatographic techniques that have been used in achiral (Fig. 12a) and chiral (Fig. 12b) 2D-LC. It clearly appears that RPLC has been extensively used since the beginning of 2D-LC studies, with more than 80% of 2D systems using RPLC in at least one dimension. In achiral 2D-LC, the two most common 2D systems are (i) either IEX or IPC combined with RPLC (30%) or (ii) two RPLC systems combined together (50%). In chiral 2D-LC, 85% of 2D-systems are based on the same chromatographic mode in both dimensions, the objective being to separate, in ¹D, a chiral compound from other sample components and to further separate its two enantiomeric forms in ²D. Thus, a high degree of orthogonality is not essential. The only requirement is that one of the two dimensions be enantioselective, usually the second one.

5.1. Coupling IEX or IPC with RPLC

Such 2D-systems were widely used in the 1990's for trace analysis. They were also applied to chiral analysis. The combination of RPLC with IEX or IPC usually provides a high degree of orthogonality in case of ionizable compounds due to a significant difference in retention mechanisms. This configuration was therefore widely used for the straightforward analysis of complex biological fluids (plasma, blood, urine). By removing interferences between analytes and the rest of the matrix, IEX or IPC in ¹D makes the injection of the sample possible without or with reduced sample treatment [37-42,49,50,56,63]. IEX or IPC in ²D and RPLC in ¹D was found to be convenient to focus the analytes at the head of ²D column thanks to the lower ionic strength in RPLC [43,44,57,62,88]. A few studies report the use of IPC and/or IEX in both dimensions. For instance, such 2D-systems were applied to the analysis of Efletrizine [45] and Vanillylmandelic acid [46]. In both cases, 2D-LC allowed to reach detection limits of less than 20 ng/L, despite the direct injection of urine samples. Unlike in RPLC, method development in IEX is often based on either trial and error or one factor-at-a-time approaches, both being time consuming and tedious [120]. In addition, ion pair reagents for IPC are generally expensive and not MS compatible. Furthermore column equilibration can take a long time. These reasons explain why IEX and IPC have almost disappeared in 2D-LC for pharmaceuticals.

5.2. HILIC or NPLC in one or both dimensions

Coupling RPLC with NPLC is very attractive in term of orthogonality as retention mechanisms are quite different. This can be useful for the analysis of mixtures of compounds with very different chemical properties. However, the solvents used are generally not fully miscible, thereby leading to artifacts and system peaks in the chromatogram [76]. Only three applications were reported in RPLC-NPLC, two of them being carried out in off-line 2D-LC [109,111] and the third one in on-line 2D-LC [76]. During the recent years, HILIC separations have been receiving greater attention mainly due to their versatility for the analysis of polar drugs, metabolites and biological compounds [121]. In HILIC the stationary phase is polar as in NPLC while the mobile phases remain similar to those employed in RPLC [122,123]. As a result, a high degree of orthogonality is expected between HILIC and RPLC whereas solvent miscibility issues as those encountered with the coupling of NPLC and RPLC are avoided. Furthermore, using RPLC in ¹D and HILIC in ²D provides favorable conditions for MS detection, the mobile phase in HILIC being rich in acetonitrile which is beneficial for ionization [124]. However, severe band broadening due to difference in eluent strength between first and second dimension can be observed [83]. Consequently only one study [66] deals with the coupling of HILIC with RPLC in the field of pharmaceutical analysis.

In contrast, some 2D-systems using NPLC or HILIC in both dimensions were developed [54,59,73,89,90,93,115]. The main advantage of such coupling is the absence of mobile phase incompatibility. However, the resulting degree of orthogonality is low and this coupling is therefore generally reserved for specific applications such as chiral analysis.

5.3. RPLC in both dimensions

RPLC in both dimensions is the most widely used approach for 2D-LC in pharmaceutical analysis and represent about 60% of the reported applications [38,47,48,51–53,55,58,60,61,64– 72,74,75,78–82,87,91,92,94,96–101,109,110,112–114,117]. As highlighted by Li et al. [86], such a success can be attributed to the following reasons: (i) RPLC is applicable to a large variety of samples and the retention mechanisms have been well known since several decades; (ii) RPLC columns require short equilibration times and furthermore generate high efficiency and hence high peak capacity, which is of prime interest in the context of 2D-LC analysis; (iii) several hundred stationary phases with a large variety of selectivity are commercially available. Those are different in terms of material (silica, polymer or inorganic oxide), functional groups (C18, Cyano, Phenyl, polar embedded...) and



Fig. 12. Relative distribution of reported 2D-LC techniques in (a) achiral and (b) chiral separations of pharmaceuticals. The symbol "*" is related to chiral dimension. Based on Scopus (from 1980 to April 2017).

bonding density. Moreover, RPLC mobile phases are most time compatible with MS detection.

The main issue is the lack of orthogonality between RPLC systems which may lead to lesser degree of orthogonality compared to the combination of RPLC with HILIC, NPLC or IEX [125]. However, it was shown [126] that high effective peak capacities can be obtained with some RPLC/RPLC-systems despite their rather low degree of orthogonality thanks to high column efficiency.

5.4. Other couplings

A mixed-mode stationary phase in ²D (acidic functional group embedded to the hydrophobic chain) and a RPLC stationary phase in ¹D (conventional C18-column) were recently used for pharmaceuticals in selective comprehensive 2D-LC [81]. Coeluted impurities in ¹D were resolved and detected in ²D at levels <0.05% of the major component. Only a few mixed-mode columns are commercially available so far but an increasing number of such 2D-systems can be expected in the future with further developments in this column technology.

More recently, a 2D-LC system was developed for the simultaneous separation and toxicity assessment of pharmaceuticals and related substances [77]. Ibuprofen and its impurities were separated in ¹D RPLC while LC50 values (i.e. lethal concentrations at 50%) of individual impurity were further obtained in ²D BMC (biopartitioning micellar chromatography) from quantitative retention–activity relationships.

6. Conclusions and perspectives in the field

Since the early 1990's, the heart-cut mode has been extensively used for 2D-analysis of pharmaceutical compounds. Such a success is explained by different factors: (i) By adding a multiport valve, it is quite easy to transform a conventional HPLC system into a 2D-LC system; (ii) Method development is straightforward and does not require complex procedures as in the comprehensive mode; (iii) Application fields are various (trace analysis, chiral analysis, preparative 2D-LC, MS detection) and (iv) data processing is much easier than in comprehensive 2D-LC. However, the effective peak capacity is very poor compared to that usually obtained in comprehensive 2D-LC. LC-LC is therefore well adapted to enhance sensitivity or to search for alternative selectivity for one compound or a limited number of compounds of interest. In contrast, when many compounds are to be analyzed, a very high separation power is required and LC-LC is no longer suitable.

Unlike LC-LC, LC \times LC can generate very high peak capacities, which makes it very attractive for the screening of complex sam-

ples. Recent progress in both theoretical apects and optimization approach have made $LC \times LC$ analysis more affordable, suggesting that the number of applications should increase in the coming years. The use of $LC \times LC$ could be expanded and generalized in the pharmaceutical area, first for impurity profiling and maybe later for quality control. However, some efforts have to be made in term of data processing since $LC \times LC$ generates a massive amount of data which requires sophisticated computing tools. Dedicated softwares are still uncommon, which currently represents a potential barrier to the implementation of $LC \times LC$, especially for quantitative analysis.

RPLC in both dimensions has long been proved to be a good choice due to high column efficiencies and the reliability of the technique in the context of 2D-LC analysis. Although the interest for the combination of RPLC and HILIC remains poor, it should increase in the future since HILIC is wellknown to be a valuable alternative to RPLC for separating small polar compounds [121] such as those encountered in the pharmaceutical area. Furthermore, the need for identification by hyphenating 2D-LC to Mass Spectrometry, is growing up and HILIC can provide ideal mobile phase conditions for this hyphenation. For these reasons, the number of papers dealing with RPLCxHILIC analysis should probably increase in the near future. Nevertheless, some efforts should be done to avoid band broadening (due to injection effects), and hence to maximize the potential of such 2D-LC systems. The use of trapping columns seems to be a promising solution, but that needs further theoretical studies to rationalize the choice of both the stationary phase and the column geometry. Nowadays only a few papers deal with this topic, as the study of Cao et al. [127] who proposed the development of a method for evaluation and optimization of trapping columns used in the case of 2D-LC analysis.

A final interesting point, in our opinion, is the potential use of online LCxSFC for 2D-LC pharmaceutical analysis. SFC has been widely used in the pharmaceutical area [128–131] and very recently, LC-SFC has been used for the simultaneous achiral-chiral analysis of pharmaceutical compounds with multiple chiral centers within a single run [116]. Unfortunately, the combination of LC and SFC in a comprehensive way has never been applied to pharmaceuticals although there are a few reported studies, in alternative fields, dealing with off-line or on-line approach with SFC either in the first [132–134] or in the second dimension [135,136]. These separations were essentially obtained in the fields of bio-oils and food analysis. The obtained results highlighted the high degree of orthogonality between the two techniques as well as the interest of using SFC in the second dimension (high speed analysis, low mobile phase viscosity, solvent compatibility).

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B. TRAITEMENT DES DONNEES ET ANALYSE QUANTITATIVE EN CHROMATOGRAPHIE BIDIMENSIONNELLE

Si dans les modes « heart-cutting » et « sélective compréhensive » le traitement des résultats se fait globalement avec les mêmes outils qu'en 1D-LC, le traitement d'une analyse en mode compréhensive est un exercice plus complexe. En effet, les données sont acquises sous forme d'un long chromatogramme pour l'ensemble de l'analyse bidimensionnelle, qui doit être découpé selon des longueurs de fraction précisément calculées afin de séparer les chromatogrammes de chaque échantillonnage. On obtient alors une matrice de données, qui requiert un certain nombre de manipulations avant de pouvoir exploiter les résultats. Enfin, la dernière étape consiste à relier de façon pertinente la « dimension » d'un pic 2D à la concentration, de façon à obtenir une quantification la plus fiable possible.

1. Prétraitement des Chromatogrammes

1.1. Correction du bruit de fond

En LCxLC, la correction du bruit de fond est d'une importance primordiale puisque sa contribution peut avoir un effet dramatique tant sur la détection que sur la quantification des pics. En effet, dans le cas de l'utilisation de certains outils chimiométriques pour traiter les données, un seuil peut être appliqué afin de différencier les pics du bruit de fond. La moindre variation non corrigée de la ligne de base peut avoir, dans ce cas, un impact sur la détection des pics dans le chromatogramme.

Ce bruit de fond est lié à des changements rapides et importants de l'indice de réfraction du milieu [1,2]. Il peut être découpé en trois régions distinctes [3,4], comme illustré par la Figure 1.1 : La zone A correspond au pic d'injection, lié à la différence de composition entre la phase mobile de 1^{ère} dimension et la composition initiale du gradient de 2^{ème} dimension ; La zone B correspond à la dérive de la ligne de base, qui est liée à l'évolution de la composition de la phase mobile en gradient d'élution ; La zone C est due à un retour très rapide aux conditions initiales avant l'analyse suivante.



Figure 1.1 - Structure du bruit de fond d'une analyse LCxLC représentée par (a) la superposition de trois chromatogrammes de deuxième dimension [3], et (b) le chromatogramme 3D de l'analyse d'un blanc [4].

La méthode la plus commune est d'effectuer un blanc d'analyse LCxLC, puis de soustraire ce blanc au signal de l'analyse LCxLC de l'échantillon [3]. Cette méthode est plus facile d'un point de vue du traitement des données, mais elle nécessite une analyse LCxLC supplémentaire et ne peut être utilisée que si le signal du bruit de fond est répétable. De plus, elle peut être source d'erreurs dans l'interprétation des résultats, en générant des pics artefacts sur le chromatogramme [4].

Reinchenbach et coll. [5] ont développé un algorithme permettant d'estimer le niveau du bruit de fond, basé sur une analyse statistique des pixels de l'image obtenue lors d'une analyse GCxGC. Ce bruit de fond estimé est ensuite soustrait au signal de l'analyse. Cette approche a par la suite été appliquée à la correction du bruit de fond en LCxLC [6], après des modifications permettant (1) de prendre en compte le fait que les variations de la ligne de base peuvent être négatives ou positives en gradient d'élution, et (2) de modéliser le bruit de fond dans les deux dimensions.

Une autre approche est la méthode OBGC de correction du bruit de fond orthogonale, développée par Filgueira et coll. [3]. Elle est appliquée en deux étapes : (1) une méthode spécifique est appliquée à tous les points de seconde dimension pour recréer le bruit de fond de l'analyse bidimensionnelle, (2) ce bruit de fond généré est soustrait du chromatogramme LCxLC et permet d'obtenir un chromatogramme LCxLC avec pas ou peu de bruit de fond. Cette approche a été, par la suite, appliquée avec succès au traitement des données issues de l'analyse LCxLC-DAD de vins rouges [7].

Enfin, il est également possible d'utiliser des outils chimiométriques afin d'analyser et de décomposer les données multidimensionnelles générées par une analyse LCxLC. Elles permettent d'estimer le bruit de fond, puis d'en tenir compte lors de la modélisation des données afin de le soustraire au chromatogramme 2D [8,9]. Cependant, ces approches nécessitent une grande maitrise de l'utilisation d'algorithmes et d'outils chimiométriques car elles peuvent être source d'erreurs. De plus, elles peuvent compromettre la détection des pics lors de l'analyse des résultats, du fait de la perte d'intensité du signal qu'elles peuvent générer [4].

1.2. Alignement des temps de rétention

L'une des étapes importantes de la phase de prétraitement des données est l'alignement des temps de rétention. En effet, il existe en chromatographie liquide des décalages incontrôlés, liés à des variations de température ou de pression, une dégradation de la phase stationnaire, mais également des décalages contrôlés qui peuvent provenir d'un chemin additionnel sur la vanne selon la configuration de l'interface entre les deux dimensions [10].

Plusieurs méthodes d'alignement, basées essentiellement sur des outils chimiométriques ou l'utilisation d'algorithmes peuvent être utilisées [11–19]. Cependant, aucune méthode n'est à ce jour reconnue comme étant la plus efficace.

1.3. Affectation des pics de 2ème dimension

En LCxLC, les pics du chromatogramme de 1^{ère} dimension sont échantillonnés en plusieurs fractions. Les différents pics de 2^{ème} dimension correspondant à un même composé doivent donc être regroupés, afin de pouvoir exploiter les résultats de l'analyse. Plusieurs méthodes ont été développées afin d'affecter les pics de 2^{ème} dimension au pic de 1^{ère} dimension duquel ils sont issus.

L'une des approches développées est basée sur un algorithme de détection en deux étapes [20]. Tout d'abord, les pics de 2^{ème} dimension sont détectés avec les outils classiques de traitement unidimensionnel. Puis, l'algorithme analyse ces pics et regroupe ceux qui correspondent à un même pic de 1^{ère} dimension en se basant sur deux critères : le taux de chevauchement des régions de pic, pour deux pics adjacents, et l'unimodalité.

Dans le cas de traitement des données à l'aide d'outils dédiés à l'analyse d'image, il est possible d'appliquer un algorithme appelé « Watershed algorithm » [21]. Cet algorithme permet de détecter, à partir des pixels de l'image, le sommet d'un pic, puis continu sa progression jusqu'à atteindre la ligne de base du chromatogramme. Cependant, cet algorithme peut être affecté par la variabilité des temps de rétention en 2^{ème} dimension et la largeur des pics, ce qui entraine une probabilité d'échec pouvant être supérieure à 20% [22]. En revanche, il a été montré qu'après correction et alignement des temps de rétention, le « Watershed algorithm » permettait de détecter les pics avec une plus grande précision que l'approche en deux étapes [23].

Enfin, plusieurs approches basées sur l'utilisation d'outils chimiométriques ont été proposées pour le traitement de données LCxLC. Elles peuvent être regroupées en deux grandes familles : Les méthodes qui supposent que les données d'une analyse 2D suivent un modèle trilinéaire [24–31], et les méthodes basées sur un modèle bilinéaire [19,32–38]. Cependant, l'application de telles méthodes n'est pas directe car elles sont basées sur des concepts mathématiques avancés et, beaucoup de précautions doivent être prises avant d'appliquer de tels outils.

2. Analyse quantitative en LCxLC

L'intégration des pics de 2^{ème} dimension peut être faite soit de façon manuelle en utilisant des logiciels classiques de traitement d'analyses LC unidimensionnelles, soit de façon automatique, au moyen d'algorithmes mathématiques. Dans le second cas, le principe de l'intégration est basé sur les propriétés d'un pic gaussien. En effet, il est possible à partir du pic et de ses dérivées de déterminer le début et la fin du pic, ainsi que son maximum d'intensité [10].

L'analyse quantitative peut être faite à partir de l'aire totale correspondant à un même composé. Deux grandes approches existent pour évaluer cette surface totale. La première méthode consiste à calculer la somme des aires de chaque pic de 2^{ème} dimension correspondant à un même composé. La seconde méthode, appelée approche Gaussienne [39], consiste à tracer l'évolution de l'aire des pics de 2^{ème} dimension en fonction du temps de 1^{ère} dimension, puis de modéliser cette évolution par un pic gaussien. Une étude comparative a montré que ces deux approches conduisaient à des résultats équivalents [40]. Enfin l'analyse quantitative en LCxLC peut également être faite à partir du chromatogramme 3D, en considérant les pics comme des cônes. Le volume de ces pics est alors calculé, puis relié à la concentration. Une étude comparative dans le cas d'une analyse GCxGC [41] a montré que le calcul de la somme des aires ainsi que le calcul des volumes de pics conduisaient à des résultats répétables, la somme des aires ayant tout de même un RSD légèrement meilleur.

Une stratégie un peu plus originale, la 2DALC (Two-dimensional assisted liquid chromatography) a récemment été proposée [36]. Elle est basée à la fois sur l'utilisation d'un détecteur DAD à la sortie de chaque dimension, et sur un traitement chimiométrique des données. Une analyse des données issues du détecteur de 2^{ème} dimension permet d'obtenir les spectres UV de chaque composé du mélange. Ces spectres sont ensuite utilisés dans l'analyse des données du détecteur de 1^{ère} dimension, ce qui permet d'extraire des chromatogrammes 1D-LC résolus pour chaque composé du mélange. Ces chromatogrammes extraits sont ensuite utilisés pour l'analyse quantitative de l'échantillon.

Le Tableau 1.1 regroupe toutes les applications d'analyse quantitative faites en LCxLC, tous domaines confondus, que l'on peut trouver dans la littérature. Il apparaît que l'approche basée sur les aires de pics de 2^{ème} dimension est plus souvent employée que le calcul des volumes de pics. Cette tendance peut s'expliquer par le fait que le calcul des volumes de pics nécessite l'utilisation de logiciels spécifiques comme LC-Image, ou le développement de routines sous Matlab et logiciels équivalents. Au contraire, l'intégration de LC classique et la somme des aires est ensuite calculée à l'aide d'Excel ou d'une simple calculatrice. Enfin, il est important de noter que dans toutes ces applications, les performances quantitatives de la LCxLC ont été validées par des linéarités équivalentes à la LC, et de très bonnes répétabilités des analyses avec des RSD inférieurs à 5 % sur les temps de rétention et en moyenne entre 5 et 10 % sur les aires ou les volumes.

| Echantillon | Mé | ref | |
|-----------------------------------|-------|--------|------|
| | Aires | Volume | |
| Stevia Rebaudiana | Х | | [42] |
| Mélange modèle | Х | | [40] |
| Extraits végétaux | Х | | [43] |
| Poussières | | Х | [44] |
| Aérosols | Х | | [45] |
| Extraits de Lamiacées | Х | | [46] |
| Huile de maïs | Х | | [47] |
| Urine | Х | Х | [48] |
| Extraits de graines de maïs | Х | Х | [4] |
| Comprimés de phytothérapie | Х | | [49] |
| Extraits de plantes chinoises | | Х | [50] |
| Médicament (naproxène) | Х | | [51] |
| Plasma humain | Х | | [52] |
| Huile essentielle de pamplemousse | Х | | [53] |
| Résines époxydes | Х | | [54] |
| Vins et jus de fruits | | Х | [55] |
| D- et L-phenylalanine | Х | | [56] |

| Tableau 1.1 | - Applications d | e la LCxLC à | l'analyse | quantitative | d'échantillons | complexes |
|-------------|------------------|--------------|-----------|--------------|----------------|-----------|
|-------------|------------------|--------------|-----------|--------------|----------------|-----------|

Chapitre 1 : Contexte | 32

La 2D-LC a été appliquée avec succès à l'analyse d'échantillons pharmaceutique depuis les années 1980. La grande majorité des méthodes développées est dédiée (1) à l'analyse de trace, soit dans le cadre de suivis thérapeutiques pharmacologiques soit pour la détection et l'identification d'impuretés dans les premières phases de développement du médicament ; et (2) à l'analyse d'échantillons chiraux. De nouvelles tendances sont apparues plus récemment, comme par exemple le développement de la LCxLC pour du profilage d'impuretés, ou encore l'utilisation de la LC-LC pour coupler une méthode LC non compatible avec la spectrométrie de masse.

Il apparait que la majorité de ces méthodes (environ 85 %) ont été développées dans le mode heart-cutting. Cette tendance peut avoir différentes explications parmi lesquelles on peut trouver l'optimisation des méthodes et le traitement des données qui sont beaucoup plus simples qu'en LCxLC et ne nécessitent pas l'utilisation de logiciels spécifiques ou d'outils mathématiques complexes. Cependant, les possibilités de la LC-LC en termes de pouvoir de séparation restent très limitées, c'est pourquoi des approches récentes comme le mode sélective compréhensive doivent être privilégiées pour l'industrie pharmaceutique.

Enfin, le traitement des données en LCxLC, qui est un élément clé pour l'implantation de cette technique en milieux industriels, reste à ce jour un exercice complexe. En effet, un certain nombre d'étapes de prétraitement des données est nécessaire pour éviter toute source d'erreurs dans l'interprétation des résultats. L'analyse quantitative en LCxLC est possible, à travers différentes approches. Cependant elle représente encore aujourd'hui un travail laborieux et très consommateur de temps, ce qui est un frein à son implantation dans un contexte comme l'industrie pharmaceutique, où l'analyste doit partager son temps entre développement de méthode et analyse de routine.

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CHAPITRE 2

Conditions expérimentales

Les études menées au cours de ce travail ont été réalisées sur différents échantillons, et au moyen de différents instruments. Ce chapitre décrit l'appareillage, les phases stationnaires, les phases mobiles, la nature des échantillons ainsi que les outils utilisés pour le traitement des données tout au long de ces travaux.

La première section décrit les différents instruments employés, à savoir deux systèmes de chromatographie en phase supercritique, deux systèmes de chromatographie en phase liquide bidimensionnelle, ainsi que le simple quadripôle qui a été utilisé pour coupler les analyses avec une détection par spectrométrie de masse. Les différentes interfaces utilisées dans le cas des couplages LCxLC et LCxSFC sont également décrites dans cette partie.

Les deux parties suivantes détaillent les phases stationnaires ainsi que les différentes phases mobiles employées au cours de ces travaux.

La partie D, quant à elle, précise la nature et les caractéristiques des différents échantillons utilisés lors des différentes études. Dans tous les cas, il s'agit d'échantillons pharmaceutiques qui ont été fournis par Oril Industrie. Ces composés, qui sont issus de différents projets, et de différentes voies de synthèses, sont des produits jeunes que l'on situe dans les premières phases de développement du médicament (préclinique et phase I).

Enfin, la dernière partie aborde la question du traitement et de la gestion des données. Elle précise notamment les outils et logiciels utilisés pour l'exploitation des résultats.

Nous remercions Waters pour le prêt de l'UPC² et des colonnes dédiées. Nous remercions Agilent Technologie pour le prêt de la chaîne 1260 Infinity II SFC et du système de vanne « Multiple Heart-Cutting » qui a été installé sur notre instrument 2D-LC. Nous remercions Chiral technologies (Daïcel) pour le prêt de colonnes chirales.

A. INSTRUMENTATION

1. Systèmes chromatographiques

1.1.1290 Infinity I 2D-LC (Agilent)

Ce système de chromatographie en phase liquide bidimensionnelle est composé de deux systèmes Agilent 1290 reliés par une vanne double 4 voies 2 positions résistante aux hautes pressions (jusqu'à 1000 bar). Il inclut deux systèmes de pompage binaires identiques, deux fours colonnes identiques, un passeur d'échantillon, un détecteur UV mono longueur d'onde en 1^{ère} dimension et un détecteur DAD en 2^{ème} dimension. Le système de pompage haute pression peut délivrer un débit maximal de 5 mL/min. La pression limite est de 1200 bar. La température maximale du four colonne est de 100°C. Le système d'injection est de type FTN (flow-through needle), il permet l'injection de volumes allant jusqu'à 20 μ L. Pour injecter des volumes plus grands, il est nécessaire d'ajouter un tube dimensionné selon le volume souhaité. La cellule du détecteur UV a un volume de 2 μ L, un chemin optique de 3 mm et supporte une pression maximale de 60 bar. Le DAD, lui, possède une cellule de 0.6 μ L avec un chemin optique de 10 mm supportant une pression maximale de 120 bar. L'interface est détaillée dans la partie A.2 de ce chapitre.

Le pilotage du système 2D-LC, ainsi que l'acquisition des données, sont réalisés par le logiciel OpenLab CDS (Chemstation).

Ce système peut également être utilisé pour des analyses unidimensionnelles, en mode LC-DAD. Dans ce cas, seules les pompes de 1^{ère} dimension, le passeur d'échantillon, un des fours colonnes et le DAD sont utilisés.

1.2. 2D I-Class (Waters)

Ce système est composé de deux systèmes UHPLC I-Class reliés par une interface composée de deux vannes hautes pressions 6 voies 2 positions. Il comprend deux systèmes de pompage binaire haute pression, un passeur d'échantillon, deux fours colonnes identiques, un détecteur UV mono longueur d'onde en 1^{ère} dimension et un DAD en 2^{ème} dimension. Le système de pompage peut délivrer des débits allant jusqu'à 2 mL/min. Le maximum de pression autorisée est 1200 bar et décroît au-delà de 1 mL/min en fonction du débit et de la phase mobile. Le système d'injection est de type FTN et permet l'injection de volumes allant jusqu'à 15 μ L. Une boucle d'extension optionnelle permet d'augmenter le volume injecté jusqu'à 50 μ L. La température maximale du four colonne est de 90°C. Les deux cellules de détection (UV et DAD) ont un volume de 0.5 μ L, un chemin optique de 10 mm et supportent une pression de maximale de 70 bar. L'interface d'origine de ce système n'a pas été utilisée au cours de ces travaux, il ne sera donc pas détaillé.

Ce système 2D-LC est piloté par le logiciel MassLynx qui permet également l'acquisition des données.

Ce système a été utilisé uniquement en mode LC-DAD/MS dans le cadre de l'étude préliminaire du Chapitre 3 et en première dimension pour le couplage RPLCxSFC (Chapitre 5 Partie A). Dans les deux cas, seules les pompes de 1^{ère} dimension, le passeur d'échantillon, un des fours colonnes et le DAD ont été utilisés.

1.3. UPC² (Waters)

Ce système de chromatographie en phase supercritique (SFC) inclut un système de pompage binaire haute pression, un passeur d'échantillon, deux fours colonnes identiques avec un système de sélection de colonnes (switch colonne) composé de deux vannes 6 ports 6 positions, un détecteur DAD et un module permettant la régulation de la contre pression (ABPR). Le système de pompage peut délivrer jusqu'à 4 mL/min. Le maximum de pression autorisée est de 414 bar pour des débits inférieurs à 3.25 mL/min et décroît linéairement à 293 bar à 4 mL/min. Le volume de la boucle d'injection est de 10 μ L. La température maximale du four colonne est de 90°C. Le détecteur DAD possède une cellule 8 μ L, un chemin optique de 10 mm et supporte une pression de 414 bar maximum.

Le pilotage de ce système SFC est fait par le logiciel Empower, qui permet également l'acquisition des données.

1.4. 1260 Infinity II SFC (Agilent)

Ce système de chromatographie en phase supercritique (SFC) inclut un système de pompage binaire haute pression, un passeur d'échantillon, un four colonne, un détecteur DAD et un module de contrôle SFC permettant à la fois le pré-conditionnement du CO₂ (booster pump) avant son entrée dans le système et la régulation de la contre pression (BPR) à la sortie du détecteur. Le système de pompage peut délivrer jusqu'à 5 mL/min. Le maximum de pression autorisée est de 600 bar. Le système d'injection est doté de la technologie « Feed injection ». L'échantillon est prélevé à pression atmosphérique via l'aiguille et le piston de prélèvement, puis pressurisé à l'intérieur d'une boucle et enfin mis au contact du débit de CO₂ venant de la pompe et dirigé vers la colonne. Cette technologie permet l'injection de volumes allant de 0.1 à 100 μ L. La température maximale du four colonne est de 85°C. Le détecteur DAD possède une cellule 2 μ L, un chemin optique de 3 mm et supporte une pression de 400 bar maximum.

Le système Infinity II SFC est piloté par le logiciel OpenLab CDS, qui permet également l'acquisition des données.

1.5. Couplages LCxSFC

Contrairement aux couplages LCxLC pour lesquels il existe des instruments commerciaux dédiés, les couplages LCxSFC nécessitent de relier deux instruments différents entre eux. Deux montages différents ont été utilisés dans le cadre de ces travaux.

1.5.1. LCxSFC achirale

La première dimension est composée de plusieurs modules du 2D I-Class, en particulier le système de pompage et le four colonne de première dimension, le passeur d'échantillon et le détecteur DAD. La deuxième dimension est constituée de tout le système UPC², à l'exception du passeur d'échantillon, et du sélecteur de colonne qui a été bypassé afin de travailler avec le volume de délai le plus petit possible. Une vanne 10 voies 2 positions équipée de deux boucles de 5 μ L est utilisée pour relier les deux dimensions. Cette interface est détaillée dans la partie A.2 de ce chapitre.

Le logiciel Masslynx est utilisé pour le pilotage et l'acquisition des données de la première dimension, ainsi que le pilotage de la vanne 10 voies. Le logiciel Empower, quant à lui, permet le pilotage et l'acquisition des données de la deuxième dimension. Les deux systèmes sont synchronisés en connectant électriquement les deux appareils et en programmant des évènements externes à partir de la première dimension dans Masslynx.

1.5.2. sLCxSFC chirale

Plusieurs modules de la chaîne Infinity I 2D-LC ont été utilisés en première dimension ainsi que pour l'interface, à savoir le système de pompage et le four de première dimension, le passeur d'échantillon, le détecteur UV et la vanne double 4 voies 2 positions équipée du système « Multiple heart-cutting » (MHC) d'Agilent. Cette interface particulière est décrite en détail dans la partie A.2 de ce chapitre. La deuxième dimension comprend tous les modules de le la chaîne Infinity II SFC, à l'exception du passeur d'échantillon.

Chacune des deux dimensions est pilotée par le logiciel OpenLab CDS, qui permet également l'acquisition des données, mais via deux ordinateurs différents. En effet, le logiciel ne supportant pas de gérer à la fois le module de contrôle SFC et la configuration 2D avec pilotage de la vanne, l'ordinateur de la chaîne Infinity I 2D-LC est utilisé pour piloter la première dimension et l'interface tandis que l'ordinateur de la chaîne SFC gère la seconde dimension. La synchronisation entre les deux systèmes est obtenue en connectant électriquement les deux instruments via une connexion « Remote ».

2. Interfaces

L'interface joue un rôle primordial dans le déroulement d'une analyse bidimensionnelle dite « compréhensive ». Equipée en général de deux boucles identiques, l'interface assure à la fois l'échantillonnage de la séparation issue de la première dimension et le transfert des fractions collectées vers la deuxième dimension. Afin de minimiser la dispersion du créneau d'injection dans les boucles, celles-ci sont remplies et vidées en sens inverse (« back-flush »). Les branchements sur les différents ports des vannes sont faits de façon à travailler avec des interfaces symétriques, pour lesquelles le trajet des composés est le même pour les deux boucles. Cela permet d'éviter les décalages d'une fraction à l'autre[1].

2.1. Vanne double 4 voies

Cette interface, présentée Figure 2.1, est spécifique du système 2D-LC Agilent. Elle présente l'avantage de pouvoir travailler en configuration symétrique sans avoir besoin de

chemins additionnels. Cela permet notamment de ne pas avoir à recaler/réaligner les pics de deuxième dimension, et facilite donc le retraitement des données. De plus, aucun problème de synchronisation n'est à craindre entre les deux positions puisque l'interface n'est composée que d'une seule vanne.



Figure 2.1 - Vanne double 4 voies 2 positions équipée de deux boucles d'échantillonnage

2.2. Vanne 10 voies

Cette interface a été utilisée pour le couplage LCxSFC. Il s'agit d'une vanne 10 voies 2 positions Valco Cheminert UHPLC pouvant résister à des pressions jusqu'à 1000 bar. Afin de pouvoir travailler en configuration symétrique, il est nécessaire d'ajouter un raccord entre les positions 4 et 9 de la vanne (Figure 2.2), l'échantillon ne parcourt donc pas le même trajet en position A et position B lors de l'injection en deuxième dimension. En position A, le raccord intervient après la boucle, donc le chemin parcouru est plus important qu'en position B où le raccord est situé avant la boucle. Une correction est donc nécessaire pour tenir compte de ce décalage, et il faut réaligner les pics des fractions paires et impaires pendant le traitement des données.





2.3. Système « multiple heart-cutting » (MHC)

Cette interface particulière est spécifique du système 2D-LC Agilent (Figure 2.3). Elle a été utilisée pour le couplage sLCxSFC chirale. Elle est composée de la vanne double 4 voies Agilent décrite précédemment. Deux vannes 14 voies 6 positions, appelées Deck A et Deck B, viennent remplacer les boucles d'échantillonnage. Chacune de ces deux vannes est équipée de six boucles identiques, ce qui permet de stocker temporairement jusqu'à dix fractions. En position A, les boucles 2 à 6 du Deck A sont remplies pendant que le contenu des boucles 1 à 5 du Deck B est injecté en deuxième dimension, et inversement lorsque l'on passe en position B. La boucle 1 du Deck A et la boucle 6 du Deck B servent à assurer une circulation des phases mobiles en continu et ne peuvent donc pas être utilisées pour stocker des fractions.

Un algorithme intégré au logiciel Openlab CDS permet de minimiser le nombre de rotations des vannes. Ainsi, lorsque l'on est en position A, le remplissage des boucles du Deck A se fait de la boucle n°1 à la boucle n°5. Puis la vanne double 4 voies passe en position B, et l'analyse des boucles du Deck A se fait de la boucle n°5 à la boucle n°1.



Figure 2.3 - Vanne double 4 voies 2 positions équipée du système "multiple heart-cutting"

3. Spectromètre de masse QDa (Waters)

Le QDa est un simple quadripôle équipé d'une source electrospray (ESI) qui possède une gamme dynamique allant de 50 à 1250 Th. Il est piloté par le logiciel Masslynx. Les réglages de sources sont listés dans le Tableau 2.1. Les conditions spécifiques à chaque composé sont mentionnées dans le chapitre concerné.

| Polarité | ESI + |
|------------------------------|-----------|
| Tension du capillaire | 0.8 kV |
| Température de désolvatation | 500°C |
| Format des données | Continuum |

Tableau 2.1 - Réglages du spectromètre de masse QDa pour l'analyse de composés pharmaceutiques

Les acquisitions ont été réalisées en mode Scan, qui permet de balayer une gamme de m/z choisie. Le signal enregistré représente alors la somme de toutes les masses (Courant ionique total, TIC). Il est ensuite possible soit d'extraire le spectre de masse d'un composé, soit d'extraire spécifiquement une ou plusieurs m/z (EIC).

Ce détecteur a été couplé au système 2D I-Class utilisé en LC-MS ainsi qu'au 1290 Infinity 2D-LC pour les analyses LCxLC-UV/MS.

4. Caractéristiques des systèmes

4.1. Volume de délai et volume externe

Le volume de délai correspond au volume compris entre le point de rencontre des solvants et leur arrivée en tête de colonne. Ce paramètre est caractéristique d'un système et intervient lors d'analyses en gradient d'élution. Il est mesuré en remplaçant la colonne par un raccord union à zéro volume mort, et en appliquant une montée linéaire d'un solvant A à un solvant B. En LC, A contient du méthanol et B du méthanol avec 0.1 % d'acétone en volume, et la montée linéaire va de 1 à 99 % B [2]. En SFC, A contient du CO₂ et B du méthanol avec 0.1 % d'acétone en volume. Dans ce cas la montée linéaire se fait de 69 à 99 % B car il a été montré que la mesure de volume de délai était plus fiable lorsque l'on se place en conditions quasi-liquides [1].

Le volume externe, quant à lui, correspond à la somme des volumes situés entre l'injecteur et la colonne d'une part et entre la colonne et le détecteur d'autre part. Il est mesuré en considérant le moment statistique centré d'ordre 1 par injection d'1 μ L d'ethylparabène, une union à zéro volume mort remplaçant la colonne.

| | | V _{délai} (µL) | V _{externe} (µL) |
|-----------------------|----------------------------|-------------------------|---------------------------|
| 2D I-Class | 1 ^{ère} dimension | 110 | 12 |
| | 2 ^{ème} dimension | 120 | 6.5* |
| 1290 Infinity I 2D-LC | 1 ^{ère} dimension | 140 | 22 |
| | 2 ^{ème} dimension | 65 | 8.5* |
| UPC ² | | 300** | 83 |
| 1260 Infinity II SFC | | 600 | 40 |

Tableau 2.2 - Caractéristiques des différents systèmes utilisés. *Le volume indiqué ne tient pas compte du volume des boucles situées au niveau l'interface, **le volume indiqué ne tient pas compte du sélecteur de colonne, qui a été bypassé pendant les manipulations.

4.2. Variance extra-colonne

La variance extra-colonne comprend les dispersions provenant de l'injection, des tubes et de la détection. Elle est mesurée à partir du moment statistique centré d'ordre 2 [3].

Lorsque le système Infinity 2D-LC est utilisé en système unidimensionnel, la première dimension est directement connectée au détecteur DAD (Figure 2.4a). Dans cette configuration, le système génère une variance 2 à 3 fois moins importante que lorsqu'il est utilisé en configuration 2D dans laquelle le DAD est utilisé comme détecteur de 2^{ème} dimension pendant que le détecteur UV est connecté à la 1^{ère} dimension. Cette différence s'explique par le fait que les deux cellules de détection n'ont pas le même volume (2 μ L et 0.6 μ L pour l'UV et le DAD, respectivement), et génèrent donc des dispersions différentes.





La variance extra-colonne mesurée en 2^{ème} dimension (Figure 2.4b) est quant à elle de l'ordre de 5 μ L² pour des débits allant jusqu'à 2 mL/min. Ces faibles valeurs sont essentielles en 2^{ème} dimension, car elles permettent de travailler avec des colonnes sub-2 μ m de faibles volumes mort (\leq 100 μ L), qui sont nécessaires pour les analyses 2D-LC.

En SFC, la variance extra-colonne a été mesurée en conditions supercritiques et en conditions quasi-liquides. Les résultats de ces deux mesures sont superposés sur la Figure 2.5. En conditions supercritiques cette variance est de l'ordre de 30 μ L² alors qu'en conditions quasi-liquides elle varie de 100 à 150 μ L² pour des débits supérieurs à 1 mL/min. Cette différence vient du fait que les coefficients de diffusion sont bien plus importants en CO₂ supercritique qu'en méthanol. On parle d'un facteur 10 à 15 entre les deux, qui peut être divisé par deux dès lors que l'on ajoute 5 % de méthanol dans le CO₂ [4]. Or la variance est inversement proportionnelle au coefficient de diffusion [5].



Figure 2.5 - Evolution de la variance extra-colonne en fonction du débit pour le système Infinity II SFC. Injection de 1 μL d'une solution d'acétone à 15 % dans le méthanol. Phases mobiles : CO₂/MeOH 95/5 (v/v) en conditions supercritiques et CO₂/MeOH 1/99 (v/v) en conditions quasi-liquides. BPR = 140 bar.

4.3. Test des interfaces

Afin de vérifier que les interfaces fonctionnement correctement, quelques tests préliminaires ont été menés. Dans le cas du système « MHC » en particulier, il est important de s'assurer que toutes les boucles sont bien identiques. Pour cela, de la caféine est ajoutée à la phase mobile de ¹D afin de servir de marqueur UV. Le débit est fixé à 0.1 mL/min et plusieurs rotations de vannes sont programmées, avec un temps d'échantillonnage de 2.5 sec. Ainsi, toutes les boucles de chaque Deck sont remplies puis analysées trois fois. La répétabilité des fractions est vérifiée (1) en superposant les chromatogrammes d'une série à l'autre et (2) en comparant l'aire des pics de toutes les fractions (Figure 2.6).



Figure 2.6 - Superposition de 3 séries de collecte/analyse avec le système MHC pour tester la répétabilité des fractions. ¹D : sans colonne ; 0.1 mL/min ; 30°C ; isocratique eau/ACN 50/50 (v/v). ²D : Acquity CSH C18 50x2.1 mm 1.7 μm ; 0.4 mL/min ; 30°C ; isocratique eau/ACN 30/70 (v/v) pendant 0.5 min.

On obtient ainsi un coefficient de variation de l'ordre de 4 % sur l'aire des pics ce qui permet de conclure que les fractions sont répétables et donc que les boucles sont identiques.

B. PHASES STATIONNAIRES

Les différentes phases stationnaires utilisées au cours de ce travail sont listées dans le Tableau 2.3. Elles sont toutes à particules totalement poreuses, à l'exception de la Nucleoshell HILIC ZW qui est à particule core-shell (noyau dur recouvert d'une couche poreuse).

| | Nom | Support | Fournisseur | L (mm) | di (mm) | dp (µm) | Chap |
|---------------|-------------------------|------------------|----------------|-----------|------------|------------|------|
| SPLC | Acquity CSH C18 | Si | Waters | 100 | 2,1 | 1,7 | 3 |
| | Acquity CSH PFP | Si | Waters | 50 | 2,1 | 1,7 | 3, 4 |
| | Acquity BEH C18 | Si | Waters | 50 | 2,1 | 1,7 | 3, 4 |
| | Acquity BEH Shield RP18 | Si | Waters | 50 | 2,1 | 1,7 | 3 |
| | Hypercab | Graphite | Thermo Fisher | 50 | 1 | 3 | 3 |
| | Acquity BEH Phenyl | Si | Waters | 50 | 2,1 | 1,7 | 3, 4 |
| | Acquity CSH C18 | Si | Waters | 30 | 2,1 | 1,7 | 3, 4 |
| HILIC | Acquity BEH HILIC | Si | Waters | 50 | 2,1 | 1,7 | 3 |
| RPLC & SFC | Discovery Zr-Carb | ZrO ₂ | Sigma-Aldrich | 50 | 4,6 | 3 | 3, 5 |
| | Zorbax SB-CN | Si | Agilent | 150 | 4,6 | 5 | 3, 5 |
| | Resolve Spherical C18 | Si | Waters | 150 | 3,9 | 5 | 5 |
| | Zorbax RX-SIL | Si | Agilent | 150 | 4,6 | 5 | 5 |
| | Viridis Silica | Si | Waters | 50 | 3 | 5 | 5 |
| | Nucleoshell HILIC ZW | Si | Macherey Nagel | 100 | 2 | 2,7 | 5 |
| | Discovery PBD | ZrO ₂ | Sigma-Aldrich | 50 | 4,6 | 3 | 5 |
| SFC | Synergie Polar RP | Si | Phenomenex | 50 | 3 | 2,5 | 5 |
| | Synergie Hydro RP | Si | Phenomenex | 50 | 3 | 2,5 | 5 |
| | Torus 2-PIC | Si | Waters | 50 | 3 | 1,7 | 5 |
| | SFC-A | Si | Daïcel | 50 | 3 | 3 | 5 |
| | SFC-B | Si | Daïcel | 100 | 3 | 5 | 5 |
| | Chiralpack IC | Si | Daïcel | 150 | 4,6 | 3 | 5 |

Tableau 2.3 - Colonnes utilisées pour les différentes études. Si : silice ; ZrO2 : oxyde de zirconium

C. PHASES MOBILES

L'eau utilisée pour la préparation des phases mobiles est issue d'un système de purification Elga (Purelab classic UV) permettant d'obtenir une conductivité de 18 MΩ.cm⁻¹. Les bactéries présentes dans l'eau sont détruites par rayonnement UV. Les phases mobiles organiques sont préparées à partir d'acétonitrile et de méthanol qualité HPLC ou LC-MS fournis par Sigma Aldrich.

1. Chromatographie en phase liquide

Divers additifs sont ajoutés pour contrôler le pH (Tableau 2.4) : acide méthanesulfonique (AMS), acide formique (FA), acétate d'ammonium (AA), formate d'ammonium (AF) et hydroxyde d'ammonium (NH₄OH). Ces additifs sont ajoutés dans les phases mobiles aqueuses et organiques afin de maintenir la force ionique constante, à l'exception des sels d'ammonium car leur solubilité dans les solvants organiques est trop faible [6]. Les phases aqueuses préparées à base de sels sont filtrées sur 0.22 μ m avec un système de filtration Millipore avant utilisation.

| Additif | Concentration | рН |
|-------------------------|---------------|-----------------|
| Acide méthanesulfonique | 0.1 % (v/v) | 2.0 |
| Acide formique | 0.1 % (v/v) | 2.7 |
| Acétate d'ammonium | 5 mM | 6.8 |
| Formate d'ammonium | 5 mM | 3.5 ajusté (FA) |
| Hydroxyde d'ammonium | 0.1 % (v/v) | 10.4 |

Tableau 2.4 - Caractéristiques des phases mobiles utilisées

Les mesures et ajustements de pH sont effectués à l'aide d'un pH-mètre (Mettler Toledo) équipé d'une électrode de pH combinée avec sonde de température (InLab Expert Pro-ISM). Les phases mobiles sont préparées de façon quotidienne.

2. Chromatographie en fluide supercritique

En SFC achirale, un co-solvant MeOH/H₂O 98/2 (v/v) contenant 20 mM d'acétate d'ammonium est utilisé avec le CO_2 en mode d'élution gradient. Afin d'éviter d'éventuels problèmes de solubilité, une solution aqueuse d'acétate d'ammonium à 1 M est préparée, puis diluée dans du méthanol.

En SFC chirale, du méthanol pur ainsi qu'un mélange MeOH/H₂O 98/2 (v/v) ont été utilisés comme co-solvant, en mode d'élution isocratique et gradient.

D. ECHANTILLONS

Toutes les études réalisées au cours de ce travail ont été menées sur des échantillons de mélanges pharmaceutiques fournis par Oril Industrie (Bolbec). Pour chaque échantillon, les différents composés (principe actif et impuretés) ont été reçus sous forme isolée et non solubilisée, puis mis en solution avec le solvant adéquat. Ces solutions ont été conservées au réfrigérateur à 4°C, et leur stabilité a été vérifiée de façon hebdomadaire à l'aide des méthodes de contrôle qualité fournies par Oril, durant tout le temps de leur utilisation.

1. Projet S56922

Cet échantillon est issu d'une synthèse multi-étapes, il est constitué du principe actif et de huit intermédiaires de synthèse. La plupart des composés de ce projet sont des composés halogénés, contenant des atomes de Brome ou de Fluor.

Pour les études présentées dans les Chapitres 3 et 5A, des solutions individuelles à 0.1 mg/mL ainsi qu'un mélange contenant le principe actif et les impuretés à une concentration identique de 0.1 mg/mL sont préparés en solubilisant 1 mg de composé dans un mélange eau/ACN 50/50 (v/v) acidifié avec 0.1 % d'acide méthanesulfonique.

Pour l'étude du Chapitre 4, différents mélanges sont préparés dans le même solvant, avec une concentration en principe actif de 0.5 mg/mL et des niveaux de concentration plus faibles (0.05 % à 2 %) pour les impuretés.

2. Projet S68502

Cet échantillon est également issu d'une synthèse multi-étapes, mais différente de la précédente. Il contient un principe actif ainsi que neufs intermédiaires de synthèse. Il a été utilisé pour les études des Chapitres 3 et 5A, ainsi que pour la comparaison instrumentale présentée Chapitre 4C.

Le mélange contenant le principe actif et les impuretés à une concentration identique de 0.1 mg/mL est directement préparé par pesée en mélangeant 1 mg de chaque composé. La mise en solution est faite dans un mélange eau/ACN 20/80 (v/v) acidifié avec 0.1 % d'acide méthanesulfonique.

3. Echantillon Chiral

Cet échantillon a été utilisé pour l'étude présentée dans le Chapitre 5B. Les deux énantiomères du principe actif sont disponibles séparément sous forme de poudre, l'énantiomère S étant la forme « active ». Cela permet de pouvoir préparer des solutions individuelles de chaque énantiomère à 0.2 mg/mL en solubilisant 2 mg de produit dans 10 mL d'acétonitrile.

Pour la mise en œuvre des analyses sLCxSFC, ainsi que l'étude de performances et la comparaison avec la 1D-SFC, plusieurs mélanges ont été préparés avec l'énantiomère S à une

concentration de 0.4 mg/mL et l'énantiomère R à des niveaux de concentration plus faibles (5 % à 0.5 %) dans 100 % d'acétonitrile.

4. Autres échantillons

Un échantillon supplémentaire a été utilisé pour challenger les systèmes LCxLC génériques tout en travaillant sur l'aspect quantitatif des méthodes développées (voir Chapitre 4). Il s'agit d'un produit très « jeune » qui a été reçu sous forme de poudre, et qui correspond à un lot de fabrication. La mise en solution est faite dans 100 % d'acétonitrile, selon les indications fournies par Oril lors de l'envoi.

Le methylparabène et l'ethylparabène ont été utilisés comme composés modèles dans le Chapitre 4 Partie A, pour des études préliminaires. Les différentes solutions ont été préparées par dissolution de ces composés dans un mélange eau/ACN 50/50 (v/v)

E. TRAITEMENT DES DONNEES ET CALCULS

1. Optimisation de méthodes LCxLC

Les calculs effectués pour l'optimisation et le dimensionnement des méthodes RPLCxRPLC mises en place dans les chapitres 3 et 4 sont réalisés à l'aide d'un outil développé sur Excel au laboratoire [7,8]. Il permet entre autre de choisir les paramètres chromatographiques (débits, température, échantillonnage, etc.) les plus adaptés pour l'analyse bidimensionnelle que l'on cherche à développer.

2. Données 1D

Les données acquises en 1D-LC et 1D-SFC sont traitées soit directement sur le logiciel dédié au pilotage de l'appareil ou le logiciel Azur, soit sur Excel après export des données brutes.

3. Données 2D

Une analyse bidimensionnelle génère un grand nombre de données qu'il faut ensuite traiter. Ces données contiennent trois informations différentes, à savoir le temps de rétention dans chaque dimension et le signal du détecteur. De plus, ces données sont obtenues sous forme de matrice, ce qui ne facilite pas l'exploitation de résultats.

En général, le chromatogramme obtenu en sortie du détecteur de deuxième dimension correspond à l'analyse successive des fractions de première dimension. Ce chromatogramme doit dans un premier temps être fractionné. Après une étape de réalignement si besoin, les différentes fractions peuvent alors être superposées afin de pouvoir interpréter les résultats. Ces différentes étapes sont réalisées à l'aide d'une macro Excel développée au laboratoire. Enfin, les données peuvent être visualisées sous la forme d'un « contour-plot » ou d'un chromatogramme 3D. Ces représentations sont obtenues directement à partir de la matrice des données, grâce à une routine Matlab développée au laboratoire. Les différentes étapes du traitement sont illustrées Figure 2.7.

Bien qu'il existe quelques logiciels commerciaux destinés à la représentation de données LCxLC, leur utilisation n'est pas simple et des progrès restent à faire quant à l'exploitation des données dans une optique d'analyse de routine.


Figure 2.7 - Traitement et représentation des données d'une analyse 2D. Toutes les fractions du chromatogramme de ¹D (a,b) sont successivement analysées en ²D (c). Après découpe et superposition des fractions (d), les résultats sont visualisés sous forme de contour plot (e) ou de chromatogramme 3D (f).

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CHAPITRE 3

Mise en place d'une stratégie 2D-LC générique. Partie 1 : Sélection de systèmes bidimensionnels intéressants

Ce chapitre a fait l'objet d'une publication :

Article 2

"Comprehensive two dimensional liquid chromatography as analytical strategy for pharmaceutical analysis"

M. Iguiniz, F. Rouvière, E. Corbel, N. Roques, S. Heinisch, Journal of Chromatography A, 1536 (2018) 195–204. https://doi.org/10.1016/j.chroma.2017.08.070.

Chapitre 3 : Mise en place d'une stratégie 2D-LC générique. Partie 1 | 60

En LCxLC, la capacité de pics théorique est le produit des capacités de pics dans chaque dimension, ce qui permet d'atteindre des valeurs impressionnantes et fait de la LCxLC une technique particulièrement attractive pour l'analyse d'échantillons complexes. Cependant, en pratique, cette multiplication est corrigée par plusieurs facteurs dont les valeurs sont comprises entre 0 et 1. Ces facteurs correctifs sont au nombre de trois, et permettent de prendre en compte (1) le taux d'occupation de l'espace de séparation par les composés (γ); (2) le sous échantillonnage de la première dimension (α); (3) les effets à l'injection pouvant être observés lors du transfert des fractions de la ¹D vers la ²D (β). L'optimisation des paramètres α et β se fait généralement lors du dimensionnement de l'analyse [1,2], tandis que γ reflète l'orthogonalité entre les deux dimensions d'un système 2D-LC et dépend essentiellement du choix des conditions phase stationnaire/phase mobile dans chaque dimension.

Dans ce chapitre nous nous sommes intéressés à ce dernier facteur, avec comme objectif de trouver un set de quelques systèmes LCxLC génériques qui permettrait l'analyse d'échantillons pharmaceutiques variés. Pour cela, nous avons utilisé une méthodologie développée au laboratoire [3], qui tient compte à la fois du degré d'orthogonalité (O_d) et de la capacité de pic pratique (n_{2D} *) générés par un système bidimensionnel.

La première étape consiste à acquérir un grand nombre de données, en testant différentes conditions chromatographiques, puis à étudier le potentiel des différentes combinaisons possibles par le biais du calcul de O_d et n_{2D}^* . Cette étude a été faite sur deux échantillons différents, et les résultats ont été comparés aux conclusions tirées lors de l'étude initiale [3]. Enfin, les systèmes LCxLC sélectionnés ont été appliqués à l'analyse des deux échantillons utilisés pour l'étude. Afin de pouvoir plus facilement interpréter les résultats et comparer les systèmes LCxLC, le principe actif et les impuretés sont présents à la même concentration dans les échantillons. Des proportions plus réalistes, à savoir le principe actif majoritaire et les impuretés présentes à de faibles niveaux de concentrations, seront utilisées dans le Chapitre 4 qui a pour objectif d'évaluer le potentiel de la LCxLC pour des analyses quantitatives.

Article 2

"Comprehensive two dimensional liquid chromatography as analytical strategy for pharmaceutical analysis"

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Comprehensive two dimensional liquid chromatography as analytical strategy for pharmaceutical analysis



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ABSTRACT

Comprehensive on-line two-dimensional liquid chromatography (LCxLC) is expected to generate impressive peak capacities, which makes it a method of choice for the analysis of complex samples such as pharmaceuticals. A comparative study of different sets of chromatographic conditions including stationary phase, pH additive and organic modifier was carried out with two real pharmaceutical samples in order to find out the best analytical conditions for implementation of one or several generic on-line LCxLC separations. Our choice was based on the evaluation of both degree of orthogonality and practical sample peak capacity under linear gradient conditions. The potential of 190 combinations of chromatographic systems was compared. A set of 3 RPLCxRPLC configurations was found to be very attractive for both samples and in good agreement with the findings of a previous study carried out with 17 model compounds, thereby supporting the idea of using generic LCxLC conditions in the pharmaceutical area. The three selected 2D-systems were implemented for the on-line RPLCxRPLC-UV/MS analysis of two pharmaceutical samples. It was shown, for each sample, that these 2D-systems were able to generate an effective peak capacity close to 1000 in less than 50 min. For each sample, baseline separation was obtained for every known compound and furthermore a large number of unknown impurities could also be separated and identified. Finally, in the proposed conditions, the total number of compounds detected was significantly improved from one RPLC separation to one RPLCxRPLC separation. Only a small additional gain was observed by performing a second RPLCxRPLC separation or even a third one.

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1. Introduction

For pharmaceuticals, safeness and efficacy are mandatory. This naturally places high demands on drug development to consider these guarantees of quality. The safeness of a drug product is dependent not only on the toxicological properties of the active substance itself, but also on the impurities and degradation products that it contains. Thus, according to International Conference on Harmonization (ICH) guidelines, it is advisable to consider 0.05% as reporting limit, 0.1% as identification limit and 0.15% as quantification limit for impurity control of both drug substances and drug products [1,2]. The specificity is also of prime importance. Identification methods have therefore to be able to discriminate between compounds having similar structures. The coupling of High Performance Liquid Chromatography (HPLC) or Ultra High Performance

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https://doi.org/10.1016/j.chroma.2017.08.070 0021-9673/© 2017 Elsevier B.V. All rights reserved. Liquid Chromatography (UHPLC) with different detection techniques (DAD,MS,...) has been proved to be a technique of choice for both impurity analysis [3,4], and pharmacokinetic or metabolism studies [5,6]. Indeed, new efficient RPLC columns with more stable packing materials are continuously introduced by manufacturers. Such columns can withstand extreme chromatographic conditions (i.e. large pH and temperature ranges and/or fully aqueous mobile phases). However, a unique separation is often insufficient to be sure that all impurities, whose number is unknown and whose structures are usually similar to that of the active substance, were identified. Thus, ICH guidelines [7] recommends the use of several different chromatographic techniques, offering different retention mechanisms. Indeed, additional data obtained from different separations can be very useful to enhance the separation power provided that the separation conditions are orthogonal enough [8]. However this approach results in long development time and additional costs. Actually a set of generic 1D-LC conditions able to deal with a large amount of analytical problems associated with drug development has not been established so far.

196

Two-dimensional liquid chromatography (2D-LC) can be helpful to solve these analytical problems. This technique has been used for several years in pharmaceutical analysis for various purposes: (i) increasing the separation power within an acceptable analysis time [9], (ii) enhancing the specificity of the method in the early phase of drug development [10], or (iii) reducing matrix effects such as ion suppression in LC–MS analysis [11,12]. However, most 2D-separations, in the pharmaceutical area, were developed in the heart-cutting mode (LC-LC) whereas on-line comprehensive two-dimensional liquid chromatography (LCxLC) has received very little attention. Yet, LCxLC can be very attractive, due to its large separation power. Furthermore this technique could be very useful to support degradation studies by comparing resulting 2Dcontour-plots obtained for the same sample before and after forced degradation, as illustrated in some recent studies [13–15].

The main challenge when developing LCxLC methods is to find orthogonal conditions between both dimensions. Whereas there is no controversy about the concept of full orthogonality, quite different approaches were proposed to assess the degree of orthogonality which results in distinct ways to consider orthogonality. A certain degree of orthogonality is achieved as soon as there are some differences in selectivity between both dimensions. The problem is to define a suitable tool to quantify the difference in selectivity between two LC conditions, in order to compare several possibilities and combinations for 2D-LC development. Different methods to evaluate the degree of orthogonality can be found in the literature. These methods can be divided into two different categories. First category corresponds to methods which consider retention data only (thermodynamic approach), including linear regression of retention data as well as more complex chemometric methods [16–18], but needing visual information of retention data to avoid ambiguous conclusion resulting, for example, from clustered data. The second category includes methods which also take into account peak band broadening (thermodynamic and kinetic approach). Those are generally based on a geometric approach [19–21]. They consist in assessing the two dimensional space occupied by peaks in the retention diagram. However they require a large number of representative compounds, which make their implementation not straightforward. Recently, an alternative method was proposed for selecting LCxLC conditions from a limited number of representative compounds [22]. The potential of various possible LCxLC configurations was evaluated, based on the use of two descriptors, the degree of orthogonality and the practical sample peak capacity. The practical sample peak capacity, n^{*}_{2D}, was used to assess the separation power of a given LCxLC configuration. Based on the assumption that the degree of orthogonality must reflect the extent to which the selectivity between two given LC conditions is different, a new descriptor, Od, based on the gradient theory, was proposed to assess the potential change in selectivity between two LC chromatographic systems that can be combined [22]. Both descriptors were used to evaluate various combinations of RPLC and HILIC systems with the aim of separating charged compounds (pharmaceuticals or peptides) in LCxLC. The most promising RPLCxRPLC and RPLCxHILIC combinations that were found for peptides, were further compared in the case of the separation of a tryptic digest of proteins [23]. More recently, the optimization of 2D parameters for the on-line RPLCxRPLC separation of peptides, permitted to achieve in 30 min a 3-fold higher effective peak capacity (i.e. 900) than the one obtained in optimized RPLC conditions [24]. This approach was also successfully applied to the selection of a RPLCxRPLC configuration for the separation of complex samples containing neutral compounds such as bio-oils [25].

The objective of this work was to find one or more generic 2Dsystems able to significantly improve the knowledge on impurities in the course of drug development while keeping acceptable analysis times. The search for 2D-systems was carried out by applying the above approach on two real pharmaceutical samples, both originated from the early stages of drug development. The first part of this work was dedicated to the orthogonality study, considering a set of several LC-systems. Both the degree of orthogonality and the practical peak capacity were considered for the choice of the RPLCxRPLC configurations. The obtained results were compared to those previously obtained on a sample of representative compounds. In the second part, three RPLCxRPLC configurations were selected according to appropriate considerations and hyphenated to a simple quad mass spectrometer (RPLCxRPLC-UV/MS) for impurity identification in the two real samples. Finally this 2D-approach is compared to the usual LC-approach and an analysis strategy is proposed for drug development.

2. Material and methods

2.1. Chemical and samples preparation

Organic solvents (acetonitrile and methanol) were LC-MS grade from Sigma-Aldrich (Steinheim, Germany). Water was obtained from an Elga Purelab Classic UV purification system (Veolia water STI, Le Plessis Robinson, France). Various additives were added in mobile phase: formic acid (0.1% - pH 2.7 measured in aqueous phase), methanesulfonic acid (MSA) (0.1% – pH 2.0 measured in aqueous phase), ammonium acetate (AA) (5 mM in aqueous phase – pH 6.8), ammonium hydroxide (0.1% – pH 10.4 measured in aqueous phase) and ammonium formate (5 mM in aqueous phase) adjusted with formic acid (FA) at pH 3.5. All these additives were obtained from Sigma-Aldrich (Steinheim, Germany). Aqueous eluents prepared from salts were filtered on 0.22 µm nylon filter before use. This study was performed with real pharmaceutical samples provided by Oril Industrie (Servier laboratories, Bolbec, France). Due to confidentiality agreement, the molecular structures cannot be provided. Both samples (Sample #1 and Sample #2) consisted in a mixture of active pharmaceutical ingredient (API) and related synthesis intermediates. Solutions were prepared every month at a concentration of 0.1 mg/mL in water/acetonitrile (50/50 v/v) and (20/80 v/v) for Sample #1 and Sample #2 respectively. Solutions were stored at 4 °C. Sample stability was checked every week with Oril quality control method.

2.2. Instruments

The preliminary screening of LC conditions was carried out with an IClass liquid chromatographic system hyphenated to a QDA mass detector, both from Waters (Milford, United States). I-class instrument includes a high-pressure binary solvent pump, an autosampler with a flow-through needle of $15 \,\mu$ L, a column manager composed of two independent column ovens with a maximum temperature of 90 °C, and a diode array detector, equipped with 500 nL flow-cell withstanding pressure up to 70 bar. The measured dwell volume was $110 \,\mu$ L. The measured extra-volume was $12 \,\mu$ L. Detector wavelength was set at 210 nm, with an acquisition rate of 40 Hz. Data acquisition and instrument control were performed with Masslynx software (Waters).

LCxLC separations were performed with an Infinity 1290 2D-LC system from Agilent Technologies (Waldbronn, Germany). This instrument includes two high-pressure binary solvent delivery pumps, an autosampler with a flow-through needle of 20 μ L, a column oven with a maximum temperature of 100 °C, a UV detector and a diode array detector equipped with 2 μ L and 0.6 μ L flow-cells respectively. A 2-position/4-port duo valve was used as interface between the two dimensions, and was equipped with two 20 μ L sampling loops. Dwell volumes, extra-column volumes and extracolumn variances were determined using a zero dead volume union

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connector in place of the column. The measured dwell volumes were 140 μ L in the first and 65 μ L in the second dimension (measured without the interface between both dimensions). A total extra-column volume of 22 μL and 8.5 μL and an extra-column variance of $12 \,\mu L^2$ and $4.5 \,\mu L^2$ were determined in first and second dimension respectively. Detector wavelengths were set at 210 nm. The acquisition rates were set at 10Hz and 80Hz in the first and second dimension respectively. Data acquisition and instrument control were performed by OpenLab software (Agilent). LCxLC data were processed using Excel sheet and Matlab V7.12.0635.

In addition to UV, both LC and LCxLC systems were hyphenated to a QDA mass spectrometry detector from Waters (Milford, United States), working in scan mode with an acquisition rate of 20 Hz.

2.3. Columns and gradient conditions

Various columns and mobile phases were investigated for preliminary screening. Column characteristics, and operating conditions can be found in Table 1. All experiments were performed under linear gradient conditions from 5% B to 95% B in RPLC (A: water + additive and B: organic solvent + additive) and from 2% B to 35% B in HILIC (A: Acetonitrile and B: water + additive). Five column volumes were used for column equilibration in initial gradient conditions. The injected volume represented 0.5% of the column dead volume. Flow-rates were close to optimum values and hence dependent on both column dimensions and temperature. An initial isocratic step was programmed. Its duration, tini, was adjusted so that the ratio $(t_D + t_{ini})/t_0$ (t_D and t_0 being the dwell time and the column dead time, respectively) was kept constant for all columns conditions. Other operating conditions are given in Table 1.

2.4. Calculations

The volume fraction of strong eluent at elution, φ_e , for a given solute was calculated using the following relationship:

$$\phi_e = \phi_i + s \times \left(\frac{t_R}{t_0} - \frac{t_D + t_{ini}}{t_0} - 1\right) \tag{1}$$

Where t_R , t_D , t_{ini} and t_0 are the solute retention time, the system dwell time, the initial isocratic step duration and the column dead time respectively. φ_i is the initial composition and s is the normalized gradient slope given by

$$s = (\phi_f - \phi_i) \times \frac{t_0}{t_G}$$
⁽²⁾

with $\phi_{f},$ the final composition and $t_{G},$ the gradient time.

In this work, we used the Linear Solvent Strength Theory (LSST) [26,27] where the solute retention (log(k)) is assumed to be a linear function of the volume fraction of the strong eluent " ϕ ":

$$\log(k) = \log(k_0) - S\phi \tag{3}$$

k₀, being the solute retention factor in the weak solvent and S, the solvent strength parameter. For each chromatographic system, two gradient runs with two different normalized gradient slopes, s (i.e. s = 0.02 and s = 0.06) were performed for both samples. The normalized gradient slope was kept constant by changing the gradient time when the column dimension and hence the column dead time changed. The two gradient data series allowed to determine S and $log(k_0)$ values for each compound and then, the average S value for a given chromatographic system. This was done by means of a modeling software (OSIRIS 4.2, Datalys, Grenoble, France) [28].

Experimental sample peak capacities were calculated according

to (4)

$$n = 1 + (t_n - t_1) / w$$

| Lharacteristics of the station. | iary pnases and of | perating conditions. | | | | | | | | |
|---------------------------------|--------------------|----------------------|---|----------------------------------|----------------------------------|-----------------------|--------|--------------------|---------------------------|------------------------------|
| Column name | Abbreviation | Manufacturer | Stationary phase chemistry | d _c ^a (mm) | L _c ^b (cm) | d _p c (μm) | T (°C) | Flow-rate (mL/min) | pH additives ^d | Organic solvent ^d |
| Acquity CSH C18 | CSH C18 | Waters | Octadecyl (silica) | 2.1 | 10 | 1.7 | 40 | 0.4 | MSA, FA, AA | ACN |
| Acquity CSH PFP | PFP | Waters | Pentafluorophenyl (silica) | 2.1 | 5 | 1.7 | 40 | 0.4 | FA, AA | ACN, MeOH |
| Acquity BEH C18 | BEH C18 | Waters | Octadecyl silica (silica) | 2.1 | 5 | 1.7 | 40 | 0.4 | AA, NH4OH | ACN |
| Discovery Zr-Carbon C18 | Zr Carb | Supelco | Octadecyl-modified carbon-clad (zirconia) | 4.6 | 5 | c | 06 | 2 | FA, AA | ACN |
| Acquity BEH Shield RP18 | Shield | Waters | Polar embedded-octadecyl (silica) | 2.1 | 5 | 1.7 | 40 | 0.4 | FA, AA | ACN |
| Zorbax SB CN | CN | Agilent | Propyl cyano (silica) | 4.6 | 150 | 5 | 40 | 1 | FA | ACN |
| Hypercarb | Hypercarb | Thermo Fischer | Porous graphitic carbon | 1 | 5 | c | 06 | 0.3 | FA, AA | ACN, ACN/THF |
| Acquity BEH HILIC | HILIC | Waters | Unbonded silica | 2.1 | 5 | 1.7 | 40 | 0.4 | AA, FA+AF | ACN |
| Acquity BEH Phenyl | Phenyl | Waters | Phenyl hexyl (silica) | 2.1 | 5 | 1.7 | 40 | 0.4 | FA, AA, NH₄OH | ACN, MeOH |
| | | | | | | | | | | |

Column internal diameter

Table 1

Column length.

Particle diameter.

MSA, methanesulfonic acid; FA, formic acid; AA, ammonium acetate; NH40H, ammonia; AF, ammonium formate; ACN, acetonitrile; MeOH, methanol; THF, tetrahydrofurane

With t_n and t_1 being the retention times of the last and the first eluted compounds respectively and w, the average 4σ peak width (13.4% of peak height). Sample peak capacities were normalized to a 5 cm column length and a 1.7 µm particle size for more suitable comparison of the different chromatographic systems (except for the zirconia-based column which did not exist in this particle format).This was done by multiplying the obtained peak capacities

by $\sqrt{\frac{5dp}{1.7L}}$, dp being the particle size expressed in μ m and, L, the column length expressed in cm.

The separation power of each LCxLC configuration was evaluated according to a procedure previously reported [22]. This procedure involves two descriptors: the degree of orthogonality, O_d and the practical sample peak capacity, n^*_{2D} . Both were calculated, for each combination of two LC chromatographic systems, using the following Eqs. (5) and (6): The separation power of each LCxLC configuration was evaluated according to a procedure previously reported [22]. This procedure involves two descriptors: the degree of orthogonality, O_d and the practical sample peak capacity, n^*_{2D} . Both were calculated, for each combination of two LC chromatographic systems, using the following Eqs. (5) and (6):

$$O_{d} = \gamma \times {}^{1}S \times {}^{2}S \times \Delta^{1}\phi_{e} \times \Delta^{2}\phi_{e} \tag{5}$$

Where the superscript stands for the dimension number. γ is the correction factor corresponding to the retention space coverage (ratio of the practical to the theoretical retention area). A schematic representation of the retention space coverage is given in the supplementary material (Fig.S1). Whereas the theoretical sample peak capacity of a 2D-system is the product of sample peak capacities in each dimension ($n_{2D,theoretical} = {}^{1}n^{2}n$), the practical sample peak capacity is given by

$$n_{2D}^* = {}^1 n^2 n^* \tag{6}$$

where ${}^{2}n^{*}$ represents the practical sample peak capacity in the second dimension. It is given by

$${}^{2}n^{*} = 1 + \gamma({}^{2}n - 1) \tag{7}$$

1D-data were exported to an Excel sheet in order to calculate the degree of orthogonality and the practical peak capacity using calculation tools developed under Excel 2010. 2D-data were exported to a Microsoft Excel sheet to construct a matrix and were further processed with Matlab V7.12.0635 for the construction of 2D-contour plots.

The conditions of LCxLC separations (presented in Table 3) were optimized using a home-made procedure aiming, for a given analysis time, at finding the best compromise between two criteria: higher peak capacity and lower dilution [24,29]. This calculation method, developed under Microsoft Excel, consists in a three input level procedure based on both theoretical relationships and Pareto-optimality approach.

The effective sample peak capacity, $n_{2D,effective}$, was evaluated, for each 2D-separation, from experimental chromatograms according to the following equation

$$n_{2D,effective} = {}^{1}n.\alpha.(1-\gamma) + {}^{1}n.{}^{2}n.\alpha.\gamma$$
(8)

where α is the undersampling correction factor, introduced by Davis et al. [30]. It was calculated from the sampling time, ts and the average peak width, according to

$$\alpha = \frac{1}{\sqrt{1 + 0.21(4t_{\rm s}/w_{4\sigma})^2}} \tag{9}$$

¹n and ²n were calculated according to Eq. (4) with ¹w and ²w measured in ¹D and ²D respectively. γ was graphically measured in the same way as in Fig.S1 by replacing compositions at elution by retention times.

3. Results and discussion

The first part of this study concerns the search for two or more 2D-systems that may each provide a large separation power and furthermore that are sufficiently different from each other to provide complementary information. The search was carried out with two real samples, both originating from the early stages of drug development. The second part is devoted to the evaluation of the selected 2D-systems by way of on-line LCxLC-UV/MS analysis.

3.1. Search for 2D-systems

A large variety of stationary phases and pH additives was studied. The resulting different conditions are reported in Table 1. Most stationary phases were silica-based. However the possibility of using non-silica based stationary phases in RPLC (i.e. porous graphitic carbon and zirconia based columns) at high temperature was also investigated. Acetonitrile was mainly used but Methanol was also tested as solvent B with Acquity CSH PFP and Acquity BEH Phenyl in order to assess the effect of the organic modifier. When mass spectrometry detection was not possible due to non MS-compatible additives (e.g. MSA additive), solutes were injected separately for unambiguous peak tracking.

A first real sample (Sample #1) containing API, synthesis intermediates and related unknown impurities was analyzed with all investigated LC-systems under linear gradient conditions and a normalized gradient slope of 0.06. Some LC-systems were rapidly abandoned due to either an excess or an absence of retention. The Hypercarb column was too retentive for most components. Even with ACN/THF (80/20, v/v) as strong solvent, 3 compounds over 9 did not elute during the gradient step but during the final isocratic step. Conversely, in HILIC, almost all compounds were eluted in the dead volume. As a result, these two columns were not taken into account for the rest of this study. The compositions at elution for each solute as well as the sample peak capacity were calculated for the 20 remaining RPLC-systems according to Eqs. 1 and 4. A second gradient run with a different normalized gradient slope (i.e. 0.02) made it possible to determine average S value for each RPLC-system. The obtained values are listed in Table 2. They are in the order 4–5 with all stationary phases except with Discovery Zr-Carbon C18 for which S values were close to 3. For all studied stationary phases S values appear to be affected to a very limited extent by pH additive and/or organic modifier.

The 20 selected LC conditions led to 190 possible LCxLC configurations. The potential of these combinations was compared by means of two descriptors, the degree of orthogonality, O_d (Eq. (5)) and the practical sample peak capacity, n_{2D}^{*} (Eq. (6)) [22]. Average S values were of prime importance because they have a direct impact on O_d as shown in Eq. (5), high S values leading to high Od values. For sake of consistency, both descriptors were calculated for all LC-conditions from gradient data obtained with the same normalized gradient slope of 0.06. It was previously shown that these descriptors were fully complementary [22], O_d being related to the variation of the selectivity between the first and the second RP-system, and n^{*}_{2D} representing the overall separation power of the studied 2D-system. Calculated O_d and n^{*}_{2D} values are listed in the supplementary material (Table S1) for each possible 2D-system. Fig. 1 gives a representation of n_{2D}^* as a function of O_d for the 190 possible 2D-systems obtained with Sample #1 (blue triangle). Each spot is related to a given 2D-system. In case of Sample #1, Od varies from 0.5 to 10 while n_{2D}^* varies from 500 to 12000. The low value for the coefficient of determination (i.e. <0.5) supports the idea that both descriptors can be considered as complementary. As highlighted in Fig. 1, some data are located along the best-fit line while others deviate significantly from it. These latter can be divided into 3 different groups circled by different colors in Fig. 1. Group A

M. Iguiniz et al. / J. Chromatogr. A 1536 (2018) 195-204

Table 2

S values determined from two gradient runs with two different slopes. Calculated with Osiris software. Abbreviations are given in the text.

| Stationary phase | Mobile phas | e conditions | | | | | |
|-------------------------|--------------|--------------|-----|--------------------|----------|-----|--------------------|
| | Acetonitrile | | | | Methanol | | |
| | MSA | FA | AA | NH ₄ OH | FA | AA | NH ₄ OH |
| Acquity CSH C18 | 4.4 | 4.1 | 5.4 | па | na | na | na |
| Acquity CSH PFP | na | 4.7 | 4.9 | na | 4.7 | 4.3 | na |
| Acquity BEH C18 | na | na | 4.6 | 4.4 | па | па | па |
| Discovery Zr-Carbon C18 | na | 3.2 | 3.0 | na | па | па | па |
| Acquity BEH Shield RP18 | па | 4.4 | 4.4 | na | па | па | па |
| Xselect HSS CN | па | 3.0 | na | na | па | па | па |
| Acquity BEH Phenyl | na | 4.9 | 4.9 | 5.0 | 4.7 | 4.7 | 4.8 |

na: not available.

Table 3

Optimized RPLCxRPLC conditions for the 3 selected 2D-systems. Conditions in ²D differ only by a slight difference in the gradient time. 2D-systems #24, #25 and #36 are listed in Table S1.

| | ¹ D | | | ² D |
|------------------------|-----------------------------------|----------------------------|------------------------------|--|
| | 2D-System #24 | 2D-System #25 | 2D-System #36 | |
| Stationary phase | Xselect HSS PFP | Xbridge BEH C18 | Acquity BEH Phenyl | Acquity CSH C18 |
| Column geometry | $50mm \times 2,1mm$, 3,5 μm | $50mm \times 2,1mm,5\mu m$ | $50mm \times 2,1mm,1,7\mu m$ | $30mm \times 2,1mm,1,7\mu m$ |
| Mobile phase | A: Water + AA 5 mM | A: Water + 0,1% NH4OH | A: Water + 0,1% NH4OH | A: Water + 0,1% FA |
| | B: MeOH | B: ACN + 0,1% NH4OH | B: ACN + 0,1% NH4OH | B: ACN + 0,1% FA |
| Flow-rate | 0,1 mL/min | 0,1 mL/min | 0,04 mL/min | 1,8 mL/min |
| Gradient conditions | 27–92% (B) in 60 min | 15–100% (B) in 60 min | 15–100% (B) in 60 min | 1-86% (B) in 0,17 min (S #24); 0,15 min (S #25) and 0,18 min (S #36) |
| Sampling time | 0.32 min | 0.32 min | 0.33 min | |
| Split ratio | 1:3 | 1:3 | 1:3 | |
| Temperature | 30 °C | 30 °C | 30°C | 80 ° C |
| Injected | 2 μL | 2 μL | 2 μL | 11 μL (S #24); 10 μL (S #25); |
| Volume | | | | 4.4 μL (S #36) |
| UV | 210 nm | 210 nm | 210 nm | 210 nm |
| MS | - | - | - | Scan (150–500) |



Fig. 1. Plots of the practical peak capacity in 2D versus the degree of orthogonality for 190 2D-systems tested with Sample #1 (values given in table S2). Orange squares correspond to 2D-systems also tested with Sample #2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

is located below the straight line. It includes 2DLC-systems that provide a very high degree of orthogonality (>7) but a rather low practical peak capacity (<4000). Such 2D-systems present a large difference in selectivity between the two LC-systems. However at least one stationary phase provides poor column efficiency, typically a non-silica based column such as Discovery Zr-Carbon C18 or a silica grafted to a pentafluorophenyl group. Carbon phases are known to be significantly different from silica-based ones in term of selectivity, but the difference seems to be enhanced with Discovery Zr-Carbon C18 in acidic mobile phase conditions. The above observations are in very good agreement with previous ones obtained with a set of representative charged compounds [22]. Group B includes 2D-systems offering high practical peak capacity (>5000) in spite of rather low degree of orthogonality (<7). It concerns 2Dsystems with two different highly efficient silica-based columns, typically Acquity BEH phenyl in the first dimension and Acquity CSH C18 in the second dimension working at two different pH (acidic and neutral or neutral and basic). Finally Group C includes the most attractive 2D-configurations offering a high degree of orthogonality (close to 9) and a very high practical peak capacity (in the range 7000-12000). All these configurations concern a large difference in pH between both mobile phases (ammonia in the first dimension and either formic acid or Methane sulfonic acid in the second dimension) and highly efficient columns in both dimensions (typically Acquity BEH phenyl in the first dimension and Acquity CSH C18 in the second one). 2D-systems with ammonia in one dimension lead to high O_d values, probably due to the high retention of basic compounds, mainly present in drug mixtures, thereby leading to a large range of compositions at elution.

The results obtained with Sample #1 were compared with those obtained with a second real sample, Sample #2, quite different from the former in terms of both molecular structures and drug development. The study was carried out with a reduced set of 10 RPLC-systems, carefully selected so that the corresponding data for Sample #1 were spread over the graph in Fig. 1. Since both n^*_{2D} and O_d values are expected to be dependent on the sample, normalized values instead of absolute ones were considered to evaluate the reliability of this approach. The results are shown in Fig. 2. A small difference in O_d can be observed between the two samples for 2D-



Fig. 2. Comparison of (a) normalized O_d values and (b) normalized n_{2D}^* values between Sample #1 (blue) and Sample #2 (red) for 10 possible LCxLC configurations identified by their number on X-axis (see Table S1 for the corresponding LCxLC configurations). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

systems #102, #73, #100. This can be easily explained by the nature of the compounds in Sample #1 which contains halogens which may strongly interact with the Acquity CSH PFP stationary phase due to its pentafluorophenyl groups, as already mentioned [31,32]. That results for this sample in an additional change in selectivity between this stationary phase and another one. Apart from this, it is very interesting to notice that, for both descriptors, the trend for Sample #1 is very similar to that for Sample #2, suggesting that a suitable selection of 2D-systems for a given sample is expected to be applicable to other pharmaceutical samples. This very good news is also supported by the fact that the present evaluation of 2D-systems from two real samples is in good agreement with the conclusions that were drawn from a previous study on 17 representative compounds [22]. The common conclusions of those studies are: (i) 2D-systems offering a high degree of orthogonality may lead to a low practical peak capacity and reciprocally some 2D-systems are attractive in term of peak capacity but not attractive in term of orthogonality. For example, using ammonium acetate in the first dimension and formic acid in the second is not very advantageous in term of orthogonality but attractive in term of peak capacity (ii) considering the studied conditions, the best 2D-system for pharmaceutical compounds corresponds to a first dimension with Acquity BEH Phenyl and ammonia as pH additive and a second dimension with Acquity CSH C18 and formic acid as pH additive (i.e. 2D-system #36 in Table S1). This first study allowed us to select 3 RPLCxRPLC configurations out of 190 possible candidates with a view to provide 3 quite different RPLCxRPLC separations. Our choice was driven by the following requirements: (i) the first selected LCxLC configuration had to provide a very high practical peak capacity, and hence its corresponding spot had be located in Group C of Fig. 1 (i.e. possible combinations #18, #36, #53 or #144 in Table S1); (ii) the two other selected LCxLC configurations had to provide a sufficient degree of orthogonality (i.e. >6) between first and second dimensions. Indeed whereas the practical peak capacity is a key descriptor for complex samples, a high degree of orthogonality (i.e. a large change in selectivity between both dimensions) is of prime importance for pharmaceutical samples to ensure a greater chance of separating

all impurities; (iii) the second dimension column had to withstand high temperatures (e.g. 80 °C), a high temperature being a prerequisite for very fast separation. As a result, ammonia as pH additive and PFP column should be used in the first dimension only; (iv) the second dimension conditions had to be MS-compatible making the use of MSA impossible and (v) the same second dimension for the 3 LCxLC configurations was required in order to simplify peak tracking and hence to better assist MS in solute identification. With this requirements in mind, the final objective was to find ¹D configurations providing sufficient degree of orthogonality between themselves.

In view of all these considerations and of the results presented in Table S1, our three selected LCxLC configurations were those reported as #36, #25 and #24 in Table S1. For all of them, the second dimension consisted in an Acquity CSH C18 column performed at 80 °C, with formic acid as pH additive and acetonitrile as organic modifier. The relevance of this selection was established by comparing their first dimensions two by two, considering the degree of orthogonality obtained for each combination. As shown in Table S1, O_d values of 8.0, 8.8 and 3.3 were respectively found for the three resulting 2D-combinations, (#100, #111 and #123) which was considered as in line with our objectives despite the somewhat low third value.

3.2. Application to LCxLC-UV/MS analysis

The operating conditions for the selected LCxLC configurations were optimized according to a procedure previously developed for RPLC x RPLC separations [23,24]. The optimized conditions included the sampling rate, the flow-rates in both dimensions, the gradient conditions in ²D and the split ratio between ¹D and ²D. Some conditions were fixed prior to optimizing. They include ¹D gradient time which was fixed at 60 min, column dimensions and column temperatures in both dimensions. These conditions were applied to on-line RPLCxRPLC-UV/MS analysis of two real pharmaceutical samples (Sample #1 and Sample #2). Operating conditions for the three selected 2D-systems are given in Table 3.

The different steps leading to the identification of peaks obtained in the second dimension are reported in Fig. 3 for the particular case of Sample #1 separated with LCxLC #25. The first step consisted in tracking known analytes (API and synthesis intermediates) on UV- and MS-2D-chromatograms (Fig. 3a). For this purpose, we used the Extracted Ion Chromatograms (EIC) corresponding to the known mass over charge ratio (m/z). EIC data were then summed, in order to obtain a full EIC contour-plot. The comparison of both UV and TIC contour plots with the full EIC contour plot, made it possible to affect retention times to known compounds (see blue spots in Fig. 3a) and then to locate unknown impurities in UV and MS contour plots. Fig. 3b displays several overlaid ²D chromatograms, each corresponding to ¹D fractions containing impurities of interest. Unknown 2D-peaks, present inside the coloured area in Fig. 3a, are clearly visible in Fig. 3b and could be further identified by extracting MS spectra (Fig. 3c). It is important to highlight the complementarity of both detection modes (UV and MS) which permitted to detect in one mode some compounds that could not be detected in the other one. Finally, once an impurity was located in the first LCxLC contour plot and its m/z value was determined, EIC chromatograms could be processed from other LCxLC data in order to advance in the knowledge of all detected impurities. Such a procedure allowed to monitor impurities from one set of RPLCxRPLC conditions to another one, which was helpful for comparing several 2D-separations of the same sample. Fig. 4 shows the separations of Sample #1 with the three selected configurations. As can be observed, the peak distribution varies depending on the selected configuration, highlighting the relevance of this selection. Due to the basic properties of most components, the change in both



Fig. 3. Successive steps leading to the identification of impurities by RPLC x RPLC-UV/MS. (a) First step: Tracking known compounds in both UV-contour plot and TIC-contour plot and comparison with EIC-contour plot, (b) second step: locating unknown impurities; example of overlaid ²D TIC chromatograms of ¹D fractions corresponding to the green area, and (c) third step: extracting corresponding MS spectra for identification of unknown impurities. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4

Experimental results obtained from 3 optimized RPLCxRPLC configurations (2D-systems #24, #25 and #36 listed in Table S1) for the separation of two real pharmaceutical samples (Sample #1 and #2). Experimental conditions given in Table 3.

| 2D-System | Sample #1 | | | | Sample #2 | 2 | | |
|-----------|-----------|------|-----------------------------|---------------------------|-----------|------|-----------------------------|---------------------------|
| | γ | α | n _{2D,theoretical} | n _{2D,effective} | γ | α | n _{2D,theoretical} | n _{2D,effective} |
| #24 | 0.66 | 0.73 | 1797 | 891 | 0.64 | 0.66 | 2736 | 1176 |
| #25 | 0.66 | 0.64 | 2033 | 893 | 0.61 | 0.64 | 2153 | 878 |
| #36 | 0.66 | 0.7 | 1719 | 824 | 0.66 | 0.7 | 1734 | 822 |

mobile phase pH and stationary phase chemistry between the two dimensions led to a large retention space coverage. The retention surface, delimited by dashed lines, is very similar for the three 2Dsystems which is confirmed by the same γ value of 0.66 (Table 4). As expected, the retention surface is fully diagonalized and no cluster can be observed, thereby validating the present approach for γ calculation. The effective peak capacities are reported in Table 4. It is very interesting to note that for the three LCxLC conditions, the effective peak capacity was close to 900 while the analysis time was shorter than 50 min. Some peaks were detected either in UV or in MS which shows the complementarity of both detection modes. An example pointing out the advantage of using both detections, is given in Fig. 5, showing overlaid ²D separations of ¹D fractions obtained within a period of 3 min (coloured areas in Fig. 4a and b). The comparison of UV (Fig. 5a) and MS (Fig. 5b) chromatograms shows that two compounds (circled in red in Fig. 5b) were only detected in MS. The first peak was not detected in UV because not retained and hence hidden by unretained compounds present in the injection solvent. The second one was likely to be not concentrated enough for UV detection while correctly detected in MS. Another interesting point highlighted in Fig. 5 by the overlay of the successive separations, is the high repeatability in ²D, absolutely necessary for the purpose of impurity identification.

Similar conclusions were drawn from the results obtained with Sample #2 and the 3 selected systems (chromatograms given in the supplementary material in Fig.S2 and S3). The peak distribution seems to be a little more diagonalized compared to that of Sample #1 which can be explained by the fact that this sample contained fewer compounds with basic properties. As a result, unlike with Sample #1 for which pH was a key parameter, the change in stationary phase between both dimensions, played the predominant role with Sample #2, by varying selectivity and hence maximizing the retention surface coverage. As reported in Table 4, the use of a Phenyl column in ¹D and a C18 column in ²D (system #36) provides the highest γ value (about 0.7) whereas changing pH while using similar stationary phases in both dimension (Configuration #25) leads to slightly lower γ values (about 0.6). Nevertheless, a value of 0.6 is very attractive considering the fact that the same retention mechanism (i.e. RPLC) was applied in both dimensions. Effective peak capacities in the range 800-1200 were obtained in less than 50 min which is guite impressive compared to those that can be obtained in 1D-LC for the same analysis time. It should be noted that, for both samples, these experimental values are much lower than those predicted in Table S1. This is easily explained by the fact that predicted practical peak capacities were calculated in both dimensions with a normalized gradient slope of 0.05, sub 2 µm particles, 5 cm as column length and under optimum flowrate conditions. However, such ideal conditions cannot be used in on-line LCxLC as shown in Table 3. In addition, both undersampling in ¹D and injection effects in ²D also contribute to lessen peak



Fig. 4. Contour plots of optimized on-line RPLCxRPLC separations of Sample #1 performed with LCxLC configurations (a, b) #24, (c, d) #25 and (e, f) #36. UV detection at 210 nm (a, c, e) and MS-TIC (b, d, f). Dotted lines represent the retention area for the calculation of γ (see Fig.S1 for explanation). Experimental conditions are given in Table 2.

capacity in LCxLC. As a result of this, despite the optimization of 2Dparameters, effective peak capacities are always much lower than practical peak capacities. Consequently, in the research of best 2Dsystems, relative values for practical peak capacity (Fig. 2) are more relevant than absolute ones. As for Sample #1, the observation of overlaid ²D separations of ¹D fractions (Fig.S3) highlights the interest of combining both UV and MS detection modes for the research of unknown impurities. Indeed, some peaks were not detected in UV but detected in MS while others could be detected in UV only. The absence of signal in MS for some impurities present in Sample #2 was further attributed to their specific structure which include nitrile functions, not easily ionizable in the electrospray source.

Finally, by cross-checking the data (i.e. extracted mass spectra and 2D-retention times), obtained in the three 2D-systems, it was possible to know the exact number of impurities detected. It appears, for both samples, that most impurities could be detected with each of the three sets of 2D-conditions, a few of them being detected with one set only, Configuration #36 being the most attractive in this respect. As an example, only one LCxLC configuration (i.e. #36) was sufficient, for Sample #1, to detect 84% of the total number of compounds while an additional LCxLC configuration (i.e. #24) increased this percentage to 97%. For Sample #2, in spite of the difficulty to extract mass spectra of some impurities present at a very low concentration, the results were similar for the remaining identified impurities. It was interesting to assess the contribution of 1D-LC alone. This was done by counting the number of compounds detected in 1D-LC, for Sample #1, underbest 1D-conditions (Fig.S4). These conditions were those used for the study on orthogonality. In all cases, LCxLC configuration #36 outperformed by about 40% the number of compounds detected in 1D-LC as highlighted



Fig. 5. Overlaid ²D separations of ¹D fractions, indicated by blue areas in Fig. 4. (a) UV detection and (b) MS-TIC detection. Circled are detected in MS while not detected in UV. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Fig. 6. Increase in the total number of compounds detected in Sample #1 depending on the type and the number of LC-configurations used. The results for one LC set-up were those obtained with best 1D-LC-UV/MS conditions. Cumulative results for RPLCxRPLC set-ups correspond to (1) configuration # 36, (2) configurations #36 and #24, (3) configurations #36, #24 and #25. See Table 2 for experimental conditions.

in Fig. 6. The additional increase is reduced to 12% and 3% with one and two additional LCxLC separations respectively. From the above results, it can be concluded that one or possibly two of the three proposed RPLCxRPLC configurations could be efficiently used as generic systems for pharmaceutical analysis throughout drug development.

4. Conclusions

The objective of this study was to find suitable on-line LCxLC conditions able to separate pharmaceutical samples with a view to detecting and identifying impurities.

An orthogonality study based on the screening of many different LC conditions was performed with two real pharmaceutical samples. Both the degree of orthogonality and the practical peak capacity were used as descriptors for assessing the separation power of two given LC conditions. The present study leads to similar conclusions as those previously drawn from an experimental study on representative compounds. These conclusions suggest that a few combinations of LC-systems could be appropriate for any pharmaceutical sample. In particular, the use of a phenyl hexyl column with ammonia as pH additive in ¹D and an Acquity CSH C18 column with formic acid in ²D is found to be one of the most promising combinations over the 190 studied. More generally, any combination of a C18 column and a different stationary phase chemistry, associated with a variation in mobile phase pH between both dimensions represent the most attractive 2D-systems for defining generic analytical 2D-conditions. Considering the descriptor values of 190 possible 2D-systems, this study permitted to define three relevant ones to be used in an analytical strategy for drug development. A C18 column with formic acid is recommended as second dimension for the three 2D-systems in order to facilitate peak tracking in 2D-contour plots.

These selected 2D-LC conditions were applied to on-line LCxLC separations of two real pharmaceutical samples. An effective peak capacity close to 1000 in less than 50 min was achieved in all cases, thereby highlighting the relevance of these systems. Thanks to a dual UV/MS detection, a large number of impurities could be detected. Finally it was that one LCxLC-UV/MS system outperforms by 40% the number of compounds detected with one 1D-LC-UV/MS. An additional increase of 12% with a second 2D-system and again 3% with a third one suggest that a good analytical strategy throughout drug development may be to use one or possibly two different sets of LCxLC conditions as those proposed in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.08.070.

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Supplementary information for

Comprehensive two dimensional liquid chromatography as analytical strategy for pharmaceutical analysis

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Figure S1:

Schematic representation of theoretical (hatched rectangle) and practical (delimited by blue straight lines) retention areas for a series of data (red squares), expressed as ${}^{1}\phi_{e}$ vs ${}^{2}\phi_{e}$, obtained from two different chromatographic systems. The practical retention area is delimited by blue straight lines, representing the confidence envelope of the data, related to a confidence level of 95%. $\Delta^{1}\phi_{e}$ and $\Delta^{2}\phi_{e}$ represent the ranges of compositions at elution covered by the components of the sample. They are indicated by double arrows.



Figure S2: Contour plots of optimized on-line RPLCxRPLC separations of Sample #2 performed with (a, b) 2D-system #24, (c, d) 2D-system #25 and (e, f) 2D-system #36. UV at 210nm (a, c, e) and MS-TIC (b, d, f). Dotted lines represent the retention area for the the calculation of γ (see Fig.S1). Experimental conditions given in Table 3.



Figure S3: Overlaid 2D separations of 1D fractions of Sample #2, corresponding to colored areas in (a) Fig. S2.c) with UV detection, and (b) Fig. S2.d) with MS-TIC detection. It should be noted that some peaks in 2D, circled in red, were not detected in UV but detected in MS. Conversely, one peak (circled in purple) was only detected in UV. The absence of signal in MS for some impurities present in Sample #2 was further attributed to their specific structure which include nitrile functions, not easily ionizable in the electrospray source.



Figure S4: Separations of Sample #1 and Sample #2 in the best LC-conditions.

Conditions for Sample #1: Acquity CSH C18 (100x2,1mm, 1,7 μ m); Mobile phases : A= water + 0.1% FA – B= ACN + 0.1% FA ; gradient : 5 to 95% in7min ; 0,4mL/min; 40°C; UV detection 210nm.

Conditions for Sample #2: Acquity CSH C18 (50x2,1mm, $1,7\mu m$); Mobile phases : A= water + 0.1% FA; Solvent B= ACN + 0,1% FA; gradient : 5 to 95% in 3,5min ; 0,4mL/min; 40°C; UV detection 210nm.

Table S2: Separation power (degree of orthogonality and practical sample peak capacity) for 190 possible LCxLC configurations, tested with Sample #1 (see Table 1 for abbreviations). Conditions expressed as "Stationary phase-organic modifier-pH additive".

* LCxLC configurations that were also tested with Sample #2.

** LCxLC configurations present in Group C in Fig.1.

| n° | Possible co | ombinations | Od | n* _{2D} |
|------|----------------|-------------------|-----|------------------|
| 1 | CSHC18-ACN-MSA | CSHC18-ACN-FA | 3.3 | 5303 |
| 2 | CSHC18-ACN-MSA | CSHC18-ACN-AA | 6.8 | 8309 |
| 3 | CSHC18-ACN-MSA | PFP-ACN-FA | 2.5 | 2329 |
| 4 | CSHC18-ACN-MSA | PFP-ACN-AA | 5.9 | 4708 |
| 5 | CSHC18-ACN-MSA | PFP-MeOH-FA | 4.5 | 2899 |
| 6 | CSHC18-ACN-MSA | PFP-MeOH-AA | 7.1 | 4739 |
| 7 | CSHC18-ACN-MSA | BEHC18-ACN-NH4OH | 7.7 | 6856 |
| 8 | CSHC18-ACN-MSA | BEHC18-ACN-AA | 5.6 | 4923 |
| 9 | CSHC18-ACN-MSA | Shield-ACN-FA | 2.6 | 2883 |
| 10 | CSHC18-ACN-MSA | Shield-ACN-AA | 5.6 | 5534 |
| 11 | CSHC18-ACN-MSA | ZrCarb-ACN-FA | 7.9 | 7261 |
| 12 | CSHC18-ACN-MSA | ZrCarb-ACN-AA | 4.3 | 4147 |
| 13 | CSHC18-ACN-MSA | CN-ACN-FA | 1.5 | 2304 |
| 14 | CSHC18-ACN-MSA | Phenyl-ACN-FA | 2.8 | 3127 |
| 15 | CSHC18-ACN-MSA | Phenyl-MeOH-FA | 3.7 | 2874 |
| 16 | CSHC18-ACN-MSA | Phenyl-ACN-AA | 4.7 | 6280 |
| 17 | CSHC18-ACN-MSA | Phenyl-MeOH-AA | 5.2 | 5048 |
| 18** | CSHC18-ACN-MSA | Phenyl-ACN-NH4OH | 9.0 | 12375 |
| 19 | CSHC18-ACN-MSA | Phenyl-MeOH-NH4OH | 6.0 | 6988 |
| 20 | CSHC18-ACN-FA | CSHC18-ACN-AA | 4.3 | 5061 |
| 21 | CSHC18-ACN-FA | PFP-ACN-FA | 2.8 | 2454 |
| 22* | CSHC18-ACN-FA | PFP-ACN-AA | 4.9 | 3710 |
| 23 | CSHC18-ACN-FA | PFP-MeOH-FA | 4.2 | 2522 |
| 24* | CSHC18-ACN-FA | PFP-MeOH-AA | 6.5 | 4105 |
| 25* | CSHC18-ACN-FA | BEHC18-ACN-NH4OH | 7.3 | 6125 |
| 26 | CSHC18-ACN-FA | BEHC18-ACN-AA | 3.5 | 2951 |
| 27* | CSHC18-ACN-FA | Shield-ACN-FA | 1.2 | 1352 |
| 28 | CSHC18-ACN-FA | Shield-ACN-AA | 3.7 | 3481 |
| 29* | CSHC18-ACN-FA | ZrCarb-ACN-FA | 6.7 | 5831 |
| 30 | CSHC18-ACN-FA | ZrCarb-ACN-AA | 4.0 | 3677 |
| 31 | CSHC18-ACN-FA | CN-ACN-FA | 2.0 | 2852 |
| 32 | CSHC18-ACN-FA | Phenyl-ACN-FA | 1.9 | 1996 |
| 33 | CSHC18-ACN-FA | Phenyl-MeOH-FA | 2.6 | 1935 |
| 34 | CSHC18-ACN-FA | Phenyl-ACN-AA | 3.1 | 3917 |
| 35 | CSHC18-ACN-FA | Phenyl-MeOH-AA | 3.9 | 3554 |
| 36** | CSHC18-ACN-FA | Phenyl-ACN-NH4OH | 8.7 | 11224 |
| 37 | CSHC18-ACN-FA | Phenyl-MeOH-NH4OH | 5.7 | 6220 |
| 38 | CSHC18-ACN-AA | PFP-ACN-FA | 6.2 | 4020 |
| 39 | CSHC18-ACN-AA | PFP-ACN-AA | 4.1 | 2382 |
| 40 | CSHC18-ACN-AA | PFP-MeOH-FA | 7.8 | 3543 |
| 41 | CSHC18-ACN-AA | PFP-MeOH-AA | 6.5 | 3136 |
| 42 | CSHC18-ACN-AA | BEHC18-ACN-NH4OH | 7.9 | 5026 |
| 43 | CSHC18-ACN-AA | BEHC18-ACN-AA | 0.6 | 493 |
| 44 | CSHC18-ACN-AA | Shield-ACN-FA | 5.0 | 3836 |

| 45 | CSHC18-ACN-AA | Shield-ACN-AA | 1.1 | 856 |
|------|---------------|-------------------|-----|------|
| 46 | CSHC18-ACN-AA | ZrCarb-ACN-FA | 7.3 | 4785 |
| 47 | CSHC18-ACN-AA | ZrCarb-ACN-AA | 4.1 | 2857 |
| 48 | CSHC18-ACN-AA | CN-ACN-FA | 3.7 | 3921 |
| 49 | CSHC18-ACN-AA | Phenyl-ACN-FA | 3.7 | 2906 |
| 50 | CSHC18-ACN-AA | Phenyl-MeOH-FA | 3.9 | 2169 |
| 51 | CSHC18-ACN-AA | Phenyl-ACN-AA | 1.2 | 1243 |
| 52 | CSHC18-ACN-AA | Phenyl-MeOH-AA | 2.7 | 1893 |
| 53** | CSHC18-ACN-AA | Phenyl-ACN-NH4OH | 9.6 | 9403 |
| 54 | CSHC18-ACN-AA | Phenyl-MeOH-NH4OH | 6.0 | 4998 |
| 55 | PFP-ACN-FA | PFP-ACN-AA | 5.4 | 2267 |
| 56 | PFP-ACN-FA | PFP-MeOH-FA | 2.4 | 844 |
| 57 | PFP-ACN-FA | PFP-MeOH-AA | 6.4 | 2263 |
| 58 | PFP-ACN-FA | BEHC18-ACN-NH4OH | 7.4 | 3441 |
| 59 | PFP-ACN-FA | BEHC18-ACN-AA | 5.2 | 2392 |
| 60 | PFP-ACN-FA | Shield-ACN-FA | 2.8 | 1579 |
| 61 | PFP-ACN-FA | Shield-ACN-AA | 5.1 | 2678 |
| 62 | PFP-ACN-FA | ZrCarb-ACN-FA | 6.7 | 3204 |
| 63 | PFP-ACN-FA | ZrCarb-ACN-AA | 4.1 | 2069 |
| 64 | PFP-ACN-FA | CN-ACN-FA | 1.3 | 1042 |
| 65 | PFP-ACN-FA | Phenyl-ACN-FA | 3.0 | 1724 |
| 66 | PFP-ACN-FA | Phenyl-MeOH-FA | 3.5 | 1413 |
| 67 | PFP-ACN-FA | Phenyl-ACN-AA | 4.3 | 3031 |
| 68 | PFP-ACN-FA | Phenyl-MeOH-AA | 4.7 | 2408 |
| 69 | PFP-ACN-FA | Phenyl-ACN-NH4OH | 8.4 | 6035 |
| 70 | PFP-ACN-FA | Phenyl-MeOH-NH4OH | 5.7 | 3478 |
| 71 | PFP-ACN-AA | PFP-MeOH-FA | 6.5 | 1920 |
| 72* | PFP-ACN-AA | PFP-MeOH-AA | 2.4 | 791 |
| 73* | PFP-ACN-AA | BEHC18-ACN-NH4OH | 6.7 | 2827 |
| 74 | PFP-ACN-AA | BEHC18-ACN-AA | 3.8 | 1577 |
| 75* | PFP-ACN-AA | Shield-ACN-FA | 5.1 | 2557 |
| 76 | PFP-ACN-AA | Shield-ACN-AA | 2.9 | 1361 |
| 77* | PFP-ACN-AA | ZrCarb-ACN-FA | 5.7 | 2440 |
| 78 | PFP-ACN-AA | ZrCarb-ACN-AA | 2.7 | 1231 |
| 79 | PFP-ACN-AA | CN-ACN-FA | 3.0 | 2069 |
| 80 | PFP-ACN-AA | Phenyl-ACN-FA | 3.9 | 2005 |
| 81 | PFP-ACN-AA | Phenyl-MeOH-FA | 3.1 | 1129 |
| 82 | PFP-ACN-AA | Phenyl-ACN-AA | 2.8 | 1794 |
| 83 | PFP-ACN-AA | Phenyl-MeOH-AA | 1.4 | 667 |
| 84 | PFP-ACN-AA | Phenyl-ACN-NH4OH | 7.5 | 4864 |
| 85 | PFP-ACN-AA | Phenyl-MeOH-NH4OH | 4.9 | 2667 |
| 86 | PFP-MeOH-FA | PFP-MeOH-AA | 7.6 | 1889 |
| 87 | PFP-MeOH-FA | BEHC18-ACN-NH4OH | 9.7 | 3170 |
| 88 | PFP-MeOH-FA | BEHC18-ACN-AA | 6.6 | 2154 |
| 89 | PFP-MeOH-FA | Shield-ACN-FA | 4.2 | 1666 |
| 90 | PFP-MeOH-FA | Shield-ACN-AA | 6.4 | 2174 |
| 91 | PFP-MeOH-FA | ZrCarb-ACN-FA | 7.5 | 2536 |
| 92 | PFP-MeOH-FA | ZrCarb-ACN-AA | 5.0 | 1777 |
| 93 | PFP-MeOH-FA | CN-ACN-FA | 1.9 | 1050 |
| 94 | PFP-MeOH-FA | Phenyl-ACN-FA | 4.4 | 1774 |

| 95 | PFP-MeOH-FA | Phenyl-MeOH-FA | 4.0 | 1145 |
|-------|-------------------|-------------------|------|------|
| 96 | PFP-MeOH-FA | Phenyl-ACN-AA | 5.6 | 2723 |
| 97 | PFP-MeOH-FA | Phenyl-MeOH-AA | 5.6 | 2008 |
| 98 | PFP-MeOH-FA | Phenyl-ACN-NH4OH | 10.7 | 5395 |
| 99 | PFP-MeOH-FA | Phenyl-MeOH-NH4OH | 7.4 | 3152 |
| 100* | PFP-MeOH-AA | BEHC18-ACN-NH4OH | 8.0 | 2799 |
| 101 | PFP-MeOH-AA | BEHC18-ACN-AA | 5.9 | 2053 |
| 102* | PFP-MeOH-AA | Shield-ACN-FA | 6.9 | 2871 |
| 103 | PFP-MeOH-AA | Shield-ACN-AA | 5.1 | 1999 |
| 104* | PFP-MeOH-AA | ZrCarb-ACN-FA | 6.6 | 2376 |
| 105 | PFP-MeOH-AA | ZrCarb-ACN-AA | 2.9 | 1119 |
| 106 | PFP-MeOH-AA | CN-ACN-FA | 3.7 | 2131 |
| 107 | PFP-MeOH-AA | Phenyl-ACN-FA | 5.4 | 2333 |
| 108 | PFP-MeOH-AA | Phenyl-MeOH-FA | 4.6 | 1397 |
| 109 | PFP-MeOH-AA | Phenyl-ACN-AA | 4.8 | 2487 |
| 110 | PFP-MeOH-AA | Phenyl-MeOH-AA | 3.5 | 1340 |
| 111 | PFP-MeOH-AA | Phenyl-ACN-NH4OH | 8.8 | 4721 |
| 112 | PFP-MeOH-AA | Phenyl-MeOH-NH4OH | 5.8 | 2674 |
| 113 | BEHC18-ACN-NH4OH | BEHC18-ACN-AA | 6.6 | 3043 |
| 114** | BEH C18-ACN-NH4OH | Shield-ACN-FA | 7.6 | 4214 |
| 115 | BEH C18-ACN-NH4OH | Shield-ACN-AA | 6.6 | 3393 |
| 116* | BEH C18-ACN-NH4OH | ZrCarb-ACN-FA | 8.9 | 4226 |
| 117 | BEH C18-ACN-NH4OH | ZrCarb-ACN-AA | 4.4 | 2209 |
| 118 | BEH C18-ACN-NH4OH | CN-ACN-FA | 4.3 | 3282 |
| 119 | BEH C18-ACN-NH4OH | Phenyl-ACN-FA | 5.9 | 3352 |
| 120 | BEH C18-ACN-NH4OH | Phenyl-MeOH-FA | 6.7 | 2653 |
| 121 | BEH C18-ACN-NH4OH | Phenyl-ACN-AA | 5.5 | 3825 |
| 122 | BEH C18-ACN-NH4OH | Phenyl-MeOH-AA | 6.2 | 3116 |
| 123 | BEH C18-ACN-NH4OH | Phenyl-ACN-NH4OH | 3.3 | 2410 |
| 124 | BEH C18-ACN-NH4OH | Phenyl-MeOH-NH4OH | 1.7 | 1105 |
| 125 | BEH C18-ACN-AA | Shield-ACN-FA | 4.0 | 2236 |
| 126 | BEH C18-ACN-AA | Shield-ACN-AA | 1.3 | 706 |
| 127 | BEH C18-ACN-AA | ZrCarb-ACN-FA | 6.3 | 2969 |
| 128 | BEH C18-ACN-AA | ZrCarb-ACN-AA | 3.5 | 1754 |
| 129 | BEH C18-ACN-AA | CN-ACN-FA | 3.1 | 2359 |
| 130 | BEH C18-ACN-AA | Phenyl-ACN-FA | 2.9 | 1688 |
| 131 | BEH C18-ACN-AA | Phenyl-MeOH-FA | 3.3 | 1339 |
| 132 | BEH C18-ACN-AA | Phenyl-ACN-AA | 1.1 | 836 |
| 133 | BEH C18-ACN-AA | Phenyl-MeOH-AA | 2.5 | 1301 |
| 134 | BEH C18-ACN-AA | Phenyl-ACN-NH4OH | 8.0 | 5703 |
| 135 | BEH C18-ACN-AA | Phenyl-MeOH-NH4OH | 5.0 | 3023 |
| 136 | Shield-ACN-FA | Shield-ACN-AA | 4.1 | 2549 |
| 137* | Shield-ACN-FA | ZrCarb-ACN-FA | 7.3 | 4172 |
| 138 | Shield-ACN-FA | ZrCarb-ACN-AA | 4.1 | 2450 |
| 139 | Shield-ACN-FA | CN-ACN-FA | 1.7 | 1602 |
| 140 | Shield-ACN-FA | Phenyl-ACN-FA | 1.5 | 1098 |
| 141 | Shield-ACN-FA | Phenyl-MeOH-FA | 2.4 | 1186 |
| 142 | Shield-ACN-FA | Phenyl-ACN-AA | 3.4 | 2869 |
| 143 | Shield-ACN-FA | Phenyl-MeOH-AA | 4.1 | 2484 |
| 144** | Shield-ACN-FA | Phenyl-ACN-NH4OH | 8.9 | 7631 |

| 145 | Shield-ACN-FA | Phenyl-MeOH-NH4OH | 5.8 | 4219 |
|-----|------------------|-------------------|-----|------|
| 146 | Shield-ACN-AA | ZrCarb-ACN-FA | 6.4 | 3190 |
| 147 | Shield-ACN-AA | ZrCarb-ACN-AA | 3.4 | 1813 |
| 148 | Shield-ACN-AA | CN-ACN-FA | 3.2 | 2561 |
| 149 | Shield-ACN-AA | Phenyl-ACN-FA | 3.2 | 1943 |
| 150 | Shield-ACN-AA | Phenyl-MeOH-FA | 3.1 | 1305 |
| 151 | Shield-ACN-AA | Phenyl-ACN-AA | 1.2 | 886 |
| 152 | Shield-ACN-AA | Phenyl-MeOH-AA | 1.7 | 947 |
| 153 | Shield-ACN-AA | Phenyl-ACN-NH4OH | 8.4 | 6236 |
| 154 | Shield-ACN-AA | Phenyl-MeOH-NH4OH | 5.2 | 3308 |
| 155 | Zr Carb-ACN-FA | ZrCarb-ACN-AA | 4.2 | 2174 |
| 156 | Zr Carb-ACN-FA | CN-ACN-FA | 4.2 | 3307 |
| 157 | Zr Carb-ACN-FA | Phenyl-ACN-FA | 6.3 | 3690 |
| 158 | Zr Carb-ACN-FA | Phenyl-MeOH-FA | 5.9 | 2446 |
| 159 | Zr Carb-ACN-FA | Phenyl-ACN-AA | 5.6 | 4017 |
| 160 | Zr Carb-ACN-FA | Phenyl-MeOH-AA | 5.2 | 2700 |
| 161 | Zr Carb-ACN-FA | Phenyl-ACN-NH4OH | 9.8 | 7236 |
| 162 | Zr Carb-ACN-FA | Phenyl-MeOH-NH4OH | 6.7 | 4201 |
| 163 | Zr Carb-ACN-AA | CN-ACN-FA | 2.3 | 1944 |
| 164 | Zr Carb-ACN-AA | Phenyl-ACN-FA | 3.4 | 2089 |
| 165 | Zr Carb-ACN-AA | Phenyl-MeOH-FA | 3.3 | 1408 |
| 166 | Zr Carb-ACN-AA | Phenyl-ACN-AA | 3.1 | 2314 |
| 167 | Zr Carb-ACN-AA | Phenyl-MeOH-AA | 2.8 | 1533 |
| 168 | Zr Carb-ACN-AA | Phenyl-ACN-NH4OH | 5.0 | 3851 |
| 169 | Zr Carb-ACN-AA | Phenyl-MeOH-NH4OH | 3.3 | 2162 |
| 170 | CN-ACN-FA | Phenyl-ACN-FA | 1.4 | 1507 |
| 171 | CN-ACN-FA | Phenyl-MeOH-FA | 1.4 | 1122 |
| 172 | CN-ACN-FA | Phenyl-ACN-AA | 2.5 | 2875 |
| 173 | CN-ACN-FA | Phenyl-MeOH-AA | 2.3 | 2194 |
| 174 | CN-ACN-FA | Phenyl-ACN-NH4OH | 4.1 | 5664 |
| 175 | CN-ACN-FA | Phenyl-MeOH-NH4OH | 2.8 | 3222 |
| 176 | Phenyl-ACN-FA | Phenyl-MeOH-FA | 2.0 | 999 |
| 177 | Phenyl-ACN-FA | Phenyl-ACN-AA | 2.3 | 1974 |
| 178 | Phenyl-ACN-FA | Phenyl-MeOH-AA | 3.1 | 1921 |
| 179 | Phenyl-ACN-FA | Phenyl-ACN-NH4OH | 7.1 | 6220 |
| 180 | Phenyl-ACN-FA | Phenyl-MeOH-NH4OH | 4.5 | 3325 |
| 181 | Phenyl-MeOH-FA | Phenyl-ACN-AA | 2.6 | 1544 |
| 182 | Phenyl-MeOH-FA | Phenyl-MeOH-AA | 2.4 | 1046 |
| 183 | Phenyl-MeOH-FA | Phenyl-ACN-NH4OH | 7.7 | 4723 |
| 184 | Phenyl-MeOH-FA | Phenyl-MeOH-NH4OH | 5.0 | 2585 |
| 185 | Phenyl-ACN-AA | Phenyl-MeOH-AA | 1.8 | 1411 |
| 186 | Phenyl-ACN-AA | Phenyl-ACN-NH4OH | 6.7 | 7179 |
| 187 | Phenyl-ACN-AA | Phenyl-MeOH-NH4OH | 4.1 | 3725 |
| 188 | Phenyl-MeOH-AA | Phenyl-ACN-NH4OH | 7.2 | 5555 |
| 189 | Phenyl-MeOH-AA | Phenyl-MeOH-NH4OH | 4.6 | 2997 |
| 190 | Phenyl-ACN-NH4OH | Phenyl-MeOH-NH4OH | 2.2 | 2175 |

Une étude d'orthogonalité, basée à la fois sur le calcul du degré d'orthogonalité et de la capacité de pic pratique a été menée sur deux échantillons pharmaceutiques. Un screening de conditions 1D-LC a permis d'évaluer le potentiel de presque 200 combinaisons possibles pour la mise en place d'analyses 2D-LC. Cette étude préliminaire a conduit à des conclusions similaires à celle tirées lors d'une étude antérieure qui avait été menée sur un mélange de composés modèles. Il apparaît que (1) le changement de pH entre les phases mobiles de ¹D et de ²D a un impact très important sur les valeurs de O_d et n_{2D}* ; (2) l'utilisation d'ammoniaque comme additif de phase mobile dans l'une des deux dimensions conduit aux capacités de pics les plus élevées ; (3) les systèmes les plus orthogonaux ne sont pas nécessairement ceux qui génèrent les plus grandes capacités de pics.

En tenant compte de critères additionnels comme les températures maximales d'utilisation des colonnes et la compatibilité du système 2D-LC avec une détection par spectrométrie de masse, un set de 3 systèmes RPLCxRPLC a été retenu. Ces systèmes ont été appliqués à la séparation 2D-LC de deux échantillons pharmaceutiques, et ont permis d'obtenir des capacités de pic effectives proches de 1000 en moins d'une heure. De plus, l'intérêt d'utiliser une double détection UV et MS a été mis en avant, puisque certains composés n'ont été détectés que dans un seul de ces deux modes de détection.

Finalement, une étude comparative a permis de montrer qu'une analyse LCxLC-UV/MS permettait de détecter 40% de composés de plus qu'une analyse LC-UV/MS classique. La combinaison de deux analyses LCxLC-UV/MS, quant à elle, permet de séparer et détecter plus de 95% des composés présents dans un échantillon. L'utilisation d'une plateforme de 2 à 3 systèmes RPLCxRPLC-UV/MS semble donc être une stratégie analytique viable pour l'analyse de composés pharmaceutiques.

Une question reste cependant en suspens : Dans le cas d'un échantillon un peu plus réaliste où les impuretés sont présentes à de très faibles concentrations, les méthodes LCxLC développées seront-elles capable de détecter et quantifier ces impuretés de façon fiable ?

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Chapitre 3 : Mise en place d'une stratégie 2D-LC générique. Partie 1 | 84

CHAPITRE 4

Mise en place d'une stratégie 2D-LC générique. Partie 2 : Aspects quantitatifs et éléments de validation

Ce chapitre a fait l'objet d'une publication :

Article 3

"Quantitative aspects in comprehensive on-line two dimensional liquid chromatography for pharmaceutical applications"

M. Iguiniz, E. Corbel, N. Roques, S. Heinisch, Talanta 195 (2019) 272–280. https://doi.org/10.1016/j.talanta.2018.11.030.

Chapitre 4 : Mise en place d'une stratégie 2D-LC générique. Partie 2 | 86

L'étude menée dans le Chapitre 3 a montré l'intérêt de la LCxLC pour l'analyse de composés pharmaceutiques en termes de sélectivité et de pouvoir de séparation. Cependant, dans l'industrie pharmaceutique, une méthode d'analyse doit remplir d'autres critères que la séparation de tous les composés d'un mélange.

En effet, la sensibilité des méthodes développées est également très importante, puisque les textes règlementaires [1,2] imposent des limites strictes quant à la présence d'impuretés dans les substances actives à usage humain :

| Dose journalière | | Seuils** | |
|------------------|-------------|--|--|
| maximale | Déclaration | Identification | Qualification |
| ≤ 2 g/jour | > 0.05 % | Soit > 0.10 % soit > 1.0 mg par jour* | Soit > 0.15 % soit > 1.0 mg par jour* |
| > 2 g/jour | > 0.03 % | > 0.05 % | > 0.05% |

Tableau 4.1 - Déclaration, identification et qualification des impuretés organiques dans les substances actives. *en prenant le plus petit des deux, ** des seuils spécifiques peuvent s'appliquer dans des cas particuliers.

Un autre point capital est de vérifier les performances quantitatives des méthodes développées. Pour cela, chaque méthode destinée à l'analyse pharmaceutique doit passer une étape de validation [3,4].

La LCxLC a très souvent été utilisée pour les capacités de pics très impressionnantes qu'elle peut générer par rapport à la LC. En revanche, de même que pour la capacité de pics, le facteur de dilution en LCxLC peut être assimilé au produit des facteurs de dilution dans chaque dimension [5]. La LCxLC est donc une technique plus dilutive que la LC, et peu d'études se sont penchées sur la sensibilité des méthodes développées en LCxLC. De plus, le traitement des données générées par une analyse LCxLC n'est pas un exercice évident (voir Chapitres 1 et 2), en particulier dans le cas d'une analyse quantitative. Cela peut compliquer l'évaluation des critères de validation d'une méthode et expliquer qu'il existe très peu de publications, tous domaines confondus, dans lesquelles on peut retrouver ces éléments de validation [6–9].

L'objectif de ce chapitre est donc de travailler sur l'application de la LCxLC dans un contexte industriel aux exigences règlementaires strictes telles que l'industrie pharmaceutique. Pour cela, une première étape consiste à étudier le potentiel de la LCxLC pour l'analyse quantitative d'échantillons pharmaceutiques réels, dans lesquels les impuretés sont présentes à de faibles niveaux de concentration. Une étude de la sensibilité des méthodes développées dans le Chapitre 3 est faite sur un mélange modèle, puis la capacité de la LCxLC à générer des résultats quantitatifs fiables est évaluée en déterminant des éléments de validation de méthode. Dans un deuxième temps, une stratégie générique est proposée pour la mise en place d'analyses 2D-LC d'échantillons pharmaceutiques. Enfin, le problème du transfert de méthodes est abordé, puisque lorsqu'une méthode est développée puis validée dans l'industrie, elle doit en général pouvoir être transférée d'un instrument à l'autre voire d'un laboratoire à l'autre.

Chapitre 4 : Mise en place d'une stratégie 2D-LC générique. Partie 2 | 88

A. SENSIBILITE ET ASPECTS QUANTITATIFS EN LCXLC

Article 3

"Quantitative aspects in comprehensive on-line two dimensional liquid chromatography for pharmaceutical applications"

M. Iguiniz, E. Corbel, N. Roques, S. Heinisch, Talanta (2018), to be submitted

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Quantitative aspects in on-line comprehensive two-dimensional liquid chromatography for pharmaceutical applications



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ABSTRACT

On-line comprehensive two-dimensional liquid chromatography (on-line LCxLC) is expected to offer impressive effective peak capacity. That makes it an attractive technique for the analysis of complex samples such as pharmaceuticals, for which impurities are often unknown both in number and in structure, and can be either totally different or similar to the active substance. A study on comprehensive on-line RPLCxRPLC (reversed phase liquid chromatography in both dimensions) with respect to quantitative aspect at low concentration levels was carried out with the objective of finding conditions able to meet regulatory requirements for the control of pharmaceutical impurities. Preliminary studies showed that the best approach for quantitative purpose was to relate the impurity concentration to the sum of the second dimension peak areas. RPLCxRPLC conditions were optimized according to a Pareto-optimality approach with a focus on sensitivity, thereby favouring low dilution factor. The resulting RPLCxRPLC separation led to a detection limit below 0.05% for impurities (compared with API). Furthermore, the properties of this analytical method was found to be satisfying in terms of linearity $(R^2 > 0.999)$, repeatability (RSD < 1% for second dimension retention times) and precision (RSD < 1.5% for the sum of second dimension peak areas). Finally, this on-line RPLCxRPLC approach was applied to the analysis of a batch of drug substance in the early stages of drug development. The method was able to detect impurities at concentrations as low as 0.04% (signal-to-noise ratio of 3). All these results clearly demonstrate the power of online RPLCxRPLC for the quantitative analysis of pharmaceutical samples.

1. Introduction

On-line comprehensive two-dimensional liquid chromatography (on-line LCxLC) has been applied to the analysis of complex samples in many application fields over the last decades [1-3]. In LCxLC, the whole sample is subjected to two different separations. The theoretical peak capacity represents the product of the peak capacities in both dimensions. As a result this may lead to impressive theoretical values which cannot be reached with conventional one-dimensional chromatography (1D-LC) [4]. This has made LCxLC a very attractive technique for the analysis of biological samples, natural products, peptides or bio-oils [5–8]. In the past few years, a number of scientists have worked on the theoretical understanding of LCxLC separations as well as on the optimization of chromatographic conditions [9–13]. However, in most studies, the primary objective was to achieve very large peak capacities whereas solute dilution was of less concern. The dilution in on-line LCxLC is often more critical than in 1D-LC as highlighted by Schure [14] who pointed out that, under isocratic conditions in both dimensions, an analyte band could be 100-fold less concentrated when it reached the second dimension detector than when it reached the detector in conventional 1D-LC. Usual strong dilution along with far more complex implementation can explain why on-line comprehensive approach remains very little used for pharmaceutical analysis compared to a heart-cut approach (LC-LC) [15]. Pharmaceutical samples are generally composed of an active pharmaceutical ingredient (API) and its related impurities, comparatively present at very low concentrations. For sake of safeness, the regulation for the control of impurities and degradation products imposes strict limits. According to ICH guidelines [16,17], it is recommended, for impurities and related substances of both drug substances and drug products, to consider 0.05% (compared with API) as reporting threshold, 0.1% as identification limit and 0.15% as qualification limit. Such requirements imply that any developed method should achieve a limit of quantitation (LOQ) of at least 0.05% (compared with API) which makes the high dilution involved in on-line LCxLC a major obstacle although this technique is expected to be the technique

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of choice in the pharmaceutical field with respect to both orthogonality and selectivity [18].

Parameters that were recommended to consider for reducing the analyte dilution include, the sampling rate, the split ratio and the mechanisms used in both dimensions [19]. The use of more complex interfaces between both dimensions, with either trapping columns or make-up flow or both [20–22] was also shown to be an efficient way to limit injection issues in the second dimension and thus, to reach low limits of detection and quantitation. Although such interface configurations were first developed to circumvent solvent compatibility issues between the mobile phases of first and second dimensions [23–25], they are also currently used to improve peak intensity by promoting analyte focusing on the second dimension column inlet [26,27]. However, they involve additional instrument devices, and also additional parameters that need to be optimized including the make-up flow rate, the make-up composition, the stationary phase and the dimensions of the trapping columns.

The aim of this study was to minimize the dilution on the basis of an optimization approach previously proposed for on-line LCxLC with reversed phase liquid chromatography (RPLC) in both dimensions [10,12]. This approach is attractive since it does not require additional devices for the interface but only a 2-position/4-port dual switching valve with two similar empty loops. The objective of the developed online LCxLC separation was to meet the ICH recommendations for pharmaceutical compounds, using usual UV detection, while keeping a peak capacity significantly higher than the one achieved in 1D-LC. After carrying out preliminary tests to select the best method for quantitative analysis, two different sets of optimized RPLCxRPLC conditions were compared. The first set was designed to provide a good trade-off between a large peak capacity and a low dilution factor as usually recommended for the analysis of complex samples such as proteins and peptides [10]. The second set was designed to minimize the dilution factor regardless of the peak capacity. Finally, the second approach was applied to three generic 2D-systems proposed in a previous work [18]. The best RPLCxRPLC conditions were applied to the quantitative analysis of a batch of drug substance in the early stages of drug development.

2. Material and methods

2.1. Chemical and samples preparation

Acetonitrile (ACN) and methanol (MeOH) were LC-MS grade from Sigma–Aldrich (Steinheim, Germany). Water was obtained from an Elga Purelab Classic UV purification system (Veolia water STI, Le Plessis Robinson, France). Several additives were added in the mobile phase: formic acid (0.1% - pH 2.7 measured in aqueous phase), ammonium acetate (AA) (5 mM in aqueous phase - pH 6.8), and ammonium hydroxide (0.1% - pH 10.4 measured in aqueous phase). All of them were obtained from Sigma–Aldrich (Steinheim, Germany). Aqueous eluents prepared from salts were filtered on 0.22 µm nylon filter before immediate use.

For preliminary study, methylparaben and ethylparaben were used as model compounds. They were obtained from Sigma–Aldrich (Steinheim, Germany). First quantitative study was performed with methylparaben solutions at concentrations ranging from 0.01 to 0.5 mg/mL in H₂O/ACN 50/50 (v/v). The comparative study between the peak volume and the sum of peak areas for quantitative analysis was carried out with 3 different samples of 0.5 mg/mL methylparaben in water/ACN 50/50 (v/v) containing 50% ethylparaben (i.e. 0.25 mg/ mL), 1% ethyl paraben (i.e. 5 µg/mL) and 0.05% ethylparaben (i.e. 0.25 µg/mL).

Pharmaceutical compounds (Drug substance A and Drug substance B) where provided by Oril Industrie (Servier laboratories, Bolbec, France). Due to confidentiality agreement, the molecular structures could not be given. Samples of Drug substance A at 0.5 mg/mL in

Water/ACN 50/50 (v/v) containing different concentrations of three synthetic intermediates were used first to evaluate the impact of optimized 2D-LC separation on sensitivity, and then to assess some critical parameters such as linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision. Finally, LCxLC-UV/MS analysis were carried out on a real sample (batch analysis) of Drug substance B at concentration of 0.5 mg/mL in ACN.

2.2. Instruments

LCxLC separations were performed with an Infinity 1290 2D-LC system from Agilent Technologies (Waldbronn, Germany). This instrument includes two high-pressure binary solvent delivery pumps, an autosampler with a flow-through needle of 20 µL, a column oven with a maximum temperature of 100 °C, a UV detector and a diode array detector equipped with 2 µL and 0.6 µL flow-cells, respectively. A 2-position/4-port dual valve was used as interface between the two dimensions, and was equipped with two 80 µL sampling loops. Dwell volumes, extra-column volumes and extra-column variances were determined using a zero dead volume union connector in place of the column. The measured dwell volumes were 140 μ L in the first and 65 μ L in the second dimension (measured without the interface between both dimensions). A total extra-column volume of 22 μL and 8.5 μL and an extra-column variance of $12\,\mu\text{L}^2$ and $4.5\,\mu\text{L}^2$ were determined in first and second dimension respectively. Detector wavelengths were set at 210 nm or 254 nm depending on the experiments. The acquisition rates were set at 10 Hz and 80 Hz in the first and second dimension respectively. Data acquisition and instrument control were performed by OpenLab software (Agilent).

For the analysis of batch sample, LCxLC systems were hyphenated to a QDA mass spectrometer from Waters (Milford, United States), working in scan mode with an acquisition rate of 20 Hz. Data acquisition and instrument control were performed by MassLynx software (Waters). Synchronization between both instruments was obtained by electrical connection.

2.3. Chromatographic conditions

For the comparison of the linear range between 1D-LC-UV and online LCxLC-UV, experiments were carried out without columns (i.e. replaced by zero-dead volume unions). In the first dimension, the temperature, the flow rate, the wavelength and the acquisition rate were 30 °C, 0.01 mL/min, 254 nm and 20 Hz respectively. The mobile phase in both dimensions was a mixture of water/Acetonitrile 50/50 (v/v). The sampling time was 0.3 min (i.e. 3 μ L injected in the second dimension). In the second dimension and in 1D-LC-UV, the temperature, the flow rate, the wavelength and the acquisition rate were 30 °C, 1.5 mL/min in, 254 nm and 80 Hz respectively. $1 \,\mu$ L of a solution of methylparaben was injected with concentrations ranging from 0.01 to 0.5 mg/mL. The comparison of the results obtained from the peak volumes and the sum of peak areas was carried out under isocratic conditions in both dimensions. In the first dimension, the conditions were similar to those mentioned above. In the second dimension, an Acquity CSH C18 column (30 mm x 2.1 mm; 1.7 µm) from Waters (Milford, MA, USA) was used. The temperature, the flow rate, the wavelength and the sampling rate were 30 $^\circ\text{C},\,1.5\,\text{mL/min},\,254\,\text{nm}$ and 80 Hz respectively. The mobile phase was a mixture of water/acetonitrile 70/30 (v/v) with 0.1% formic acid. The sampling time was 0.5 min and the injected volume was 1 µL.

For the rest of the study, three different sets of conditions (stationary phases, mobile phases, additives) were used as optimized in a previous work [18] with a view to define a generic analytical strategy for pharmaceuticals (2D-systems #1, #2 and #3, respectively). 2Dsystem #2 was used to evaluate the impact of optimized chromatographic conditions on the sensitivity in on-line LCxLC. The conditions used in the two different set-up are given Table 1. Finally, a
(3)

Table 1

Optimized RPLCxRPLC conditions for the separation of API and related impurities. Set-up #1 focuses on peak capacity while Set-up #2 focuses on sensitivity. See text for more explanations on the condition selection.

| | Set-up #1 | | Set-up #2 | |
|---------------------|-----------------------|------------------------|-----------------------|------------------------|
| | ¹ D | ² D | ¹ D | ² D |
| Stationary phase | Xbridge BEH C18 | Acquity CSH C18 | Xbridge BEH C18 | Acquity CSH C18 |
| Column geometry | 50 mm x 2.1 mm, 5 μm | 30 mm x 2.1 mm, 1.7 μm | 50 mm x 2.1 mm, 5 μm | 30 mm x 2.1 mm, 1.7 μm |
| Mobile phase | A: Water + 0,1% NH4OH | A: Water + 0,1% FA | A: Water + 0,1% NH4OH | A: Water + 0,1% FA |
| | B: ACN + 0,1% NH4OH | B: ACN + 0,1% FA | B: ACN + 0,1% NH4OH | B: ACN + 0,1% FA |
| Flow-rate | 0.1 mL/min | 1.8 mL/min | 0.15 mL/min | 1.8 mL/min |
| Gradient conditions | 15–100% (B) in 60 min | 5–90% (B) in 0.15 min | 15-100% (B) in 60 min | 5–90% (B) in 0.11 min |
| Sampling time | 0.3 min | | 0.3 min | |
| Split ratio | 1:3 | | 1:1 | |
| Temperature | 30 °C | 80 °C | 30 °C | 80 °C |
| Injected Volume | 3 μL | 10 μL | 3 μL | 45.58 μL |

Table 2

RPLCxRPLC conditions for the separation of pharmaceutical samples using the three 2D-systems previously proposed [14] with identical conditions in the second dimension. See text for more explanations on the condition selection.

| | First dimension | | | Second dimension |
|---------------------|------------------------|-----------------------|------------------------|--|
| | 2D-System #1 | 2D-System #2 | 2D-System #3 | 2D-Systems #1, #2 and #3 |
| Stationary phase | Xselect HSS PFP | Xbridge BEH C18 | Acquity BEH Phenyl | Acquity CSH C18 |
| Column geometry | 50 mm x 2,1 mm, 3,5 µm | 50 mm x 2,1 mm, 5 μm | 50 mm x 2,1 mm, 1,7 μm | 30 mm x 2,1 mm, 1,7 μm |
| Mobile phase | A: Water + AA 5 mM | A: Water + 0,1% NH4OH | A: Water + 0,1% NH4OH | A: Water + 0,1% FA |
| | B: MeOH | B: ACN + 0,1% NH4OH | B: ACN + 0,1% NH4OH | B: ACN + 0,1% FA |
| Flow-rate | 0,15 mL/min | 0,15 mL/min | 0,3 mL/min | 1,8 mL/min |
| Gradient conditions | 27-92% (B) in 60 min | 15–100% (B) in 60 min | 15-100% (B) in 60 min | 5–90% (B) in 0,13 min (S #1 and #3); and 0.11 min (S #2) |
| Sampling time | 0.33 min | 0.3 min | 0.33 min | |
| Split ratio | - | - | 1:2 | |
| Temperature | 30 °C | 30 °C | 30 °C | 80 °C |
| Injected Volume | 3 μL | 3 μL | 3 μL | 49.35 μL (S #1); 45.58 μL (S #2); |
| | | | | 49.15 μL (S #3) |
| UV | 210 nm | 210 nm | 210 nm | 210 nm |
| MS | - | - | - | Scan (100–700) |
| | | | | |

pharmaceutical sample was analyzed using the three 2D-systems with optimized conditions (Table 2).

2.4. Calculations

The conditions for LCxLC separations were optimized using a homemade procedure developed with Microsoft Excel [10,12]. This calculation method consists in a three input level procedure based on both theoretical relationships and Pareto-optimality approach.

The dilution factor, Df, was calculated in each dimension with

$$Df = \frac{\sigma_v \times \sqrt{2\pi}}{V_i} \tag{1}$$

 σ_v being the peak standard deviation in volume units and V_i, the injected volume. The dilution factor in LCxLC was assumed to be the product of the dilution factors in each dimension [14].

The effective peak capacity $(n_{2D,eff})$ in LCxLC was predicted using the following relationship [12]

$$n_{2D,eff} = \alpha \times \beta \times \gamma \times {}^{1}n \times {}^{2}n$$
⁽²⁾

where ¹n and ²n are the peak capacities in the first and second dimension respectively. The coefficients α , β and γ are correction factors between 0 and 1. α corrects ¹n for undersampling [28], β corrects ²n for non-ideal transfer of the sample fraction from ¹D to ²D [12] and γ allows to takes into account the retention surface coverage [29].

Both peak standard deviations and peak capacities were predicted according to relationships given in previous studies [10,12].

The signal-to-noise ratio (S/N) was calculated for minor compounds, according to

$$S/N = 2H/h$$

where H is the peak height and h, the difference between the highest and the lowest noise values observed over a distance \geq 5 peak widths (measured at half peak height) around the peak of interest.

LCxLC data were processed using Microsoft Excel and Matlab V7.12.0635. Azur software (Datalys, Grenoble, France) was used to measure peak areas and peak heights. Peak volumes were obtained from LC Image software (GC Image, LLC, Lincoln, USA).

3. Results and discussion

3.1. Preliminary study on quantitative aspects

One major question raised by quantitative analysis in on-line LCxLC is how to perform data processing. According to published results, there are two different ways to proceed. The first approach consists in summing the successive second dimension peak areas related to the same first dimension peak [30,31]. The second approach consists in calculating the volume of the three dimensional peak by considering it as a cone [32]. A third method might consist in considering the peak area of the main fraction but it is clear that an excellent repeatability from run to run should be required in this case. The three methods were first evaluated through a simple LCxLC set-up with the columns in both dimensions replaced by zero dead volume unions. LCxLC experiments were carried out with samples containing methylparaben in various concentrations. In addition to the results obtained from the three above methods, Fig. 1 also shows the variation of the detector response in the first dimension as a function of the solute concentration. Fig. 1 leads to the following comments: (1) not surprisingly, whereas the response in the first dimension is no more linear above 0.2 mg/mL, it is still linear



Fig. 1. Illustration of the response in the second dimension depending on the calculation method. Normalized response values against solute concentration considering the first dimension and the peak area (black) or the second dimension and the peak area of the main fraction (green), the sum of the peak areas (blue) or the peak volume (red). Determination coefficient are specified in the figure. Solute: methylparaben; 1 μ L injected; 254 nm; no column in both dimensions. Other conditions given in Section 2.3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

above 0.5 mg/mL in the second one. This can be easily explained by the dilution due both to the sampling of the first dimension peak and to the band broadening due to the injection process in the second dimension; (2) the coefficients of determination are higher than 0.999 for methods involving either the sum of the peak areas or the peak volume; (3) the third method involving the area of the main fraction seems to be less suitable with a determination coefficient lower than 0.999. This is likely to be due to some very small variations of the fractions between the different LCxLC runs. This third method was therefore abandoned.

In order to choose between the two other methods, samples composed of methylparaben and ethylparaben in different proportions were injected in on-line LCxLC without column in the first dimension but with a reversed phase column in the second one. The absence of column in the first dimension allowed to ensure a constant ratio, all along the first dimension peak, between the weight concentrations of ethylparaben and methylparaben. As a result, any fraction and, in particular the most intense one, can be considered as representative of the whole sample. As a result, the reference concentration ratio was considered as the ratio of the peak areas obtained during the separation of the most intense fraction. Three samples composed of methylparaben (0.5 mg/ mL) and of various concentrations of ethylparaben (i.e. 47.9%, 0.9% and 0.04% compared to methylparaben) where separated with the objective of (1) evaluating both LOD and LOQ, (2) comparing the two studied methods (i.e. based on the sum of peak areas and on the peak volume) under conditions similar to those encountered in pharmaceutical analysis and (3) evaluating the impact of the concentration ratio on the resulting error. Fig. 2 shows the first dimension separation with the fractions sent to the second dimension delimited by dotted lines (Fig. 2a) along with the overlaid second dimension separations of the different fractions for the three samples (Fig. 2b, c and d). The Signalto-Noise ratios (S/N), calculated from the peak of ethylparaben in the most intense fraction were found to be 6170, 130 and 7 respectively, thereby suggesting that LOD was lower than 0.04% while LOQ was slightly higher. Calculated concentration ratios were obtained from the two studied quantitation methods and compared to the reference values provided by the ratio of the two peak areas in the most intense fraction as shown in Table 3. Not surprisingly, the error increases for both methods when the concentration ratio decreases due to the fact that the impact of the baseline noise on the calculation of the peak area (or the peak volume) is more significant for less intense peaks. However, the

results in Table 3 clearly show that the error with the sum of the peak areas was lower than 5% in the worst case (i.e. a concentration ratio of 0.04%) while it attained 30% with the method based on the peak volume. It should be noticed that quantitation based on the peak volume tends to overestimate the ehtylparaben concentration because (1) a cone is likely to be inappropriate for representing a three-dimensional gaussian peak and (2) the number of fractions per ¹D-peak is small (i.e. from 2 to 5 only) which is probably not sufficient to properly define the peak, leading to a significant underestimation of the major peak volume (i.e. methylparaben) and hence an overestimation of the concentration ratio.

This first study points out that on-line RPLCxRPLC can meet some of the ICH recommendations and that the method based on the sum of peak areas is much more reliable than the two other studied ones and hence should be used for quantitation purpose in pharmaceutical analysis.

3.2. Quantitative results on pharmaceutical samples

3.2.1. Optimization of RPLCxRPLC conditions

Most often, on-line LCxLC has been applied to the analysis of very complex samples such as natural products, peptides or bio-oils [5,6,23,33]. Once a combination of two LC-systems has been selected, with both dimensions (mobile and stationary phases) defined with respect to their degree of orthogonality, the key objective in optimizing the conditions of LCxLC, usually consists in finding the best compromise between a high peak capacity, a low dilution and a short analysis time [10,12,13]. We recently defined, three combinations of two RPLC-systems (three 2D-systems) able to meet most analytical needs in pharmaceutical development [18]. It was shown on real pharmaceutical samples that any of these combinations can achieve an effective peak capacity of 1000 in less than 60 min. Furthermore it was suggested that using one or possibly two 2D-systems, with both UV and MS detection, represented a good analytical strategy throughout drug development. In the present study 2D-conditions were optimized for these 2D-systems, according to a predictive approach recently developed for peptide analysis [10,12]. The objective was to achieve a maximum level of sensitivity for quantitative analysis by minimizing the dilution factor while keeping a high enough effective peak capacity. The optimization procedure, based on both predictive calculation tools and a Paretooptimality approach, defines, for a given analysis time, the best set of 2D-conditions, considering both the effective peak capacity and the dilution factor. In this approach, it was shown that β (Eq. (2)) and hence the additional band broadening due to strong injection solvent (Compression factor < 1) or the focusing effect due to weak injection solvent (Compression factor > 1) could be correctly predicted, for peptide mapping, using the following equation for the compression factor [10]:

$$C_F = \frac{{^2k_{e,1}}}{{^2k_{e,2}}}$$
(4)

Where C_F , is the compression factor, ${}^2k_{e,1}$ and ${}^2k_{e,2}$ are the solute retention factors obtained with the second dimension stationary phase, the injection solvent (first dimension mobile phase) and the second dimension mobile phase respectively.

The same approach was applied to pharmaceutical compounds, considering an analysis time of 60 min. The parameters that were simultaneously optimized included the first dimension flow-rate (from 15 to 1000 μ L/min), the split ratio between both dimensions (from 1:1–1:10), the second dimension flow-rate (from 1000 to 3000 μ L/min with a maximum pressure of 1000 bar), the sampling rate (from 1 to 3 fractions per peak) and the second dimension column length (3 cm and 5 cm). For calculations, a value of 0.6 was used for γ (Eq. (2)) according to previous results on pharmaceuticals [18]. Furthermore the extracolumn band broadening in both tubing and detector cell was



Fig. 2. LCxLC separations of samples composed of methyparaben and ethylparaben in various proportions. (a) First dimension peak with the fractions sent to the second dimension delimited by dotted lines (the most intense fraction is colored in green) and overlaid second dimension separations of the fractions for samples composed of (b) 50% methylparaben and 50% ethylparaben, (c) 99% methylparaben and 1% ethylparaben, and (d) 99.95% methylparaben and 0,05% ethylparaben. MP and EP refer to methylparaben and ethylparaben, respectively. Other conditions given in Section 2.3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3

Comparison of two methods of quantitation in on-line LCxLC. Determination of the concentration ratios between ethylparaben and methylparaben. The reference concentration ratio is assumed to be the ratio of the peak areas obtained from the separation of the most intense fraction.

| | Reference ratio | Method based on the sum of peak areas | | Method based on the peak volume | | |
|-------------------------------------|--------------------------|---------------------------------------|------------------------|---------------------------------|------------------------|--|
| | | Calculated ratio | Error (%) | Calculated ratio | Error (%) | |
| Sample #1 Sample #2 Sample #3 | 47.89% 0.89% 0.04% | 47.90% 0.91% 0.039% | 0.02 2.25 - 2.50 | 48.81% 1.03% 0.05% | 1.92 15.73 32.50 | |



Fig. 3. Full Pareto plots of the dilution factor versus the effective peak capacity for an analysis time of 60 min. Partial Pareto-curves are given for different sets of second dimension column length and flow-rate: (-----) 5 cm and 1 mL/min; (-----) 3 cm and 1 mL/min; (-----) 3 cm and 1.8 mL/min and (-----) 3 cm and 3 mL/min. Empty and full symbols correspond to a second dimension column length of 5 cm and 3 cm respectively. The full Pareto curve is in black. Black circles correspond to the two selected sets of conditions (Set-up #1 and Set-up #2). See the text for explanations and Sections 2.3 and 2.4 for calculations and other conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

considered depending on the second dimension flow-rate. The resulting partial and full Pareto-curves are shown in Fig. 3. It can be concluded from these curves that reducing the second dimension flow-rate is beneficial to low dilution but at the expense of a severe decrease in effective peak capacity. In contrast, reducing the column length while increasing the flow-rate until the maximum pressure is reached, is very attractive for the effective peak capacity but detrimental for the dilution factor. In order to have a good trade-off between dilution and effective peak capacity, we selected two sets of conditions, the first one favoured a high effective capacity while the second one favoured a low dilution factor. Both are indicated by black circles in Fig. 3. Their corresponding conditions are listed in Table 1. On-line RPLCxRPLC separations (Fig. 4) were carried out with Drug substance A containing 0.5 mg/mL of API and 3 isolated impurities with concentrations relative to API varying from 2% to 0.05%. The two 3D-representations in Fig. 4 illustrate the significant difference in sensitivity depending on the set of conditions. The peak intensities of impurities #1, #2 and #3 are indeed three to four-fold higher with Set-up #2 compared to with Set-up #1 (for impurities at 1% level), which was in good agreement with the expected increase in dilution factor (i.e. from 2.7 to 10) shown in Fig. 3. It is important to note that, at the same time, the predicted effective peak capacity decreases from 2500 to 1500, 1500 being still much higher than the effective peak capacity that can be achieved in 1D-LC. S/N values were calculated for the three impurities at different concentration levels for the two RPLCxRPLC separations. As shown by the resulting values in Table 4, impurities at a concentration level as low as 0.05% could not be detected with Set-up #1. A three-fold and a four to five-fold increase in S/N was observed with Set-up #2 for impurity #2 and #3 respectively. The increase was less significant for impurity #1 (less than two-fold), which might be due to its low absorbance, thereby enhancing the noise impact. According to ICH guidelines [34] which recommend S/N values of 10 for LOQ and 3 for LOD, it appears that LOD with Set-up #1 was between 0.05% and 0.1% (impurity #2 and #3) and even higher than 0.1% (impurities #1) while it is always lower than 0.05% with Set-up #2. Similarly, LOQ was higher than 0.2% with Set-up #1 while between 0.05% and 0.1% with Set-up #2 and even lower for impurity #3. These results emphasize the great benefit of this predictive approach in RPLCxRPLC to maximize sensitivity in order to meet ICH requirements for the control of impurities [16,17,34] while keeping the impressive separation power offered by on-line LCxLC.



Fig. 4. On-line RPLCxRPLC separations of a synthetic sample (Drug substance A) containing 3 impurities at 1% level with (a) set-up #1 and (b) set-up #2. UV detection at 210 nm. See Table 1 for RPLCxRPLC-conditions.

Table 4

S/N values obtained for three impurities of Drug Substance A, with concentrations (relative to API) in the range 2–0.05%. On-line RPLCxRPLC conditions given in Table 1.

| | Set-up # | Set-up #1 | | | Set-up #2 | | |
|---------------|----------|-----------|--------|--------|-----------|--------|--|
| Concentration | Imp #1 | Imp #2 | Imp #3 | Imp #1 | Imp #2 | Imp #3 | |
| 2% | 76 | 177 | 99 | 98 | 512 | 690 | |
| 1% | 33 | 84 | 64 | 42 | 294 | 344 | |
| 0.5% | 16 | 56 | 35 | 19 | 126 | 192 | |
| 0.2% | 8 | 18 | 11 | 9 | 55 | 66 | |
| 0.1% | 2 | 6 | 8 | 4 | 19 | 36 | |
| 0.05% | - | - | - | 3 | 9 | 18 | |

3.2.2. Some validation aspects

The above RPLCxRPLC-UV method was evaluated with respect to some critical validation parameters (linearity, precision, retention time repeatability, LOD and LOQ) in order to test the reliability of this 2D-LC method with respect to quantitative analysis. Each impurity peak present in the first dimension was distributed into several peaks in the second dimension corresponding to the different cuts. These second dimension peaks have to be merged for calculations. In view of the above study, the sum of the peak areas was applied to quantitative analysis. Linearity was evaluated by plotting the sum of 2D peak areas against the impurity concentration (ranging from 0.2% to 2% for impurity #1 and from 0.05% to 2% for impurities #2 and #3). The retention time repeatability and the precision of the method was tested from six on-line RPLCxRPLC-experiments carried out with six mixed standard solutions prepared independently. The resulting straight line equations, R² values, LOD, LOQ and RSD values for both the second dimension retention times and the sum of peak areas are summarized in Table 5. As previously discussed, LODs and LOQs meet ICH requirements. In all cases, R² values were higher than 0.999, which confirms (1) the ability of RPLCxRPLC to provide quantitative results, and (2) the reliability of the sum of second dimension peak areas for quantification with RSD values lower than 1% for second dimension retention times, thereby highlighting a good repeatability from run-to-run in RPLCxRPLC while RSD values were lower than 1.5% for the sum of peak areas, thereby showing a good precision in quantitative results. In conclusion, all these results showed that the present RPLCxRPLC method leads to appropriate linearity, sensitivity and precision for quantitative analysis of pharmaceutical compounds. The last step of this study was to apply this optimization approach to the analysis of a real pharmaceutical sample using one of the three generic 2D-systems previously selected [18].

3.3. Application to the analysis of a pharmaceutical sample

The optimized conditions found above (Set-up #2) were applied to the three combinations of RPLC systems previously proposed as a generic analytical strategy in drug development [18] for analyzing a real batch of Drug substance B. 2D-conditions are given in Table 2 for the three 2D-systems. Fig. 5 shows MS-TIC contour plots for the 3 RPLCxRPLC separations. As can be seen, the first 2D-system (Fig. 5a) is not suitable for the analysis of this Drug substance B as highlighted by the intense non retained peak which might be due to a breakthrough phenomenon in case of API. This made quantitative analysis impossible since a significant part of this analyte was eluted in the column dead time. Such phenomenon can be explained by the fact that API is strongly retained on the first dimension column, hence eluted with a high acetonitrile content in the mobile phase (i.e > 65%). This latter becomes a strong injection solvent in the second dimension, thus leading to a breakthrough phenomenon. In addition to this, the number of detected compounds is much lower than with the two other 2Dsystems (Fig. 5b and c), probably due to strong interactions between the fluorinated compounds present in Drug substance B and the

Table 5

Quantitative performance of on-line RPLCxRPLC for the analysis of three impurities in Drug sample A at 0.5 mg/mL. 2D-Conditions given in Table 1 (Set-up #2).

| Impurity | ¹ tr ^a | ² tr ^b | Linearity | inearity | | LOD | LOQ | RSD (%) | |
|-------------------|------------------------------|------------------------------|---|---------------------------------------|----------------------------|-----------------------------------|--------------------------|------------------------------|----------------------|
| | (min) | (s) | Standard curve ^c | Range | R ² | | | ² tr ^b | Sum of peak areas |
| # 1 # 2 # 3 | 6 17 22 | 10.85 11.96 14.16 | y = 43394x - 41.296 y = 259884x - 122.04 y = 218422x - 34.108 | 0.2% - 2% 0.05% - 2% 0.05% - 2% | 0.9996 0.9992 0.9991 | 0.05% < 0.05% < 0.05% | 0.2% 0.05% < 0.05% | 0.88 0.38 0.27 | 0.91 1.09 1.32 |

^a : Retention time in the first dimension.

^b : Retention time in the second dimension.

^c: Based on 4 points (impurity #1) and 6 points (impurities #2 and #3).



Fig. 5. On-line RPLCxRPLC-MS-TIC of a real sample (Drug substance B) with conditions optimized for (a) 2D-system #1, (b) 2D-system #2 and (c) 2D-system #3. The impurities circled in red are well separated with 2D-system #2 while not 2D-system #3. Experimental conditions for the three 2D-systems are given Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Fig. 6. On-line RPLCxRPLC separation of Drug substance B using 2D-system #2 with (a) UV detection (210 nm), and (b) MS-TIC detection. Experimental conditions are given Table 2.

pentafluorophenyl groups of the stationary phase. As a result, most impurities were probably not eluted from the first dimension stationary phase. This first 2D-system cannot be therefore selected for quantitative analysis. The two other RPLCxRPLC separations show quite similar peak distribution over the separation space which was not surprising considering that the two 2D-systems only differ by their first dimension stationary phase (see Table 2) and that, in the present specific case where API and most impurities are basic, selectivity is expected to be essentially governed by the difference in pH between both mobile phases. However, some impurities, circled in red in Fig. 5b and c, were well separated with 2D-system #2 while not separated with 2D-system #3. 2D-system #2 was therefore considered as the most appropriate for the quantitative analysis of Drug substance B. Fig. 6 shows the contour plots obtained with UV (Fig. 6a) and MS (Fig. 6b) detection, all detected impurities being specified in the figure. The comparison of both contour plots shows that some too little concentrated impurities (e.g. #9 and #10), were detected with MS only. On the other hand, due to ionization issues, impurity #3 were detected with UV only. These observations

highlight the benefit of combining both UV and MS detection when looking for impurities in pharmaceutical samples, as previously recommended [18]. Furthermore, the advantage of using on-line LCxLC instead of 1D-LC is emphasized by the observation of some coelutions in each dimension (e.g. API and impurity #4 in ¹D; impurities #7 and #8 in ²D) whereas all the peaks are well separated in RPLCxRPLC (Fig. 6). The concentration ratio of the detected impurities (compared with API) was estimated with the sum of peak areas using UV data. API purity was estimated at 91.82%, which is close to the known value provided by Oril industrie for this batch. Some impurities (i.e. #1, #4 and #6), with S/N > 10, could be quantified (assuming that all of them had the same UV molar absorptivity, as usually done for pharmaceuticals). Their concentrations (compared with API) were found to be 0.12%, 0.10%, and 7.64% respectively. Impurities #2, #3, #5, #7 and #8 with S/N ratios between 4 and 8.5 could be detected. Their concentrations were estimated between 0.04% and 0.09%. Finally, impurities #9 and #10 could only be detected with MS detection since they were present at too low concentration (< 0.02%) for UV detection. These quantitative results, in good agreement with the conclusions of Section 2.3, clearly show that on-line RPLCxRPLC is able to meet ICH requirement for the control of impurities in pharmaceutical samples.

4. Conclusions

The objective of this study was to assess the quantitative performances of on-line RPLCxRPLC in the context of pharmaceutical analysis considering the low detection and quantitation limits required by ICH guidelines.

Preliminary studies showed that (1) summing the second dimension peak areas was more reliable than calculating peak volumes for quantitative purpose, particularly at low concentration levels, and (2) online LCxLC could achieve LOD below 0.04%, in good agreement with ICH guidelines for the analysis of impurities and related substances of APIs.

A home-made procedure, based on both predictive calculation tools and Pareto-optimality approach, was applied to the optimization of RPLCxRPLC conditions. Two sets of conditions were selected, one in favour of high effective peak capacity, the other one in favour of low dilution (Set-ups #1 and #2, respectively). The resulting RPLCxRPLC separations showed that (1) peak intensities, for impurities at low level, were three to four-fold higher with Set-up #2 compared to Set-up #1, (2) unlike Set-up #1, Set-up #2 was able to detect impurities at concentrations as low as 0.05% with LOQ between 0.05% and 0.1% (compared with API). The evaluation of quantitative performances allowed to highlight (1) a good linearity ($R^2 > 0.999$) provided that the sum of peak areas was used for quantitative analysis; (2) a good repeatability on the retention times in both dimensions (RSD < 1%); and (3) a good precision on the sum of peak areas (RSD < 1.5%).

The optimized conditions were applied to the analysis of a real pharmaceutical sample (Drug substance B). Impurities present at concentrations as low as 0.04% (relative to API) were successfully detected with S/N ratios higher than 3 while impurities present at concentrations as low as 0.1% were detected with S/N ratios higher than 10. This real application made it possible to confirm the great potential of RPLCxRPLC-UV/MS for impurity control in drug development. Finally, it is important to note that this 2D-LC approach does not need complex additional set-up such as make-up solvent and/or focusing columns, thereby making the implementation of 2D-LC much easier in an industrial environment.

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M. Iguiniz et al.

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B. PROPOSITION D'UNE STRATEGIE D'ANALYSE

L'étude présentée dans la partie A de ce chapitre a permis de mettre en avant que la LCxLC en ligne, optimisée de façon à privilégier un faible facteur de dilution au détriment d'une très grande capacité de pics, est suffisamment sensible pour détecter des impuretés présentes à 0.05 % (par rapport au principe actif). Ainsi, il est possible de proposer une stratégie générique globale pour la mise en place d'analyses 2D-LC dans l'industrie pharmaceutique. Cette stratégie est présentée Figure 4.1, et peut être divisée en deux étapes.

La première étape (en bleu) consiste à sélectionner, parmi le set de trois combinaisons RPLC/RPLC développées dans le Chapitre 3, le jeu de conditions le plus adapté à l'échantillon que l'on veut analyser. Pour cela, l'échantillon est analysé en RPLCxRPLC-UV/MS avec les trois systèmes génériques, et les contour plots obtenus sont comparés afin de déterminer quel jeux de conditions est le plus intéressant. La comparaison peut se faire sur des critères comme le nombre de pics détectés ou encore la répartition des composés dans l'espace de séparation (une séparation LCxLC où les composés sont diagonalisés sera moins intéressante en termes de sélectivité, et le risque de co-élution sera élevé). Finalement, seul le jeu de conditions le plus adapté à l'échantillon est retenu pour la suite du processus.

La deuxième étape (en vert), quant à elle, consiste à choisir le mode 2D-LC à utiliser en fonction des besoins de l'analyse. La première question à se poser est « est-ce que l'analyse est quantitative ou qualitative ? ». Dans le cas d'une analyse qualitative, on peut distinguer deux types de tests. Tout d'abord le test d'identification, qui a pour objectif de déterminer la structure d'une impureté, par spectrométrie de masse. Dans ce cas, une analyse en mode heart-cutting peut être suffisante puisque l'objectif est d'isoler l'impureté que l'on veut identifier, avant d'effectuer une analyse structurale par spectrométrie de masse. Le second type de test est souvent appelé « Test limite » [3,4], il consiste à déterminer s'il y a présence ou absence d'impuretés au-dessus d'un certain seuil. Ce seuil peut donc être considéré comme une limite de détection (LOD), et une analyse en mode compréhensive est alors préconisée. En effet, ce mode 2D-LC permet (1) d'avoir un grand pouvoir de séparation, et donc des chances plus élevées de résoudre les co-élutions potentielles de l'échantillon, (2) de développer des analyses suffisamment sensibles pour atteindre des limites de détection ≤ 0.05 %. Ce type de méthodes, peut être utilisé pour pouvoir comparer, par exemple, différents lots d'un même échantillon, ou encore le contenu d'un même échantillon avant et après dégradation. L'étude consiste alors en une comparaison « d'images », faite à l'aide des contour plots. Enfin, dans le cas d'une analyse quantitative, qu'il s'agisse du dosage d'une impureté spécifique ou du contrôle d'un principe actif, c'est le mode sélective compréhensive (sLCxLC) qui est le plus adapté. En effet, par rapport au mode heart-cutting, ce mode permet (1) de transférer l'intégralité du pic de 1^{ère} dimension vers la 2^{ème} dimension, sans que le volume injecté en ²D soit trop élevé, et (2) de maintenir la séparation obtenue dans la première dimension. De plus, la contrainte de temps pour laquelle le temps d'analyse de seconde dimension est le temps de fractionnement de la première [10] n'est pas appliquée en sLCxLC, ce qui permet de choisir le temps de gradient de 2^{ème} dimension afin d'avoir une analyse plus sensible et plus efficace.



Figure 4.1 - Schéma décisionnel pour la mise en place d'analyses 2D-LC sur des produits en phase de développement

Evidemment, cette stratégie sert de guide mais peut être adaptée au cas par cas. Par exemple, dans le cas d'un échantillon avec un grand nombre d'impuretés co-éluées, et si la sensibilité est suffisante, on pourra utiliser le mode LCxLC pour le contrôle du principe actif au lieu du mode sLCxLC.

Pour finir, chaque méthode développée, qu'elle soit en mode LC-LC, LCxLC ou sLCxLC, peut être validée selon les exigences règlementaires. Les critères à valider peuvent varier selon le type de méthode analytique développée. En effet, toutes les caractéristiques d'une méthode (exactitude, robustesse, répétabilité, linéarité, sélectivité, sensibilité, etc.) ne sont pas obligatoirement à prendre en compte dans tous les cas. Le Tableau 4.2, issu des textes règlementaires, peut servir de guide pour déterminer les caractéristiques à valider pour chaque type de méthode [3,4].

| | Class A | Clas | s B | Class C | Class D |
|-------------------|---------|--------------|-------------|---------|---------|
| | | quantitative | qualitative | | |
| Accuracy | | Х | | Х | Х |
| Precision | | | | | |
| Repeatability | | Х | | Х | Х |
| Interm. Precision | | Х | | Х | Х |
| Specificity | Х | Х | Х | Х | Х |
| LOD | Х | | Х | | |
| LOQ | | Х | | | |
| Linearity & range | | Х | | Х | Х |

Tableau 4.2 - Caractéristiques à prendre en compte pour les différents types de méthodes analytiques.

Les différentes classes du tableau correspondent aux différents types de méthodes analytiques que l'on peut retrouver dans l'analyse pharmaceutique, à savoir :

- → Classe A : méthodes visant à établir l'identité d'un analyte dans une préparation pharmaceutique.
- → Classe B : méthodes destinées à détecter et doser les impuretés dans une préparation. Ces méthodes peuvent être quantitatives (dosage des impuretés) ou qualitatives (tests limites).
- → Classe C et D : méthodes visant à déterminer quantitativement la concentration d'un constituant principal (C) ou les caractéristiques (D) d'une préparation pharmaceutique.

C. TRANSFERT DE METHODE EN 2D-LC

La dernière question à se poser quant à l'application de la 2D-LC dans l'industrie pharmaceutique est le transfert de méthode. En effet, il a été montré dans ce chapitre et le précédent que la 2D-LC avait du potentiel pour l'analyse de composés pharmaceutiques, tant en termes de sélectivité que de sensibilité. Cependant, lorsqu'une méthode est développée puis validée, il se peut qu'elle soit transférée sur plusieurs instruments différents, dont les caractéristiques peuvent changer légèrement (volume de délai, volume externe, dispersion, etc.).

En 1D-LC, en gradient d'élution, il existe des règles pour pouvoir transférer une méthode tout en conservant la qualité de la séparation. En suivant ces règles, on peut alors transférer une méthode de l'HPLC à l'UHPLC, ou tenir compte d'une différence de volume de délai entre deux appareils. En effet, il est possible de modifier les dimensions et/ou le diamètre des particules de la colonne, le débit de phase mobile, ou encore l'appareillage utilisé, et de conserver la même résolution et la même sensibilité à condition de :

- travailler à t₀/t_G constant
- maintenir (t_D + t_{iso})/t₀ constant
- conserver le rapport $\sigma^2_{ext}/\sigma^2_{col}$ constant

En 2D-LC, tout va dépendre du mode dans lequel on travaille. Dans les modes « heartcutting » et « sélective compréhensive », les temps sont découplés grâce à un stockage temporaire des fractions, c'est-à-dire que le temps d'échantillonnage et le temps d'analyse de 2^{ème} dimension ne sont pas forcément égaux. Ainsi, il est possible d'optimiser indépendamment chacune des dimensions et donc d'appliquer les règles de transfert de méthode mentionnées précédemment. Dans le cas où les changements induisent une variation des temps de rétention en 1^{ère} dimension, il faut bien évidemment adapter les temps de début et de fin de collecte. En mode sélective compréhensive, une attention particulière devra tout de même être portée au taux d'échantillonnage, afin de conserver le même nombre de fraction par pic de 1^{ère} dimension.

En mode compréhensive, le problème est un peu plus compliqué. En effet, les deux dimensions ne doivent plus être considérées de façon indépendante mais comme un seul système. Lors du dimensionnement d'un tel système, tous les facteurs (conditions opératoires et contraintes instrumentales) sont interconnectés, et leur optimisation est loin d'être simple puisqu'ils influencent de façon contradictoire les différents critères de satisfaction d'une méthode chromatographique qui sont (1) une capacité de pics élevée, (2) une faible dilution et (3) un temps d'analyse acceptable [11,12].

Afin de mettre en avant les problèmes qui peuvent être engendrés par des variations instrumentales, un mélange PA et intermédiaires de synthèse du projet S68502 a été analysé en RPLCxRPLC-UV/MS avec l'un des systèmes 2D génériques du Chapitre 3. Trois configurations instrumentales différentes ont été utilisées et la comparaison des contour plots obtenus a permis de mettre en évidence l'impact de la variation de certains facteurs sur la qualité de la séparation. Le détail des conditions LCxLC est donné dans le Tableau 4.3.

| | 1 ^{ere} dimension | 2 ^{eme} dimension |
|-----------------------|--------------------------------|---------------------------------|
| Phase stationnaire | Xselect HSS PFP | Acquity CSH C18 |
| Dimensions de colonne | 50 x 2.1 mm ; 3.5 μm | 30 x 2.1 mm ; 1.7 μm |
| Phases Mobiles | A : H ₂ O + 5 mM AA | A : H ₂ O + 0.1 % FA |
| | B : ACN | B : ACN + 0.1 % FA |
| Débit | 0.1 mL/min | 1.8 mL/min |
| Gradient d'élution | 27 à 92 % (B) en 60 min | 5 à 90 % (B) en 0.17 min |
| Température | 30°C | 80°C |
| V _{inj} | 2 μL | 11 μL |
| Split | 1:3 | - |
| Détection | UV 210 nm (10 Hz) | UV 210 nm (40 Hz) |
| | | MS scan de 200 à 550 (20 Hz) |

Tableau 4.3 - Conditions RPLCxRPLC-UV/MS pour l'analyse de l'échantillon S68502 avec différentes configurations instrumentales

La Figure 4.2 représente, pour la même analyse et le même échantillon, les séparations obtenues dans le cas où (1) des systèmes de pompage différents sont utilisés en 1^{ère} dimension, et (2) les tubes utilisés en 2^{ème} dimension sont légèrement différents. La Figure 4.3, quant à elle, montre les contour plots obtenus pour la même analyse et le même échantillon, dans le cas où les instruments sont similaires en 1^{ère} dimension, mais présentent d'importantes différences en 2^{ème} dimension.

De très légères différences, lorsqu'elles sont opérées en 1^{ère} dimension, peuvent être gérables. En effet, dans le cas de l'instrument 1 (Figure 4.2a) la pompe de 1^{ère} dimension est un système binaire et le volume de délai est de 140 μ L, tandis que pour l'instrument 2 (Figure 4.2b) il s'agit d'un système quaternaire, ce qui induit un volume de délai plus important de l'ordre de 600 μ L. Cette différence entraîne décalage des temps de rétention le long de la 1^{ère} dimension, mais cela n'a qu'un impact négligeable sur la séparation LCxLC puisque les pics ont la même largeur en 1^{ère} dimension, donc le taux d'échantillonnage est inchangé. Une simple application des règles de transfert de méthode, comme l'ajout d'un palier isocratique modulable avant le début du gradient de 1^{ère} dimension permettrait de prendre en compte cette différence de volume de délai, et de pouvoir obtenir des contour plots superposables.

En revanche, lorsque les variations sont faites sur la 2^{ème} dimension, le problème est un peu plus complexe. En examinant de plus près la 2^{ème} dimension des analyses présentées Figure 4.2, on note un décalage des temps de rétention d'environ 2 secondes entre les deux instruments, ainsi qu'une largeur de pic plus importante pour l'instrument 2 (Figure 4.2b). Ces différences sont liées à un changement des tubes en 2^{ème} dimension, qui génèrent un volume de délai et une dispersion plus importants. L'utilisation des règles de transfert permettant de prendre en compte ce délai consisterait à rajouter un palier isocratique en début de gradient de 2^{ème} dimension pour l'instrument 1. Cependant, cela implique une augmentation du temps d'analyse en 2^{ème} dimension, qui est lié aux conditions de 1^{ère} dimension. La meilleure solution reste donc de ré optimiser l'analyse LCxLC. De plus, on peut noter que ce changement de tube entraîne une légère augmentation de la largeur des pics de 2^{ème} dimension, du fait d'une dispersion extra-colonne plus importante, ce qui a pour effet une diminution de la capacité de pic en 2^{ème} dimension.



Figure 4.2 - Séparations RPLCxRPLC du mélange contenant le PA et les intermédiaires¹ de synthèse du projet S68502 avec un système de pompage de ¹D (a) binaire et (b) quaternaire et des tubes légèrement différents en ²D. Contour plots tracés avec les données UV à 210 nm

Enfin, le cas extrême est illustré par la Figure 4.3, qui présente les contour plots obtenus pour la même analyse et le même échantillon, dans le cas où les instruments présentent d'importantes différences en 2^{ème} dimension. Par rapport à l'instrument 1 (Figure 4.3a), les tubes de 2^{ème} dimension de l'instrument 3 (Figure 4.3b) ont un diamètre interne plus faible. Cela génère des problèmes de pression qui empêchent de travailler à un débit aussi élevé qu'avec l'instrument 1 (F = 1.4 mL/min au lieu de 1.8 mL/min). L'utilisation des règles de transfert permettant de prendre en compte ce changement de débit est ici impossible car elle impliquerait une augmentation du temps de gradient et donc du temps d'analyse en 2^{ème} dimension, or ce temps d'analyse est également lié aux conditions de 1^{ère} dimension. L'analyse a donc été lancée avec une diminution du débit, sans adapter le temps de gradient en 2^{ème} dimension. La principale conséquence est que la pente normalisée du gradient n'est plus la même, ce qui va avoir un impact sur la composition à l'élution des composés et donc sur la qualité de la séparation et la capacité de pics en 2^{ème} dimension. De plus, le diamètre interne des tubes utilisés pour les boucles d'échantillonnage de l'interface est deux fois plus grand pour l'instrument 3, ce qui génère une dispersion extra-colonne beaucoup plus importante. En conséquence, malgré un volume de boucle identique, les pics de 2^e dimension sont beaucoup plus larges avec l'instrument 3, ce qui a pour effet de diminuer de façon drastique la capacité de pics de la 2^{ème} dimension.

¹ Les légères différences que l'on peut observer entre les deux contour plots de la figure (taches présentes sur l'un des contour plots mais par sur l'autre) sont liées à la préparation des échantillons



Figure 4.3 - Séparations RPLCxRPLC du mélange contenant le PA et les intermédiaires de synthèse du projet S68502 avec deux instruments présentant des différences de diamètres de tube sur la 2^{ème} dimension. Contour plots tracés avec les données MS (TIC)

En conclusion, lorsque de légères variations sont faites sur la 1^{ère} dimension, mais que la 2^{ème} dimension reste rigoureusement identique, le transfert de méthode LCxLC d'un appareil à l'autre est possible, mais il faut tout de même rester vigilant quant à la conservation du taux d'échantillonnage des pics de 1^{ère} dimension. En revanche, dans le cas où les variations instrumentales sont situées au niveau de la 2^{ème} dimension, il est très compliqué voire impossible de transférer la méthode simplement en appliquant les règles de transfert couramment utilisées en 1D-LC. Cela est essentiellement lié à l'interconnexion d'un grand nombre de facteurs entrant en jeux dans l'optimisation de méthodes LCxLC. De ce fait, la moindre modification d'un paramètre, même de 2^{ème} dimension, a un impact non négligeable sur la séparation bidimensionnelle dans sa globalité. Dans ce cas, il est nécessaire de redimensionner le système LCxLC afin d'obtenir des performances équivalentes.

Une étude a été menée afin d'évaluer les performances quantitatives de la LCxLC dans l'objectif de mettre en place une stratégie 2D-LC générique pour l'analyse d'échantillons pharmaceutiques.

Deux approches différentes ont été appliquées au dimensionnement d'un même système 2D-LC : (1) une optimisation visant à trouver le meilleur compromis entre capacité de pics élevée et faible dilution, et (2) une optimisation ayant pour objectif de minimiser le facteur de dilution au détriment de la capacité de pics. La comparaison des séparations LCxLC-UV obtenues a montré que seule la deuxième approche permettait d'atteindre une sensibilité suffisante pour pouvoir satisfaire aux exigences règlementaires. Les limites de détection et de quantification ont été estimées, et les analyses ont montré une linéarité équivalente à la LC, ainsi qu'une très bonne répétabilité. Finalement, les trois systèmes LCxLC génériques développés dans le Chapitre 3 ont été optimisés selon cette deuxième approche. Appliqués à l'analyse LCxLC-UV/MS d'un lot de principe actif, ils ont permis de détecter des impuretés présentes à des concentrations ≤ 0.05%.

En tenant compte de ces performances, et des exigences règlementaires pour l'analyse pharmaceutique, une stratégie générique en deux étapes a été proposée pour la mise en place d'analyses 2D-LC. Un screening avec les trois systèmes RPLCxRPLC génériques permet de trouver les conditions chromatographiques adaptées à l'échantillon. Puis un arbre de décision permet de choisir le mode de 2D-LC à utiliser en fonction des besoins de la méthode. En effet, dans le cas d'une méthode destinée au dosage le mode sLCxLC sera le choix le plus judicieux tandis que pour une méthode de type « test limite » visant à déterminer l'absence ou la présence d'un composé au-dessus d'un certain seuil le mode LCxLC sera préconisé. Enfin le mode LC-LC, quant à lui, est adapté à des analyses qualitatives visant à « isoler » une impureté en vue d'en identifier la structure par spectrométrie de masse.

Enfin, la question du transfert de méthode a été abordée. En LC-LC et sLCxLC, le transfert de méthode peut être envisagé en appliquant les règles de transfert destinées à la LC. En revanche, la comparaison de la même analyse LCxLC effectuée sur différents instruments a permis de mettre en évidence que le transfert de méthode pouvait être difficile, voire impossible, en LCxLC. En effet, du fait de l'interconnexion d'un grand nombre de facteurs entrant en jeux dans l'optimisation de méthodes LCxLC, la moindre modification d'un paramètre, a un impact non négligeable sur l'analyse LCxLC dans sa globalité.

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Chapitre 4 : Mise en place d'une stratégie 2D-LC générique. Partie 2 | 108

CHAPITRE 5

Intérêt et développement du couplage entre la LC et la SFC pour l'analyse d'échantillons pharmaceutiques

Ce chapitre a fait l'objet d'une publication :

Article 4

"On-line coupling of achiral Reversed Phase Liquid Chromatography and chiral Supercritical Fluid Chromatography for the analysis of pharmaceutical compounds"

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Au cours des dernières années, la Chromatographie en Fluide Supercritique (SFC) a connu un regain d'intérêt considérable. Ce changement a notamment été stimulé par l'introduction d'une nouvelle génération d'appareils provenant des principaux fournisseurs d'instrumentation chromatographique, décidés à faire avancer cette technologie.

Dès lors de nombreux travaux ont été menés dans le but de mettre en avant les avantages de la SFC dans de nombreux domaines d'application tels que l'analyse de produits pétroliers, de produits naturels ou encore l'analyse alimentaire [1].

Pour ce qui est de l'industrie pharmaceutique, la SFC s'est avérée être une alternative intéressante à la LC pour l'analyse de composés chiraux [2,3]. Mais c'est également une technique complémentaire à la LC dans le cas de l'analyse achirale d'échantillons pharmaceutiques [4,5].

Dans ce chapitre, la possibilité de coupler la SFC et la RPLC en ligne pour l'analyse d'échantillons pharmaceutiques a été explorée à travers deux cas d'application différents :

- (1) Un couplage RPLCxSFC en mode « compréhensive » dans le cadre de la mise en place d'analyses 2D génériques (Chapitres 3 et 4). Le potentiel de ce couplage a été évalué en termes d'orthogonalité et de capacité de pics, et comparé à celui du couplage RPLCxRPLC.
- (2) Un couplage sRPLCxSFC chirale en mode « sélective compréhensive » pour l'analyse de composé chiraux. Les possibilités offertes par la 2D-LC pour l'analyse chirale étant connues depuis de nombreuses années, c'est ici le potentiel de la SFC utilisée en 2^{ème} dimension qui a été mis en avant.

A. LCxSFC POUR L'ANALYSE DE COMPOSES PHARMACEUTIQUES ET COMPARAISON AVEC LA LCxLC

1. Recherche de conditions intéressantes

1.1. Protocole expérimental

La première étape consiste à acquérir les données de rétention dans différentes conditions chromatographiques, pour les deux échantillons S56922 et S68502 (également utilisés dans le Chapitre 3). La description de ces échantillons a été donnée précédemment (cf. Chapitre 2 Partie D). La phase mobile a été choisie d'après une étude récente de l'équipe de C. West [6]. Au cours de ces travaux sur le développement d'une méthode d'analyse de composés pharmaceutiques en SFC achirale, différents additifs ont été testés dans la phase mobile et les résultats obtenus ont été comparés avec ceux d'une analyse faite sans additif. L'application de fonctions de désirabilité de Derringer a permis de comparer les performances des différentes conditions testées. L'acétate d'ammonium et l'hydroxyde d'ammonium semblent donner les meilleurs résultats en termes de performance chromatographique.

Les séparations sont effectuées en gradient d'élution de 5 à 50 % de co-solvant avec une pente normalisée de 5 %. La température est restée fixée à 30°C. Les phases stationnaires utilisées pour ce screening de conditions sont à base de matériaux différents : silice, polymère organique, zircone. Les différentes colonnes utilisées sont listées dans le Chapitre 2.

Dans une deuxième étape, des diagrammes de rétention en deux dimensions sont tracés pour chaque combinaison LC/SFC (en utilisant les données de l'étude du Chapitre 3) et SFC/SFC. Chaque axe du diagramme correspond aux compositions à l'élution des composés dans le système chromatographique sélectionné. Le calcul du degré d'orthogonalité (Od) et l'évaluation de la capacité de pics pratique sont fait de la même façon que dans le Chapitre 3.

1.2. Orthogonalité et capacité de pic pratique

Après un premier examen des résultats, plusieurs colonnes sont retirées de l'étude. En effet, les colonnes Hypercarb, Discovery Zr Carb C18 et Discovery Zr PBD se sont avérées beaucoup trop rétentives, avec une majorité de composés élués après la montée du gradient. Au contraire, les colonnes Synergie Hydro RP, Torus 2-PIC, Daicel SFC-A et Daicel SFC-B se sont montrées trop peu rétentives, avec une majorité de composés co-élués sur le palier isocratique pour les deux échantillons testés.

Les résultats obtenus pour l'échantillon S56922 sont illustrés par la Figure 5.1 qui représente la capacité de pic pratique (n_{2D}^*) en fonction du degré d'orthogonalité (O_d) . Chaque point du graphique représente une combinaison [dimension1] x [dimension2]. Les différentes possibilités de combinaisons LC/LC, LC/SFC et SFC/SFC ont été superposées dans le but de pouvoir comparer les différents couplages. Les valeurs obtenues dans le cas des

combinaisons LC/LC sont issues du Chapitre 3, et le détail des valeurs obtenues pour les combinaisons LC/SFC et SFC/SFC est donné en Annexe 1.



Figure 5.1 - Capacité de pic pratique en fonction du degré d'orthogonalité pour l'échantillon S56922.

Dans le cas des combinaisons LC/LC (triangles bleus) les valeurs de degré d'orthogonalité sont comprises entre 0.5 et 10 et la capacité de pic pratique varie de 500 à 12000. Des explications détaillées sur les trois groupes de systèmes 2D (A, B et C) entourés sur la Figure 5.1 sont données dans le Chapitre 3. Les combinaisons couplant la LC et la SFC (losanges violets) génèrent des valeurs comprises entre 1 et 4.5 pour O_d et des valeurs de n_{2D}* allant de 500 à 7000. En conséquence, la majorité des combinaisons LC/SFC se situent en dehors des zones d'intérêt (groupes A, B et C) de la Figure 5.1. En d'autres termes, le couplage LC/SFC semble moins intéressant que le couplage LC/LC pour la mise en place de méthodes 2D génériques pour l'analyse de composés pharmaceutiques. Cela peut sembler surprenant puisque la SFC est connue pour avoir des sélectivités différentes de la RPLC. On pourrait donc s'attendre à ce qu'un couplage RPLC/SFC génère un degré d'orthogonalité plus élevé qu'un couplage RPLC/RPLC.

L'explication est donnée par la formule de calcul du degré d'orthogonalité (Eq. 5 Chap. 3) qui prend notamment en compte les plages de compositions à l'élution des composés dans les deux dimensions. La différence entre les combinaisons RPLC/RPLC et RPLC/SFC est illustrée par la Figure 5.2 qui présente un exemple de diagramme de rétention en deux dimensions obtenus pour ces deux couplages. Afin de permettre une meilleure comparaison, les mêmes conditions chromatographiques sont envisagées en première dimension (axe des abscisses). Dans le cas de la combinaison RPLC/RPLC, les plages de compositions à l'élution en 1^{ère} et 2^{ème} dimensions sont d'environ 70 %. Ainsi, bien que le taux d'occupation de l'espace de séparation γ soit de 0.8, le degré d'orthogonalité atteint une valeur de 6.4. Dans le cas de la combinaison RPLC/SFC, la plage de compositions à l'élution en première dimension est de 70 % mais celle de deuxième dimension n'est que de 23 %. Cela explique pourquoi, malgré un meilleur taux d'occupation de l'espace ($\gamma = 1$), le degré d'orthogonalité généré par la combinaison RPLC/SFC est bien inférieur (Od = 2.6) à celui généré par la combinaison RPLC/RPLC. Il devient donc aisé de comprendre le positionnement des combinaisons SFC/SFC (carrés oranges) sur le graphique n_{2D}* = f(O_d) présenté Figure 5.1.



Figure 5.2 - Diagrammes de rétention en deux dimensions obtenus dans le cas (a) d'une combinaison RPLC/RPLC et (b) d'une combinaison RPLC/SFC.

Quelques combinaisons RPLC/SFC restent néanmoins intéressantes et en compétition avec les combinaisons RPLC/RPLC. Il s'agit des systèmes localisés dans le groupe B du graphique $n_{2D}^* = f(O_d)$. Ces combinaisons sont listées dans le Tableau 5.1.

| Combinaison | Dimension 1 | Dimension 2 |
|-------------|--------------------------|---------------|
| #1 | CSH C18 - ACN / AF | Zorbax Rx SIL |
| #2 | CSH C18 - ACN / AMS | Zorbax Rx SIL |
| #3 | BEH Phenyl - ACN / NH₄OH | Zorbax Rx SIL |
| #4 | CSH C18 - ACN / AA | Zorbax Rx SIL |
| #5 | CSH C18 - ACN / AF | Zorbax SB CN |
| #6 | CSH C18 - ACN / AMS | Zorbax SB CN |

Tableau 5.1 - Combinaisons intéressantes pour un couplage en ligne RPLCxSFC

Il apparaît que les combinaisons RPLC/SFC qui sont compétitives avec les couplages RPLC/RPLC correspondent à l'utilisation de colonnes polaires, de type phase normale, utilisées en mode SFC en seconde dimension. Les autres phases stationnaires testées en SFC, qui sont plutôt de type phase inverse, ne permettent pas de générer des degrés d'orthogonalité suffisants pour pouvoir être en compétition avec les combinaisons RPLC/RPLC. La combinaison #2 a été retenue pour la mise en œuvre de l'analyse RPLCxSFC en ligne d'un échantillon pharmaceutique.

Cette étude a également été menée sur un deuxième échantillon (S68502), avec un set réduit de conditions LC et la totalité des conditions SFC. Les mêmes conclusions ont été tirées

pour la comparaison des combinaisons RPLC/RPLC, RPLC/SFC et SFC/SFC en termes de degré d'orthogonalité. Les combinaisons du Tableau 5.1 sont également les combinaisons qui sont ressorties comme étant les plus compétitives par rapport à un couplage RPLC/RPLC.

2. Mise en place du couplage RPLCxSFC

2.1. Etude préliminaire

Lors d'une analyse 2D-LC en ligne, le solvant d'injection de 2^{ème} dimension est l'éluant provenant de la 1^{ère} dimension. Dans le cas d'un couplage RPLC/SFC, cela signifie que l'on va injecter en SFC un mélange eau/acétonitrile, ce qui n'est pas courant. Plusieurs études récentes ont montré qu'en SFC le solvant d'injection avait un impact significatif sur la forme de pics, et donc sur la qualité du chromatogramme obtenu lors de l'analyse [7–10].

De plus, dans le cas d'une analyse bidimensionnelle en ligne, le volume injecté en 2^{ime} dimension ($^{2}V_{inj}$) est directement lié au débit de 1^{ire} dimension (^{1}F) et au temps d'échantillonnage (t_{s}) par l'équation suivante :

$${}^{2}V_{inj} = t_{s} \times {}^{1}F \times z$$
 Eq. 1

avec z le ratio de split réduisant le débit au niveau de l'interface ($0 \le z \le 1$). Cependant, dans le cas d'un couplage RPLC/SFC en ligne, il est impossible d'utiliser un split entre les deux dimensions, du fait de la dépressurisation du CO₂ lorsque la vanne (interface) change de position. Il est donc important de déterminer quel est le volume maximum que l'on peut injecter en ²D SFC, sans déformation de pics, afin de pouvoir dimensionner l'analyse bidimensionnelle.

Pour cela, une étude préliminaire a été réalisée en SFC, dans les conditions initiales de 2^{ème} dimension (8 % B), sur l'un des composés du projet S56922. Il s'agit d'un composé peu retenu en 2^{ème} dimension, qui est donc plus susceptible de subir les effets à l'injection liés au volume injecté et au solvant d'injection qu'un composé très retenu sur la colonne. Le soluté a été dissous dans différents mélanges H₂O/ACN qui correspondent à la composition de la phase mobile au début, au milieu et à la fin du gradient de première dimension (5 %, 50 % et 95 % ACN). Les résultats de cette étude sont présentés sur la Figure 5.3, qui montre la superposition des chromatogrammes obtenus dans les différents solvants d'injection testés, pour des volumes injectés de 1 μ L, 5 μ L, et 10 μ L.

Jusqu'à 5 μ L injectés (soit 2.5 % du volume mort de la colonne), aucune dégradation significative de la forme de pic n'est observée, même si l'on note une légère déformation de pic pour le solvant d'injection contenant le moins d'acétonitrile. Lorsque le volume injecté est de 10 μ L (5 % du volume mort de la colonne), on observe de très mauvaises formes de pics, pour des solvants d'injection contenant 50 à 95 % d'eau. De plus, la Figure 5.3 met en évidence la présence d'un décalage de la rétention, qui augmente avec le pourcentage d'eau contenue dans le solvant d'injection et le volume injecté. Ce décalage peut être attribué à deux phénomènes combinés : (1) une affinité importante entre l'eau et les sites polaires de la phase stationnaire et (2) une forte affinité du composé pour l'eau. Par conséquent, lorsque le créneau d'injection entre dans la colonne, l'analyte va interagir préférentiellement avec la phase stationnaire ce qui a pour effet une augmentation de la rétention [10].



Figure 5.3 - Effets du solvant d'injection et du volume injecté sur la forme de pic en SFC. Soluté : Impureté isolée du S56922. Conditions : Viridis Silica 50x3 mm 5 μm ; 2.5 mL/min ; 30°C ; BPR = 150 bar ; isocratique 8 % co-solvant MeOH/H₂O 98/2 (v/v) + 20 mM AA ; UV 210 nm

Au vu de ces résultats, le volume maximum injecté en ²D-SFC est fixé à 5 μ L, dans le but de limiter les effets à l'injection que l'on pourrait observer en 2^{ème} dimension. Le dimensionnement de l'analyse bidimensionnelle est donc fait à partir de cette valeur.

2.2. Dimensionnement

Le dimensionnement d'une analyse bidimensionnelle prend en compte un certain nombre de paramètres parmi lesquels certains sont fixes (soit liés à l'appareillage soit fixés par l'utilisateur) et d'autres peuvent être ajustés. Dans le cas de l'analyse RPLCxSFC développée dans ce chapitre, les paramètres fixes sont les suivants :

| Volume injecté en ² D : | 5 μL |
|---|---|
| Débit ² D : | 3.5 mL/min |
| Volume mort de la colonne en ² D : | 212 μ L (soit ² t ₀ = 0.06 min) |
| Volume de délai de la ² D : | 300 μ L (soit ² t _D = 0.09 min) |

| Tableau 5.2 - Paramètres fixés pour l | e dimensionnement | de l'analyse RPLCxSFC |
|---------------------------------------|-------------------|-----------------------|
|---------------------------------------|-------------------|-----------------------|

En travaillant avec un débit de 10 μ L/min, on obtient d'après l'équation 1 un temps d'échantillonnage de 0.5 min, qui correspond également au temps d'analyse en 2^{ème} dimension (²t). Ce temps peut être décomposé de la façon suivante :

$${}^{2}t = {}^{2}t_{0} + {}^{2}t_{D} + {}^{2}t_{G} + {}^{2}t_{desc} + {}^{2}t_{seq}$$
 Eq. 2

Avec ${}^{2}t_{0}$ le temps mort de la colonne, ${}^{2}t_{D}$ le temps de délai en 2^{ème} dimension. ${}^{2}t_{G}$ et ${}^{2}t_{desc}$ correspondent respectivement aux temps de gradient et de retour aux conditions initiales en 2^{ème} dimension, ce dernier est fixé à 0.01 min. Enfin ${}^{2}t_{seq}$ est un temps additionnel qui est lié au montage utilisé pour cette analyse. En effet, comme il n'existe pas d'instrument commercial dédié à ce genre de couplage, deux appareils différents sont utilisés (cf. Chapitre 2 Partie A) et la programmation des analyses de 2^{ème} dimension est faite sous forme de séquence. ${}^{2}t_{seq}$ correspond au temps que met le logiciel de pilotage de l'instrument pour passer d'une ligne de la séquence à la suivante, il a été évalué à 0.2 min. Ce temps est incompressible, mais peut être utilisé pour le rééquilibrage de la colonne de 2^{ème} dimension à la fin du gradient.

D'après l'équation 2, sur les 0.5 min de temps d'analyse de 2^{ème} dimension, 0.36 min sont occupées par des temps incompressibles (²t₀, ²t_D, ²t_{seq} et ²t_{desc}), il reste donc 0.14 min pour le gradient d'élution.

2.3. Séparation RPLCxSFC d'un échantillon pharmaceutique

L'analyse RPLCxSFC-UV a été mise en place sur l'échantillon S56922. Le mélange est constitué du principe actif (PA) et de ses intermédiaires de synthèses, disponibles sous forme isolée. Afin de faciliter l'interprétation des résultats, et de pouvoir comparer facilement l'analyse RPLCxSFC avec les systèmes RPLCxRPLC mis en place dans les Chapitres 3 et 4, tous les composés sont introduits dans le mélange à la même concentration de 0.1 mg/mL. Le but de cette étude étant de comparer le potentiel de différents couplages en termes d'orthogonalité et de capacité de pic, il n'est pas nécessaire d'avoir un mélange représentatif d'un échantillon pharmaceutique en termes de rapports de concentration PA/Impuretés. Le détail des conditions est donné dans le Tableau 5.3.

| | 1 ^{ère} dimension | 2 ^{ème} dimension | |
|-----------------------|----------------------------------|---|--|
| Phase stationnaire | Xbridge C18 | Viridis silica | |
| Dimensions de colonne | 50 x 1 mm ; 3.5 μm | 50 x 3 mm ; 5 μm | |
| Phasas mobiles | A : H ₂ O + 0.1 % AMS | A : CO ₂ | |
| Phases mobiles | B : ACN + 0.1 % AMS | B : MeOH/H ₂ O 98/2 (v/v) + AA 20 mM | |
| Débit | 0.01 mL/min | 3.5 mL/min | |
| Gradient d'élution | 10 à 80 % (B) en 84 min | 10 à 55 % (B) en 0.14 min | |
| Température | 30°C | 30°C | |
| V _{inj} | 3 μL | 5 μL | |
| Détection | UV 210 nm (10 Hz) | UV 210 nm (40 Hz) | |

Tableau 5.3 - Conditions d'analyses RPLCxSFC-UV pour l'analyse de l'échantillon S56922

La séparation RPLCxSFC obtenue est présentée Figure 5.4 sous la forme d'un contour plot tracé à partir des données UV. Par rapport aux séparations RPLCxRPLC obtenues sur le même échantillon au Chapitre 3, le nombre de composés détectés est beaucoup moins élevé. Cela peut être attribué à une dilution plus importante dans le cas d'une analyse RPLCxSFC, qui résulte de l'effet cumulé de plusieurs facteurs tels que le volume externe de la 2^{ème} dimension qui est dix fois plus grand en RPLCxSFC qu'en RPLCxRPLC (83 µL vs 8.5 µL), ainsi que le volume

de cellule du détecteur de 2^e dimension qui est de 8 µL en SFC contre 0.6 µL en RPLC. Ces différences instrumentales induisent une variance extra-colonne cinq à six fois plus élevée lorsque la SFC est utilisée en 2^{ème} dimension. Cette variance entraîne donc une dilution importante des composés, ce qui peut expliquer la perte de sensibilité en RPLCxSFC par rapport à une analyse RPLCxRPLC. Cette différence de sensibilité avait déjà été notée lors de travaux antérieurs, visant à étudier le potentiel du couplage RPLCxSFC en ligne pour l'analyse d'une bio-huile [10]. Cependant, pour ce qui est des composés que l'on arrive à détecter, leur répartition dans l'espace de séparation est assez aléatoire, ce qui est en accord avec les valeurs de γ trouvées pour les combinaisons RPLC/SFC dans l'étude menée pour rechercher des conditions intéressantes en 1^{ère} et en 2^{ème} dimension (Partie A.1).



Figure 5.4 - Séparation RPLCxSFC-UV du mélange contenant le PA et les intermédiaires de synthèse du projet S56922. Les conditions sont données dans le Tableau 5.3, et le montage utilisé est détaillé dans le Chapitre 2

Enfin, la capacité de pic effective a été évaluée à $n_{2D,eff} \approx 500$ pour un temps d'analyse de 85 min. Ces performances sont en dessous de celles obtenues en RPLCxRPLC (Chapitre 3), puisque des capacités de pic effectives de l'ordre de 1000 ont été atteintes en 50 min quel que soit le système LCxLC considéré. Ces observations sont également en accord avec les résultats théoriques prédits lors de l'étude préliminaire faite dans la Partie A.1. Cette différence de performances peut être en partie attribuée aux difficultés instrumentales liées au montage, comme une dispersion extra-colonne beaucoup plus importante qui entraîne une perte d'efficacité en ²D-SFC par rapport à une ²D-RPLC.

B. sLCxSFC POUR L'ANALYSE ACHIRALE-CHIRALE DE COMPOSES PHARMACEUTIQUES

Article 4

"On-line coupling of achiral Reversed Phase Liquid Chromatography and chiral Supercritical Fluid Chromatography for the analysis of pharmaceutical compounds"

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On-line coupling of achiral Reversed Phase Liquid Chromatography and chiral Supercritical Fluid Chromatography for the analysis of pharmaceutical compounds



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ABSTRACT

On-line selective comprehensive two-dimensional chromatography combining Reversed Phase Liquid Chromatography and Supercritical Fluid Chromatography (sRPLCxSFC) was investigated for the analysis of chiral pharmaceutical compounds. Preliminary studies were carried out with the aim of overcoming instrumental constraints which are related to such 2D-coupling. The impact of both injection solvent and injection volume on the chiral SFC second separation was assessed with a view to limiting injection effects due to mobile phase compatibility issues between both dimensions. The resulting on-line sRPLCxSFC system was applied to the achiral x chiral analysis of a pharmaceutical sample. Using an Acquity BEH C18 column in the first dimension and a Chiralpak IC column in the second one, both chemical (achiral) and enantiomeric (chiral) purities could be evaluated in less than 50 min within a single run. Under such conditions, a detection limit of about 0.5% for R-enantiomer could be obtained with UV detection. The results obtained in sRPLCxSFC were compared to those obtained in conventional chiral 1D-SFC. Baseline resolution was obtained in both cases and the linearity in the detector response was on the same order of magnitude (R² > 0.99). Finally, despite current instrumental limitations (no commercially available system for sLCxSFC, large dwell volume and large extra-column volume in SFC), the on-line coupling of RPLC and SFC appears to be attractive and promising for rapid achiral/chiral analysis of complex pharmaceutical samples.

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1. Introduction

Chirality is a fundamental characteristic of living organisms. That is why our body is able to recognize a chiral component and why essential physiological processes are based on enantioselectivity. There can be marked differences between the pharmacological activity of enantiomers. In many cases, whereas one enantiomer generates the desired therapeutic response (eutomer), the other one is either less active, inactive or even toxic (distomer) [1]. Several examples were reported in the literature, including some of the most famous, such as the thalidomide disaster [2] or the amphetamine case [3].

Nowadays, regulatory authorities impose strict guidelines for the commercialization of chiral drug substances [4–6]. First of all, it is recommended that only the therapeutically active enantiomer of a chiral drug can be brought to market. It is therefore

* Corresponding author. *E-mail address*: sabine.heinisch@univ-lyon1.fr (S. Heinisch). necessary to study the pharmacological properties and metabolic pathways of each enantiomer separately before making a decision. Furthermore, in pharmaceutical industry, Active Pharmaceutical Ingredients (APIs) have to be fully characterized according to ICH guidelines [7]. For chiral compounds, such requirement leads to the development of, at least, two different analytical methods: an achiral (most often RPLC) method, generally used to assess the chemical purity (amount of achiral impurities and related substances) and a chiral method to assess the enantiomeric purity (amount of undesired enantiomer). However, such analytical procedure makes the API process development time consuming and laborious as soon as API and/or related compounds are chiral, which is often the case.

Over the last decades multidimensional chromatography has emerged as an alternative for the analysis of complex samples when one-dimensional chromatography fails to obtain satisfactory separation. 2D-LC separations can be heart-cutting (LC-LC), comprehensive (LCxLC) or selective comprehensive (sLCxLC) in approach [8,9]. In LC-LC, a fraction of the first dimension separation is transferred to the second one whereas in LCxLC the entire sample is subjected to a separation in both dimensions. This is done

https://doi.org/10.1016/j.jpba.2018.06.058 0731-7085/© 2018 Elsevier B.V. All rights reserved. by storing and analysing, one after the other, the successive fractions from the first dimension. In sLCxLC, a selected region of the first dimension is sampled in a comprehensive way and transferred to the second dimension. In the pharmaceutical industry, 2D-LC has been successfully applied to enantioselective analysis as well as to the direct determination of enantiomeric excess in reaction mixtures [10–13]. However, the achiral quantitative analysis in the first dimension has been little considered so far. 2D-LC approach able to assess both chemical and enantiomeric purities together should be very advantageous during API process development thereby significantly reducing sample preparation, analysis time and data treatment. Furthermore, its development should be facilitated by ultrafast chiral chromatography which has been recently proposed [14]

Whereas SFC is becoming the preferred choice, some chiral separations are still performed in Normal Phase Liquid Chromatography (NPLC). However this separation technique is poorly compatible with RPLC for a RPLCxNPLC separation perspective. Firstly, the nonmiscibility of solvents can cause severe band broadening, peak distortions or artifacts [15]. Secondly, potentially long equilibration and analysis times are usually required in chiral NPLC and thirdly the use of large volumes of toxic solvents in NPLC can also be mentioned as an environmental issue. In the recent past years, SFC has been found to be an excellent alternative to NPLC for the enantioseparation of pharmaceutical compounds, and has gradually replaced NPLC in both analytical and purification areas [16-20]. In addition to being a greener technique compared to NPLC, SFC provides higher efficiency with faster analyses and requires much lower equilibration times. Furthermore chiral SFC usually outperforms chiral RPLC in terms of selectivity and analysis time [21].

The potential of on-line RPLCxSFC was first investigated by Sarrut et al. for bio-oil analysis using conventional sample loops [22] and more recently for depolymerised lignin samples using trapping columns [23]. In both cases, the authors highlighted the very high degree of orthogonality between first and second dimension, but also the difficulties involved by CO₂ depressurization that requires full loop injection and also by the presence of hydro-organic solvents in RPLC that requires low injection volumes in SFC. Recently, achiral RPLC and chiral SFC were combined for on-line multiple heart-cutting analysis (mRPLC-SFC) of pharmaceutical compounds using trapping columns for the interface [24]. This 2D approach was successfully applied to the separation of 8 stereoisomers of a drug substance containing multiple chiral centers.

The objective of our work was to develop a selective comprehensive set-up for the achiral/chiral analysis of pharmaceuticals. The main advantage of using a selective comprehensive approach is that the resolution achieved in the first dimension can be essentially maintained in the second dimension. This is necessary in case of complex samples containing achiral impurities which may coelute with the chiral API in the first dimension. Preliminary studies were carried out to address injection effects in chiral SFC with hydro-organic sample solvents and to assess the interface components with respect to quantitative analysis. A proof-of-concept was first successfully carried out on a simple sample, with an attempt to compare the achieved sensitivity between 1D-SFC-UV and sLCxSFC-UV. Then, the developed sLCxSFC separation was applied to the achiral/chiral analysis of a complex pharmaceutical sample.

2. Experimental

2.1. Material and reagent

Acetonitrile (ACN), methanol (MeOH) and acetone were LC grade from Sigma–Aldrich (Steinheim, Germany). Water was obtained from an Elga Purelab Classic UV purification system (Veolia water STI, Le Plessis Robinson, France). Methanesulfonic acid (MSA) (0.1% – pH 2.0 measured in aqueous phase), was obtained from Sigma–Aldrich (Steinheim, Germany). Pressurized liquid CO₂ (99.9%) was obtained from Air Liquide (Pierre Bénite, France).

The pharmaceutical sample was provided by Oril Industrie (Servier laboratories, Bolbec, France). It consisted in a chiral active pharmaceutical ingredient (API) and its related impurities, S enantiomer being the active form of the drug. Due to confidentiality agreement, the molecular structures cannot be provided. Solutions were prepared every month at concentrations of 0.4 mg/mL, 0.2 mg/mL or 0.1 mg/mL (depending on the assays) in ACN and were



Fig. 1. sLCxSFC setup using Multiple Heart-Cut (MHC) valve system, equipped with 12 sample loops. (a) The fractions from the first dimension are successively stored in the loops #2 to #6 (Deck A) while the contents of the loops #1 to #5 (Deck B) are successively analyzed in the second dimension and (b) vice versa after valve switching. Loop #1 (Deck A) and loop #6 (Deck B) are only used to ensure uninterrupted mobile phase flow.

stored at 4°C. Sample stability was checked every week according to the Oril quality control method.

2.2. Instruments

2.2.1. 1D-SFC system

The Agilent 1260 Infinity II SFC system (Agilent Technologies, Waldbronn, Germany) was equipped with a binary solvent delivery pump, a 400 µL mixing chamber, an autosampler with the Feed Injection Technology, allowing injection volumes from 0.1 to 90 µL, a column oven compatible with temperature up to 85 °C, a diode array detector with a 2 µL flow-cell withstanding pressure up to 400 bar and a SFC control module whose functions are (i) CO₂ conditioning upstream of the SFC pump and (ii) backpressure regulating downstream of the detector. The maximum allowable flow rate is 5 mL/min. The maximum allowable pressure is 600 bar with flowrates up to 5 mL/min. Data acquisition was performed by OpenLab CDS software (Agilent Technologies). The extra-column volume and the extra-column variance, measured under supercritical conditions, are 40 μ L and 30 μ L² respectively. The system dwell volume was estimated at 600 μL , using CO $_2$ as solvent A and MeOH + 0.1% acetone as solvent B. The gradient was started with a high percentage of B (i.e. 69% B) to ensure a quasi-liquid phase from initial gradient composition as previously recommended to get reliable measurements [22].

2.2.2. sLCxSFC system

The sRPLCxSFC setup is shown in Fig. 1. The first dimension is the first dimension of the 1290 Infinity I 2D-LC system from Agilent technologies. It includes a high-pressure binary solvent delivery pump, an autosampler with a flow-through needle of 20 µL, a column oven with a maximum temperature of 100 °C, and a UV detector equipped with a 2 µL flow-cell. The maximum allowable pressure is 1200 bar up to 5 mL/min. The second dimension is composed of the binary solvent delivery pump, the column oven, the diode array detector and the SFC control module of the 1260 Infinity II SFC apparatus. The interface between the two dimensions is the "Multiple Heart-cutting" (MHC) system from Agilent Technologies. It consists in a 2-positions/4-port duo valve with two parking decks, each equipped with six 10 µL sampling loops. Data acquisition and instrument control were performed by OpenLab software (Agilent Technologies). The synchronization between both dimensions was obtained by electrically connecting the two systems.

2.3. Chromatographic conditions

An Acquity BEH C18 column ($50 \text{ mm} \times 2.1 \text{ mm}$; $1.7 \mu \text{m}$) from Waters (Milford, MA, USA) was used in the first dimension. Two Chiralpak IC columns ($100 \text{ mm} \times 3 \text{ mm}$; $3 \mu \text{m}$ and $150 \text{ mm} \times 4.6 \text{ mm}$; $3 \mu \text{m}$) from Chiral Technologies (West Chester, PA, USA) were used in SFC conditions for preliminary 1D-SFC study and for the second dimension in sLCxSFC.

2.3.1. 1D-SFC experiments

The compatibility of the Chiralpack IC column (100 mm \times 3 mm; 3 μ m) with the injection solvent (RPLC eluent) was studied in isocratic conditions. In the Oril quality control separation, the composition at elution of the compound of interest was nearly 70% ACN. A solution of S enantiomer at 0.1 mg/mL in this solvent composition was prepared. Different volumes were injected (from 1 μ L to 5 μ L). Temperature, flow rate, BPR, wavelength and frequency were set at 40 °C, 2 mL/min, 130 bar, 210 nm and 20 Hz respectively. Pure MeOH and a mixture of MeOH/H₂O 98/2 (v/v) were used as co-solvent B.

SFC chiral experiments were carried out on the Chiralpack IC column (150 mm \times 4.6 mm; 3 μ m) in order to compare conventional

SFC and sLCxSFC. The flow rate was 2.5 mL/min. Other conditions were the same as above. The solvent B was a mixture of MeOH/H₂O 98/2 (v/v) and the gradient was 0 min, 1% B; 2.9 min, 30% B; 3.4 min, 1% B; 6 min, 1% B. Standard solutions of API containing different levels of undesired enantiomer (0.5–5%) were prepared in ACN. The injection volume was 2 μ L.

2.3.2. 2D sLCxSFC experiments

2D experiments were carried out under isocratic conditions in both dimension in order to assess the repeatability of full sample loop injections. The columns were replaced by zero-dead volume unions. Acetone was used as model compound. In the first dimension, the temperature, the flow rate, the wavelength and the sampling rate were $30 \degree C$, 0.1 mL/min, 270 nm and 20 Hz respectively. The mobile phase was a mixture of 5% acetone in MeOH. In the second dimension, the flow rate was 2 mL/min with a BPR of 140 bar. The temperature, the wavelength and the sampling rate were $40 \degree C$, 270 nm and 20 Hz respectively. The mobile phase was composed of $CO_2/MeOH$ 95/5 (v/v). The sampling time was 6 s.

The conditions for the sRPLCxSFC achiral/chiral separations are given in Table 1. A proof-of-concept was carried out with a simple sample under isocratic conditions in ²D while a pharmaceutical sample was analyzed under gradient conditions in ²D.

3. Results and discussion

3.1. Optimization of injection volume in ²D-SFC

In SFC, recent studies [22,25,26] showed that both the sample solvent and the injection volume have a strong effect on peak shapes. Polar solvent such as dimethyl sulfoxyde or methanol were generally found to be the worst option, leading to significant peak distortions even for small injection volumes. On the other hand, aprotic solvents (e.g. methyl tert-butyl ether, dichloromethane) were found to be more adapted to large injection volumes. It was also highlighted that the obtained results were dependent on both the nature of the analyte and the nature of the stationary phase [22,26]. Abrahamson and Sandhal [27] recently showed that the injection solvent may interact with the stationary phase and that can lead to either positive or negative effect on peak shapes depending on the hydrogen bonding capabilities of both injection solvent and stationary phase (e.g. with grafted-silica polar injection solvent may interact with free silanols, thereby locally limiting secondary interactions). Solubility issues of the injection solvent into the methanol-modified carbon dioxide were also highlighted [22,27]. We therefore studied the effect of injection solvent on SFC peak shapes in our 2D-conditions with the aim of injecting the largest possible volume onto the SFC column. In on-line LCxSFC, the injection solvent in ²D-SFC is the mobile phase coming from ¹D-RPLC, namely a mixture of water and acetonitrile. The volume fraction of the strong eluent at peak elution, φ_e , was calculated for API using the following relationship:

$$\varphi_{e} = \varphi_{i} + s \times (\frac{t_{R}}{t_{0}} - \frac{t_{D} + t_{ini}}{t_{0}} - 1)$$
(1)

Where t_R , t_D , t_{ini} and t_0 are the solute retention time, the system dwell time, the initial isocratic step duration and the column dead time respectively. φ_i is the initial composition and s is the normalized gradient slope (i.e. $t_0 x \Delta C/t_G$ were ΔC and t_G are the composition range and the gradient time respectively). In the original RPLC quality control separation, used in this study as first dimension, φ_e value was 70% ACN for API. We therefore studied the impact of the injection volume on the peak shape with API dissolved in 70% ACN. Based upon a preliminary study on several alcohols (ethanol, methanol, isopropanol) with the Chiralpak IC column (100 mm × 3 mm; 3 µm), pure methanol was first chosen

Table 1

Experimental conditions used for sRPLCxSFC separations.

| | ¹ D RPLC | ² D Chiral SFC |
|------------------|---|---|
| Stationary phase | Acquity BEH C18 | Chiralpack IC |
| Column geometry | 50 mm × 2.1 mm 1.7 μm | 150 mm × 4.6 mm 3 μm |
| Mobile phase | A: H ₂ O/ACN 98/2 (v/v) + 0,1% MSA | A: CO ₂ |
| | B: ACN/H ₂ O 98/2 (v/v) + 0,1% MSA | B: MeOH/H ₂ O 98/2 (v/v) |
| Flow rate | 0.2 mL/min | 2.5 mL/min |
| Elution mode | Gradient 5 to 95% (B) in 14 min | (1) preliminary study: 6% (B) ^a |
| | | (2) real sample: gradient 1 to 30% (B) in 2.9 min |
| BPR | / | 130 bar |
| Temperature | 40 °C | 40 ° C |
| UV wavelength | 210 nm | 210 nm |
| Injected volume | 2 µL | 10 µL ^b |

^a ²D analysis time : 3 min.

^b 0.05 min as sampling time.



Fig. 2. Effect of both injection solvent and injected volume on the peak shape. Solute: S-enantiomer. Injection solvent composition: H₂O/ACN 30/70 (v/v). Column: Chiralpak IC (100 × 3mm; 3 µm); mobile phase: CO₂/co-solvent 94/6 (v/v) with co-solvent: (a) MeOH and (b) MeOH/H₂O 98/2 (v/v); 2 mL/min; 40 °C; BPR 130 bar; 210 nm.

as co-solvent. The obtained results for injection volumes ranging from 1 μ L to 5 μ L are displayed in Fig. 2a. As can be seen, the volume that can be injected in these conditions, without strong peak distortion, should not exceed 2 μ L which is very low considering the column dead volume (i.e. less than 0.5% of the column dead volume). That can be critical for sensitivity. In on-line twodimensional chromatography, modifying the injection solvent or decreasing the injection volume while keeping the same injected amount is possible only by adding a suitable make-up solvent or by using appropriate trapping columns respectively. Provided that such actions can improve the peak shapes, they require more complex interface which may add further element of uncertainty in quantitative analysis.

In order to increase the injection volume without modifying the interface and hence without modifying the injection solvent, we studied the effect of the nature of the co-solvent on the peak shape. A small content of water (i.e. 2%) was added to methanol thereby slightly increasing the co-solvent polarity. The peaks obtained in these mobile phase conditions are shown in Fig. 2b and can be compared to those obtained with pure methanol as co-solvent (Fig. 2a). As can be observed, the peak shapes are significantly improved by a low content of water in the co-solvent. Although broader for large injection volumes, the peaks are kept symmetrical up to $5 \mu L$ injected. The beneficial effect of a small amount of water in the cosolvent, reducing secondary interactions is now well known and has been reported in different studies [28–30]. This phenomenon can be ascribed to the fact that the surface chemistry of the stationary phase is slightly altered by the presence of water. At the same time, this may lead to a slight change in solute retention as can be observed by comparing Fig. 2a and b. However, in our present case, peak shapes are not degraded, in the absence of water, as long as the injection volume is kept low enough (i.e. $\leq 2 \mu L$). The presence of water becomes clearly advantageous for larger volumes. This suggests that the present peak broadening caused by large injection volumes in polar solvent is quite different to that caused by

secondary interactions with the stationary phase. We think that this may result from two distinct phenomenon, both involving the adsorption of water onto the stationary phase:

- (1) A large and polar injection volume is expected to be poorly solubilized in a mobile phase mainly composed of apolar CO₂. The presence of a small amount of water may result in a thin layer of water dynamically adsorbed onto the stationary phase which could enhance the solubility.
- (2) The injection solvent plays the role of a strong eluent during the injection duration. With pure MeOH as co-solvent, the small amount of water contained in the injection solvent is gradually adsorbed onto the stationary phase surface which in turn is gradually deactivated, thereby leading to a decrease in solute retention during the injection process. This phenomenon could cause severe injection effects. The water-rich layer formed with the presence of water in the co-solvent could avoid the progressive reduction of retention during the injection process, thereby limiting peak distortions.

In view of these results, MeOH/H₂O 98/2 (v/v) was used as co-solvent, and an injection volume of 5 μ L on the Chiralpak IC (100 mm × 3 mm; 3 μ m) was considered as the best option. However, due to the fact that the smallest loop volume commercially available for the "Multiple Heart-cutting" interface was 10 μ L, a 150 × 4.6 mm column was used thereby allowing to inject 10 μ L instead of 5 μ L. It is important to note that due to CO₂ depressurization, flow splitting between ¹D and ²D was not possible. As a result, the injection volume in ²D, ²V₁, is directly related to the flow-rate in the first dimension, ¹F, and to the sampling time, t_s according to:

$${}^{2}V_{i} = t_{s} \times {}^{1}F \tag{2}$$

The ${}^{1}F$ and t_{s} were set at 0.2 mL/min and 0.05 min respectively in order to inject 10 μ L in ${}^{2}D$. The gradient time in the first dimension was scaled up from original gradient conditions to fit with the



Fig. 3. Overlay of 3 chromatograms obtained in sLCxSFC conditions with zero dead volume unions in place of columns in both dimensions. ¹D: no injection; mobile phase: MeOH + 5% Acetone; 0,1 mL/min; 30 °C; 270 nm $-^{2}$ D: mobile phase: CO₂/MeOH 95/5 (v/v); 2 mL/min; 40 °C analysis time: 0.2 min; BPR: 130 bar; 270 nm; sampling time = 6 s.

flow-rate of 0.2 mL/min. In these conditions, the sampling rate was approximatively 4–5 fractions per peak for the main compound hence allowing to maintain the first dimension separation without too much dilution. It is also important to specify that in selective LCxLC the sampling time does not necessarily correspond to the analysis time in the second dimension. Thus, it is possible to work with a very short sampling time (i.e. a few seconds) while keeping an acceptable analysis time in the second dimension (i.e. a few minutes).

3.2. Impact of full loop injection on sample volume repeatability

In LCxLC, partial loop injection is usually preferred in order to avoid any sample loss due to parabolic flow profile. In LCxSFC, full loop injection is mandatory. In case of partial loop injection and due to CO₂ depressurization after valve switching, a portion of the loop is filled by the sample while the other one is filled by both air and cosolvent droplets which cover the loop walls. That may lead to some severe issues as highlighted by Sarrut et al. [22]. Full loop injection in the second SFC dimension is expected to be less detrimental than in LC since the presence of air provides a flow with nearly flat profile, allowing to entirely fill the loop with one volume only whereas 3 to 5 volumes are required in LC due to parabolic profile. The interface provided for sLCxSFC is equipped with 12 sample loops which should be strictly identical for the purpose of quantitative analysis. We therefore studied the repeatability of the collected sample volumes, under full loop injection conditions with our sLCxSFC setup. It is important to specify that here the term repeatability is not used in the sense of method validation, but only to check that the 10 loops (among 12) used for the collection of the fractions have the same volume. Thus, the acceptance criteria from ICH guidelines or equivalent are not those required in this study.

Three consecutive sLCxSFC experiments were carried out, with acetone dissolved in ¹D- mobile phase, and zero-dead volume unions in place of columns in both dimensions, for peak area assessment. As can be seen in Fig. 3, the resulting overlaid ²D chromatograms are very similar. The peak area relative standard deviations (RSD), calculated by considering (i) all the loops of the two decks (10 loops) and (ii) the loops of each deck separately (5 loops) are given in Table 2. The RSD value for Deck B (9.5%) is twice as high as that for Deck A, suggesting some differences in loop sizes in Deck B. Nevertheless, when the two decks are considered together, the RSD value is below 10% which was quite satisfying considering the two major issues we faced: (i) Only one loop volume was expected to entirely fill the loop as discussed above; (ii) Compared to 1D-instruments, additional tubing and additional valves were likely to introduce further uncertainties on peak area measurements.

Table 2

Peak areas and RSD values obtained from the 10 identical sample loops of $10 \,\mu$ L equipping the two parking decks of the MHC interface. Acetone used as model compound. Experimental conditions given in Fig. 3.

| | Deck A | Deck B | Deck A + Deck B |
|--------------------|--------|--------|-----------------|
| Average peak area | 58527 | 53837 | 56182 |
| Standard deviation | 2202 | 5092 | 4533 |
| RSD (%) | 3.8 | 9.5 | 8.1 |



Fig. 4. Detailed achiral-chiral analysis of simple API sample by sRPLCxSFC with isocratic elution in ²D-SFC: ¹D-RPLC chromatogram (top) and ²D-SFC chromatograms of the 5 five consecutive fractions (bottom); Sample: 0.4 mg/mL of S-enantiomer with 5% R-enantiomer. All experimental conditions are given in Table 1.

In view of these results, the MHC interface was considered as suitable for sLCxSFC analysis.

3.3. Application to achiral-chiral sLCxSFC analysis

3.3.1. Preliminary study on a simple sample

In order to validate our choices, a preliminary study was carried out in on-line sRPLCxSFC with a simple sample containing 5% of undesired R-enantiomer and the Chiralpak IC column (4.6 mm i.d.). The experimental conditions are given in Table 1 and the resulting separation is presented in Fig. 4. It is interesting to notice the absence of significant injection effects, with quite satisfactory peak shapes in all the five ²D-separations (see Fig. 4 bottom) corresponding to the five fractions of the API peak (see Fig. 4 top). These results



Fig. 5. Achiral-chiral analysis of a pharmaceutical sample by sRPLCxSFC with gradient elution in ²D-SFC: ¹D-RPLC chromatogram for chemical purity (top); zoom on chiral compound peaks (middle) and ²D-SFC chromatograms of the different consecutive fractions for enantiomeric purity (bottom). An impurity separated from API in SFC is circled in pink. All experimental conditions are given in Table 1.

are in very good agreement with the preceding study. Furthermore, baseline resolution between the two enantiomers could be obtained in less than 3 min. Finally, using this sLCxSFC setup it was possible to separate in ¹D-RPLC, API from its synthetic intermediate (see Fig. 4top) and to separate, in ²D-SFC, S-enantiomer from undesired R-enantiomer (see Fig. 4 bottom), within a single experiment lasting less than 40 min.

3.3.2. Achiral-chiral analysis of a pharmaceutical sample

A complete characterization of active principle is mandatory to ensure the quality of drug products. As previously pointed out, for chiral compounds, the characterization can be both time consuming and laborious. RPLC techniques are most often used to assess the chemical purity of drug products. However, enantiomers cannot be differentiated by conventional RPLC. Chiral stationary phases are able to separate enantiomers but usually, they offer poor selectivity for achiral compounds. As a result, whereas both techniques are required for a complete characterization, their successive use can be troublesome due to the necessity of performing two different sample preparations (one per method) and/or to the risk of not separating API from achiral impurities in chiral analysis. An illustration of this issue is given in supplementary section with two 1D-separations: achiral RPLC (Fig. S1) and chiral SFC (Fig. S2). In the present study, the use of a 2D approach to overcome these drawbacks was investigated. The conditions of the first dimension were those of the validated method developed for API quality control. The flow-rate was slowed down to 0.2 mL/min in order to fit with on-line sLCxSFC implementation and the gradient time was increased so that the normalized gradient slope (gradient slope x column dead time) was kept constant. Unlike the preceding separation (Fig. 4) for which ²D-SFC experiments were carried out under isocratic elution, a gradient elution from 1% to 30% MeOH/H₂O 98/2 (v/v) was necessary in order to elute all compounds in appropriate retention conditions on the Chiralpak IC column (4.6 mm i.d.). The obtained results are shown in Fig. 5. The RPLC separation (top chromatogram) allowed to separate a chiral synthetic intermediate as well as other process-related impurities from API, hence providing chemical purity (also called achiral purity). From the obtained chromatogram, the calculated API chemical purity was found to

be 86.0%. API and the chiral synthetic intermediate were transferred and then analyzed in the second chiral dimension. As can be observed in Fig. 5, S-enantiomer is well separated from undesired R-enantiomer for both chiral compounds (bottom chromatograms). The two enantiomers are eluted in reversed order for the synthetic intermediate and API, not surprising with compounds of different nature. From these second dimension chromatograms, the calculated enantiomeric purities for synthetic intermediate and API were found to be 94.0% and 93.1% respectively. The peak circled in pink (Fig. 5) corresponds to an achiral impurity that was not separated from API in RPLC but was well separated in SFC. It is important to underline that, while this result is achievable with a comprehensive approach, it is unlikely that a heart-cutting approach could have permitted such good separation because its principle consists in sending only one fraction of the first dimension to the second one and of course, the sent fraction may not contain the searched impurity. Furthermore, the impurity may be partially separated in RPLC (as is the case here wherein the impurity appears in the third fraction only). The large fractions generally sent to ²D in heart-cutting may remix the compounds, making the separation in the second dimension more difficult to achieve, considering the poor selectivity of chiral columns for achiral separation In sLCxLC, the resolution achieved in ¹D is essentially maintained in ²D due to an appropriate sampling rate (3-4 fractions per peak), which enhances the chance of separating the compounds. And last, but not least, the observation of this additional impurity in the second dimension made it possible to correct the calculated chemical purity which was finally found to be 84.1% instead of 86.0% as first calculated.

3.4. Comparison of sLCxSFC and 1D-SFC

To assess the possibilities offered by on-line sLCxSFC for achiralchiral analysis, we compared both sensitivity and peak resolution between on-line sLCxSFC and conventional 1D-SFC, using the same Chiralpak IC column (4.6 mm i.d.). 2 μ L of solutions of S-enantiomer with 5 different percentages of undesired R-enantiomer were analyzed, on the one hand, by conventional 1D-SFC operated in the same conditions as in ²D-SFC and on the other hand, by sLCxSFC. The corresponding overlaid chromatograms are shown in Fig. 6a and



Fig. 6. Comparison of the separations of R and S with different concentration ratios of R over S (0.5%–5%) using (a) 1D-SFC and (b) sLCxSFC (most intense ¹D-fraction shown). Experimental conditions are given in Section 2.3 for 1D-experiments and Table 1 for 2D-experiments. For a better comparison, the same volumes of 2 µL were injected in both cases.

b respectively. In both cases, the two enantiomers were baseline resolved and the detector response was linear within the studied range of concentrations (R² values upper than 0.999). The resulting linear curve equations were guite similar (i.e. 279.16x + 121.85 and 279.45x + 110.74 respectively). A notable difference in peak intensity can be observed depending on the separation technique (i.e. factor 3-4 in favor of 1D-SFC). This result is not surprising considering that: (i) Extra-column dispersion is more significant in sLCxSFC, due to additional extra-column volumes (tubing, valves and loops) and to larger injection volumes, resulting in larger peaks as highlighted by comparing the average 4σ peak widths in both chromatograms (i.e. 0.045 min in 1D-SFC while 0.085 min in sLCxSFC). This difference is significant enough to have an impact on both resolution and sensitivity in sLCxSFC; (ii) 2D-chromatography is a dilutive technique with ¹D peaks divided into several fractions that are injected in ²D. The resulting dilution factor is close to the product of the dilution factors in each dimension.

The signal-to-noise ratios (S/N) were calculated, according to US pharmacopeia (S/N = 2xH/h, with H and h being the peak height and the noise amplitude respectively), for both techniques from the peak corresponding to the lowest R-concentration (i.e 0.5%). The obtained S/N values were 3.2 in sLCxSFC while 8.7 in 1D-SFC. Thus 0.5% R seems to be the detection limit in the former case. This value is slightly higher than the limit value usually requested in the pharmaceutical area (0.1% as quantitation limit). Nevertheless, this value was found to be quite satisfying considering that (i) the requirement for this specific sample was to detect at least 1% of the undesired enantiomer, and (ii) the detection limit of 0.5% was found with UV detection and it is likely that MS detection operated in SIM mode would significantly reduce this value to reach the detection limit recommended in pharmaceutical analysis.

4. Conclusions

The goal of this work was to evaluate the potential of on-line sRPLCxSFC for the achiral/chiral analysis of pharmaceutical compounds. The key device of this 2D setup is the interface which includes parking decks equipped with several sample loops. This interface enables to work in selective comprehensive mode with less constraints than in comprehensive mode, thanks to a temporary storage of the collected fractions into ten sample loops. Preliminary studies were carried out with the aim of limiting injection effects in ²D chiral SFC, due to the lack of compatibility between mobile phases used in first and second dimension. With a mixture of water/acetonitrile as sample solvent, it was shown that the addition of only 2% water in the co-solvent (MeOH) allowed to inject larger volumes than with pure MeOH as co-solvent. The adsorption of a thin layer of water onto the stationary phase was given as tentative explanation.

The on-line achiral/chiral sRPLCxSFC separation of a complex pharmaceutical sample was successfully achieved in less than 50 min

The performance of achiral/chiral sRPLCxSFC, as presently developed, was compared to that of conventional chiral 1D-SFC. In terms of resolution and linearity, we found that the two techniques were similar with satisfying resolution between R and S enantiomers in both cases. Furthermore the resulting calibration curves could be overlaid suggesting that sLCxSFC was quite reliable in term of quantitative analysis. However, the peak intensities in sLCxSFC were found to be lower by a factor of 3 to 4 than those in 1D-SFC. This difference can be explained by extra-column dispersion in the interface (additional valves and tubing) and by dilution arising from the two-dimensional process. Despite this, a detection limit as low as 0.5% was reached in sLCxSFC with UV detection which was in good agreement with the specific recommendations for this sample (i.e. LOD of 1%).

Finally, in the light of these results, the on-line coupling of RPLC and SFC can be considered as an attractive solution for the simultaneous achiral/chiral analysis of pharmaceuticals.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.06.058.

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Supplementary information for

On-line coupling of achiral Reversed Phase Liquid Chromatography and chiral Supercritical Fluid Chromatography for the analysis of pharmaceutical compounds

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Analysis of the pharmaceutical sample with quality control method. Conditions: Acquity BEH C18 (50x2.1 mm 1.7 μ m); F = 0.8 mL/min; T = 40°C; Vi = 2 μ L; Mobile phases: A = H₂O/ACN (98/2) + 0.1% MSA, B = ACN/H₂O (98/2) + 0.1% MSA ; 5 to 95% (B) in 3.5 min; UV 210 nm. RPLC separation is able to resolve API from its synthetic intermediate and other related impurities, but is not suitable for the separation of the different enantiomers of API or its intermediate.





Chiral analysis of the pharmaceutical sample using SFC. Conditions: Chiralpak IC3 (150x4.6 mm 3 μ m); F = 2.5 mL/min; T = 40°C; Vi = 2 μ L; Mobile phases: A = CO₂, B = MeOH/H₂O (98/2); 1 to 30% (B) in 2.9 min; BPR = 130 bar; UV 210 nm. Chiral SFC separation well resolves the R and S enantiomers for the two target compounds (namely API and synthetic intermediate). However, compared to chromatogram shown in Fig. S1 it clearly appears that the selectivity of the column is not sufficient to resolve the other related impurities.

CONCLUSIONS

Deux types différents de couplages RPLC/SFC en ligne ont été appliqués à l'analyse d'échantillons pharmaceutiques.

Dans le cas d'un couplage RPLCxSFC pour la mise en place d'une analyse générique, le taux d'occupation de l'espace de séparation est plus intéressant avec une combinaison RPLC/SFC, mais le degré d'orthogonalité, lui, reste plus élevé avec des combinaisons RPLC/RPLC. En effet, bien que la SFC permette d'obtenir des sélectivités très différentes de la RPLC, les plages de compositions à l'élution sont généralement plus faibles qu'en RPLC. Cela a pour conséquence de générer un espace de séparation plus restreint. Même si certaines combinaisons RPLC/SFC restent compétitives d'un point de vue théorique, le gain final en termes de capacité de pics et de sensibilité est limité par un certain nombre de contraintes instrumentales, telles que les problèmes de compatibilité de phase mobile, ou encore la variance extra-colonne et le volume de délai qui sont bien plus importants en ²D-SFC qu'en ²D-RPLC.

En revanche, dans le cas d'une analyse où le besoin en sélectivité est le premier objectif, comme pour l'analyse de composés chiraux, le couplage sRPLCxSFC s'avère être très intéressant. L'analyse peut alors se faire en mode « sélective compréhensive », ce qui permet de découpler les temps d'échantillonnage et d'analyse de 2^{ème} dimension. La ²D-SFC chirale peut alors être optimisée indépendamment de la 1^e dimension, ce qui permet de moins subir les contraintes instrumentales évoquées dans le paragraphe précédent. Il est alors possible de développer des méthodes quantitatives permettant de profiter à la fois de l'efficacité d'une ¹D-RPLC et de la sélectivité d'une ²D-SFC chirale.

Finalement, dans le domaine pharmaceutique, le couplage LC/SFC est une solution viable et intéressante dans les cas où l'on ne s'intéressera qu'à une ou quelques zones du chromatogramme de 1^{ère} dimension qui nécessitent une grosse différence de sélectivité entre les deux dimensions. C'est le cas par exemple de l'analyse de composés chiraux, mais cela pourrait aussi être appliqué à la séparation d'atropoisomères ou d'isomères de positions non résolus en 1^{ère} dimension. En revanche, si l'objectif est de développer une méthode générique avec un grand pouvoir de séparation, on se dirigera plutôt vers une analyse RPLCxRPLC.

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Les développements et recherches effectués au cours de ces travaux de thèse ont pu mettre en avant l'intérêt des méthodes bidimensionnelles pour l'analyse d'échantillons pharmaceutiques. En effet, quel que soit le mode 2D employé, cette technique séparative à très fort potentiel permet de répondre à différents challenges analytiques rencontrés dans l'industrie pharmaceutique.

Tout d'abord, il a été montré que l'utilisation d'une méthodologie basée à la fois sur l'acquisition d'un grand nombre de données en 1D-LC et sur le calcul de deux descripteurs complémentaires, le degré d'orthogonalité et la capacité de pics pratique, était une approche pertinente pour la sélection de conditions intéressantes en 2D-LC. Cette étude, qui a été menée sur deux échantillons pharmaceutiques aux propriétés et aux structures différentes, a conduit à des conclusions similaires à celles initialement obtenues sur un mélange de composés modèles, à savoir : (1) les systèmes qui ont les degrés d'orthogonalité les plus élevés ne sont pas ceux qui génèrent les plus grandes capacités de pics, il y a donc un compromis à trouver selon les besoins et les objectifs de l'analyse ; (2) l'additif, et donc le pH de la phase mobile, est le paramètre qui a le plus d'influence sur le degré d'orthogonalité dans le cas des composés pharmaceutiques ; et (3) les systèmes bidimensionnels utilisant une phase mobile basique dans l'une des deux dimensions sont en général ceux qui conduisent aux capacités de pics les plus élevées. Ces travaux ont abouti à la sélection de trois systèmes RPLCxRPLC dans le cadre de la mise en place d'une stratégie 2D générique pour l'analyse d'échantillons pharmaceutiques dans les premières phases de développement du médicament. Les séparations RPLCxRPLC-UV/MS obtenues ont montré que l'utilisation d'un à deux système 2D permettait de détecter plus de 95% des impuretés présentes, assurant ainsi une bonne caractérisation de l'échantillon.

Une étude ciblée sur les performances quantitatives de ces systèmes 2D-LC génériques a ensuite été menée dans le but (1) de vérifier que la LCxLC en ligne était applicable à l'analyse quantitative d'échantillons pharmaceutiques, qui est soumise à des exigences règlementaires strictes ; et (2) d'affiner la stratégie de mise en place d'analyses 2D dans l'industrie pharmaceutique. Une étude sur les aspects quantitatifs de la LCxLC en ligne a permis de montrer que cette technique était tout à fait apte à fournir des résultats quantitatifs, avec une linéarité équivalente à celle que l'on peut obtenir en 1D-LC. L'utilisation d'une approche « Pareto-optimal », développée au laboratoire, a permis d'optimiser les conditions LCxLC en privilégiant la minimisation du facteur de dilution, tout en conservant une capacité de pics largement supérieure à ce que l'on peut attendre en 1D-LC. Les séparations RPLCxRPLC-UV/MS ainsi optimisées ont permis d'atteindre des limites de détection, voire de quantification, inférieures à 0.05% d'impureté (par rapport au PA) avec une répétabilité satisfaisante (RSD < 2 %), que ce soit sur les temps de rétention ou les sommes d'aires de pics en 2^{ème} dimension. Ces résultats nous ont également conduit à proposer une méthodologie générique, en deux étapes, pour la mise en place d'analyses 2D dans le milieu pharmaceutique. La première étape, basée sur un screening des trois systèmes RPLCxRPLC génériques proposés, permet de choisir le jeu de conditions RPLC/RPLC (phases stationnaires et phases mobiles) le plus adapté à l'échantillon. La seconde étape a pour but de guider l'analyste dans le choix du mode 2D-LC (LC-LC, sLCxLC ou LCxLC) à utiliser, en fonction des objectifs et besoins de l'analyse.

Enfin le potentiel du couplage en ligne de la RPLC et de la SFC a été évalué, pour répondre à deux challenges différents. Tout d'abord, dans le cadre du développement de méthodes 2D génériques, une analyse SFC achirale a été envisagée en 2^{ème} dimension, puisque la SFC présente l'avantage (1) de fournir des sélectivités très différentes de la RPLC, et (2) de permettre des analyses très rapides du fait de la faible viscosité de la phase mobile. Une étude préliminaire visant à trouver des conditions intéressantes en se basant sur le calcul du degré d'orthogonalité et de la capacité de pics pratique a été menée. Les résultats ont montré qu'étonnement les combinaisons RPLC/SFC généraient des degrés d'orthogonalité moins élevés que les combinaisons RPLC/RPLC, ce qui s'explique par la plage de composition à l'élution, bien plus faible en SFC (souvent < 30%). Cependant guelques systèmes RPLCxSFC restent compétitifs par rapport aux systèmes RPLCxRPLC. La séparation RPLCxSFC d'un échantillon pharmaceutique a été comparée aux séparations RPLCxRPLC obtenues sur ce même échantillon. Malgré un excellent taux d'occupation de l'espace de séparation en RPLCxSFC, la capacité de pics obtenue ainsi que le manque de sensibilité ont montré que dans le cadre de la mise en place de méthodes génériques, les couplages RPLCxRPLC restent les plus intéressants. Dans un deuxième temps, la SFC chirale a été envisagée en 2^{ème} dimension d'une analyse achirale/chirale, en mode 2D sélective compréhensive. Dans le domaine de l'analyse chirale, la SFC est une technique de choix puisque (1) elle permet des analyses plus rapides qu'en mode NPLC, avec l'utilisation de solvants moins toxiques, et (2) elle surpasse la RPLC en termes de sélectivité et de temps d'analyse. Un travail préliminaire concernant la compatibilité des phases mobiles et les effets à l'injection en ²D-SFC a montré que l'ajout d'une faible quantité d'eau dans le co-solvant permettait d'augmenter de façon considérable le volume injecté en 2^{ème} dimension, tout en conservant une forme de pic satisfaisante. L'analyse sRPLCxSFC-UV ainsi développée a été appliquée à l'analyse achirale/chirale d'un échantillon pharmaceutique, permettant de déterminer, en une seule analyse et en moins de 50 minutes, les puretés chimiques et énantiomériques du principe actif. Enfin, une étude des performances quantitatives de la méthode a montré qu'il était possible d'atteindre une LOD de 0.5% pour l'énantiomère non désiré, et que la linéarité était comparable à celle obtenue en SFC chirale unidimensionnelle. Ces résultats montrent que le couplage sLCxSFC est une solution intéressante pour l'analyse d'échantillons chiraux. Cependant, une réduction de volumes instrumentaux (volume de délai, volume extra-colonne) en SFC reste nécessaire pour accroître la sensibilité de ce type de méthode, et ainsi satisfaire aux exigences règlementaires.

Les différentes études présentées dans ce travail conduisent à envisager plusieurs perspectives :

(1) Le développement et la mise en place d'analyses bidimensionnelles, en particulier en mode LCxLC, reste à l'heure actuelle très complexe. C'est pourquoi les travaux d'optimisation doivent être poursuivis, afin d'une part d'affiner et améliorer les outils prédictifs, et d'autre part d'approfondir les connaissances des phénomènes en jeu dans une analyse bidimensionnelle. L'objectif serait, dans l'idéal, de pouvoir développer des outils prédictifs aussi performants que les logiciels d'optimisation LC que l'on peut trouver sur le marché. Dans l'idée d'implanter cette technique dans le milieu industriel, un axe de travail majeur serait donc de développer des outils permettant de faciliter l'optimisation des méthodes 2D-LC, afin que cette technique soit à la portée de tous.

(2) Le traitement des données, quant à lui, est encore compliqué et laborieux, ce qui représente un frein au développement de la 2D-LC chez les industriels. Bien que des efforts soient faits par les équipementiers pour proposer des solutions de traitement des résultats, comme par exemple le logiciel LC-Image, cela reste insuffisant. En effet, ces outils sont d'une aide précieuse pour visualiser et exploiter les résultats dans le cas d'analyses qualitatives, cependant des efforts doivent être faits quant au traitement d'analyses quantitatives. Dans ce dernier cas, le travail reste encore très laborieux et consommateur de temps, dès lors que l'on veut utiliser certaines approches, comme la somme des aires de pics au lieu du volume. Le développement d'un outil convivial permettant de choisir l'approche à utiliser, d'automatiser les étapes d'intégration et de regroupement des pics de 2^{ème} dimension, ou d'évaluer des critères chromatographiques comme la résolution ou le rapport signal sur bruit, serait un atout majeur pour de développement de la 2D-LC.

(3) Enfin, le couplage avec la spectrométrie de masse peut également faire l'objet d'un axe de travail et d'amélioration. En effet, ce couplage peut être d'un grand intérêt pour l'identification de nouvelles impuretés dans les substances pharmaceutiques, notamment en associant l'analyse 2D-LC à un détecteur par spectrométrie de masse haute résolution (HRMS). Cependant, le couplage de ces deux techniques nécessite encore des compromis :

- sur le débit de la phase mobile de deuxième dimension, en général trop élevé pour le détecteur HRMS. L'approche actuelle consistant à placer un split avant le détecteur permet de réduire ce débit, mais a pour conséquence une perte en sensibilité de l'analyse

- sur la fréquence d'acquisition (temps de balayage) qui doit être très élevée pour décrire correctement un pic de 2^e dimension (>20-30Hz), ce qui généralement nuit à la sensibilité du détecteur HRMS.

ANNEXES

ANNEXE 1 : Valeurs des degrés d'orthogonalité et des capacités de pic calculés

pour le chapitre 05 partie A

Echantillon S56922

Combinaisons LC/SFC

| Dimension 1 (LC) | Dimension 2 (SFC) | Od | nc* _{2D} | Δc _{e,1} (%) | Δc _{e,2} * (%) | γ |
|-------------------|-------------------|-----|-------------------|-----------------------|-------------------------|-----|
| CSH C18-ACN-AMS | Resolve C18 | 1.9 | 3738 | 70.3 | 16.9 | 1.0 |
| CSH C18-ACN-AMS | Zorbax RX-SIL | 3.8 | 7134 | 70.3 | 33.9 | 0.9 |
| CSH C18-ACN-AMS | Nucleoshell HILIC | 4.1 | 2311 | 70.3 | 36.0 | 1.0 |
| CSH C18-ACN-AMS | Zorbax SB CN | 2.6 | 5076 | 70.3 | 22.8 | 1.0 |
| CSH C18-ACN-AMS | Synergie Polar RP | 2.1 | 2243 | 70.3 | 18.8 | 1.0 |
| CSH C18-ACN-AF | Resolve C18 | 1.9 | 3313 | 73.0 | 16.3 | 0.9 |
| CSH C18-ACN-AF | Zorbax RX-SIL | 3.8 | 6268 | 73.0 | 32.5 | 0.9 |
| CSH C18-ACN-AF | Nucleoshell HILIC | 4.0 | 2016 | 73.0 | 34.3 | 0.9 |
| CSH C18-ACN-AF | Zorbax SB CN | 2.7 | 4648 | 73.0 | 22.8 | 1.0 |
| CSH C18-ACN-AF | Synergie Polar RP | 2.2 | 2090 | 73.0 | 19.2 | 1.0 |
| CSH C18-ACN-AA | Resolve C18 | 1.6 | 2702 | 61.1 | 15.9 | 0.9 |
| CSH C18-ACN-AA | Zorbax RX-SIL | 3.1 | 5125 | 61.1 | 31.8 | 0.9 |
| CSH C18-ACN-AA | Nucleoshell HILIC | 3.3 | 1657 | 61.1 | 33.6 | 0.9 |
| CSH C18-ACN-AA | Zorbax SB CN | 2.2 | 3785 | 61.1 | 22.2 | 0.9 |
| CSH C18-ACN-AA | Synergie Polar RP | 1.9 | 1760 | 61.1 | 19.3 | 1.0 |
| CSH PFP-ACN-AF | Resolve C18 | 1.6 | 1749 | 58.6 | 16.9 | 1.0 |
| CSH PFP-ACN-AF | Zorbax RX-SIL | 3.1 | 3289 | 58.6 | 33.4 | 0.9 |
| CSH PFP-ACN-AF | Nucleoshell HILIC | 3.3 | 1058 | 58.6 | 35.2 | 0.9 |
| CSH PFP-ACN-AF | Zorbax SB CN | 2.2 | 2385 | 58.6 | 22.9 | 1.0 |
| CSH PFP-ACN-AF | Synergie Polar RP | 1.7 | 1034 | 58.6 | 18.6 | 1.0 |
| CSH PFP-ACN-AA | Resolve C18 | 1.4 | 1468 | 50.9 | 17.5 | 1.0 |
| CSH PFP-ACN-AA | Zorbax RX-SIL | 2.9 | 2827 | 50.9 | 35.3 | 1.0 |
| CSH PFP-ACN-AA | Nucleoshell HILIC | 2.9 | 877 | 50.9 | 35.9 | 1.0 |
| CSH PFP-ACN-AA | Zorbax SB CN | 1.9 | 1973 | 50.9 | 23.3 | 1.0 |
| CSH PFP-ACN-AA | Synergie Polar RP | 1.5 | 852 | 50.9 | 18.8 | 1.0 |
| CSH PFP-MeOH-AF | Resolve C18 | 2.0 | 1579 | 73.5 | 17.4 | 1.0 |
| CSH PFP-MeOH-AF | Zorbax RX-SIL | 4.0 | 2958 | 73.5 | 34.2 | 0.9 |
| CSH PFP-MeOH-AF | Nucleoshell HILIC | 4.1 | 931 | 73.5 | 35.2 | 0.9 |
| CSH PFP-MeOH-AF | Zorbax SB CN | 2.7 | 2123 | 73.5 | 23.2 | 1.0 |
| CSH PFP-MeOH-AF | Synergie Polar RP | 2.0 | 852 | 73.5 | 17.3 | 0.9 |
| CSH PFP-MeOH-AA | Resolve C18 | 1.7 | 1322 | 64.0 | 17.0 | 1.0 |
| CSH PFP-MeOH-AA | Zorbax RX-SIL | 3.8 | 2704 | 64.0 | 36.7 | 1.0 |
| CSH PFP-MeOH-AA | Nucleoshell HILIC | 3.8 | 834 | 64.0 | 37.2 | 1.0 |
| CSH PFP-MeOH-AA | Zorbax SB CN | 2.3 | 1717 | 64.0 | 22.0 | 0.9 |
| CSH PFP-MeOH-AA | Synergie Polar RP | 1.4 | 569 | 64.0 | 13.3 | 0.7 |
| BEH C18-ACN-NH4OH | Resolve C18 | 1.4 | 1491 | 70.7 | 12.7 | 0.7 |
| BEH C18-ACN-NH4OH | Zorbax RX-SIL | 3.5 | 3466 | 70.7 | 31.3 | 0.8 |
| BEH C18-ACN-NH4OH | Nucleoshell HILIC | 2.9 | 883 | 52.2 | 35.3 | 0.9 |
| BEH C18-ACN-NH4OH | Zorbax SB CN | 1.8 | 1901 | 52.2 | 21.9 | 0.9 |

| BEH C18-ACN-NH4OH | Synergie Polar RP | 2.1 | 1144 | 70.7 | 18.3 | 0.9 |
|--------------------|-------------------|-----|------|------|------|-----|
| BEH C18-ACN-AA | Resolve C18 | 1.5 | 1630 | 60.7 | 15.7 | 0.9 |
| BEH C18-ACN-AA | Zorbax RX-SIL | 3.1 | 3105 | 60.7 | 31.5 | 0.9 |
| BEH C18-ACN-AA | Nucleoshell HILIC | 3.3 | 1008 | 60.7 | 33.5 | 0.9 |
| BEH C18-ACN-AA | Zorbax SB CN | 2.1 | 2296 | 60.7 | 22.1 | 0.9 |
| BEH C18-ACN-AA | Synergie Polar RP | 1.9 | 1072 | 60.7 | 19.3 | 1.0 |
| BEH Shield-ACN-AF | Resolve C18 | 1.9 | 2290 | 70.2 | 16.6 | 0.9 |
| BEH Shield-ACN-AF | Zorbax RX-SIL | 3.8 | 4406 | 70.2 | 33.5 | 0.9 |
| BEH Shield-ACN-AF | Nucleoshell HILIC | 4.0 | 1417 | 70.2 | 35.4 | 1.0 |
| BEH Shield-ACN-AF | Zorbax SB CN | 2.6 | 3198 | 70.2 | 23.0 | 1.0 |
| BEH Shield-ACN-AF | Synergie Polar RP | 2.1 | 1419 | 70.2 | 19.1 | 1.0 |
| BEH Shield-ACN-AA | Resolve C18 | 1.6 | 1720 | 62.2 | 16.2 | 0.9 |
| BEH Shield-ACN-AA | Zorbax RX-SIL | 3.2 | 3284 | 62.2 | 32.6 | 0.9 |
| BEH Shield-ACN-AA | Nucleoshell HILIC | 3.4 | 1054 | 62.2 | 34.2 | 0.9 |
| BEH Shield-ACN-AA | Zorbax SB CN | 2.1 | 2223 | 70.3 | 18.4 | 1.0 |
| BEH Shield-ACN-AA | Synergie Polar RP | 1.9 | 1101 | 62.2 | 19.3 | 1.0 |
| Zr Carb-ACN-AF | Resolve C18 | 2.5 | 1941 | 90.0 | 17.5 | 1.0 |
| Zr Carb-ACN-AF | Zorbax RX-SIL | 4.4 | 3198 | 90.0 | 30.3 | 0.8 |
| Zr Carb-ACN-AF | Nucleoshell HILIC | 3.9 | 880 | 90.0 | 26.9 | 0.7 |
| Zr Carb-ACN-AF | Zorbax SB CN | 3.0 | 2343 | 90.0 | 21.0 | 0.9 |
| Zr Carb-ACN-AF | Synergie Polar RP | 2.7 | 1132 | 90.0 | 19.0 | 1.0 |
| Zr Carb-ACN-AA | Resolve C18 | 1.5 | 1130 | 53.5 | 17.4 | 1.0 |
| Zr Carb-ACN-AA | Zorbax RX-SIL | 3.0 | 2156 | 53.5 | 34.8 | 0.9 |
| Zr Carb-ACN-AA | Nucleoshell HILIC | 3.2 | 697 | 53.5 | 37.0 | 1.0 |
| Zr Carb-ACN-AA | Zorbax SB CN | 2.0 | 1511 | 53.5 | 23.1 | 1.0 |
| Zr Carb-ACN-AA | Synergie Polar RP | 1.7 | 677 | 53.5 | 19.3 | 1.0 |
| Zorbax CN-ACN-AF | Resolve C18 | 1.5 | 1726 | 53.8 | 17.3 | 1.0 |
| Zorbax CN-ACN-AF | Zorbax RX-SIL | 3.0 | 3300 | 53.8 | 34.8 | 0.9 |
| Zorbax CN-ACN-AF | Nucleoshell HILIC | 3.1 | 1057 | 53.8 | 36.6 | 1.0 |
| Zorbax CN-ACN-AF | Zorbax SB CN | 1.9 | 2250 | 53.8 | 22.4 | 1.0 |
| Zorbax CN-ACN-AF | Synergie Polar RP | 1.5 | 934 | 53.8 | 17.3 | 0.9 |
| BEH Phenyl-ACN-AF | Resolve C18 | 1.3 | 1880 | 51.2 | 16.4 | 0.9 |
| BEH Phenyl-ACN-AF | Zorbax RX-SIL | 2.8 | 3665 | 51.2 | 33.6 | 0.9 |
| BEH Phenyl-ACN-AF | Nucleoshell HILIC | 2.9 | 1181 | 51.2 | 35.5 | 1.0 |
| BEH Phenyl-ACN-AF | Zorbax SB CN | 1.9 | 2641 | 51.2 | 22.9 | 1.0 |
| BEH Phenyl-ACN-AF | Synergie Polar RP | 1.6 | 1186 | 51.2 | 19.2 | 1.0 |
| BEH Phenyl-MeOH-AF | Resolve C18 | 1.6 | 1457 | 56.6 | 17.2 | 1.0 |
| BEH Phenyl-MeOH-AF | Zorbax RX-SIL | 3.1 | 2/9/ | 56.6 | 34.6 | 0.9 |
| BEH Phenyl-MeOH-AF | Nucleoshell HILIC | 3.2 | 875 | 56.6 | 35.5 | 1.0 |
| BEH Phenyl-MeOH-AF | Zorbax SB CN | 2.1 | 1985 | 56.6 | 23.2 | 1.0 |
| BEH Phenyl-MeOH-AF | Synergie Polar RP | 1.7 | 848 | 56.6 | 18.5 | 1.0 |
| BEH Phenyl-ACN-AA | Resolve C18 | 1.2 | 2112 | 48.6 | 15.9 | 0.9 |
| BEH Phenyl-ACN-AA | | 2.5 | 4101 | 48.6 | 32.4 | 0.9 |
| BEH Phenyl-ACN-AA | | 2.6 | 1309 | 48.6 | 33.9 | 0.9 |
| BEH Phenyl-ACN-AA | ZORDAX SB CN | 1.8 | 3015 | 48.6 | 22.6 | 1.0 |
| BEH Phenyl-ACN-AA | Synergie Polar RP | 1.5 | 1380 | 48.6 | 19.3 | 1.0 |
| BEH Phenyl-MeOH-AA | Resolve C18 | 1.4 | 1684 | 51.9 | 1/.1 | 1.0 |

| BEH Phenyl-MeOH-AA | Zorbax RX-SIL | 2.8 | 3207 | 51.9 | 34.1 | 0.9 |
|-----------------------|-------------------|-----|------|------|------|-----|
| BEH Phenyl-MeOH-AA | Nucleoshell HILIC | 2.9 | 1008 | 51.9 | 35.2 | 0.9 |
| BEH Phenyl-MeOH-AA | Zorbax SB CN | 1.9 | 2305 | 51.9 | 23.2 | 1.0 |
| BEH Phenyl-MeOH-AA | Synergie Polar RP | 1.6 | 1016 | 51.9 | 19.1 | 1.0 |
| BEH Phenyl-ACN-NH4OH | Resolve C18 | 1.5 | 2680 | 68.7 | 13.4 | 0.8 |
| BEH Phenyl-ACN-NH4OH | Zorbax RX-SIL | 3.4 | 5854 | 68.7 | 31.1 | 0.8 |
| BEH Phenyl-ACN-NH4OH | Nucleoshell HILIC | 2.3 | 1216 | 40.9 | 35.3 | 1.0 |
| BEH Phenyl-ACN-NH4OH | Zorbax SB CN | 1.5 | 2665 | 40.9 | 22.3 | 1.0 |
| BEH Phenyl-ACN-NH4OH | Synergie Polar RP | 2.0 | 1952 | 68.7 | 18.3 | 0.9 |
| BEH Phenyl-MeOH-NH4OH | Resolve C18 | 1.1 | 1623 | 48.9 | 14.0 | 0.8 |
| BEH Phenyl-MeOH-NH4OH | Zorbax RX-SIL | 2.5 | 3530 | 48.9 | 32.3 | 0.9 |
| BEH Phenyl-MeOH-NH4OH | Nucleoshell HILIC | 2.1 | 882 | 36.0 | 35.8 | 1.0 |
| BEH Phenyl-MeOH-NH4OH | Zorbax SB CN | 1.3 | 1943 | 36.0 | 22.7 | 1.0 |
| BEH Phenyl-MeOH-NH4OH | Synergie Polar RP | 1.5 | 1156 | 48.9 | 18.7 | 1.0 |

Combinaisons SFC/SFC

| Dimension 1 (SFC) | Dimension 2 (SFC) | Od | nc* _{2D} | Δc _{e,1} (%) | Δc _{e,2} * (%) | γ |
|-------------------|-------------------|-----|-------------------|-----------------------|-------------------------|-----|
| Resolve C18 | Zorbax RX-SIL | 0.8 | 1396 | 17.6 | 27.6 | 0.7 |
| Resolve C18 | Nucleoshell HILIC | 0.9 | 469 | 15.6 | 34.0 | 0.9 |
| Resolve C18 | Zorbax SB CN | 0.4 | 729 | 15.6 | 15.1 | 0.6 |
| Resolve C18 | Synergie Polar RP | 0.4 | 451 | 17.6 | 15.7 | 0.8 |
| Zorbax RX-SIL | Nucleoshell HILIC | 0.8 | 456 | 36.9 | 13.7 | 0.4 |
| Zorbax RX-SIL | Zorbax SB CN | 0.7 | 1276 | 36.9 | 11.7 | 0.5 |
| Zorbax RX-SIL | Synergie Polar RP | 1.1 | 1068 | 36.9 | 18.8 | 1.0 |
| Nucleoshell HILIC | Zorbax SB CN | 1.0 | 568 | 37.2 | 17.3 | 0.7 |
| Nucleoshell HILIC | Synergie Polar RP | 1.1 | 319 | 37.2 | 18.2 | 0.9 |
| Zorbax SB CN | Synergie Polar RP | 0.6 | 645 | 23.4 | 16.8 | 0.9 |

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Combinaisons LC/SFC

| Dimension 1 (LC) | Dimension 2 (SFC) | Od | nc* _{2D} | Δc _{e,1} (%) | Δc _{e,2} * (%) | γ |
|------------------|-------------------|-----|-------------------|-----------------------|-------------------------|------|
| CSH C18-ACN-AF | Resolve C18 | 1.5 | 3016 | 52.2 | 17.9 | 0.79 |
| CSH C18-ACN-AF | Zorbax RX-SIL | 2.5 | 4160 | 52.2 | 29.7 | 0.80 |
| CSH C18-ACN-AF | Nucleoshell HILIC | 1.0 | 400 | 52.2 | 11.7 | 0.69 |
| CSH C18-ACN-AF | Zorbax SB CN | 1.6 | 3050 | 52.2 | 19.5 | 0.79 |
| CSH C18-ACN-AF | Synergie Polar RP | 1.5 | 1170 | 52.2 | 17.4 | 0.96 |
| CSH PFP-ACN-AA | Resolve C18 | 1.2 | 1723 | 34.5 | 22.6 | 0.99 |
| CSH PFP-ACN-AA | Zorbax RX-SIL | 2.0 | 2067 | 34.5 | 37.0 | 0.99 |
| CSH PFP-ACN-AA | Nucleoshell HILIC | 0.9 | 216 | 34.5 | 17.0 | 1.00 |
| CSH PFP-ACN-AA | Zorbax SB CN | 1.2 | 1493 | 34.5 | 21.0 | 0.85 |
| CSH PFP-ACN-AA | Synergie Polar RP | 1.0 | 549 | 34.5 | 18.0 | 0.99 |
| CSH PFP-MeOH-AA | Resolve C18 | 1.4 | 1583 | 39.1 | 21.8 | 0.96 |
| CSH PFP-MeOH-AA | Zorbax RX-SIL | 2.2 | 1917 | 39.1 | 36.0 | 0.97 |

| CSH PFP-MeOH-AA | Nucleoshell HILIC | 1.1 | 205 | 39.1 | 16.9 | 1.00 |
|-------------------|-------------------|-----|------|------|------|------|
| CSH PFP-MeOH-AA | Zorbax SB CN | 0.6 | 715 | 39.1 | 10.3 | 0.42 |
| CSH PFP-MeOH-AA | Synergie Polar RP | 1.1 | 528 | 39.1 | 18.2 | 1.00 |
| BEH C18-ACN-NH4OH | Resolve C18 | 2.0 | 3147 | 58.9 | 21.6 | 0.95 |
| BEH C18-ACN-NH4OH | Zorbax RX-SIL | 3.3 | 3716 | 58.9 | 34.8 | 0.94 |
| BEH C18-ACN-NH4OH | Nucleoshell HILIC | 1.6 | 408 | 58.9 | 16.7 | 0.99 |
| BEH C18-ACN-NH4OH | Zorbax SB CN | 2.3 | 3288 | 58.9 | 24.3 | 0.98 |
| BEH C18-ACN-NH4OH | Synergie Polar RP | 1.5 | 935 | 58.9 | 15.9 | 0.87 |
| BEH Shield-ACN-AF | Resolve C18 | 1.3 | 1963 | 44.0 | 17.9 | 0.79 |
| BEH Shield-ACN-AF | Zorbax RX-SIL | 2.1 | 2381 | 44.0 | 29.6 | 0.80 |
| BEH Shield-ACN-AF | Nucleoshell HILIC | 0.8 | 234 | 44.0 | 12.0 | 0.70 |
| BEH Shield-ACN-AF | Zorbax SB CN | 1.4 | 2000 | 44.0 | 19.6 | 0.79 |
| BEH Shield-ACN-AF | Synergie Polar RP | 1.2 | 765 | 44.0 | 17.5 | 0.96 |
| Zr Carb-ACN-AF | Resolve C18 | 1.6 | 2139 | 46.8 | 21.6 | 0.95 |
| Zr Carb-ACN-AF | Zorbax RX-SIL | 2.6 | 2503 | 46.8 | 34.5 | 0.93 |
| Zr Carb-ACN-AF | Nucleoshell HILIC | 1.1 | 244 | 46.8 | 14.3 | 0.84 |
| Zr Carb-ACN-AF | Zorbax SB CN | 1.8 | 2202 | 46.8 | 23.9 | 0.97 |
| Zr Carb-ACN-AF | Synergie Polar RP | 1.3 | 696 | 46.8 | 17.5 | 0.96 |

Combinaisons SFC/SFC

| Dimension 1 (SFC) | Dimension 2 (SFC) | Od | nc* _{2D} | Δc _{e,1} (%) | Δc _{e,2} * (%) | γ |
|-------------------|-------------------|-----|-------------------|-----------------------|-------------------------|-----|
| Resolve C18 | Zorbax RX-SIL | 0.4 | 815 | 22.7 | 11.7 | 0.3 |
| Resolve C18 | Nucleoshell HILIC | 0.4 | 185 | 22.7 | 11.1 | 0.7 |
| Resolve C18 | Zorbax SB CN | 0.2 | 616 | 22.7 | 6.9 | 0.3 |
| Resolve C18 | Synergi Polar RP | 0.5 | 487 | 22.7 | 12.9 | 0.7 |
| Zorbax RX-SIL | Nucleoshell HILIC | 0.7 | 243 | 37.2 | 12.5 | 0.7 |
| Zorbax RX-SIL | Zorbax SB CN | 0.4 | 770 | 37.2 | 7.2 | 0.3 |
| Zorbax RX-SIL | Synergi Polar RP | 0.8 | 585 | 37.2 | 13.0 | 0.7 |
| Nucleoshell HILIC | Zorbax SB CN | 0.4 | 168 | 17.0 | 15.7 | 0.6 |
| Nucleoshell HILIC | Synergi Polar RP | 0.4 | 68 | 17.0 | 14.6 | 0.8 |
| Zorbax SB CN | Synergi Polar RP | 0.5 | 508 | 24.6 | 13.5 | 0.7 |

ANNEXE 2 : Essais inter-laboratoire démontrant le potentiel de la SFC utilisée

comme méthode de contrôle d'impuretés pharmaceutiques

Article:

"First inter-laboratory study of a Supercritical Fluid Chromatography method for the determination of pharmaceutical impurities"

A. Raimbault, T. Leek, M. Hill, P. Hubert, P. Henning, C. West, C. Brunelli, M. Wong, R. Yadav, Y. Vander Heyden, E. Lesellier, L. Villemet, M. Sarrut, L. Dai, A. Grand-Guillaume-Perrenoud, N. Roques, K. Zhang, F. Mauge, N. Da Costa, S. Bertin, D. Guillarme, S. Rane, M. Iguiniz, S. Heinisch, E. Corbel, E. Lemasson, M. Grangrade, S. Sadaphule, S. Shringare, W. Farrell, N. Deshpande, R. Marini, V. Desfontaine, J-L. Veuthey, D. Kotoni, L. G. Losacco, A. Clarke, C. Muscat Galea, D. Mangelings, B. Jocher, E. Regalado, K. Plachka, L. Novakova, B. Wuyts, I. Francois, M. Gray, A. Aubin, A. Tarafder, M. Cazes, J. Lefèvre, C. Desvignes, Journal of Pharmaceutical and Biomedical Analysis, submitted

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First inter-laboratory study of a Supercritical Fluid Chromatography method for the determination of pharmaceutical impurities



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ABSTRACT

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Supercritical Fluid Chromatography (SFC) has known a strong regain of interest for the last 10 years, especially in the field of pharmaceutical analysis. Besides the development and validation of the SFC method in one individual laboratory, it is also important to demonstrate its applicability and transferability to various laboratories around the world. Therefore, an inter-laboratory study was conducted and published for the first time in SFC, to assess method reproducibility, and evaluate whether this chromatographic technique could become a reference method for quality control (QC) laboratories.

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Keywords: Supercritical Fluid Chromatography (SFC) Inter-laboratory study Collaborative study Reproducibility Pharmaceutical impurities Salbutamol sulfate This study involved 19 participating laboratories from 4 continents and 9 different countries. It included 5 academic groups, 3 demonstration laboratories at analytical instrument companies, 10 pharmaceutical companies and 1 food company. In the initial analysis of the study results, consistencies within- and between-laboratories were deeply examined. In the subsequent analysis, the method reproducibility was estimated taking into account variances in replicates, between-days and between-laboratories. The results obtained were compared with the literature values for liquid chromatography (LC) in the context of impurities determination. Repeatability and reproducibility variances were found to be similar or better than those described for LC methods, and highlighted the adequacy of the SFC method for QC analyses. The results demonstrated the excellent and robust quantitative performance of SFC. Consequently, this complementary technique is recognized on equal merit to other chromatographic techniques.

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1. Introduction

From its first commercialization in the 1980s, SFC has the reputation to be poorly reproducible and robust. However, the performance of modern SFC instruments has significantly improved since 2012 and it can now be considered as a well-established technology in pharmaceutical research/discovery environments [1,2]. Despite these instrumental advances, its application in more regulated laboratories still seems to be considered risky and this perception may continually hamper the implementation of routine SFC methods in QC environments.

Recently, several studies highlighted the excellent quantitative performance of SFC in the pharmaceutical domain [3-6]. The published methods were fully validated according to ICH Q2 guidelines and demonstrated the applicability of modern SFC in the context of pharmaceutical quality control. Nevertheless, the evaluation of method precision was limited to repeatability and intermediate precision, as the validation protocol included only one equipment/laboratory. To properly evaluate method reproducibility, the between-laboratories variability should also be studied by means of an inter-laboratory assay [7,8]. This evaluation is required when the analytical method has to be transferred and used in different laboratories, or is introduced as a reference method in a monograph. Inter-laboratory studies are well described in the literature for chromatographic techniques, especially liquid chromatography [9–11]. To the best of our knowledge, such data have never been published for SFC.

In a previous paper, a robust SFC method was developed for the determination of salbutamol sulfate related impurities according to the Quality by Design principles [6]. For this purpose, Design Space determination was employed to find out a robust working zone [12]. The optimization of a robust method was indeed a keystone to guarantee successful method transfer to several laboratories. Moreover, the developed method was fully validated according to the total error approach for the quantitative determination of impurities B, D, F and G, down to a concentration level of 0.3% of active pharmaceutical ingredient, in agreement with the specifications of pharmacopoeai method. This predictive validation strategy follows the requirements of ICH Q2 (R1) guideline and ensures that every future analysis result will fall within the acceptance limits (i.e. +-15% for impurities determination) with at least a probability of 95%. Method development and validation were performed considering the salbutamol impurities available as chemical reference standard (namely impurity B, D, F, I and G). To propose this method as a normative method, an inter-laboratory study is a mandatory step.

The objective of the present study was to estimate the precision (repeatability and reproducibility) of the results obtained for the determination of impurity D in salbutamol sulfate samples. The study protocol was proposed following the ISO 5725-2 international standard [8]. A detailed protocol was established to study the sources of variability at different levels, i.e. replicates, days and

laboratories. In this study, all experiments were conducted on one single type of instrument to avoid potential problems related to the delivery of a compressible fluid, backpressure control, and injection mode, often observed in SFC. Several academic, demonstration and industrial laboratories equipped with SFC technology were selected to take part in this study.

To ensure proper instrument handling and method set-up, a preliminary method test was organized to get familiar with the method and to verify various criteria, i.e. method selectivity, sensitivity and system repeatability. The results of this test were collated and evaluated by the study coordinator before starting the quantitative study.

In the following study the content of impurity D was evaluated in three independent salbutamol sulfate samples. These samples at different concentration levels for impurity D aimed at covering the validated dosing range. The results of this study were analysed according to the ISO guidelines [8]. Finally, the quantitative data issued from this study were used to assess measurement uncertainty.

2. Material and methods

2.1. Chemicals and reagents

Salbutamol hemisulfate (> 98.0%) was purchased from TCI Europe (Zwijndrecht, Belgium) and used as salbutamol hemisulfate standard. Related impurities B, D, F, G and I were provided by EDQM (Strasbourg, France). Salbutamol hemisulfate was split in three batches and each batch was spiked with different amount of related impurity D to get three salbutamol samples.

The minimal quality requirements for solvents and reagents were: methanol gradient grade, 2-propanol analytical grade, water ULC-MS/SFC grade, ammonium hydroxide 25 or 28% w/w analytical grade, carbon dioxide 99.995%.

2.2. Instrumentation

Each laboratory used a Waters Acquity UPC^{2®} equipped with a PDA detector (Waters, Milford, MA, USA). If MS or another detectors were hyphenated to the chromatographic system, they were disconnected prior to the experiments. The injector was equipped with a 5 or 10 μ L loop operating in the partial loop with needle overfill mode. 2-propanol (900 μ L) and water/methanol (50/50, v/v) (500 μ L) were used as weak and strong needle wash solvents, respectively. Chromatograms were recorded at 220 nm in compensated mode (310–410 nm) with an acquisition frequency of 20 Hz, a resolution of 1.2 nm and a filter time constant of 0.5 s.

a resolution of 1.2 nm and a filter time constant of 0.5 s. Masslynx[™] or Empower[™] software was used to control the system and acquire the data.

2.3. Chromatographic conditions

SFC conditions were reported in a previous publication [6]. The UPC² Torus Diethylamine (DEA) 100 × 3.0 mm (particle size of 1.7 μ m) analytical column was used. One new column was provided to each laboratory and was used immediately to perform the present study. The experiments were executed at a flow rate of 1.5 mL/min and 0.1% v/v ammonium hydroxide in methanol was used as modifier. The gradient mode was applied, with an initial modifier fraction of 2%, followed by a linear increase to 35% in 6.5 min. Post-run: the initial mobile phase conditions were reached within 0.5 min followed by 3 min of re-equilibration (total run time 10 min). The backpressure regulator was set at 135 bar (1958 psi). The autosampler temperature and the injected volume were set at 6 °C and 2 μ L, respectively.

2.4. Sample preparation

All solutions were prepared in water/methanol 20/80 v/v. After preparation, all solutions were stored in the dark at 5 °C (\pm 3 °C).

2.4.1. Preliminary testing

Stock solution containing impurities was prepared by transferring accurately weighed amounts of 5 mg impurity B, 5 mg impurity D, 5 mg impurity F and 5 mg impurity G in a volumetric flask of 50.0 mL. Intermediate solution was prepared by weighing an accurate amount of 20 mg salbutamol sulfate and adding $600 \,\mu$ L stock solution in a volumetric flask of 10.0 mL. Then the content of one vial of impurity I was dissolved with 1.0 mL of intermediate solution. This latter solution containing salbutamol sulfate and all related impurities is used to perform the preliminary test (system suitability test (SST) solution).

2.4.2. Inter-laboratory study

Stock solution of impurity D was prepared by adding an accurately weighed amount of 5 mg of impurity D in a volumetric flask of 5.0 mL. Then, calibration standards at 4, 6 and $8 \,\mu g \,m L^{-1}$ were prepared by means of dilutions (40, 60 and 80 μL of stock solution respectively in a volumetric flask of 10.0 mL).

Each lab received three salbutamol samples labelled sample A, B and C. These latters contained different amount of impurity D to evaluate the validated dosing range during this study: 0.2% of impurity D in salbutamol sulfate (sample B), 0.3% of impurity D (sample C) and 0.4% of impurity D (sample A). The study was performed in a blind way as the laboratories did not known samples concentration and level. Sample solution was prepared by adding an accurately weighed amount of 20 mg salbutamol sulfate unknown solid sample in a volumetric flask of 10.0 mL. Three independent solutions

were prepared for each sample and this protocol was repeated on three days.

2.5. Preliminary testing

The first step of this collaborative study was to perform a familiarisation experiment, which allowed also checking the reliability of SFC instruments for further quantitative analysis. This preliminary testing was implemented to verify several performance criteria: selectivity, retention times stability, peak area variability and sensitivity. To verify method selectivity and sensitivity, SST solution was injected 6 times using the above described SFC method. The RSD values should be < 1% for retention times (for all compounds) and < 2% for peak areas (only for impurities). The signal-to-noise ratio (S/N) was calculated for impurity D as described by USP:

$$S/N=2H/h$$
(1)

where *H* is the height of the peak measured from the peak apex to a baseline extrapolated over a distance \geq 5 times the peak width at its half-height; and *h* is the amplitude of the noise values observed over a distance \geq 5 times the peak width at half-height and, if possible, situated equally around the peak of interest. The S/N ratio should be higher than 25.

2.6. Set-up of the inter-laboratory study

The study involved 19 participating analytical laboratories (p = 19): 5 academic (universities), 3 demonstration laboratories at analytical instrument company, 1 food and 10 pharmaceutical companies. These laboratories are located on 4 continents and in 9 countries. These 19 sites present different quality standard: GMP for the sending lab, ISO 9001 for the food company lab, GMP for several pharmaceutical companies and R&D instrument in GMP environment for the others. One demonstration lab has ISO 9001 and ISO 13485 certifications. Academic and the two other demonstration laboratories do not have any certification. Each laboratory performed the analyses in three different days (series) (c=3). Per day, the samples were prepared and analysed independently in triplicate (g=3) considering 3 concentrations levels (q=3) by means of samples A, B and C. The study layout per concentration (sample) is summarized in Fig. 1.

This study layout enables the inclusion of day/series variability as generally done in a method validation protocol. It allows estimating the intermediate precision for each laboratory, which is the sum of intra-day and inter-day variances. The study layout provides information on three sources of variability (i.e. replicates, days, laboratories), which are the main components of method reproducibility. Each laboratory reported raw data in a validated



Fig. 1. Set up of the collaborative study per sample: p = 19 laboratories, c = 3 series (days), g = 3 replicate measurements.

and locked Excel file. The study coordinator performed all data and statistical analyses using Excel (Microsoft Excel[®] for Mac 2011) followed by a report verification by the study supervisors.

2.7. Statistical analysis

2.7.1. Scrutiny of results for consistency and outliers

First, the results were critically examined for outliers and stragglers regarding between-laboratory and within-laboratory consistency. This examination was done by graphical consistency techniques and numerical outlier tests specified in the ISO guide-lines [8,13]. Tables with critical values for all mentioned tests can be found in the ISO guidelines [8]. Mandel's *k* plotting and Cochran's test were used to verify whether the within-laboratory variances of some laboratories. Mandel's *k* statistic was calculated as:

$$k_{ij} = \frac{s_{ij}\sqrt{p_j}}{\sqrt{\Sigma s_{ij}^2}} \tag{2}$$

where s_{ij} is the standard deviation within one cell (laboratory) at concentration level j and p_j is the number of laboratory reporting test result for concentration level j.

Mandel's k values were plotted to graphically evaluate the within-laboratory variation. The indicator values at 1% and 5% significance levels were drawn on the Mandel's plots.

The Cochran's test was applied as numerical outlier test and calculated as followed:

$$C = \frac{s_{max}^2}{\sum_{i=1}^p s_i^2}$$
(3)

where s_{max} is the highest variance obtained for one sample and s_i^2 is the variance within one laboratory for this sample. The variance is considered to be an outlier when C is larger than the 1% critical value and a straggler when C is smaller than the 1% critical value but larger than the 5% one. Outliers were noted ** and stragglers * in the results tables.

Mandel's h plotting and Grubb's tests were used to verify whether laboratories with deviating results compared to those of the others (between-laboratory variance consistency) occur. Mandel's h statistic was calculated as:

$$h_{ij} = \frac{\bar{x_{ij}} - x_j}{\sqrt{\frac{1}{p_j - 1} \sum_{i=1}^{p_j} (\bar{x_{ij}} - x_j)^2}}$$
(4)

where $\bar{x_{ij}}$ represents a cell (laboratory) mean and x_j the general mean for concentration level j. Mandel's *h* values were also plotted to graphically evaluate the between-laboratory variation. The Grubb's tests were finally used as a numerical outlier tests. They

are structured in four subsequent tests. First, the test to determine whether the largest observation (x_p) is an outlier:

$$G_{p} = \frac{\bar{x_{p}} - x_{j}}{\sqrt{\frac{1}{p_{j} - 1} \sum_{i=1}^{p_{j}} (\bar{x_{ij}} - x_{j})^{2}}}$$
(5)

Simultaneously, the test is used to determine whether the smallest observation $(\bar{x_1})$ is an outlier:

$$G_{1} = \frac{x_{j} - \bar{x_{1}}}{\sqrt{\frac{1}{p_{j} - 1} \sum_{i=1}^{p_{j}} (\bar{x_{ij}} - x_{j})^{2}}}$$
(6)

When the single Grubb's test is negative, then the equivalent double Grubb's test is performed. This Grubb's test is used to examine whether either the two largest $(G_{p-1,p})$ or two smallest $(G_{1,2})$ observations are outliers

$$G_{p-1,p} = \frac{\sum_{i=1}^{p-2} (\bar{x_{ij}} - x_{p-1,p})^2}{\sum_{i=1}^{p_j} (\bar{x_{ij}} - x_j)^2}$$
(7)
$$G_{1,2} = \frac{\sum_{i=3}^{p} (\bar{x_{ij}} - x_{1,2})^2}{\sum_{i=1}^{p_j} (\bar{x_{ij}} - x_j)^2}$$
(8)

where $x_{p-1,p}$ is the average of the two largest observations and $x_{1,2}$ of the two smallest observations in the data set. For the single Grubb's test, outliers and stragglers gives rise to values exceeding the 1% and 5% critical values respectively. For the double Grubb's test, outliers and stragglers gives rise to values smaller than the 1% and 5% critical values respectively.

2.7.2. Variances estimation

After testing and discarding the outliers, the mean squares between laboratories ($MS_{laboratories}$), between days (MS_{days}) and between replicates ($MS_{replicates}$) were calculated applying the variance analysis detailed in Table 1.

From the means squares, the repeatability (s_r^2) , betweenlaboratories $(s_{laboratories}^2)$ and reproducibility variances (s_R^2) were estimated [9].

According to the ISO 5725-2 guidelines, the calculation of the repeatability (s_r^2) and reproducibility (s_R^2) estimates were performed using the following equations:

$$s_r^2 = s_{replicates}^2 \tag{9}$$

$$s_R^2 = s_{replicates}^2 + s_{laboratories}^2 \tag{10}$$

Table 1

Analysis of variance components (p = number of laboratories, c = number of days per laboratory, g = number of replicates per day).

| Sources of variability | Mean squares | Estimated variance |
|------------------------|--|---|
| Laboratories | $MS_{laboratories} = \frac{cg \Sigma(\tilde{x}_i - \tilde{x})^2}{p-1}$ | $s_{laboratories}^2 = \frac{MS_{laboratories} - MS_{days}}{cg}$ |
| Days | $MS_{days} = \frac{g\Sigma\Sigma(\bar{x}_{ij} - \bar{x}_i)^2}{p(c-1)}$ | $S_{days}^2 = \frac{MS_{days} - MS_{replicates}}{g}$ |
| Replicates | $MS_{replicates} = \frac{\sum \sum \hat{(x_{ijk} - \bar{x}_{ij})^2}}{pc(g-1)}$ | $s_{replicates}^2 = s_r^2 = MS_{replicates}$ |



Fig. 2. Representative SFC chromatogram of salbutamol sulfate and its related impurities. Experimental conditions: see text.

In the present study, the protocol layout involved three independent series for each laboratory by means of three different days. Consequently, reproducibility was estimated according to [9]:

$$s_R^2 = s_{replicates}^2 + s_{days}^2 + s_{laboratories}^2 \tag{11}$$

2.7.3. Uncertainty estimation

The reproducibility variance allowed the estimation of the standard uncertainty u_x using the following equation:

$$u_x = \sqrt{s_R^2} \tag{12}$$

Therefore, the expanded uncertainty U_x could be calculated as:

$$U_x = 2u_x \tag{13}$$

using a coverage factor k = 2 [14].

2.7.4. Trueness criterion

The z-score gives a bias estimate of the results. An absolute z-scores below 2 are acceptable. A zone of doubtful performance exists for absolute Z-scores between 2 and 3. Those results do not necessarily have to be unacceptable, since there is some uncertainty on how close the assigned sample value is to the unknown true value. However, an absolute Z-score of 3 or more can be interpreted as an unacceptable performance [15].

For the present study, Z-score was calculated for each laboratory according to:

$$z = \frac{\bar{x}_i - \hat{x}}{\sigma} \tag{14}$$

where $\bar{x_i}$ is the mean value reported by an individual laboratory, \hat{x} is the assigned sample value, σ is the standard deviation (without outlier lab).

3. Results and discussion

3.1. Preliminary testing - performance criteria

The first step of this collaborative study was checking the ability of each laboratory to perform quantitative analysis of the salbutamol sulfate samples. A preliminary testing was done to verify several performance criteria: selectivity, retention times stability, peak area variability and sensitivity. A typical chromatogram is presented in Fig. 2. In terms of sensitivity, the signal-to-noise ratio was measured for impurity D and was always higher than 25, as highlighted for each laboratory in supplementary data Table 1. Nevertheless, a large variability in the S/N values was observed, varying from 34 to 918 with an average of 143. The UV lamp power (depending on its rated life) may partly explain this variability. A difference of noise measurement could also be suspected, especially considering that different software was used within the participant laboratories. As all laboratories fulfilled the sensitivity and variability criterion for impurity D (see below), this S/N difference was not further investigated.

Besides sensitivity, each individual laboratory also reported an adequate separation of API and related impurities (baseline separation of all peaks). The observed retention times variability with six replicates was always lower than 0.2%, as shown in supplementary data Table 1. Finally, the RSD values on the peak area for six consecutive injections was below 2.0%, except for a few values. The higher variability observed for impurity F could be explained by the more difficult peak integration (more tailing of this peak). The integration of impurity B is harder due to the baseline slope at the end of the gradient leading to higher variability. Nevertheless, this criterion was satisfied by each lab for impurity D (RSD values between 0.27 and 1.89%), which is the target analyte in the present study. All laboratories successfully passed the preliminary step and performed the collaborative study.

3.2. Inter-laboratory study – quantitative results

Using a validated and locked Excel sheet, each laboratory reported the mass content (% m/m) of impurity D in salbutamol sulfate A, B and C samples. The results are summarized in supplementary data Table 2 and supplementary Fig. 1.

3.2.1. Scrutiny of results for consistency and outliers

Within- and between-laboratories consistencies were examined by means of the graphical Mandel's methods and with numerical outlier tests.

| Table 2 | |
|--|------------|
| Within-laboratory results consistency (**outlier, *s | traggler). |

| | | Mande | el's k statistics | |
|-----------------|---------------|---|---------------------------------|---------------------------|
| Lab number | | Sample A | Sample B | Sample C |
| 01 | | 2.481** | 2.197** | 2.130** |
| 02 | | 0.386 | 0.213 | 0.391 |
| 03 | | 0.640 | 0.961 | 0.662 |
| 04 | | 0.797 | 1.123 | 0.975 |
| 05 | | 0.947 | 1.193 | 0.631 |
| 06 | | 0.494 | 0.613 | 0.370 |
| 07 | | 0.824 | 1.479* | 0.854 |
| 08 | | 0.900 | 0.823 | 1.074 |
| 09 | | 1.188 | 0.891 | 1.396 |
| 10 | | 0.963 | 0.565 | 0.585 |
| 11 | | 1.628** | 0.924 | 1.248 |
| 12 | | 0.825 | 0.874 | 0.658 |
| 13 | | 0.642 | 0.595 | 0.869 |
| 14 | | 0.543 | 0.616 | 0.877 |
| 15 | | 0.494 | 0.930 | 0.834 |
| 16 | | 1.165 | 1.065 | 1.112 |
| 17 | | 0.379 | 0.715 | 1.173 |
| 18 | | 0.672 | 0.751 | 0.562 |
| 19 | | 0.668 | 0.859 | 1.036 |
| Indicator valu | es for Mande | 1's k statistics (p=19, n=9 | 9) | |
| 5 % level | 1.38 | | <i>č</i> | |
| 1 % level | 1.56 | | | |
| | | Coc | hran's test | |
| | | Sample A | Sample B | Sample C |
| С | | 0.3240** (outlier lab 0) | 1) 0.2540** (outlier lab 01) | 0.2389** (outlier lab 01) |
| Critical values | s (p=19, n=9) | | | |
| 5 % level | 0.1500 | | | |
| 1 % level | 0.1738 | | | |
| | | Second Cochran's test | (after elimination of outliers) | |
| C | | 0.2064** (outlier lab 1) | 1) 0.1544 | 0.1347 |
| Critical values | s (p=18, n=9) | | | |
| 5 % level | 0.1579 | | | |
| 1 % level | 0.1829 | | | |
| | | Third Cochran's test (| (after elimination of outliers) | |
| C | | 0.1386 | nd | nd |
| Critical values | s (p=17, n=9) | | | |
| 5 % level | 0.1658 | | | |
| 1 % level | 0.1920 | | | |
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Fig. 3. Mandel's *k* plotting – within-laboratory consistency. Samples A, B and C were represented in orange, purple and blue, respectively. Indicator lines at 1% (red) and 5% (black) significance levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Sample A Sample B Sample C

Table 3

Between-laboratories results consistency.

| Mandel's <i>h</i> statistics | | | | | | |
|------------------------------|-------------------|-----------------------|---------------------------------|----------|--|--|
| Lab number | | Sample A | Sample B | Sample C | | |
| 01 | | 1.978* | 2.260* | 2.159* | | |
| 02 | | 0.560 | 0.781 | 0.828 | | |
| 03 | | 0.398 | 0.318 | 0.570 | | |
| 04 | | 0.566 | 1.267 | 0.841 | | |
| 05 | | -0.861 | -0.404 | -0.872 | | |
| 06 | | -0.036 | 0.206 | -0.246 | | |
| 07 | | -0.658 | 0.262 | 0.075 | | |
| 08 | | -0.801 | -0.371 | -0.342 | | |
| 09 | | -1.344 | -1.102 | -0.518 | | |
| 10 | | -0.203 | 0.213 | -0.044 | | |
| 11 | | 2.095* | 1.672 | 2.299* | | |
| 12 | | -1.388 | -1.611 | -1.441 | | |
| 13 | | 0.064 | -0.733 | -0.374 | | |
| 14 | | -1.115 | -1.035 | -1.178 | | |
| 15 | | -0.942 | -0.956 | -0.896 | | |
| 16 | | -0.108 | 0.160 | -0.161 | | |
| 17 | | -0.605 | -0.957 | -0.773 | | |
| 18 | | 0.570 | 0.168 | 0.252 | | |
| 19 | | 0.691 | -0.136 | -0.180 | | |
| Indicator values f | for Mandel's h st | atistics (p=19) | | | | |
| 5 % level | | (P ->) | 2.37 | | | |
| 1 % level | | | 1.88 | | | |
| | Gru | bb's test on lab mea | n (one outlying observation) | | | |
| | | Sample A | Sample B | Sample C | | |
| Gn | | 2.159 | 2.624 | 2.299 | | |
| G_1^r | | 1.331 | 1.871 | 1.441 | | |
| Critical values (p | = 19) | | | | | |
| 1 % level | 2.968 | | | | | |
| 5 % level | 2.681 | | | | | |
| | Gru | bb's test on lab mear | 1 (two outlying observations) | | | |
| | | Sample A | Sample B | Sample C | | |
| Gn-1 n | | 1.099 | 1.056 | 1.099 | | |
| G ₁₂ | | 0.986 | 1.034 | 0.986 | | |
| Critical values (p | = 19) | | | | | |
| 1 % level | 0.3398 | | | | | |
| 5 % level | 0.4214 | | | | | |
| | Grubb's tes | t on individual meas | urements (one outlying observat | ion) | | |
| | | Sample A | Sample B | Sample C | | |
| G _p | | 4.276** | 3.223** | 3.474** | | |
| \mathbf{G}_{1}^{r} | | 1.863 | 1.988 | 1.877 | | |
| Outlier | | Lab 01 | Lab 01 | Lab 01 | | |
| Critical values (p | = 19) | | | | | |
| 1 % level | 2.968 | | | | | |
| 5 % level | 2.681 | | | | | |
| | Grubb's test | on individual measu | rements (two outlying observat | ions) | | |
| | | Sample A | Sample B | Sample C | | |
| G _{n-1 n} | | 0.923 | 0.916 | 1.001 | | |
| G ₁ 2 | | 0.922 | 0.923 | 1.000 | | |
| Critical values (n | = 19) | | | | | |
| 1 % level | ~) | | 0.3398 | | | |
| 5 % level | | | 0.4214 | | | |
| | | | | | | |

3.2.1.1. Within-laboratory variance tests. Mandel's *k* plotting and Cochran's test were used to verify whether the within-laboratory variance (repeatability) of all laboratories can be considered equal. These tests were performed considering 9 measurements for each sample in each laboratory. Results have been reported in Table 2 and Fig. 3.

On the Mandel's *k* plot (Fig. 3), indicator lines at 1 and 5% significance levels were drawn. Laboratory 01 tends to show a higher repeatability variance. The same observation was made for laboratory 11 regarding sample A (borderline case). Numerical outlier testing was also performed by means of a Cochran's test (see Table 2). This test highlighted outlier values for laboratory 01 at all concentration levels (samples A, B, C). A second Cochran's test indicated an outlier for sample A at laboratory 11. The third Cochran's test did not suggest outlier or straggler. However, it is important to keep in mind that the repetition of statistical tests may lead to excessive rejection. Moreover, for laboratory 11, an outlier value

was only observed for one sample and this value was much more acceptable than in laboratory 01. In this context, laboratory 01 can be considered as an outlying laboratory, while sample A analysed in laboratory 11 was not discarded at this stage.

3.2.1.2. Between laboratories variance tests. Mandel's *h* plotting and Grubbs' tests were used to verify whether laboratories with deviating results occur. Results have been reported in Table 3 and Fig. 4. On the Mandel's *h* plot, indicator lines at 1 and 5% significance levels were drawn.

As illustrated in Fig. 4, laboratories 01 and 11 tend to report higher concentrations than the other laboratories, but Mandel's *h* remains below the 5% significance level. The raw data of these two laboratories were thoroughly investigated. For laboratory 01, the three calibration curves obtained were not linear, probably due to a standard preparation issue when making the dilutions. For laboratory 11, a systematic lower AUC of calibration standards led to



Fig. 4. Mandel's *h* plotting – between-laboratories consistency. Samples A, B and C were represented in orange, purple and blue, respectively. Indicator lines at 1% (red) and 5% (black) significance levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4

Cochran's test results without outlying values (**outlier).

| Cochran's test | | | |
|-------------------------------------|--------------------------------|---------------------------|---------------------------|
| | Sample A | Sample B | Sample C |
| C Critical values (p = 19, n = 8 | 0.2148** (outlier lab 01)) | 0.2353** (outlier lab 01) | 0.1918** (outlier lab 01) |
| 5 % level 1 % level | | 0.1583 0.1844 | |

an overestimation of sample content. The preparation of impurity D stock solution seemed to be the source of this issue. It is important to notice that the root causes are not related to the analytical technique (SFC) but to sample and/or standard preparation.

This Mandel's *h* plot also highlighted the quite balanced distribution of reported values around the mean value. Grubb's tests were performed on the means of the values reported by each laboratory. As illustrated in Table 3, no outlier or straggler value was reported.

These Grubb's tests could also be performed on the individual measurements (individual measurements reported) where the Cochran's test has shown the lab variance was suspicious. These results were also reported in Table 4. Grubb's test highlighted one outlying value for an individual measurement reported by laboratory 01. Consequently, this value was discarded and the Cochran's test was repeated on the remaining data set. Without this individual outlying value, the within-laboratory variance of laboratory 01 still remains significantly higher than in other laboratories, as shown in Table 4.

As already mentioned, an in-depth evaluation of individual laboratory reports showed that laboratory 01 obtained non-linear calibration curves for two series, but the curve profiles for both series were different. According to the laboratory report, accurate balance and appropriate glassware and pipettes were used. However, regarding the calibration curves, an inadequate weighing of standards and/or inappropriate use of the automatic pipette were suspected The random errors were mainly explained by an operator training/ability to work with low mass weighing (5 mg) and accurate dilutions. This observation is also corroborated by the function of the operator, which was an not a qualified and fully trained analyst. Consequently, this laboratory data cannot be considered as reliable and the lab 01 was definitively discarded for the evaluation of method precision.

3.2.1.3. Results consistency. As required by the ISO guidelines, results were removed from the original data set when they were outliers with the numerical technique, or when they were stragglers with the numerical technique and they exceed the 1% critical level on the Mandel plot. Table 5 summarized the results and outliers values. As above explained, outlier values (lab 01) were discarded for method variances estimation. The outlier values obtained were mainly explained by samples and standards preparation. This step of the analytical protocol is similar whatever the analytical technique used for the quantitative analysis (i.e. LC or SFC or other technique).

3.2.2. Variances estimation

The final objective of this inter-laboratory study was to estimate the method variance and variance components. These results were summarized in Table 6.

As shown in Table 6, the total method variability was mainly due to the "laboratory" factor (contribution around 70% at all concentration levels). The contributions from the "day" and "replicate" factors were quite similar (10 to 15% of the total variance), with a slightly larger impact of the day factor, except at the highest concentration level. It is often expected to have the reproducibility about 2 to

Summary of labs results (**outlier).

| | Average impurity D content in salbutamol sulfate (% m/m | | | | | | | | |
|------------|---|----------|----------|--|--|--|--|--|--|
| Lab number | Sample A | Sample B | Sample C | | | | | | |
| 01 | 0.4384** | 0.2444** | 0.3689** | | | | | | |
| 02 | 0.3960 | 0.2124 | 0.3346 | | | | | | |
| 03 | 0.3912 | 0.2024 | 0.3280 | | | | | | |
| 04 | 0.3962 | 0.2229 | 0.3349 | | | | | | |
| 05 | 0.3536 | 0.1868 | 0.2908 | | | | | | |
| 06 | 0.3782 | 0.2000 | 0.3070 | | | | | | |
| 07 | 0.3596 | 0.2012 | 0.3152 | | | | | | |
| 08 | 0.3553 | 0.1875 | 0.3045 | | | | | | |
| 09 | 0.3680 | 0.1717 | 0.3000 | | | | | | |
| 10 | 0.3732 | 0.2001 | 0.3122 | | | | | | |
| 11 | 0.4419 | 0.2317 | 0.3725 | | | | | | |
| 12 | 0.3378 | 0.1607 | 0.2762 | | | | | | |
| 13 | 0.3812 | 0.1797 | 0.3037 | | | | | | |
| 14 | 0.3460 | 0.1731 | 0.2830 | | | | | | |
| 15 | 0.3511 | 0.1748 | 0.2902 | | | | | | |
| 16 | 0.3761 | 0.1990 | 0.3091 | | | | | | |
| 17 | 0.3612 | 0.1748 | 0.2934 | | | | | | |
| 18 | 0.3963 | 0.1992 | 0.3198 | | | | | | |
| 19 | 0.3999 | 0.1926 | 0.3087 | | | | | | |
| Mean | 0.3790 | 0.1955 | 0.3133 | | | | | | |

4 times higher than the repeatability, when considering the standard deviations [16]. In the present study, ratios close to 3 were observed for the whole dosing range (i.e. from 0.2 to 0.4% of impurity D). Considering variances (s^2), ratios between reproducibility and repeatability were within the range 6–10 (a 4–9 range is often advised [13]). However, it is important to notice that both ratios within or above this range have reported in the literature for LC method, including ratios close to 80 for the determination of impurities [9].

The reproducibility variance was 2-3 times larger than the intermediate precision (repeatability variance+days variance), confirming the important contribution of the "laboratory" to the total variability. This laboratory contribution to the total variance could be explained by (i) the use of various SFC systems (not evaluated during method validation performed using only one equipment), (ii) the recent SFC implementation in many participating laboratories, (iii) the difficulty to handle low masses and low dilution volumes, (iv) the CO₂ supply that was not evaluated during method optimization and validation. The contribution of different equipment/systems and some technical aspects related to samples and standards preparation are analytical aspects that need to be considered independent of the separation techniques. Nevertheless the reproducibility values, that take into account all variability components, are close to or even lower than those reported for LC impurities determination. Our results obtained with several modern SFC systems in several laboratories highlighted the reliability of this technique.

Table 6

Estimation of the variance components (p = 18).

Finally, to present some more intuitive values, standard deviations and relative standard deviations were calculated for both repeatability and reproducibility. The relationship between standard deviation and impurity D concentration was presented in Fig. 5. As expected, the standard deviation was proportional to the concentration (linear relationship) while the relative standard deviation was rather constant within the validated dosing range (Table 6).

RSD reproducibility values close to or below 10% were obtained in this study. Considering all sources of variability, i.e. replicates, days and 18 laboratories (meaning 18 instruments and 18 operators), these good RSD values again clearly highlighted the reliability of this SFC method for the quantification of salbutamol sulfate impurity D.

3.2.3. Measurement uncertainty evaluation

The expanded uncertainty values are described in Table 7. For a non-conform sample (0.4% of impurity D), the result was expected to have an expanded uncertainty of 0.058% m/m. Therefore, 95% of the reported values are expected to be comprised between 0.342 and 0.458%. As illustrated in supplementary Table 2, the individual measurements fulfilled this expectation, since 9 out of 171 measurements in sample A(5%) were outside the expanded-uncertainty range. Using the mean value of each laboratory, only one laboratory was outside the range for concentrations of 0.2 and 0.3% (samples C and B) and two laboratories were outside the range for concentration of 0.4%. The laboratory outside the range was the one previously discarded by the outlier statistical tests (lab 01). As observed for the variance estimation, the relative expanded uncertainty values were also lower or equivalent those described in the literature for LC methods using a similar study protocol [9].

3.2.4. Trueness criterion

In the present study, the "true" value of the impurity D content in the three salbutamol sulfate samples is unknown. Consequently, to estimate the trueness, Z-scores were calculated using the general mean (without outlier) as assigned value (see supplementary data Table 3). Fig. 6 demonstrates that the laboratories with the highest |z-scores| were those highlighted during the outliers evaluation. In conclusion, during the preliminary screening, none of the participating laboratories, except laboratories 01 and 11 showed a significant bias.

4. Conclusion

A collaborative study was carried out on the SFC method to determine the content of impurity D in salbutamol sulfate API. After the development and validation of a robust SFC method in one single laboratory (the development lab), the precision of this method in various laboratories around the world was demonstrated. It is

| Sources of variability | Impurity D at 0.2 % (sample B) | Impurity D at 0.3 % (sample C) | Impurity D at 0.4 % (sample A) |
|-------------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Variances | | | |
| Laboratories $(s_{laboratories}^2)$ | $3.23 	imes 10^{-4}$ | $4.67 	imes 10^{-4}$ | $5.83	imes10^{-4}$ |
| Days (s_{days}^2) | $5.96 	imes 10^{-5}$ | $1.04 	imes 10^{-4}$ | $1.27 	imes 10^{-4}$ |
| Replicates $(s_{replicates}^2)$ | 4.19×10^{-5} | 7.37×10^{-5} | $1.26 	imes 10^{-4}$ |
| Repeatability variance (s_r^2) | $4.19 	imes 10^{-5}$ | 7.37×10^{-5} | 1.26×10^{-4} |
| Reproducibility variance (s_R^2) | $4.24 	imes 10^{-4}$ | $6.45 	imes 10^{-4}$ | $8.36	imes10^{-4}$ |
| Ratio $(s_R^2)/(s_r^2)$ | 10.13 | 8.75 | 6.62 |
| Repeatability sd (sr) | 6.47×10^{-3} | 8.59×10^{-3} | 1.13×10^{-2} |
| Reproducibility sd (s_R) | 2.06×10^{-2} | $2.54 	imes 10^{-2}$ | $2.89 	imes 10^{-2}$ |
| Ratio $(s_R)/(s_r)$ | 3.18 | 2.96 | 2.57 |
| Repeatability RSD (%) | 3.36 % | 2.77 % | 2.99 % |
| Reproducibility RSD (%) | 10.68 % | 8.19 % | 7.69 % |
| | | | |



Fig. 5. Standard deviations vs. concentration level relationship. Repeatability (blue triangles), reproducibility (red crosses). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 7

Estimation of the measurement uncertainty.





Fig. 6. Z-scores of the participating laboratories. Samples A, B and C were represented in orange, purple and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

important to mention that this step of reproducibility evaluation is mandatory to propose the method as an alternative to current normative methods. The method reproducibility was estimated by taking into account replicates, days and laboratories variances. The values obtained were compared with those published in the literature in the context of impurities determination [9,11]. For this SFC method, repeatability and reproducibility variances were similar or better than the ones described for LC methods. The reproducibility values highlighted the reliability of the method and its potential use in different labs for QC analysis. For the first time, the quantitative and robust performance of modern SFC was demonstrated by means of a collaborative study, showing its potential to replace to other chromatographic techniques for pharmaceutical quality control. Finally, as the study involved only Waters[®] instrumenta-

tion, an expanded study should be performed including different manufacturer's equipment.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.08.042.

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| | Retention times (min) and RSD (%, n =6) | | | | | | | Peak area RSD (%, n=6) | | | | | |
|------------|---|------------------|------------------|------------------|------------------|------------------|---|------------------------|------|------|------|------|----------|
| Lab | D | G | Ι | В | API | F | | D | G | Ι | В | F | imp D |
| 01 | 2.64 (0.10) | 3.41 (0.09) | 3.69 (0.06) | 3.96 (0.05) | 4.76 (0.04) | 6.10 (0.05) | C |).45 | 1.87 | 1.85 | 4.36 | 1.58 | 66 |
| 02 | 2.74 (0.04) | 3.46 (0.02) | 3.74 (0.02) | 3.98 (0.02) | 4.77 (0.02) | 6.11 (0.01) | C |).33 | 0.41 | 0.88 | 3.30 | 1.89 | 66 |
| 03 | 2.65 (0.05) | 3.44 (0.03) | 3.74 (0.01) | 4.11 (0.01) | 4.79 (0.01) | 6.12 (0.01) | 1 | .10 | 1.15 | 0.20 | 0.52 | 1.16 | 227 |
| 04 | 2.60 (<0.005) | 3.36 (0.12) | 3.66 (<0.005) | 4.04 (0.13) | 4.72 (<0.005) | 6.06 (<0.005) | 1 | .00 | 1.22 | 1.35 | 1.13 | 8.27 | 39 |
| 05 | 2.69 (0.13) | 3.26 (0.11) | 3.53 (0.08) | 3.95 (0.07) | 4.63 (0.07) | 5.90 (0.06) | 1 | .75 | 1.40 | 1.15 | 1.78 | 1.23 | 34 |
| 06 | 2.45 (0.10) | 3.27 (0.07) | 3.54 (0.04) | 3.95 (0.03) | 4.64 (0.03) | 6.02 (0.01) | C |).69 | 1.11 | 0.49 | 0.56 | 0.59 | 61 |
| 07 | 2.90 (<0.005) | 3.63 (<0.005) | 3.96 (0.10) | 4.28 (<0.005) | 4.95 (0.08) | 6.38 (<0.005) | 1 | .89 | 1.67 | 1.31 | 1.30 | 12.4 | 34 |
| 08 | 2.90 (<0.005) | 3.40 (<0.005) | 3.80 (<0.005) | 4.20 (<0.005) | 4.90 (<0.005) | 6.30 (<0.005) | C |).47 | 0.00 | 0.33 | 0.43 | 0.29 | 350 |
| 09 | 2.54 (0.03) | 3.33 (0.03) | 3.63 (0.02) | 4.03 (0.02) | 4.73 (0.03) | 6.11 (0.01) | 1 | .04 | 0.96 | 1.50 | 0.85 | 6.12 | 38 |
| 10 | 2.62 (0.05) | 3.49 (0.02) | 3.67 (0.04) | 4.10 (0.04) | 4.78 (0.02) | 6.09 (0.06) | C |).69 | 0.70 | 0.25 | 0.59 | 0.98 | 153 |
| 11 | 2.70 (0.03) | 3.49 (0.02) | 3.89 (0.02) | 4.07 (0.01) | 4.90 (0.03) | 6.33 (0.01) | 1 | .14 | 1.96 | 0.30 | 3.59 | 0.98 | 79 |
| 12 | 2.68 (0.04) | 3.31 (0.08) | 3.63 (0.10) | 3.95 (0.07) | 4.77 (0.05) | 6.07 (0.05) | C |).91 | 0.67 | 0.42 | 1.28 | 0.71 | 81 |
| 13 | 2.65 (0.04) | 3.36 (0.04) | 3.75 (0.02) | 4.11 (0.03) | 4.80 (0.02) | 6.23 (0.01) | 1 | .18 | 2.45 | 1.39 | 2.03 | 2.55 | 52 |
| 14 | 2.85 (0.03) | 3.46 (0.04) | 3.75 (0.03) | 4.09 (0.02) | 4.89 (0.02) | 6.17 (0.02) | C |).45 | 0.56 | 0.41 | 0.93 | 1.08 | 79 |
| 15 | 2.64 (0.05) | 3.41 (0.04) | 3.71 (0.02) | 4.09 (0.03) | 4.77 (0.02) | 6.14 (0.02) | 1 | .05 | 0.61 | 0.46 | 1.17 | 1.69 | 100 |
| 16 | 2.63 (0.04) | 3.41 (0.03) | 3.68 (0.03) | 4.06 (0.03) | 4.72 (0.02) | 6.06 (0.02) | C |).50 | 0.47 | 0.45 | 0.50 | 0.67 | 918 |
| 17 | 2.59 (0.04) | 3.41 (0.06) | 3.70 (0.03) | 3.93 (0.01) | 4.74 (0.01) | 6.11 (0.01) | C |).27 | 0.48 | 0.81 | 1.96 | 1.29 | 117 |
| 18 | 2.67 (0.16) | 3.35 (0.07) | 3.58 (0.08) | 3.92 (0.10) | 4.53 (0.14) | 5.70 (0.05) | C |).38 | 1.87 | 0.92 | 1.36 | 1.92 | 75 |
| 19 | 2.76 (0.06) | 3.47 (0.03) | 3.79 (0.01) | 4.16 (0.04) | 4.82 (0.02) | 6.22 (0.02) | C |).38 | 0.81 | 1.91 | 1.78 | 1.45 | 151 |
| Mean SD | 2.68 0.11 | 3.41 0.09 | 3.71 0.11 | 4.05 0.10 | 4.77 0.10 | 6.12 0.15 | | | | | | | |

Supplementary table 1. Results of preliminary performance testing (D, G, I, B, API, F refer to the different compounds).

| | - | Series 1 | | | Series 2 | | | Series 3 | | | |
|--------|---------------------|----------|--------|--------|----------|--------|--------|----------|--------|--------|--------|
| | Replicate Sample | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | Mean |
| | А | 0.4516 | 0.4144 | 0.4257 | 0.4827 | 0.5146 | 0.4545 | 0.3977 | 0.3978 | 0.4064 | 0.4384 |
| Lab 01 | В | 0.2236 | 0.2168 | 0.2125 | 0.2700 | 0.2597 | 0.2588 | 0.2600 | 0.2580 | 0.2405 | 0.2444 |
| | С | 0.3535 | 0.3306 | 0.3603 | 0.4095 | 0.3974 | 0.3927 | 0.3587 | 0.3720 | 0.3451 | 0.3689 |
| | А | 0.4003 | 0.4051 | 0.3984 | 0.3974 | 0.3988 | 0.3991 | 0.3852 | 0.3885 | 0.3913 | 0.3960 |
| Lab 02 | В | 0.2104 | 0.2091 | 0.2106 | 0.2133 | 0.2147 | 0.2111 | 0.2146 | 0.2142 | 0.2136 | 0.2124 |
| | С | 0.3396 | 0.3402 | 0.3348 | 0.3384 | 0.3359 | 0.3364 | 0.3301 | 0.3272 | 0.3289 | 0.3346 |
| | А | 0.4078 | 0.3949 | 0.4026 | 0.3855 | 0.3843 | 0.3961 | 0.3914 | 0.3734 | 0.3847 | 0.3912 |
| Lab 03 | В | 0.2122 | 0.2157 | 0.1923 | 0.1890 | 0.1936 | 0.2040 | 0.2071 | 0.1985 | 0.2093 | 0.2024 |
| | С | 0.3197 | 0.3397 | 0.3389 | 0.3298 | 0.3295 | 0.3237 | 0.3286 | 0.3146 | 0.3272 | 0.3280 |
| | А | 0.3934 | 0.4019 | 0.3935 | 0.4083 | 0.4045 | 0.4148 | 0.3735 | 0.3806 | 0.3952 | 0.3962 |
| Lab 04 | В | 0.2323 | 0.2334 | 0.2278 | 0.2300 | 0.2300 | 0.2244 | 0.2181 | 0.2036 | 0.2069 | 0.2229 |
| | С | 0.3486 | 0.3444 | 0.3386 | 0.3351 | 0.3516 | 0.3260 | 0.3216 | 0.3313 | 0.3173 | 0.3349 |
| | А | 0.3712 | 0.3685 | 0.3635 | 0.3668 | 0.3306 | 0.3357 | 0.3591 | 0.3479 | 0.3388 | 0.3536 |
| Lab 05 | В | 0.1890 | 0.1881 | 0.1895 | 0.1977 | 0.2059 | 0.1915 | 0.1754 | 0.1743 | 0.1695 | 0.1868 |
| | С | 0.2954 | 0.2949 | 0.3007 | 0.3014 | 0.2905 | 0.2868 | 0.2847 | 0.2796 | 0.2835 | 0.2908 |
| | А | 0.3963 | 0.3749 | 0.3784 | 0.3711 | 0.3666 | 0.3803 | 0.3787 | 0.3790 | 0.3783 | 0.3782 |
| Lab 06 | В | 0.2042 | 0.1999 | 0.2045 | 0.1887 | 0.1979 | 0.1924 | 0.2044 | 0.2065 | 0.2013 | 0.2000 |
| | С | 0.3049 | 0.3088 | 0.3029 | 0.3099 | 0.3024 | 0.3003 | 0.3142 | 0.3092 | 0.3101 | 0.3070 |
| | А | 0.3429 | 0.3653 | 0.3431 | 0.3569 | 0.3646 | 0.3868 | 0.3643 | 0.3619 | 0.3509 | 0.3596 |
| Lab 07 | В | 0.2115 | 0.2264 | 0.2109 | 0.2077 | 0.1942 | 0.1792 | 0.1976 | 0.1986 | 0.1848 | 0.2012 |
| | С | 0.3212 | 0.3103 | 0.3317 | 0.3153 | 0.3093 | 0.3167 | 0.2940 | 0.3156 | 0.3229 | 0.3152 |
| | А | 0.3467 | 0.3623 | 0.3530 | 0.3726 | 0.3742 | 0.3637 | 0.3275 | 0.3447 | 0.3534 | 0.3553 |
| Lab 08 | В | 0.1835 | 0.1881 | 0.1864 | 0.2027 | 0.1970 | 0.1829 | 0.1886 | 0.1829 | 0.1755 | 0.1875 |
| | С | 0.3093 | 0.3275 | 0.3079 | 0.3057 | 0.3072 | 0.3148 | 0.2869 | 0.2931 | 0.2879 | 0.3045 |
| | А | 0.3601 | 0.3355 | 0.3575 | 0.3822 | 0.3858 | 0.3727 | 0.3767 | 0.4024 | 0.3391 | 0.3680 |
| Lab 09 | В | 0.1614 | 0.1683 | 0.1672 | 0.1707 | 0.1792 | 0.1902 | 0.1628 | 0.1724 | 0.1730 | 0.1717 |
| | С | 0.3095 | 0.2880 | 0.2771 | 0.3083 | 0.3112 | 0.2957 | 0.3332 | 0.2907 | 0.2861 | 0.3000 |
| | А | 0.3738 | 0.4018 | 0.3846 | 0.3676 | 0.3716 | 0.3583 | 0.3671 | 0.3486 | 0.3855 | 0.3732 |
| Lab 10 | В | 0.1983 | 0.1954 | 0.2021 | 0.1968 | 0.2039 | 0.1959 | 0.1929 | 0.2081 | 0.2079 | 0.2001 |
| | С | 0.3212 | 0.3074 | 0.3149 | 0.3052 | 0.3129 | 0.3137 | 0.3001 | 0.3119 | 0.3223 | 0.3122 |
| | А | 0.4119 | 0.4139 | 0.4213 | 0.4601 | 0.4766 | 0.4569 | 0.4768 | 0.4428 | 0.4167 | 0.4419 |
| Lab 11 | В | 0.2259 | 0.2146 | 0.2302 | 0.2393 | 0.2311 | 0.2374 | 0.2471 | 0.2309 | 0.2289 | 0.2317 |
| | С | 0.3612 | 0.3522 | 0.3500 | 0.3828 | 0.3855 | 0.3929 | 0.3817 | 0.3784 | 0.3677 | 0.3725 |
| | А | 0.3203 | 0.3289 | 0.3274 | 0.3630 | 0.3419 | 0.3431 | 0.3505 | 0.3389 | 0.3263 | 0.3378 |
| Lab 12 | В | 0.1622 | 0.1637 | 0.1656 | 0.1508 | 0.1496 | 0.1498 | 0.1739 | 0.1636 | 0.1668 | 0.1607 |
| | С | 0.2784 | 0.2613 | 0.2762 | 0.2847 | 0.2759 | 0.2673 | 0.2846 | 0.2730 | 0.2843 | 0.2762 |
| | А | 0.3792 | 0.3749 | 0.3776 | 0.3950 | 0.3869 | 0.3995 | 0.3762 | 0.3672 | 0.3739 | 0.3812 |
| Lab 13 | В | 0.1785 | 0.1733 | 0.1696 | 0.1753 | 0.1871 | 0.1830 | 0.1852 | 0.1833 | 0.1816 | 0.1797 |
| | С | 0.2945 | 0.2992 | 0.2866 | 0.3108 | 0.3170 | 0.3036 | 0.3172 | 0.3089 | 0.2951 | 0.3037 |
| | А | 0.3410 | 0.3409 | 0.3654 | 0.3388 | 0.3443 | 0.3536 | 0.3502 | 0.3407 | 0.3389 | 0.3460 |
| Lab 14 | В | 0.1715 | 0.1858 | 0.1787 | 0.1667 | 0.1677 | 0.1698 | 0.1760 | 0.1711 | 0.1708 | 0.1731 |
| | С | 0.3017 | 0.2792 | 0.2911 | 0.2713 | 0.2760 | 0.2911 | 0.2749 | 0.2714 | 0.2900 | 0.2830 |

Supplementary table 2. Results of impurity D determination in salbutamol sulfate (% m/m)

| | А | 0.3618 | 0.3400 | 0.3435 | 0.3531 | 0.3504 | 0.3623 | 0.3473 | 0.3444 | 0.3574 | 0.3511 |
|--------|---|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Lab 15 | В | 0.1683 | 0.1591 | 0.1868 | 0.1805 | 0.1734 | 0.1864 | 0.1685 | 0.1714 | 0.1792 | 0.1748 |
| С | С | 0.2921 | 0.2709 | 0.3045 | 0.3002 | 0.2914 | 0.2973 | 0.2900 | 0.2827 | 0.2829 | 0.2902 |
| | А | 0.3696 | 0.3912 | 0.3922 | 0.3672 | 0.3716 | 0.4040 | 0.3403 | 0.3851 | 0.3635 | 0.3761 |
| Lab 16 | В | 0.2043 | 0.2133 | 0.1988 | 0.2068 | 0.1908 | 0.2095 | 0.1824 | 0.1974 | 0.1875 | 0.1990 |
| С | С | 0.3086 | 0.3117 | 0.2991 | 0.3284 | 0.3129 | 0.3287 | 0.3066 | 0.2862 | 0.2999 | 0.3091 |
| A | А | 0.3598 | 0.3586 | 0.3590 | 0.3570 | 0.3522 | 0.3578 | 0.3659 | 0.3689 | 0.3714 | 0.3612 |
| Lab 17 | В | 0.1825 | 0.1710 | 0.1807 | 0.1789 | 0.1846 | 0.1681 | 0.1642 | 0.1720 | 0.1713 | 0.1748 |
| | С | 0.2895 | 0.3026 | 0.2998 | 0.3176 | 0.2985 | 0.3019 | 0.2750 | 0.2752 | 0.2803 | 0.2934 |
| | А | 0.4109 | 0.4131 | 0.4022 | 0.3811 | 0.3843 | 0.3933 | 0.3887 | 0.3969 | 0.3963 | 0.3963 |
| Lab 18 | В | 0.2059 | 0.2128 | 0.2038 | 0.1877 | 0.1941 | 0.1977 | 0.1993 | 0.1951 | 0.1960 | 0.1991 |
| | С | 0.3142 | 0.3137 | 0.3177 | 0.3126 | 0.3160 | 0.3197 | 0.3231 | 0.3289 | 0.3320 | 0.3198 |
| | А | 0.4069 | 0.4095 | 0.3947 | 0.4099 | 0.4085 | 0.4085 | 0.3846 | 0.3932 | 0.3835 | 0.3999 |
| Lab 19 | В | 0.2039 | 0.2050 | 0.1959 | 0.1845 | 0.1878 | 0.1977 | 0.1811 | 0.1898 | 0.1875 | 0.1926 |
| | С | 0.3237 | 0.3233 | 0.3253 | 0.2983 | 0.3047 | 0.3110 | 0.3035 | 0.2938 | 0.2944 | 0.3087 |

| | | Z-score | |
|------------|----------|----------|----------|
| Lab number | Sample A | Sample B | Sample C |
| 01 | 3.46 | 2.77 | 2.60 |
| 02 | 0.92 | 1.05 | 1.08 |
| 03 | 0.74 | 0.52 | 0.79 |
| 04 | 0.93 | 1.62 | 1.10 |
| 05 | -0.67 | -0.32 | -0.86 |
| 06 | 0.25 | 0.38 | -0.14 |
| 07 | -0.44 | 0.45 | 0.22 |
| 08 | -0.60 | -0.28 | -0.25 |
| 09 | 0.07 | -1.13 | -0.45 |
| 10 | 0.07 | 0.39 | 0.09 |
| 11 | -0.44 | 2.09 | 2.76 |
| 12 | -1.26 | -1.72 | -1.51 |
| 13 | 0.37 | -0.71 | -0.29 |
| 14 | -0.96 | -1.06 | -1.21 |
| 15 | -0.76 | -0.96 | -0.88 |
| 16 | 0.17 | 0.33 | -0.05 |
| 17 | -0.38 | -0.97 | -0.74 |
| 18 | 0.93 | 0.34 | 0.42 |
| 19 | 1.07 | -0.01 | -0.07 |

Supplementary table 3. Z-scores using labs mean as assigned value



Supplementary figure 1. Measured impurity D content (% m/m) for salbutamol sulfate samples A, B, C and respective standard deviation per lab.



Supplementary figure 2. Salbutamol sulfate and related impurities chemical structures.