UNIVERSITÉ D'ORLÉANS

ÉCOLE DOCTORALE
SANTE, SCIENCES BIOLOGIQUES ET CHIMIE DU VIVANT

Institut de Chimie Organique et Analytique

THÈSE présentée par :

Elise LEMASSON

soutenue le : **18 janvier 2018**

pour obtenir le grade de : **Docteur de l'université d'Orléans**
Discipline/ Spécialité : Chimie Analytique

Stratégies chromatographiques en phase liquide et supercritique pour l'analyse de candidats médicaments

THÈSE dirigée par :

Dr. Caroline WEST	Maitre de conférences, Université d'Orléans

RAPPORTEURS :

Pr. Jean-Luc VEUTHEY	Professeur, Université de Genève
Pr. Christine HERRENKNECHT	Professeur, Université de Nantes

JURY

Pr. Christine HERRENKNECHT	Université de Nantes, Rapporteur
Pr. Jean-Luc VEUTHEY	Université de Genève, Rapporteur
Pr. Philippe MORIN	Université d'Orléans, Examinateur
Dr. Dominique SOTTY	Technologie Servier, Examinateur
Dr. Sophie BERTIN	Servier, Co-Encadrante
Dr. Caroline WEST	Université d'Orléans, Directrice de thèse

Remerciements

Cette grande aventure de thèse m'a amenée à rencontrer de nombreuses personnes, qui ont contribué à la réussite de ce projet. Il est temps à présent de les remercier.

Mes premiers remerciements vont à ma directrice de thèse, Caroline West. Il est difficile pour moi de résumer ici tout ce que tu m'as apporté depuis quatre ans. Tes conseils avisés, ta patience, tes encouragements et ta confiance m'ont permis de mener ce projet dans les meilleures conditions possibles. N'oublions pas que « faire, défaire et refaire c'est toujours travailler » :). Merci pour toutes les opportunités que tu m'as données pour me former et étendre mes connaissances. Outre ces aspects scientifiques, je te remercie pour les découvertes musicales que nous avons partagées.

Je tiens également à remercier sincèrement ma co-encadrante de thèse, Sophie Bertin. Sophie, merci pour ta réactivité et ta gentillesse tout au long de ces quatre ans. Tu as toujours répondu à mes questions et sollicitations avec le sourire, même quand il a fallu préparer 140 échantillons et réaliser des grosses séries de manips. Merci pour ton accueil à l'IdRS pour mes séjours ponctuels.

Un grand merci à Philippe Hennig et Eric Lesellier pour leur accompagnement tout au long de la thèse. Merci à Eric pour les différents échanges que nous avons eus pendant ces quatre années et ses précieux conseils. J'exprime ma gratitude à Philippe, pour la confiance qu'il m'a accordée lorsqu'il m'a proposé d'initier cette collaboration avec l'ICOA.

Je souhaite également remercier Fabien Mauge. Fabien, j'ai commencé la SFC à tes côtés il y a six ans et je suis heureuse d'avoir pu continuer à échanger avec toi à travers ce travail, même de manière ponctuelle.

Je tiens à remercier les Pr. Christine Herrenknecht et Jean-Luc Veuthey d'avoir accepté de juger mon travail en étant les rapporteurs de la thèse, ainsi que le Pr. Philippe Morin et le Dr. Dominique Sotty d'avoir accepté de faire partie de mon jury.

Cette thèse a été réalisée à l'ICOA, dirigé par le Pr. Pascal Bonnet, et plus particulièrement dans l'équipe « Stratégies Analytiques, Affinités et Bioactifs » dirigée par le Dr. Reine Nehmé. Je tiens à remercier l'ensemble des personnes que j'ai rencontré au laboratoire, et qui ont contribué à ce que mon passage ici soit des plus agréables. J'ai

partagé mon quotidien avec de nombreuses personnes, qu'il est important de remercier. Merci à Sophie, Lucille, David et Florian pour les très bons moments partagés et les discussions (parfois interminables) échangées avec vous aux pauses café et ailleurs. Sophie et Lucille, vous faites partie de mes plus belles rencontres et je vous souhaite de réussir dans vos nouvelles vies respectives. Emilie, merci pour tous tes petits mots et attentions qui, au quotidien, font toute la différence. Merci à Laëtitia et Sandrine de m'avoir si bien accueillie dans votre bureau, pour un thé ou un carré de chocolat réconfortants, et pour votre soutien et votre amitié. Sandrine, je n'oublie pas nos séances « formations » aux réseaux sociaux, nos sessions shopping, tes manucures toujours impeccables et tes blagues Carambar pour nous redonner le sourire, à Syntia et moi. Je remercie Alain-Michel pour son aide et sa réactivité lorsqu'il fallait changer mes bouteilles de CO_2. Lorène, je suis contente d'avoir pu te connaitre lors de tes séjours à l'ICOA et te souhaite de t'épanouir dans ta nouvelle vie à Orléans. Cette thèse a également été l'occasion de faire de belles rencontres à l'extérieur. Marion, merci pour ta bonne humeur, ta folie et ces bons moments passés à l'étranger, je te souhaite bonne chance pour la fin de ta thèse.

Je remercie les doctorants et post-doctorants de l'ICOA. Je commence par mes collègues de bureau : Justine, Johanna et Nhi. Merci au « Labo plantes » et au duo Suong et Adrien pour votre joie de vivre et les excellents moments partagés. Merci aussi à Zaineb. Je remercie également la « team supercritique » actuelle, Adrien et Angéline (à qui je souhaite bon courage pour la suite de ce projet), et passée, my dear Anna, « I wish you all the best for your PhD in Poland and see you later, alligator ». La « team supercritique » a commencé avec toi Syame, je ne te remercierai jamais assez pour ton soutien, tes conseils et ton amitié. Enfin, mes sincères remerciements vont à Syntia et Adrien, avec qui j'ai partagé de très bons moments dans mon bureau de substitution. Merci pour les jolis surnoms dont vous m'avez affublée « LMS », « HRMS », les séances (nombreuses) de gossips, les parties de badminton improvisées et les séances de sport. Syntia, alors qu'il y a peu tu passais par l'épreuve parfois compliquée de la rédaction, tu as toujours été présente et bien plus qu'une collègue, tu es une véritable amie. Adrien, bon courage pour ta publication dans Nature ;).

Je remercie mes amis « chimistes » de l'IUT, Antonin, Dylan et Quentin. C'est toujours un plaisir de vous retrouver en Suisse, en Allemagne ou ailleurs. Modesty, merci pour ta philosophie de vie et ton amitié indéfectible depuis de très nombreuses années. Mes Fofies, merci d'être une constante dans ma vie. Merci à Momo, Angélique, Anne-Lise, Laurène, Amandine, Coralie, Charlotte pour les sessions karaoké à grands renforts de

Céline et à Pierre, Antoine, Guillaume, Nicolas, Simon et Maël pour les paquitos improvisés en fin de soirée ; tous ces moments m'ont permis de changer d'air et de recharger les batteries quand cela était nécessaire. Je veux aussi remercier Lauranne, Etienne, Maryanne, Romain F., Bubu, Sarah et Romain G. pour votre amitié et votre soutien lors de la dernière ligne droite, « force et honneur ».

Mes remerciements les plus sincères vont à ma famille. A mes sœurs d'abord, Julia et Lucille, qui n'ont jamais compris ce que je faisais vraiment :). Je remercie infiniment mes parents du soutien qu'ils m'ont toujours accordé. Merci d'avoir toujours respecté nos choix d'orientation, très variés, qui nous ont permis de construire nos vies.

Enfin, je terminerai par adresser mes plus forts remerciements à Maël. Merci d'être présent à mes côtés depuis tout ce temps. Merci pour ton aide et ton soutien au quotidien. Tu as toujours respecté mes choix et tu m'as poussée à me dépasser. Si j'en suis là aujourd'hui, c'est grâce à toi et ce travail c'est aussi le tien.

Publications scientifiques

Ce travail de recherche a été réalisé à l'Institut de Chimie Organique et Analytique (Université d'Orléans), dans le cadre d'une collaboration avec l'Institut de Recherches Servier (Suresnes). Le sujet de la thèse est « Stratégies chromatographiques en phase liquide et supercritique pour l'analyse de candidats médicaments ». La thèse a été supervisée par le Dr. Caroline West (ICOA) et co-encadrée par le Dr. Sophie Bertin (Servier), avec l'accompagnement des Dr. Eric Lesellier et Philippe Hennig.

Le présent travail de thèse a fait l'objet de publications dans des journaux internationaux à comité de lecture de rang A et de rang B. Le travail exposé dans ce manuscrit a également été présenté lors de congrès nationaux et internationaux, sous la forme de communications orales ou de posters.

Publications apparaissant dans ce manuscrit

I. Impurity profiling of drug candidates: analytical strategies using reversed-phase and mixed-mode high-performance liquid chromatography method

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
Journal of Chromatography A, 1535, (2018), 101-113

II. Interest of achiral-achiral tandem columns for impurity profiling of synthetic drugs with supercritical fluid chromatography

C. West, E. Lemasson, S. Bertin, P. Hennig, E. Lesellier
Journal of Chromatography A, 1534, (2018), 161-169

III. Characterization of retention mechanisms in mixed-mode HPLC with a bimodal reversed-phase / cation-exchange stationary phase

E. Lemasson, Y. Richer, S. Bertin, P. Hennig, C. West
Chromatographia, 81, 3, (2018), 387-399

IV. Mixed-mode chromatography - A review

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
LC-GC Europe, 30, 6 (2017), 22-33

V. Comparison of ultra-high performance methods in liquid and supercritical fluid chromatography coupled to electrospray ionization − mass spectrometry for impurity profiling of drug candidates

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
Journal of Chromatography A, 1472, (2016), 117-128

VI. The use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification (Review article)

E. Lemasson, S. Bertin, C. West
Journal of Separation Science, 39, (2016), 212-233

VII. Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part II: Selection of an orthogonal set of stationary phases

E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West
Journal of Chromatography A, 1408, (2015), 227-235

VIII. Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part I: Optimization of mobile phase composition

E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West
Journal of Chromatography A, 1408, (2015), 217-226

Autres publications

I. Enantioseparation of novel chiral sulfoxides on chlorinated polysaccharide stationary phases in supercritical fluid chromatography

C. West, M-L. Konjaria, N. Shashviashvili, E. Lemasson, P. Bonnet, R. Kakava, A. Volonterio, B. Chankvetadze
Journal of Chromatography A, 1499, (2017), 174-182

II. An improved classification of stationary phases for ultra-high performance supercritical fluid chromatography

C. West, E. Lemasson, S. Bertin, E. Lesellier
Journal of Chromatography A, 1440, (2016), 212-228

III. An attempt to estimate ionic interactions with phenyl and pentafluorophenyl stationary phases in supercritical fluid chromatography

C. West, E. Lemasson, S. Khater, E. Lesellier
Journal of Chromatography A, 1412, (2015), 126-138

Communications orales

Congrès internationaux avec comité de lecture :

I. Exploring mixed-mode HPLC as an alternative to reversed phase HPLC for impurity profiling of drug candidates

E. Lemasson, Y. Richer, S. Bertin, P. Hennig, E. Lesellier, C. West
HPLC 2017, Prague (République Tchèque)

II. Ions and SFC: an improbable match ?

C. West, A. Raimbault, E. Lemasson
HPLC 2017, Prague (République Tchèque)

III. Utilisation de l'HPLC mixed-mode comme méthode alternative à l'HPLC phase inverse pour l'analyse de principes actifs pharmaceutiques

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
SEP 2017, Congrès international de l'Association francophone des sciences séparatives (AfSep), Paris (France)

IV. Improving our understanding of enantioseparation in supercritical fluid chromatography

C. West, S. Khater, E. Lemasson, A. Raimbault
Pittcon 2017, Chicago, IL (USA)

V. Interest of using both UHPLC-MS and UHPSFC-MS for impurity profiling of drug candidates

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
SFC 2016, Vienne (Autriche)

VI. Combining the expertise of academics, industrials and manufacturers to develop an achiral SFC-MS screening strategy for impurity profiling of drug candidates

C. West, E. Lemasson, S. Bertin, H. Boiteux, P. Hennig, E. Lesellier
Pacifichem 2015, Honolulu, HA (USA)

VII. Comparison of UHPLC and UHPSFC coupled to electrospray ionization mass spectrometry for impurity profiling of drug candidates

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
SFC China 2015, Shanghai (Chine)

VIII. Development of an achiral SFC-MS screening strategy for impurity profiling of drug candidates
E. Lemasson, S. Bertin, E. Lesellier, H. Boiteux, P. Hennig, C. West
HPLC 2015, Genève (Suisse)

IX. Développement d'une méthode de screening en SFC achirale pour le profilage d'impuretés de principes actifs pharmaceutiques

E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West
SEP 2015, Congrès international de l'Association francophone des sciences séparatives (AfSep), Paris (France)

Congrès nationaux avec comité de lecture :

X. Développement de méthodes chromatographiques pour le profilage d'impuretés de candidats médicaments

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
Journées de la section Régionale Centre-Ouest de la Société Chimique de France (SCF) 2017, Tours (France)

XI. Comparaison des méthodes UHPLC-MS et UHPSFC-MS pour le profilage d'impuretés de candidats médicaments

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
Journées de la section Régionale Centre-Ouest de la Société Chimique de France (SCF) 2016, Poitiers (France)

Séminaires invités :

XII. Analyse de pureté de candidats médicaments : stratégie d'utilisation de la SFC, RPLC et HPLC mixed-mode

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
Journée scientifique de l'Association francophone des sciences séparatives (AfSep), Club Ile-de-France, mai 2017, Paris (France)

XIII. Development of an achiral SFC screening strategy for impurity profiling of active pharmaceutical ingredients

E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West
User's Meeting Waters 2014, Bâle (Suisse)

Communications par affiches

I. Study of achiral selectivity of chiral columns in SFC: application for impurity profiling of drug candidates

E. Lemasson, A. Raimbault, S. Bertin, F. Mauge, P. Hennig, E. Lesellier, C. West
HPLC 2017, Prague (République Tchèque)

II. Unravelling the effects of ammonium acetate in the SFC mobile phase

E. Lemasson, H. Ansouri, J. Melin, S. Bertin, C. West
SFC 2016, Vienne (Autriche)

III. Evaluation of novel polymer-based stationary phases for achiral SFC

E. Lemasson, K. Nagai, T. Shibata, P. Franco, S. Bertin, C. West
SFC 2016, Vienne (Autriche)

IV. Comparison of UHPLC and UHPSFC coupled to electrospray ionization mass spectrometry for impurity profiling of drug candidates

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West
HTC 14 2015, Gand (Belgique)
Nominé pour le prix du meilleur poster

V. An improved classification of stationary phases for ultra-high performance supercritical fluid chromatography

C. West, E. Lemasson, E. Lesellier
HPLC 2015, Genève (Suisse)

VI. Intérêt des additifs dans la phase mobile pour l'analyse de composés pharmaceutiques en SFC-MS

E. Lemasson, S. Bertin, E. Lesellier, H. Boiteux, P. Hennig, C. West
SEP 2015, Paris (France)
Nominé pour le prix du meilleur poster

VII. Development of an achiral SFC screening strategy for impurity profiling of active pharmaceutical ingredients

E. Lemasson, S. Bertin, E. Lesellier, H. Boiteux, P. Hennig, C. West

SFC 2014, Bâle (Suisse)

Prix du meilleur poster

Sommaire

Liste des abréviations

ACN : acétonitrile

AMS : acide méthanesulfonique

As : facteur d'asymétrie

BICAR : bicarbonate d'ammonium

CAD : détecteur à aérosol chargé (Charged aerosol detection)

C_e : composition à l'élution

C_{iso} : composition isocratique

D : coefficient de diffusion

DAD : détecteur à barrettes de diodes

DEDL : détecteur évaporatif à diffusion de lumière

d_p : diamètre des particules

ESI : ionisation par électrospray

FPP : particule totalement poreuse

H ou HEPT : hauteur équivalente à un plateau théorique

HILIC : chromatographie d'interaction hydrophile

HPLC : chromatographie liquide haute performance

HRMS : spectrométrie de masse haute résolution

IEX : chromatographie d'échange d'ion

log P : coefficient de partage octanol/eau

MALDI : ionisation assistée par désorption laser (matrix assisted laser desorption ionisation)

MeOH : méthanol

MM-HPLC ou HPLC-MM : HPLC mixed-mode

MS : spectrométrie de masse

N : efficacité chromatographique ou nombre de plateaux théoriques

NPLC : chromatographie liquide à polarité de phase normale

PA : principe actif

P_c : capacité de pic

R^2 : coefficient de détermination

RMN : résonance magnétique nucléaire

RPLC : chromatographie liquide à polarité de phase inversée

SFC : chromatographie en phase supercritique

SFE : extraction sous fluide supercritique

SPP : particule superficiellement poreuse

t_D : délai de gradient (« dwell time ») du système

t_g : temps de gradient

t_R : temps de rétention

TOF-MS : spectrométrie de masse à temps de vol

u : vitesse linéaire de la phase mobile

u_{opt} : vitesse linéaire optimale de la phase mobile

UHPLC : HPLC ultra-haute-performance

UHPSFC : SFC ultra-haute-performance

UV : ultra-violet

v_{opt} : vitesse linéaire optimale réduite de la phase mobile

W_{50} : largeur du pic à mi-hauteur

Liste des figures

Introduction générale

Chapitre 1

Chapitre 2

Chapitre 3

Conclusions générales et perspectives

Liste des tableaux

Chapitre 1

Chapitre 2

Chapitre 3

Introduction générale

Le travail effectué par les chromatographistes en milieu pharmaceutique peut être divers et varié, tant à l'échelle analytique que préparative. Une des principales tâches reste le profilage d'impuretés de candidats médicaments, qui intervient à tous les stades du développement du médicament. En effet, la synthèse d'un nouveau principe actif (PA) est un procédé long, conduit en plusieurs étapes. Dans un processus complexe de développement, la présence d'impuretés résiduelles issues de la synthèse semble inévitable. C'est pourquoi l'identification et la quantification des impuretés nécessitent un contrôle strict afin de garantir l'efficacité et la toxicité limitée du candidat médicament. Le contrôle des impuretés est régulé par le Conseil international d'harmonisation des exigences techniques pour l'enregistrement des médicaments à usage humain (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use ou ICH) [1]. On comprend donc aisément que la problématique du profilage d'impuretés soit une préoccupation importante des industries pharmaceutiques.

L'Institut de Recherches Servier ne fait pas exception, avec environ 14000 échantillons traités chaque année au laboratoire de chromatographie. Les échantillons analysés sont principalement de deux types. Le premier concerne les intermédiaires de synthèse (composés de « type I »), qui sont analysés quotidiennement par la division de Physico Chimie Analytique avant d'être réintégrés pour la suite de la synthèse. La deuxième catégorie correspond aux composés de « type S », ce qui, pour le laboratoire Servier, signifie que le candidat médicament étudié se situe à un stade avancé de la Recherche et du Développement (Figure 0.1, tests précliniques). Ces produits sont ensuite destinés aux études pharmacologiques afin d'estimer leurs activités. Ils représentent 20 à 25% des composés étudiés chaque année. L'ensemble des composés analysés pour ce travail de thèse est un set de composés dit de « type S », disponibles par nature en quantités suffisantes pour mener plusieurs études contrairement aux produits I.

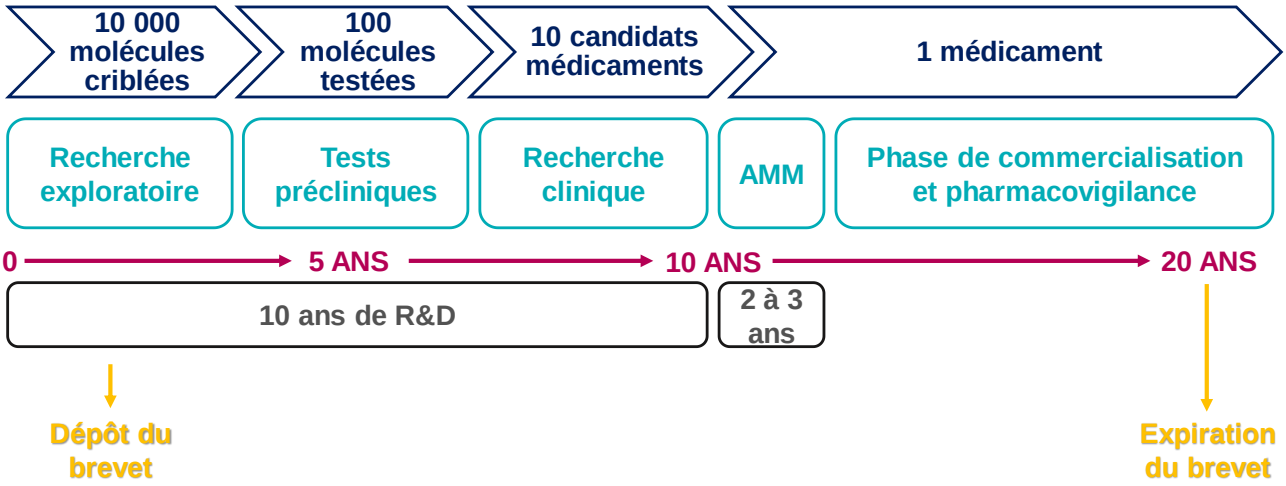


Figure 0.1 - Cycle de vie du médicament (adapté d'après : Leem, http://www.leem.org/article/recherche-developpement-0)

Ces deux types de composés sont analysés selon le même principe, avec : (i) la confirmation ou l'infirmation de l'identité du composé principal, (ii) l'estimation de la pureté relative de l'échantillon en relevant les impuretés ayant une pureté relative supérieure à 0.04% par analyse en chromatographie avec principalement une détection UV et (iii) l'identification structurale des impuretés ayant une pureté relative supérieure à 1%.

Ces analyses nécessitent de disposer de méthodes analytiques performantes, pour s'assurer que l'ensemble des impuretés est correctement identifié et qu'il n'y a pas de coélutions entre le PA et une impureté ou entre plusieurs impuretés. La méthode de choix pour le profilage d'impuretés de candidats médicament reste la chromatographie liquide en phase inverse (RPLC) couplée avec la spectrométrie de masse. À l'Institut de Recherches Servier, ce sont deux méthodes RPLC qui sont utilisées comme méthodes de référence. Ces méthodes ont été développées sur une même phase stationnaire C18 mais avec des conditions d'élution différentes (acides ou basiques). Bien que très performantes, ces méthodes échouent parfois à retenir les composés les plus polaires ou ne permettent pas d'aboutir à un résultat chromatographique acceptable (dégradation de composés, formes en équilibres etc.). Dans ces cas, il apparait essentiel de pouvoir disposer de méthodes alternatives complémentaires et de maximiser l'orthogonalité entre les méthodes [2].

L'objectif de cette thèse a donc été le développement de méthodes chromatographiques alternatives pour permettre l'analyse des candidats médicaments, lorsque les méthodes de première intention échouent.

Le premier chapitre de ce manuscrit est consacré à l'étude bibliographique des méthodes chromatographiques utilisées pour l'analyse de composés pharmaceutiques. En dehors de la phase stationnaire C18 couramment utilisée, des phases originales ont été commercialisées et utilisées en RPLC. C'est le cas des phases de silice greffée pentafluorophényle, utilisées pour augmenter la rétention des composés polaires. La chromatographie liquide à mode mixte ou mixed-mode a vu le jour plus récemment et de nouvelles phases ont été commercialisées. Ces phases, appelées bimodales ou trimodales, combinent au sein d'une même colonne plusieurs types de greffons ou plusieurs fonctionnalités sur un même greffon afin de diversifier les interactions entre phase stationnaire et analyte. La chromatographie en phase supercritique est quant à elle de plus en plus utilisée dans les laboratoires pharmaceutiques et sera également traitée dans ce chapitre.

Le deuxième chapitre de cette thèse concerne le développement de méthodes HPLC pour le profilage d'impuretés de candidats médicaments. Les méthodes RPLC de référence utilisées chez Servier seront évoquées, ainsi que la mise en œuvre d'une méthode alternative sur phase pentafluorophényle. Par la suite, le développement de méthodes HPLC mixed-mode sur colonnes bimodale et trimodale sera détaillé. Enfin, les cinq méthodes HPLC (deux méthodes de référence et trois méthodes alternatives) seront comparées en termes de performances chromatographiques et de capacité pour le profilage d'impuretés.

Le troisième chapitre traite du développement de méthodes en chromatographie en phase supercritique. Le développement de méthodes alternatives en SFC passe par le choix de la phase stationnaire et des conditions analytiques (composition de la phase mobile en gradient) adéquates. La robustesse de ces méthodes a également été testée. Une partie de ce chapitre concerne également la comparaison des méthodes SFC en gradient générique avec gradient focus ou isocratique. Le couplage de colonnes en série, en utilisant les deux meilleures méthodes SFC développées auparavant, est également détaillé. Finalement, les performances des méthodes SFC et RPLC de première intention sont comparées ainsi que leur capacité en termes de profilage d'impuretés.

La dernière partie fait un bilan de l'ensemble des méthodes analytiques (HPLC et SFC) développées dans ce travail de thèse et une stratégie d'analyse du candidat médicament est proposée.

Chapitre 1
Synthèse bibliographique des méthodes chromatographiques pour l'analyse de composés pharmaceutiques

I. Introduction

L'augmentation constante du nombre d'analytes à traiter et la complexification croissante des candidats médicaments synthétisés nécessite de disposer de méthodes chromatographiques performantes pour répondre à ces challenges analytiques.

Parmi ces méthodes, la RPLC reste sans conteste la méthode de choix, utilisée dans une majorité de laboratoires pharmaceutiques [3]. La RPLC sur phase stationnaire C18 est couramment utilisée, avec une phase mobile constituée d'un solvant organique polaire (le plus souvent ACN ou MeOH) et d'un tampon aqueux. La double détection UV et MS est largement plébiscitée [4]. Cette technique est particulièrement performante pour la séparation de composés de polarité moyenne à faible [5]. Elle est donc adaptée à l'analyse d'une majorité de composés pharmaceutiques de bas poids moléculaires, avec des log P compris entre -1 et 8 (Figure 1.2). Bien que le greffon C18 soit le plus utilisé, des phases stationnaires de type phényle (pentafluorophényle ou phényl-hexyl) ont vu le jour plus récemment et permettent parfois de retenir davantage les composés plus polaires [6]. Malgré tout, la RPLC échoue à retenir efficacement les composés polaires neutres ou chargés. En effet, les composés polaires sont parfois retenus sur les phases stationnaires par le biais d'interactions parasites avec les groupements silanol résiduels, mais ces interactions causent généralement des pics élargis et asymétriques. Différents modes chromatographiques existent et peuvent être envisagés comme alternative à la RPLC pour analyser ces composés : la chromatographie d'échange d'ion (IEX) [7], la chromatographie d'interaction hydrophile (HILIC) [8,9], la chromatographie en phase normale (NPLC), l'HPLC mixed-mode (MM-HPLC) [10] et la chromatographie en phase supercritique (SFC) [11–13] (Figure 1.2).

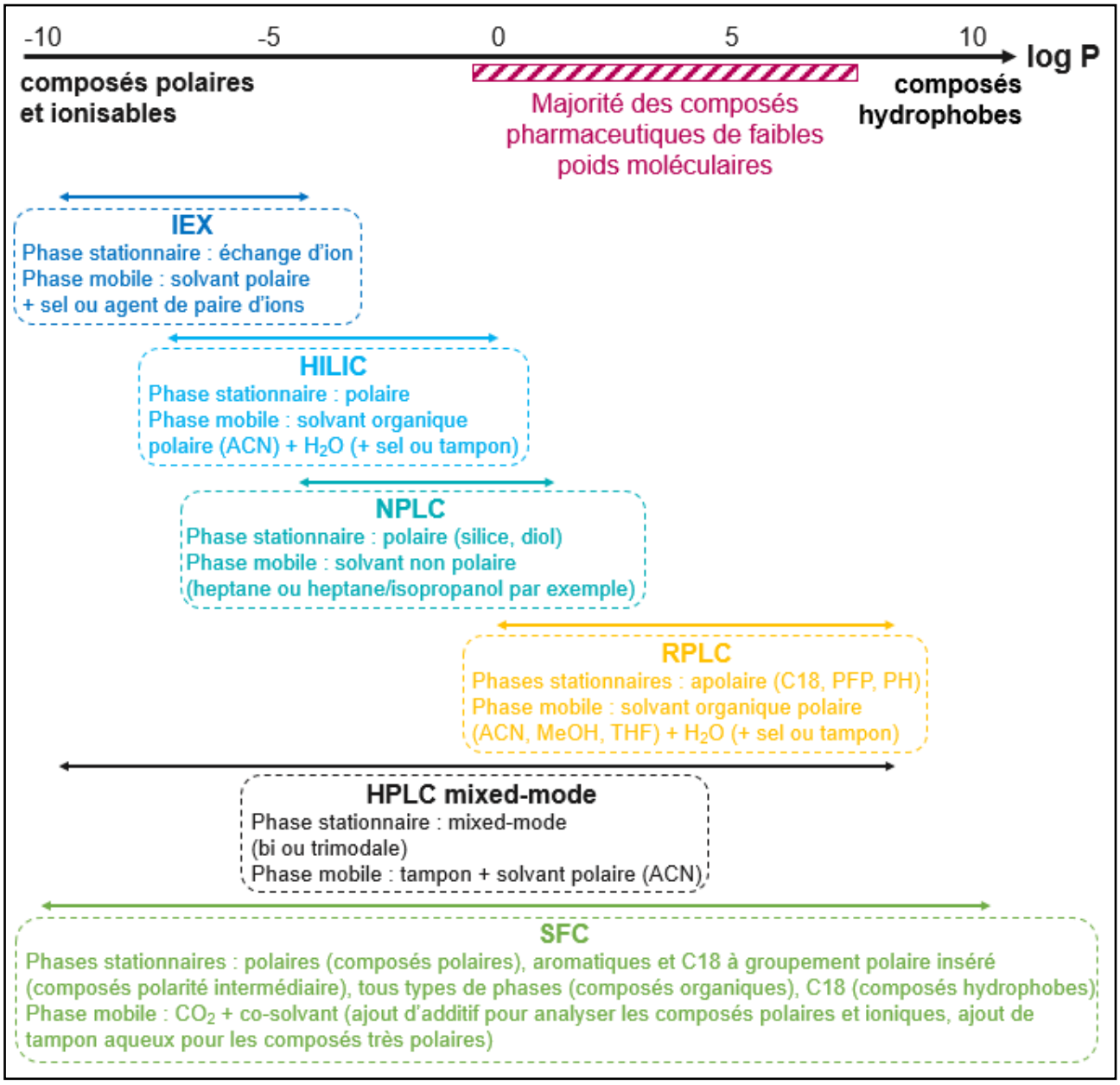


Figure 1.2 - Modes chromatographiques pouvant être utilisés pour l'analyse de composés pharmaceutiques de bas poids moléculaires en fonction de log P. Adapté de [5,14]

Cependant, certains de ces modes chromatographiques sont peu utilisés en routine au laboratoire. C'est le cas de la NPLC qui, en raison des grandes quantités de solvants organiques apolaires toxiques (hexane), était utilisée dans le passé mais a été délaissée au profit de méthodes plus vertes et moins onéreuses. La NPLC est néanmoins toujours utilisée aujourd'hui, en remplacant l'hexane par l'heptane, qui est moins toxique. L'IEX quant à elle est peu compatible avec un couplage à la spectrométrie de masse, en raison des fortes concentrations de sel utilisées dans la phase mobile, ce qui empêche toute implantation en laboratoire comme méthode de référence. La HILIC est parfaitement compatible avec un couplage MS [15,16] en raison du fort taux de solvant organique présent dans la phase mobile, facilitant la désolvatation des composés analysés et permet la séparation de composés polaires et ionisables. Cependant elle reste difficile à appliquer

en routine pour des analyses rapides au laboratoire compte tenu notamment des longs temps de rééquilibrage et de l'influence du solvant d'injection sur la séparation.

D'autres méthodes se développent et font leur apparition dans les laboratoires. C'est le cas de la SFC qui a su récemment s'imposer comme méthode alternative à la RPLC [17,18]. En effet, ces méthodes sont orthogonales [13,19] et le couplage à la spectrométrie de masse est facile et performant [20]. La SFC permet l'analyse de composés de polarités très variées (Figure 1.2) grâce à l'ajustement de la composition de la phase mobile (proportion de co-solvant, ajout d'additif ou de tampon) mais aussi aux multiples phases stationnaires disponibles. Par ailleurs, l'intérêt pour l'HPLC mixed-mode est croissant [21]. L'HPLC mixed-mode utilise différents mécanismes de rétention (modes phase inverse, HILIC, IEX) en une seule colonne afin d'augmenter le pouvoir de séparation et permet de séparer des composés polaires et apolaires grâce aux nombreuses interactions pouvant avoir lieu entre la phase stationnaire et les analytes [22]. Cette méthode est également compatible avec un couplage MS, en raison de l'utilisation de tampons volatils dans la phase mobile.

La suite de ce chapitre est consacrée aux méthodes chromatographiques citées précédemment : l'HPLC, l'HPLC mixed-mode, la SFC ainsi que leurs utilisations dans le domaine pharmaceutique seront tour à tour décrites.

II. L'HPLC

1) Histoire

A la fin des années 60, l'HPLC était pratiquée sur des colonnes de grandes dimensions et avec des temps d'analyse longs. Bien que très utilisée dans de nombreux domaines (pharmaceutique, environnemental, métabolomique, analyse de produits naturels, contrôle anti-dopage etc.), cette technique a cependant montré ses limites pour la séparation de mélanges complexes (comprenant un grand nombre de composés) et l'utilisation en routine dans les laboratoires où il est nécessaire de pouvoir traiter un nombre important d'échantillons au quotidien. Rapidement, un besoin pour des séparations rapides à haut débit s'est fait sentir et de grands efforts ont été fournis pour améliorer les performances de la technique (en termes de rapidité d'analyse, de résolution et d'efficacité) par le développement conjoint de colonnes chromatographiques de plus faibles dimensions [23], avec des particules de 5 µm jusqu'aux particules sub-2 µm, et de systèmes chromatographiques à hautes pressions [24]. On parle alors d'UHPLC, pour

chromatographie liquide à ultra-haute-pression (ou performance). En 2004, le premier appareil dédié est commercialisé par Waters Corporation sous le nom de système ACQUITY UPLC™ [25], avec une pression pouvant aller jusqu'à 1000 bar, un débit de 1 mL/min et l'emploi de colonnes remplies de particules totalement poreuses de 1.7 µm.

2) L'HPLC moderne : UHPLC

Depuis la commercialisation du premier système UHPLC en 2004, de nombreux autres systèmes ont fait leur apparition sur le marché, avec des pressions maximales comprises entre 600 et 1500 bar [26]. L'UHPLC est une méthode chromatographique largement répandue en chimie analytique et utilisée dans de nombreux domaines. Les systèmes développés ont donc été adaptés et sont compatibles avec l'utilisation de nombreux détecteurs [27]: UV, DAD, DEDL, fluorescence, CAD et MS.

2.1. Appareillage moderne

Tous les systèmes UHPLC actuels permettent de travailler à de hauts débits d'analyse sur des colonnes de faibles dimensions grâce à l'augmentation de la pression maximale de ces systèmes, jusqu'à 1500 bar. Cependant, Fekete *et al.* ont mis en lumière à plusieurs reprises les principales contraintes techniques devant être prises en compte pour tirer pleinement profit de la technologie UHPLC [26,28] : la pression et le débit maximaux supportés par le système, le volume de délai (ou délai de gradient) et la dispersion extra-colonne. Ces différents points vont être successivement abordés.

2.1.1. Pressions et débits

L'évolution principale des systèmes UHPLC par rapport aux systèmes HPLC est l'augmentation de la pression maximale des systèmes comprise entre 600 et 1400 bar. On notera l'introduction récente sur le marché du système U-HPLC Vanquish™, commercialisé par Thermo Fisher, qui permet d'atteindre une pression maximale de 1500 bar. Cette évolution vient du fait que l'on tend à utiliser des colonnes contenant des particules de très faibles dimensions, inférieures à 2 µm.

La perte de charge (ΔP) générée par l'emploi de telles particules est importante. En effet, selon la loi de Darcy (Eq. 1), ΔP est inversement proportionnelle au carré du diamètre des particules (d_p).

$$\Delta P = \frac{\emptyset * L * u * \eta}{{d_p}^2} \qquad \text{Eq. 1}$$

Où ∅ correspond au facteur de résistance à l'écoulement, L à la longueur de la colonne, u à la vitesse linéaire de la phase mobile et η à la viscosité de la phase mobile. On comprend alors que l'utilisation de colonnes contenant des particules sub-2 µm nécessite de pouvoir travailler avec des systèmes supportant des pressions maximales élevées [29]. De la même façon, l'augmentation de u (pour diminuer la durée d'analyse) ou de L (pour augmenter l'efficacité ou faire varier la sélectivité en couplant des colonnes différentes) se traduit par une augmentation de ΔP, second argument en faveur de l'augmentation de la pression limite délivrée par le système de pompage.

2.1.2. Délai de gradient

Le délai de gradient ou volume de délai correspond au temps mis par la phase mobile depuis la chambre de mélange pour atteindre l'entrée de la colonne [30]. Lors d'une analyse en gradient, ce délai va se traduire par un décalage de temps entre le gradient programmé et celui qui se déroule réellement à l'intérieur de la colonne. Dolan *et al.* [31] ont parfaitement illustré ce problème, en montrant les chromatogrammes simulés obtenus lors de la même analyse en gradient sur des systèmes ayant des délais de gradient différents (Figure 1.3).

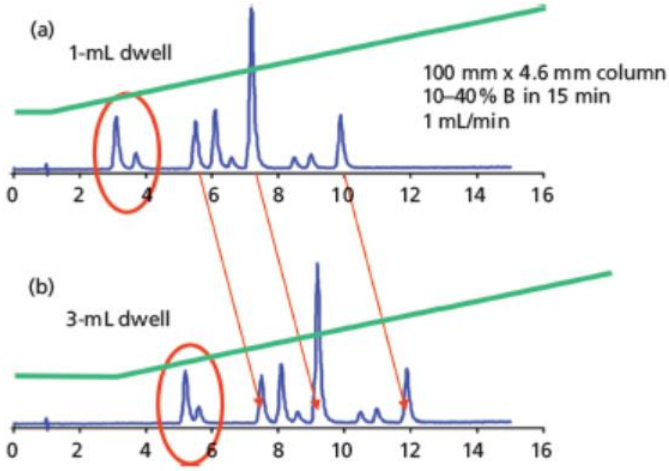


Figure 1.3 - Illustration des effets provoqués par le délai de gradient, analyse réalisée en gradient (10-40 % de solvant organique en 15 min, 1 mL/min, colonne de 100 x 4.6 mm. Chromatogrammes simulés obtenus avec des systèmes ayant des volumes de délai de (a) 1 mL, (b) 3 mL. Le gradient effectif est représenté en vert. D'après [31].

Sur la Figure 1.3, on comprend aisément que le délai de gradient d'un système chromatographique doit être connu avec précision si l'on souhaite transférer une méthode gradient d'un système à un autre et obtenir des séparations semblables et des analyses répétables. En effet, avec un délai de gradient de 3 mL (équivalent dans ce cas à 3 min car le débit est de 1 mL/min) la durée de gradient se trouve allongée de 3 min, par l'ajout d'un palier isocratique avant le gradient qui correspond au temps nécessaire à la phase mobile pour atteindre l'entrée de la colonne. Entre un délai de 1 et 3 min, les temps d'analyse augmentent et la résolution change. Bien évidemment, les volumes de délai ne sont pas aussi importants sur les systèmes UHPLC modernes (volumes compris entre 0.1 et 1.0 mL [26]) mais le problème reste identique. Dans le cas d'analyses rapides, comme souvent en UHPLC, l'ajout d'un palier isocratique en début de gradient peut être problématique et entrainer des problèmes de répétabilité entre les analyses. Pour réduire la durée de ce palier, un effort a été fait par les fabricants de matériel pour tenter de réduire les volumes des chambres de mélange et les longueurs de capillaire et ainsi diminuer le volume de délai. Nous verrons plus loin que la connaissance de ce volume de délai est également nécessaire pour mesurer avec exactitude la composition de la phase mobile au moment de l'élution d'un analyte.

2.1.3. Volumes extra-colonne

Outre la diminution de la taille des particules et la dimension des colonnes (Chapitre 1, II, 2, 2.2.), les volumes extra-colonne doivent être minimisés pour conserver une bonne efficacité [32]. Les volumes extra-colonnes sont une source indésirable d'élargissement des pics chromatographiques et concernent les volumes des tubulures du système (entre l'injecteur et l'entrée de la colonne et entre la sortie de la colonne et l'entrée du détecteur), le volume de la cellule UV ainsi que celui du système d'injection [33]. Sur les instruments de dernière génération, les volumes extra-colonne ont été réduits. Cela a été rendu possible par une diminution des volumes de l'injecteur et du détecteur ainsi que ceux des connectiques, ajoutant une perte de charge supplémentaire que seuls les systèmes UHPLC peuvent supporter, grâce à l'augmentation de la pression maximale atteignable avec ces systèmes.

2.2. Phases stationnaires pour l'UHPLC

Dès l'apparition de l'UHPLC en 2004, un effort important a été fait pour réduire le diamètre des particules contenues dans les colonnes chromatographiques afin d'augmenter les performances chromatographiques [34] et tirer pleinement profit de la technique. En 2004, le premier système UHPLC a été commercialisé avec une colonne remplie de particules de 1.7 µm (colonnes de 100 x 2.1 et 50 x 2.1 mm [25]), ce qui marque une avancée importante dans la technologie des phases stationnaires. Cette phase stationnaire commercialisée par Waters est définie comme hybride et est appelée « BEH » pour « Bridged ethylene hydrid ». La silice de cette phase stationnaire a été modifiée, par l'ajout de pont éthylènes entre les atomes de silice afin d'augmenter la stabilité de cette phase et sa résistance aux fortes pressions de l'UHPLC [35]. Le développement de l'UHPLC sur particules totalement poreuses (FPP) sub-2 µm, a été suivi par le développement des particules superficiellement poreuses (SPP) sub-3 µm [36] en 2007 et sub-2 µm en 2009 [37,38].

Il faut également mentionner les colonnes monolithiques, apparues dans les années 90 [39] et développées en vue de leur utilisation comme colonnes HPLC. La première commercialisation de colonnes monolithiques n'a eu lieu qu'en 2000 (colonnes Chromolith™ commercialisées par Merck) [40]. Ces colonnes sont constituées d'un barreau de silice contenant deux types de pores (macropores et mésopores) permettant de travailler à de hauts débits sans générer une pression trop importante [41]. De hautes efficacités (N > 100 000) ont été obtenues avec l'utilisation de colonnes couplées en série [41,42], parfois jusqu'à 10 [43]. Il parait peu probable que de telles pratiques soient utilisées dans l'optique de séparations rapides au quotidien, principalement dans l'industrie. De plus, seuls deux fabricants de colonnes proposent aujourd'hui des colonnes monolithiques (Merck et Phenomenex) avec une variété de chimies de phases très pauvre (silice, C8 et C18). Avec un champ d'applications restreint, Guillarme *et al.* ont estimé que seul 1% des spécialistes de la chromatographie utilisent en routine ce genre de colonnes [44].

A l'inverse, une grande diversité de phases stationnaires est commercialement disponible pour les phases SPP et FPP, garantissant ainsi un large spectre d'applications pour lequel l'HPLC et l'UHPLC vont donner pleinement satisfaction. Cette diversité de phases sera abordée dans le paragraphe II-3. La suite de ce paragraphe sera consacrée aux phases SPP et FPP et leurs évolutions.

2.2.1. Les colonnes FPP sub-2 µm

L'engouement pour les phases présentant des diamètres de particules de plus en plus petits s'explique simplement par cette équation (Eq. 2) :

$$N = \frac{L}{H} = \frac{L}{h * d_p} \quad \text{Eq. 2}$$

Où N correspond à l'efficacité, L à la longueur de la colonne, H (ou HEPT) à la hauteur équivalente à un plateau théorique, h à la hauteur réduite de plateau théorique et d_p au diamètre des particules. On comprend donc qu'une diminution de d_p permet une augmentation de l'efficacité. Ainsi, avec l'emploi de particules FPP ayant un diamètre compris entre 1.7 et 1.9 µm, un gain important d'efficacité peut être obtenu par rapport aux particules en usage en HPLC (généralement 3 ou 5 µm). D'après l'Eq. 2, à longueur de colonne équivalente, le fait de passer de particules de 5 à 1.7 µm permet d'augmenter N d'un facteur 3. Il est aussi possible de diminuer la longueur de colonne par 3 sans impacter la valeur de N, tout en diminuant le temps d'analyse.

Le temps d'analyse peut également être réduit lorsqu'on réduit la taille des particules via l'augmentation de la vitesse linéaire optimale (u_{opt}) (Eq. 3).

$$u_{opt} = \frac{v * D_m}{d_p} \quad \text{Eq. 3}$$

Où v_{opt} correspond à la vitesse linéaire optimale réduite, D_m au coefficient de diffusion.

Cependant, les particules FPP génèrent une pression importante (Eq. 1). Ce phénomène est exacerbé par l'emploi simultané de débits élevés (u_{opt} augmente) et la diminution de d_p. Il sera donc essentiel de pouvoir travailler sur des systèmes UHPLC avec ces colonnes. Aujourd'hui, un nombre important de colonnes FPP est disponible commercialement, et l'utilisation de telles phases est largement répandue en raison du gain d'efficacité important et des temps d'analyse réduits [45].

2.2.2. Les colonnes SPP

Les particules superficiellement poreuses (SPP) sont constituées d'un cœur solide (non poreux) entouré d'une enveloppe poreuse. Les premières particules SPP de taille réduite sont apparues en 2007, avec un d_p de 2.7 µm [36]. Ces colonnes sont couramment appelées « core-shell » ou « fused-core ». L'avantage mis en avant quant à l'utilisation de

telles colonnes, en plus du gain d'efficacité par rapport à des phases FPP de d_p identique, a été la compatibilité avec l'appareillage HPLC, grâce aux plus faibles pressions générées par ces colonnes, contrairement aux colonnes FPP sub-2 µm [36,46].

L'utilisation de colonnes contenant des particules SPP de 2.6 et 2.7 µm s'est démocratisée, au détriment des particules FPP [47]. En effet, il a été démontré qu'une efficacité supérieure à celle des FPP pouvait être obtenue avec l'emploi de SPP à taille de particule identique [36,48,49] à hauts débits, avec une perte de charge réduite (Eq.1). L'équation de Knox se décompose en trois termes : A (correspondant à l'anisotropie d'écoulement, dépend de d_p et de la qualité de remplissage de la colonne), B (correspondant à la diffusion longitudinale de l'analyte, dépend de D_m et de la qualité de remplissage de la colonne) et C (correspondant à la résistance au transfert de masse du soluté entre les phases stationnaire et mobile, terme proportionnel à (d_p^2/D_m)) (Eq. 4). L'augmentation de N dans ce cas est due à la faible valeur du terme A, qui est constante, ainsi qu'à la diminution du terme C [46,50] de l'équation lors de l'augmentation du débit [51].

$$H = A + \frac{B}{u} + C * u \qquad \text{Eq. 4}$$

Ainsi, de très hautes efficacités, de l'ordre de 300 000 plateaux/mètre, ont pu être obtenues avec ces colonnes SPP sub-3 µm [46].

Aujourd'hui, des colonnes SPP sub-2 µm ont été développées afin de repousser encore davantage les performances de ces colonnes en termes d'efficacité [52]. Le diamètre des particules a été successivement réduit, jusqu'à atteindre 1.3 µm en 2013 [53]. L'utilisation de ces colonnes a permis d'atteindre une très haute efficacité (450 000 - 500 000 plateaux/mètre) [38]. Ces colonnes génèrent cependant une pression importante, et ne sont compatibles qu'avec des systèmes UHPLC où la variance extra-colonne est réduite. Dans le futur, les colonnes SPP pourraient être amenées à se développer davantage, compte tenu des performances atteignables, à condition que l'évolution des systèmes UHPLC suive celle des colonnes chromatographiques, notamment en termes de pression maximale atteignable.

3) Choix de la phase stationnaire : à la recherche de la sélectivité

La sélectivité peut être modulée en faisant varier différents paramètres opératoires comme la température [54,55], le pH, la force éluante du solvant. Un autre paramètre clé est le choix de la phase stationnaire en HPLC.

Le mode chromatographique le plus utilisé en HPLC est le mode à polarité de phase inversée ou « reversed phase » (RPLC). A titre d'exemple, 90% des analyses de composés pharmaceutiques de faibles poids moléculaires sont réalisées en RPLC [56]. La RPLC sur phase stationnaire C18, avec une détection UV et MS, est le mode de séparation le plus fréquemment utilisé dans les entreprises pharmaceutiques [4]. Le mécanisme de rétention en RPLC est basé sur l'adsorption et le partage simultanés de l'analyte sur et dans la couche hydrophobe de la phase stationnaire [57]. Le greffon C18, qui procure une rétention importante des composés hydrophobes, est le greffon le plus utilisé devant les greffons C8, utilisés pour réduire la rétention des composés les plus hydrophobes, tout en conservant une sélectivité similaire à la phase C18. Tous les fabricants proposent aujourd'hui des phases C18 et C8 dans leurs jeux de colonnes, dont la fabrication, le greffage et le traitement varient d'un fabricant à l'autre. Aussi, la sélectivité peut varier d'une colonne à une autre même si l'appellation est la même. Aujourd'hui, il existe des classifications de colonnes C18 pouvant aider au choix de la phase stationnaire selon la nature des composés à étudier [58].

Bien que les interactions principales ayant lieu en RPLC soient des interactions intermoléculaires entre la phase stationnaire et l'analyte apolaire (Forces de London), des interactions secondaires parasites peuvent avoir lieu entre les analytes basiques et les silanols résiduels présents sur la phase stationnaire. Ces silanols peuvent établir des liaisons hydrogène et des interactions ioniques avec les analytes basiques [59]. Les impuretés métalliques sont également source d'interaction avec les composés acides. Ces interactions parasites entrainent une détérioration de la symétrie de pic des composés polaires. Pour l'analyse de composés basiques polaires en RPLC, des traitements de recouvrement, dits traitements d' « end-capping » apolaires, consistant à remplacer les atomes d'hydrogène des groupements silanol par des groupements apolaires, ont d'abord été étudiés afin de limiter les effets néfastes des silanols [60]. Le greffon le plus couramment utilisé est le triméthylsilyle [61]. Par ailleurs, des groupements polaires ont été ajoutés à la base de la chaîne alkyle (« polar embedded group ») ou à la surface de la silice (« polar end-capped »). Ce groupement polaire a pour but de limiter les interactions parasites entre les silanols et les analytes basiques en créant une pseudo-phase

stationnaire aqueuse près de la surface de silice (effet de « shield » ou écran), et favorise l'élution des composés avec une bonne symétrie [62]. Ces groupements polaires permettent aussi d'obtenir des sélectivités différentes de celles obtenues avec les phases alkyles [63,64]. La nature des groupements polaires varie [65] : on trouve des phases avec un groupement amide, carbamate, urée [66], éther ou ammonium [67].

Pour les composés aromatiques, des phases avec un groupement phényle peuvent être employées. En effet, des fortes interactions π-π peuvent avoir lieu entre le groupement phényle donneur d'électrons et les analytes [68]. Ces phases présentent une sélectivité différente des phases C18 [69]. Les fabricants de colonnes proposent des colonnes phényles, dont les différences se situent principalement au niveau du nombre de groupements phényles (simple phényle ou biphényle par exemple) et de la longueur de la chaine alkyle du bras espaceur (« spacer arm »).

L'analyse des composés polaires ionisables peut être réalisée avec une phase stationnaire pentafluorophényle (PFP). Elles sont constituées d'une chaine alkyle non fluorée et d'un groupement aromatique substitué par cinq atomes de fluor. Ces phases sont devenues populaires en raison de la sélectivité unique qu'elles offrent [6,70]. Elles sont présentées comme étant orthogonales aux phases C18 et sont aujourd'hui proposées par de nombreux fabricants, qui les intègrent à leurs jeux de colonnes au même titre qu'une phase C18. Avec ces phases, des interactions π-π peuvent avoir lieu entre les analytes riches en électrons et le greffon PFP qui est un accepteur d'électrons. En raison de la rigidité du cycle aromatique PFP, la forme de l'analyte étudié peut également influencer la rétention. Enfin, des interactions dipole-dipole et ioniques peuvent avoir lieu entre les atomes de fluor portant des charges partielles négatives et les analytes [71]. Cependant, le choix de la phase stationnaire devra être mûrement réfléchi, car les résultats obtenus d'une colonne à une autre et d'un fabricant à l'autre sont souvent très différents [6,71,72]. Nos observations faites en phase supercritique exacerbent ces différences par rapport aux observations faites en phase liquide aqueuse, mais sont néanmoins révélatrices de la diversité existant parmi ces phases : certaines sont très apolaires, proches des phases de type phenyl-hexyle, alors que d'autres sont très polaires avec des sélectivités fondamentalement différentes des premières.

4) Applications : l'HPLC pour l'analyse de produits pharmaceutiques

La RPLC, couplée à un détecteur UV ou MS, reste la technique de référence pour le profilage d'impuretés de candidats médicaments et a aujourd'hui fait ses preuves pour l'analyse de petites molécules [73–75]. Les difficultés restantes résident essentiellement dans la séparation de composés proches structurellement. Regalado *et al.* ont notamment développé une méthode HPLC sur colonne SPP pour séparer un PA, la Warfarine, de ses 5 impuretés isomères [76]. Aujourd'hui, l'HPLC est également utilisée pour détecter des impuretés génotoxiques à l'état de traces dans les PA. En effet, la législation concernant ces impuretés s'est durcie et les autorités (FDA, food and drug administration, et ANSM, agence nationale de sécurité du médicament et des produits de santé) accordent une grande importance à l'identification et la quantification des ces impuretés, la dose maximale journalière ne devant pas dépasser 1.5 µg par personne pour chaque impureté détectée [77]. Ainsi, Wang *et al.* ont développé une méthode générique en HPLC-UV pour la quantification de l'hydrazine, entité génotoxique, lors du développement de composés pharmaceutiques [78]. Grigori *et al.* ont développé et validé une méthode quantitative en HPLC-MS/MS pour la détermination de trois impuretés génotoxiques dans un PA [79]. Les méthodes HPLC développées sont de plus en plus souvent associées à la masse de haute résolution ou à la RMN pour permettre l'élucidation structurale des impuretés et confirmer l'identité du PA [80,81]. Lorsque la MS ne permet pas de différencier les impuretés, comme c'est le cas pour les impuretés isobares, la RMN se révèle très performante pour leur caractérisation structurale.

A l'opposé des petites molécules, on assiste aujourd'hui à la montée en puissance des biomolécules (encore appelées biomédicaments) qui ont tendance à les remplacer [82,83]. Contrairement aux petites molécules issues de la synthèse chimique, le PA des biomédicaments est issu d'une source biologique. Ces biomédicaments incluent les protéines et les peptides thérapeutiques, les anticorps monoclonaux (mAbs) et les anticorps conjugués ou ADCs (antibody-drug conjugates). En raison de la complexité de ces nouveaux médicaments, une grande attention est portée à leurs développement et caractérisation par les agences de régulation et de contrôle des médicaments comme l'AEM (Agence européenne des médicaments) ou la FDA [84]. Un nombre important de contrôles doit être réalisé, nécessitant l'emploi de méthodes analytiques orthogonales pour s'assurer de leurs bonne caractérisation [85,86]. Les méthodes les plus couramment utilisées sont l'HPLC (RPLC [87,88], chromatographie d'exclusion stérique (SEC) [89,90] et chromatographie d'échange d'ions (IEX) [91,92]) et l'électrophorèse capillaire [93,94]

avec une détection UV et MS, ainsi que la spectrométrie de masse (MALDI-TOF-MS) [95,96]. Le présent manuscrit traitant uniquement des petites molécules, les applications concernant les biomédicaments ne seront pas développées.

III. L'HPLC mixed-mode

La review suivante traite de la chromatographie mixed-mode. Les phases stationnaires dédiées à la MM-HPLC ainsi que les applications sont présentées :

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West

Mixed-mode chromatography – A review

LC GC, Volume 30, Issue 6 (2017), 22-33

Mixed-Mode Chromatography —A Review

Elise Lemasson[1], Sophie Bertin[2], Philippe Hennig[2], Eric Lesellier[1], and Caroline West[1], [1]University of Orleans, CNRS, Institut de Chimie Organique et Analytique (ICOA), Orléans cedex, France, [2]Institut de Recherches Servier, Suresnes, France

Mixed-mode high performance liquid chromatography (MM-HPLC) involves the combined use of two (or more) retention mechanisms in a single chromatographic system. Many original stationary phases have been proposed in recent years with promising possibilities, while applications have only started to appear in the literature. In this review, we discuss mixed-mode chromatography stationary phases. An overview of applications with mixed-mode chromatography is also covered. We finally discuss the interest of mixed-mode systems in two-dimensional chromatography.

Among the numerous available separation modes, reversed-phase liquid chromatography (LC) is the favourite HPLC mode (1,2). In reversed-phase LC, C18 columns are most frequently utilized. With this kind of phase, one type of interaction dominates: the dispersion (London) interactions, also called hydrophobic interactions, between stationary phase and analytes. Secondary "parasite" hydrophilic interactions can appear between residual silanol groups and analytes, mainly when the phase is non-endcapped, which can lead to peak shape deformation and loss of efficiency. Reversed-phase LC is particularly suited for the separation of hydrophobic compounds, but fails for the retention of polar or charged compounds.

Different chromatographic modes must be envisaged as an alternative to reversed-phase LC for the analysis of such compounds. In the past, ion-pairing reversed-phase LC methods were developed to allow for the simultaneous retention of hydrophobic analytes and ions, for instance an active pharmaceutical ingredient (API) and its counterion. However, method development may be lengthy, and compatibility to mass spectrometry (MS) is not guaranteed (3). Hydrophilic interaction liquid chromatography (HILIC) (4) is an interesting alternative. In HILIC mode, the stationary phase used is hydrophilic and the mobile phase contains a high proportion of organic solvent (typically 70–98% acetonitrile, mixed with a buffer), which provides adequate retention for polar and ionic compounds and makes it compatible with electrospray ionization mass spectrometry (ESI-MS) (5–7).

The retention mechanism is believed to be based on a combination of partitioning (between the mobile phase and a layer of water adsorbed on the stationary phase) and adsorption (onto the stationary phase surface or ligands). However, this technique also presents disadvantages: the low solubility of some compounds in high proportions of organic solvent, the long column equilibration time responsible for long method development time, and the critical influence of injection solvent on peak shape, retention, and separation, make it impractical for open-access use (8,9). Ion-exchange chromatography (IEX) allows the separation of ionic compounds based on size and charge differences (10).

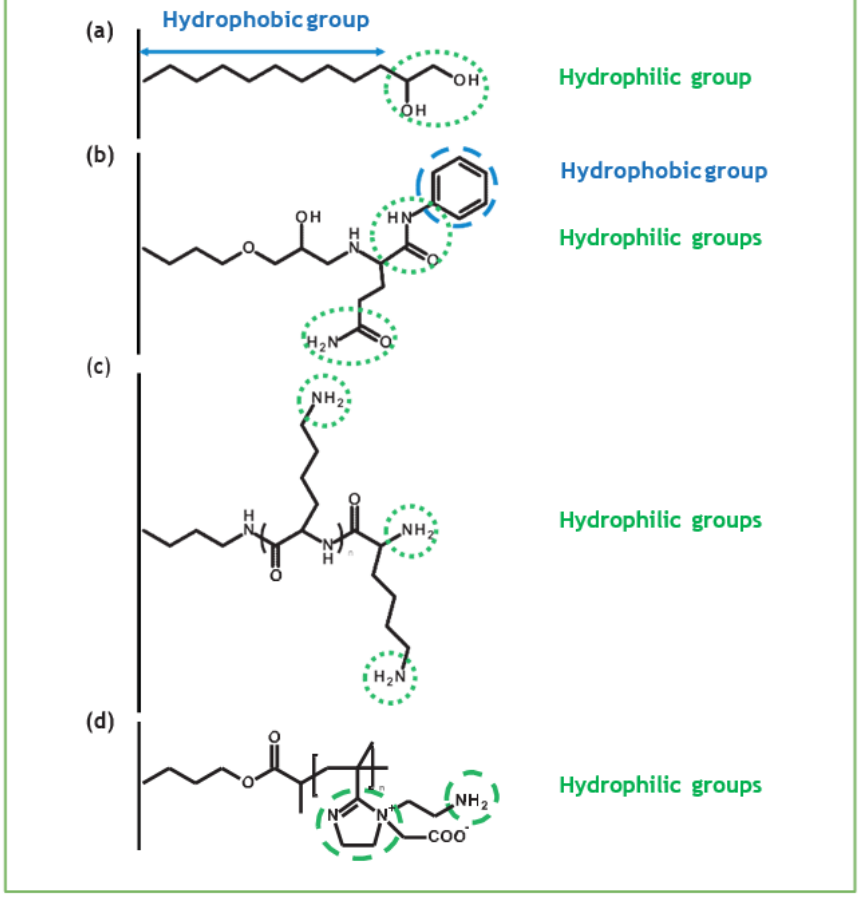
However, the compatibility with MS can be difficult because of the high concentration of buffering salts in the mobile phase. Because of the high orthogonality between supercritical fluid chromatography (SFC) and reversed-phase LC, SFC can also be envisaged as an alternative (11–13). With the selectivity offered by the numerous types of stationary phases available in SFC, not only hydrophobic but also polar and ionizable solutes may be analyzed, with adjusted mobile phase conditions (14). Unfortunately, the majority of laboratories are equipped with HPLC systems. It is therefore easier to propose methods that would be compatible with HPLC systems to ensure their applicability.

In the 1980s, chromatographers started to investigate the use of secondary parasite interactions in reversed-phase LC to increase the separation power (15). The concept of mixed-mode liquid chromatography (MM-HPLC) as we know today appeared in 1986, when the combination of reversed-phase LC and IEX modes in one column was used for the separation of proteins (16). Since this first experiment, a variety of mixed-mode stationary phases have been developed, and some of them have been commercialized. In MM-HPLC, the stationary phases may be designed according to three different procedures: (i) one ligand is functionalized with different chemical functions (17,18); (ii) several different ligands are immobilized onto one support (17,19); or (iii) different groups or particles bonded with different types of ligands are mixed together in one column (20).

The intent of MM-HPLC is to use several retention mechanisms in a single column. MM-HPLC therefore shows great flexibility and versatility in the separation of various polar and non-polar compounds, owing to the multiple possibilities for interactions taking place between stationary phase and analytes (21). In addition, MM-HPLC is also highly compatible with MS because the concentrations of salts used are lower than in IEX mode.

In this review, selected particle-based mixed-mode chromatography stationary phases are presented. An overview of applications with mixed-mode chromatography is also given. Finally, the interest of including MM-HPLC in 2D systems is discussed.

Figure 1: Different reversed-phase LC–HILIC stationary phases with (a) diol (17,25), (b) amide (26), (c) amine (27), and (d) zwitterionic polar groups (32).



Mixed-Mode Chromatography Stationary Phases

This review is not intended to be exhaustive. Indeed, MM-HPLC stationary phases have been well detailed in previous reviews (22,23). Besides, we have chosen to focus solely on MM stationary phases developed for HPLC, and not for solid-phase extraction (SPE) purposes.

Judging from the types of interactions combined, mixed-mode stationary phases can be classified into four different groups: reversed-phase–hydrophilic interaction (reversed-phase LC–HILIC), reversed-phase–ion-exchange (reversed-phase LC–IEX), hydrophilic interaction–ion-exchange (HILIC–IEX), and tri-mode MM-HPLC (with several possible combinations).

However, in practice, it often seems that only one retention mechanism can be used at the time, depending on the ratio of organic–aqueous portion in the mobile phase (20,24). Therefore, some of the stationary phases designed for MM-HPLC actually allow for multi-modal operation (for instance, reversed-phase LC or HILIC) but have not necessarily demonstrated combined mixed-mode mechanisms.

Reversed-Phase–Hydrophilic Interaction (Reversed-Phase LC–HILIC): Because reversed-phase LC is used for moderately polar and non-polar compounds and HILIC for polar compounds, the reversed-phase LC–HILIC mixed-mode presents the powerful advantage of retaining both hydrophobic and hydrophilic compounds. This combination should permit the analysis of complex mixtures with a wide range of polarities in a single run. The reversed-phase LC–HILIC stationary phases are designed by a combination of hydrophobic and polar groups. The hydrophobic portion is traditionally an alkyl chain or aromatic group. The chemical nature of the polar group varies between charge-neutral functions, like diol-, amide-, and cyano- or ionic-groups. In the latter case, the ionic functions are meant to support the aqueous pseudo-stationary phase of the HILIC retention mechanism, rather than an ion-exchange mechanism.

A typical reversed-phase LC–HILIC stationary phase is constituted of diol groups at the end of an alkyl chain (Figure 1[a]) (17,25). Aral *et al.* (26) designed a new stationary phase with two different amide groups, one was the terminal amide group and the other one was inserted between a phenyl ring and amino alcohol group (Figure 1[b]). This phase could sustain large variations of pH, which may be practical during method development, whenever ionizable species are concerned.

Another type of stationary phase employed amino acids or short peptides as ligands. Li *et al.* developed a poly-*L*-lysine-grafted silica-based stationary phase (Figure 1[c]) (27). Lysine thus provides both a polar function (terminal amine group) and an alkyl chain. In a different paper, the authors directly immobilized a small peptide Boc- Phe-Aib-Phe-OH onto silica (28).

This column demonstrated retention capability for various compounds, from hydrophobic compounds like polycyclic aromatic hydrocarbons or steroids to more polar compounds like nucleosides. In addition, the intrinsic chirality of the peptide ligand also imparted enantioselective capabilities to this phase. Surfactin, a peptide loop comprising seven amino acids and a β-hydroxyl fatty acid, was also employed as a mixed-mode ligand for reversed-phase LC–HILIC. Ohyama *et al.* (29) first reported the synthesis of a surfactin-modifed silica stationary phase. With this phase, the retention of polar solutes depended on the acetonitrile content and exhibited a reversed-phase LC–HILIC mixed-mode retention behaviour.

More recently, ionic liquids were used to develop zwitterionic ligands. The complex imidazolium organic cation and π conjugated system was commonly used to achieve the simultaneous separation of hydrophilic and hydrophobic compounds (30,31). Li *et al.* (32) used zwitterionic imidazoline to prepare a new type of stationary phase by polymerization on the silica surface with solvent-free microwave- assisted organic synthesis (Figure 1[d]). With the use of ionic liquids, multiple interactions could take place: dipole- dipole, electrostatic, π-π interaction, and hydrogen bonding. Qiao *et al.* (33) developed a new silica-based stationary phase with tricationic ionic liquid for the separation of flavonoids.

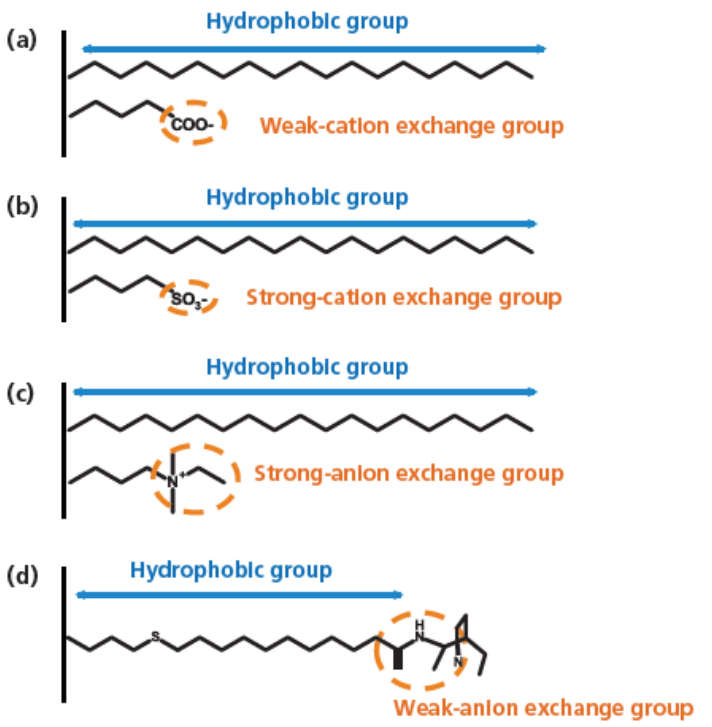
Reversed-Phase–Ion-Exchange (Reversed-Phase LC–IEX): With reversed-phase LC–IEX mixed-mode, the retention of both hydrophobic and ionic compounds is achieved. The stationary phase ligands are constituted of an alkyl chain (C8 to C18) with ionic or ionizable groups at the end of, or embedded in the alkyl chain. In this kind of MM-HPLC, four IEX modes can be differentiated: strong cation-exchange (SCX), weak cation-exchange (WCX), strong anion-exchange (SAX), and weak anion-exchange (WAX).

For cation-exchange (CEX), similarly to traditional CEX stationary phases, acidic groups were generally used: carboxylic acid for WCX (Figure 2[a]) (34) and sulfonic acid for SCX (18) (Figure 2[b]). Zhang *et al.* (35) developed an original reversed-phase LC–WCX silica-based phase with a polystyrene network comprising carboxylic groups. For basic drugs separation, this phase exhibited good orthogonality with common C18 phases.

For anion exchange (AEX), quaternary ammonium can be used for SAX (36). Wei *et al.* (37) developed a new stationary phase with copolymerization of quaternary ammonium and C18 chain on silica (Figure 2[c]). For WAX, Lämmerhofer, Lindner, and co-workers developed a novel series of phases, a surface-bonded *N*-(10-undecenyl)-3-aminoquinuclidine silica-based stationary phase (38,39). This phase showed excellent performance for oligonucleotides separation (Figure 2[d]) (40). Recently, the same team developed novel stationary phases based on thiol-ene click chemistry (41). These phases were synthesized by immobilization of *N*-undecenyl-3-α-aminotropane onto thiol-modified silica gel. The main advantages of theses phases with co-ionic endcapping were the strong reduction of retention times and the elution of acidic compounds with lower ionic strength, which enhanced the MS compatibility.

Once again, ionic liquids have been used to design imidazole-based stationary phases for reversed-phase LC–AEX (42). For example, Sun *et al.* (43,44) developed such phases for the separation of inorganic anions. The same team also synthesized more complex phases with dicationic imidazolium ionic liquids (45).

Figure 2: (a) SCX (34), (b) WCX (18), (c) WAX (37), and (d) SAX (40) reversed-phase LC–IEX stationary phases.



Hydrophilic Interaction–Ion-Exchange (HILIC–IEX): The HILIC–IEX mode is adapted to the analysis of charged polar compounds. The main application field of this mode is the analysis of proteins or peptides. In the early 1990s, Zhu *et al.* analyzed peptides on strong cation-exchange columns (24). They first observed that IEX columns exhibited two different retention mechanisms depending on the acetonitrile percentage in the mobile phase. An IEX mechanism was observed at low percentage, while a HILIC-type retention mechanism was observed with increasing acetonitrile percentage.

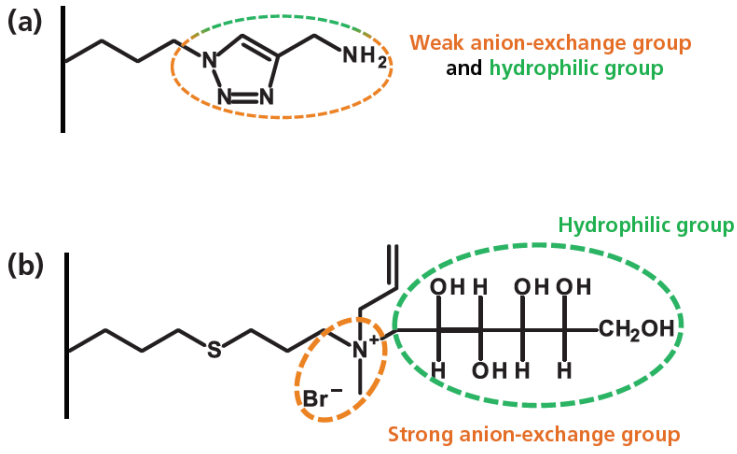
Later on, diverse stationary phases for HILIC–IEX mixed-mode were developed. The principle is always quite similar. Indeed, to obtain HILIC–IEX mixed-mode retention mechanisms, both charged (cationic, anionic, or zwitterionic) and uncharged hydrophilic groups are necessary. For HILIC–AEX mode, an amino group can be used as weak anion exchanger (Figure 3[a]) (19,46). Qiao *et al.* also used glucaminium-based ionic liquids (Figure 3[b]) for the separation of nucleosides in HILIC–SAX mode (47). Recently, Bo *et al.* (48) synthesized an original HILIC–IEC phase with adjustable selectivity by controlling the mixture ratio of two functional monomers. The application of the concept was demonstrated by the separation of nucleosides and β-agonists.

Tri-Mode MM-HPLC: As a result of the variety of analytes that may be encountered in a single sample, a need for increasingly varied interaction capabilities has arisen, and tri-mode MM-HPLC is being developed.

While the simpler mixed-mode phases described above included the combination of two retention mechanisms, the tri-mode stationary phases involve three different retention mechanisms. For this purpose, complex chemistries of stationary phases must be developed. The most common tri-mode used is the reversed-phase LC–HILIC–AEX (49).

In 2012, Qiu *et al.* (21) developed a poly(ionic liquid)-grafted silica stationary phase, using ionic liquids. This phase allowed the effective retention of hydrophobic compounds, neutral polar molecules, and anions, owing to the presence of alkyl chain, carboxyl, and imidazolium groups, respectively (Figure 4[a]). In 2014, Li *et al.* (20) proposed a novel silica-based dendritic polymer reversed-phase LC–HILIC–AEX stationary phase, which

Figure 3: (a) HILIC–AEX amino-based (19,46), (b) glucaminium-based ionic liquids (47) stationary phases.



was composed of repeated patterns comprising phenyl ring, quaternary ammonium, and hydroxyl groups (Figure 4[b]). With this material, the use of the different retention mechanisms offered a wide range of selectivities. Indeed, under reversed-phase LC conditions, the authors separated polycyclic aromatic hydrocarbons (PAH). With the IEX mechanism, the analytes were separated in acids, bases, and neutrals compounds. Finally, under HILIC conditions, neutral amides were separated. The reversed-phase LC–HILIC–CEX mode was also described for small molecules analysis (50). The analysis of proteins can also be envisaged with this tri-mode: Lin *et al.* (51) performed the separation of bovine serum albumin.

The range of applications of reversed-phase LC–AEX–CEX phases is large and allows the need for ion-pairing agents to be eliminated (52–55). Liu *et al.* (56) used a commercial tri-mode reversed-phase LC–AEX–CEX phase for the separation of pharmaceutical counterions. Another commercial tri-mode HILIC–AEX–CEX was also used for the separation of pharmaceutical counterions (57). Qiao *et al.* (58) synthesized an original tri-mode phase with 4-Chloro-6-pyrimidinylferrocene modified silica gel for the separation of PAH, phenols, and aniline compounds. With this phase, reversed-phase LC, normal-phase, and AEX modes can all be envisaged. For HILIC–CEX–AEX, Shen *et al.* developed a glutathione-based zwitterionic stationary phase (59) for the separation of oligosaccharides. Glutathione is a tripeptide containing a free amino group and two carboxyl groups. With this peptide, there were two exchange sites available (Figure 4[c]). Wang *et al.* (60) used a multi-functionalized silica synthesized via "click chemistry", which could be operated in tri-mode reversed-phase LC–CEX–HILIC, for the purification of quaternary ammonium alkaloids from plants.

One of the most complex mixed-mode phases in this category is the quinine-based zwitterionic phase developed by the group of Lindner (61,62), now available as a commercial product. In addition to hydrophilic interaction, polar organic, anion-exchange, cation-exchange, and zwitterion-exchange retention mechanisms, enantioselectivity is also possible and was demonstrated with chiral separation of amino acids, small peptides, and acidic and basic analytes.

Finally, the tri-mode phases can be used for complex samples because of the large number of interactions involved. The challenge of tri-mode is to separate anionic, cationic, and neutral species by using only one column and one set of conditions. The choice of operating conditions remains primordial to take advantage of the wide selectivity offered.

Use of Mixed-Mode HPLC for Achiral Applications

Pharmaceutical Applications:

Pharmaceutical analyses (synthetic intermediates, APIs, impurities, or degradation products) are the most popular applications of mixed-mode chromatography.

Counterions analysis is very common in pharmaceuticals. Indeed, about 50% of pharmaceutical compounds are in the salt form, to improve their physicochemical properties like solubility, purity, and stability. Zhang *et al.* developed a method for the simultaneous separation of 25 counterions, including both cations and anions, organic and inorganic, in 20 min, using a gradient elution program with a reversed-phase LC–WAX–SCX tri-mode stationary phase (63). Liu *et al.* (56) used the same stationary phase to separate 10 counterions in isocratic elution. The same team also developed a method for the separation of other counterions using a HILIC–WCX–SAX stationary phase (57). In these papers, the authors also achieved the separation of basic and acidic APIs and their associated counterions. The *European Pharmacopoeia (Ph. Eur.)* validated the method developed by Zhang *et al.* for the separation of the 25 counterions in terms of specificity, repeatability, limits of quantification, and linearity. The *Ph. Eur.* demonstrated the applicability of the method for the identification and quantification of counterions in pharmaceuticals, coupled to an MS detector (64).

The identification and quantification of impurities in pharmaceutical products must be strictly controlled to ensure the efficiency and limited toxicity of the final product. MM-HPLC has proven its worth in the field of impurity profiling of small drugs (neutral polar or charged molecules) (65,66). Zhang *et al.* (35) used the reversed-phase LC–WCX phase described above for the separation of 43 basic drugs. This phase exhibited good orthogonality with common C18 phases. Strege *et al.* proposed the use of mixed-mode anion or cation- exchange–HILIC coupled to electron light scattering detection (ELSD)-ESI-MS as an alternative to reversed-phase LC for the analysis of small molecules in drug discovery (67). With the use of a mixed-mode column, the separation selectivity was highly orthogonal to reversed-phase LC. The authors also highlighted the advantage for preparative applications. Because of the high loadability of ion-exchange packings, the loading capacity was increased 10- to 100-fold in many

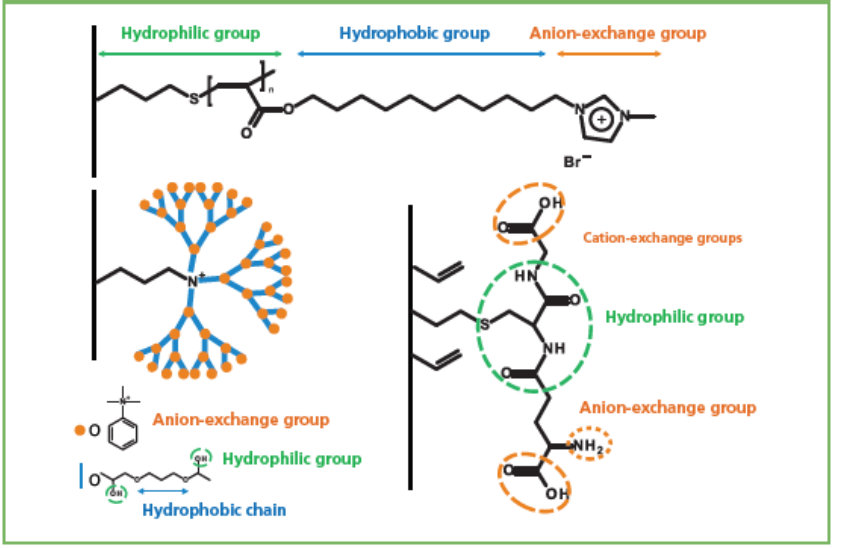
cases in comparison to reversed-phase LC. This kind of mobile phase (acetonitrile gradient with 0.05% ammonium acetate and acetic acid) was also directly compatible with ELSD and MS detectors.

The families of compounds encountered in mixed-mode achiral analysis of drug-like compounds are varied: steroids (18,28), alkaloids (60,68), non-steroidal anti-inflammatory drugs (17,60,68,69), sulfonamides (70), nitroimidazoles (71), cannabinoids (72), water and fat-soluble vitamins (70,74–76), biphosphonates (77), and amphetamines (35). Analytical conditions (stationary and mobile phases, operating conditions) reported for the separation of families of drug compounds are detailed in Table 1.

The use of peptides as therapeutics is fairly new (78). However, the range of therapeutic applications is steadily increasing: oncology, metabolic, cardiovascular diseases, and hematology are only a few examples. Lämmerhofer *et al.* (38) developed a reversed-phase LC–WAX stationary phase to separate molecules by lipophilicity and charge differences. The authors demonstrated the flexibility of retention and selectivity for peptide separations. Depending on the analytical conditions, different separation mechanisms were promoted: this stationary phase can be operated in reversed-phase LC, AEX, ion-exclusion, HILIC, and hydrophobic interaction chromatography (HIC) mode. Another mixed-mode reversed-phase LC–WAX stationary phase described above (with a *N*-(10-undecenoyl)- 3-aminoquinuclidine selector [39]) was used as an alternative to a C18 stationary phase for the separation and purification of tetrapeptide and its side products. The authors demonstrated a better selectivity and enhanced loading capacity in comparison to reversed-phase LC.

MM-HPLC has also proven beneficial for the separation and purification of proteins. Published works have reviewed the development of mixed- mode ligands (86), constituted of aliphatic or aromatic groups as the hydrophobic part and an amino, carboxyl, or sulfonic group as the ionic moiety, and their applications in the biopolymer field (87). However, because proteins are complex molecules, the development of novel stationary phases with multiple interactions is needed to provide alternative selectivity to classical reversed-phase LC and hydrophobic interactions. The use of mixed-mode columns for proteomic purposes, such as the separation of therapeutic proteins, is also growing. Wang *et al.* developed a reversed-phase LC–IEX stationary phase based on ionic liquids. The authors demonstrated the high selectivity provided by this column, with the exclusion of basic proteins and the separation with good resolution of acidic ones (79). Ding *et al.* developed a reversed-phase LC–positively charged repulsion stationary phase, with the polymerization of polar group on the surface of silica (80). By adjusting operating conditions, the separation of five proteins was achieved. They also used the stationary phase to separate degradation products of one therapeutic protein, the recombinant human growth hormone.

Figure 4: (a) Poly(ionic liquid)-grafted silica stationary phase (21), (b) dendritic polymer-modified silica (20) reversed-phase LC–HILIC–AEX stationary phases, (c) HILIC–CEX–AEX glutathione-based zwitterionic stationary phase (59).



Metabolomic Applications:

Metabolomics studies require highly efficient, precise, and selective chromatographic methods. Among them, reversed-phase LC coupled to high resolution mass spectrometry (HRMS) is the most commonly used. However, metabolomics samples usually contain numerous analytes with a wide range of polarities. The use of mixed-mode columns is therefore advisable. Wernisch *et al.* (81) presented a comparison of the chromatographic performance of C18 reversed-phase LC, four HILIC, and two mixed-mode columns for the analysis of 764 metabolite standards, including amino acids, nucleotides, sugars, and other metabolites, representing all major biological pathways and commonly observed exogenous metabolites. The authors investigated retention capabilities, selectivities, and specificity towards hydrophilic metabolites for each method. For phosphates, sugars, and amino acids, at least one of the mixed- mode columns permitted the retention of a higher percentage of metabolites with good peak shape than the C18 column. Ammann *et al.* (82) coupled two mixed-mode columns to obtain tri- modal reversed-phase LC, HILIC, and IEX separation capabilities in one run for the analysis of various metabolites: sugars, amino acids, carboxylates, fatty acids, and antioxidants. The authors also investigated the capability of a single tri-mode column for the separation of the same metabolites. The separation of 18 standards was partially achieved and the coupling with MS permitted the identification of metabolites.

Eastwood *et al.* (83) developed an analytical and semi-preparative method for the purification of nucleotides present in an enzymatic reaction mixture. The collected nucleotide was found to be pure at 99%. The dendritic

polymer described above (20) was used for the effective separation of six nucleobases and nucleosides, with the combined use of reversed-phase LC, AEX, and HILIC interactions. Qiu *et al.* also developed a reversed-phase LC–AEX–HILIC stationary phase, based on ionic liquids for the separation of similar compounds. Zimmermann *et al.* (40) used a reversed-phase LC–WAX stationary phase based on N-(10-undecenoyl)-3-aminoquinuclidine for the separation of structurally closely related oligonucleotides, with a size of 20 to 23 nucleotides. Phosphorylated carbohydrates are essential metabolites for all forms of life. The difficulty in analysis is because there is a coexistence of isomers with a similar fragmentation in mass spectrometry and the complete resolution of isomers is difficult to obtain with reversed-phase LC. Hinterwirth *et al.* (84) developed a selective method for the separation of sugar phosphates by using a reversed-phase LC–WAX stationary phase. This phase revealed remarkable selectivities for the separation of six individual isomeric hexose phosphates, which were at least partially separated.

The use of mixed-mode columns has also appeared for lipidomic purposes. Granafei *et al.* proposed a method for the separation of a complex mixture containing polar lipids, with the use of a reversed-phase LC–HILIC mixed- mode liquid chromatography coupled to a high resolution tandem mass spectrometry system (85).

Mixed-mode material has also proven its application for the analysis of polar compounds in natural products. Apfelthaler *et al.* (86) evaluated the retention properties of 79 fungal metabolites on a reversed-phase LC–WAX mixed-mode stationary phase by LC–ESI-MS/MS. Qiao *et al.* (30) investigated the analysis of highly polar compounds with the analysis of secondary metabolites of *Trichoderma*, a genus of fungi, with an imidazolium-embedded C8-based stationary phase for simultaneous reversed-phase LC–HILIC mixed-mode.

Finally, Bicker *et al.* (87) developed a method for the quantification of major chlorpyrifos metabolites, an insecticide, in human urine, by reversed-phase LC– WAX liquid chromatography coupled to ESI-MS/MS (88). Some of these metabolomics applications are detailed in Table 1.

Interest of Mixed-Mode Columns in 2D Systems

The principle of two-dimensional liquid chromatography (2D-LC) is based on the combination of two different chromatographic systems, which may be provided by different stationary phases, different elution conditions, or different chromatographic modes.

Mixed-mode columns combine multiple chromatographic modes, which are complementary or orthogonal to each other. With such columns, the analysis of complex samples with only one column has been demonstrated (21,27) because of the great flexibility and high selectivity offered. The major advantage of using one mixed-mode column for two-dimensional separations is that it can replace the dual columns used in classical 2D-LC. Thus, the entire 2D-LC operation can be accomplished on a single column in off-line or on-line mode, by adjusting the analytical conditions to promote different retention mechanisms in the two dimensions.

Stevenson *et al.* (52) developed an off-line 2D-LC separation of a β-lactoglobulin tryptic digest with a single reversed-phase LC–AEX–CAX tri-mode stationary phase. In the first dimension, the mobile phase pH was 7, while it was adjusted to 2 in the second dimension. The authors also performed the same separation with a C18 column. Greater efficiency was observed for the mixed-mode column, thereby providing larger peak capacity than the C18 column.

Gilar *et al.* (89) developed a reversed-phase LC–CEX silica- based PFP column for the pseudo 2D-LC separation of peptides with strong negative moieties, such as phosphopeptides and sialylated glycopeptides. Recently, Wang *et al.* (17,90) synthesized a reversed-phase LC–HILIC C18-diol stationary phase for the analysis of complex samples of traditional Chinese medicines. They used their column for the simultaneous separation of highly polar and hydrophobic compounds in on-line and off-line 2D-LC, using a single column but changing the ratio of organic and buffer solution. Here again, the mixed-mode column showed enhanced peak capacity and higher efficiency when compared to a C18 column. Li *et al.* (91) analyzed urinary nucleosides in off-line 2D-LC with one reversed-phase LC–AEX phase (a silica stationary phase co-functionalized with Wulff-type phenylboronate and C12).

A mixed-mode column can also be coupled to a reversed-phase LC column for comprehensive 2D-LC analysis. Li *et al.* (92) used a reversed-phase LC–SAX in the first dimension and a reversed-phase C18 in the second dimension for the simultaneous separation of ionic and non-ionic compounds with different functional groups contained in white wine. With the use of a mixed-mode column in the first dimension, higher retention and larger peak distribution areas were obtained. Li *et al.* (93) used the same configuration for the analysis of polysorbate 20, a surfactant commonly used in the formulation of monoclonal antibodies to avoid protein denaturation and aggregation. In the first dimension, the mixed-mode column was used to separate polysorbate esters from the protein. Then, the esters were separated in the second dimension with the reversed-phase column. Finally, an off-line 2D mixed- mode reversed-phase LC–MS/MS method was developed for the profiling of lipids in biological samples. In the first dimension, lipids were separated according to their polarities on a monolithic silica-based mixed-mode column. In the second dimension, the separation was improved on a C30 core–shell particle phase. The method was applied to rat plasma and liver samples and more than 800 lipids were detected.

Conclusion and Perspectives

In this review, the recent development of mixed-mode chromatography stationary phases is covered.

MM-HPLC provides unique flexibility because of the multiple retention mechanisms offered in one column.

By adjusting the ratio of organic and aqueous phases and the concentration of aqueous buffers, reversed-phase LC, HILIC, and IEX modes can be successively used. The reversed- phase LC–HILIC, reversed-phase LC–IEX, HILIC–IEX, and tri-mode MM-HPLC are the most commonly encountered.

The field of applications of MM-HPLC is wide. MM-HPLC has demonstrated its efficiency for the combined analysis of small, non- polar, polar, and charged compounds, but also for larger molecules such

Table 1: Bi-modal and tri-modal applications of mixed-mode chromatography

Family	Compounds tested	Analytes	Mode
Pharmaceuticals	Counterions	10 Counterions: choline, tromethamine, sodium, potassium, meglumine, mesylate, nitrate, chloride, bromide, iodide	Tri-modal
		12 Counterions: phosphate, sodium, potassium, chloride, malate, bromide, nitrate, citrate, fumarate, sulfate, magnesium, calcium	Tri-modal
		25 Counterions: sodium, calcium, potassium, meglumine, tromethamine, zinc, magnesium, procaine, choline chloride, sulfate, bromide, maleate, mesylate, tartrate, citrate, phosphate, fumarate, nitrate, lactate, succinate, besylate, malate, gluconate, tosylate	Tri-modal
	Steroids	4 Steroids: estriol, 17-α-estradiol, 17-β-estradiol, estrone	Bi-modal
		8 Steroids: prednisolone, corticosterone, cortisone, testosterone, estriol, estrone, 17-α-estradiol, 17-β-estradiol	Bi-modal
	Alkaloids	7 Alkaloids: tetrahydropalmatine, theophylline, gastrodin, berberine, lycorine, sinomenine, tetrandrine	Bi-modal
	Non-steroidal anti-inflammatory drugs (NSAIDs)	9 NSAIDs: fluprofen, flufenamic acid, mefenamic acid, ibuprofen, loxoprofen, ketoprofen, carprofen, indoprofen, sulindac	Bi-modal
	Sulfonamides	6 Sulfonamides: sulfapyridine, sulfamethyldiazine, sulfadoxine, sulfadimethoxine, sulfathiazole, sulfasoxazole	Bi-modal
	Nitroimidazoles	5 Nitroimidazoles: ipronidazole, ornidazole, metronidazole, ronidazole, tinidazol	Bi-modal
	Cannabinoids	5 Cannabinoids: cannabidiol, cannabinol, tetrahydro-cannabinolic acid, cannabidiolic acid, Δ9-tetrahydrocannabinol	Bi-modal
	Water-soluble vitamins	5 Compounds from vitamin B: thiamine, nicotinamide, lactoflavine, pyridoxine, cobalamin	Bi-modal
	Water and fat-soluble vitamins	10 Vitamins: B1, B6, B3-amide, B12, B2, C, B3, A-acetate, D3, K1	Bi-modal
	Biphosphonate	Etidronate tablet and related impurities or degradation products	Bi-modal
	Catecholamines and amphetamine related drugs	3-methoxytyramine, methcathinone, cathinone, ephedrine, methamphetamine, methylenedioxymethamphetamine, methylenedioxyamphetamine, methylenedioxyethylamphetamine	Bi-modal
	Peptides	Tetrapeptide (N-acetyl-Ile-Glu-Gly-Arg-p-nitroanilide) and its impurities	Bi-modal
	Proteins	5 Proteins: cytochrome, insulin, lysozyme, transferrin, bovine serum albumin	Bi-modal
Metabolomics	Nucleobases and nucleosides	6 Compounds: uracil, uridine, cytosine, cytidine, guanine, guanosine	Tri-modal
	Oligonucleotides	Mixtures of oligonucleosides with different chain lengths (20-23 nucleosides)	Bi-modal
	Phosphorylated carbohydrates	6 Individual isomeric hexose phosphates	Bi-modal
	Chlorpyrifos metabolites	Chlorpyrifos and 4 metabolites	Bi-modal

Stationary phase	Operating conditions	Detection mode	Reference
Reversed-phase LC–WAX–SCX	acetonitrile/water/100 mM ammonium acetate buffer, pH 5 (60:25:15, *v/v/v*), flow rate: 0.5 mL/min, 30 °C	CAD	56
HILIC–WCX–SAX	water/100 mM ammonium formate buffer, pH 3.65 (gradient elution program: 90/10 to 0/100 *v/v*), flow rate: 0.6 mL/min, 30 °C	CAD	57
Reversed-phase LC–WAX–SCX	acetonitrile/water/200 mM ammonium formate, pH 4.0 (gradient elution program: 60/38/2 to 60/35/5 in 7 min, then to 5/5/90 in 8 min, plateau during 5 min), flow rate: 0.5 mL/min, 35 °C	CAD	63
Reversed-phase LC–SCX (sulfonic-azobenzene-grafted silica)	methanol/water (65:35, *v/v*), flow rate: 1.0 mL/min, 30 °C	UV (254 nm)	18
Reversed-phase LC–HILIC (tripeptide-based monomeric silica)	methanol/water (30:70, *v/v*), flow rate: 1.0 mL/min, 25 °C	UV (254 nm)	28
Reversed-phase LC–HILIC (C18-dithiothreitol silica)	acetonitrile/80 mM ammonium acetate buffer, pH 4 (90:10, *v/v*), flow rate: 0.2 mL/min, 25 °C	DAD (maximum absorption)	68
Reversed-phase LC–HILIC (C18-dithiothreitol silica)	acetonitrile/40 mM ammonium acetate buffer, pH 6.8 (95:5, *v/v*), flow rate: 0.2 mL/min, 25 °C	DAD (maximum absorption)	68
HILIC/AEX (2-methylimidazolium functionalized silica)	acetonitrile/50 mM ammonium acetate (gradient elution program: 95:5 during 2 min, 95:5 to 80:20 in 5 min), flow rate: 1.0 mL/min, 35 °C	UV (254 nm)	70
Reversed-phase LC–normal-phase LC (N-ferrocenyl(benzoyl)amino-acid esters attached on silcia)	hexane/isopropanol/methanol (72:18:10, *v/v/v*), flow rate: 0.8 mL/min, 30 °C	UV (320 nm)	71
Reversed-phase LC–WAX (C18-amino silica)	A: water/methanol/acetonitrile (95:2.5:2.5, *v/v/v*) + 0.2% tetramethylammonium hydroxide (conc. 25% in methanol), B: water/methanol/acetonitrile (10:20:70, *v/v/v*) + 0.2% tetramethylammonium hydroxide (conc. 25% in methanol): gradient 100% A to 100% B in 3 min, flow rate: 1.0 mL/min, 30 °C	UV (230 nm)	72
HILIC–AEX (2-methylimidazolium functionalized silica)	acetonitrile/20 mM ammonium acetate buffer (73:27, *v/v*), flow rate: 1.0 mL/min, 35 °C	UV (272 nm)	70
Reversed-phase LC–WAX (N-(10-undecenoyl)-3-aminoquinuclidine onto thiol-modified monolithic silica)	A: water/100 mM phosphate buffer, pH 4.4/acetonitrile (85:10:5, *v/v/v*), B: water/100 mM phosphate buffer, pH 4.4/acetonitrile (18:2:80, *v/v/v*), gradient: 0% B during 2 min, 12% B at 7 min, 82% at 22 min, 97% B at 30 min, plateau at 97% B during 3 min, 0% B at 34 min, flow rate: 1.0 mL/min, 25 °C	UV (280 nm)	76
Reversed-phase LC–AEX (Primesep SB 50*3.2 mm, 5 µm)	A: acetonitrile/0.03% TFA in water (5:95, *v/v*), B: acetonitrile/0.2% TFA in water (5:95, *v/v*), gradient: 0% B to 100% B in 5 min, flow rate: 0.5 mL/min, 40 °C	CAD	77
Reversed-phase LC–WCX (polystyrene network with carboxylic groups silica-based	acetonitrile/water (40:60, *v/v*) containing 5 mM ammonium acetate, flow rate: 1.0 mL/min, 40 °C	UV (210 nm)	35
Reversed-phase LC–WAX (N-(10-undecenoyl)-3-amino-quinuclidine selector 250*4 mm, 5 µm)	acetonitrile/water/1 M formic acid, pH 4.5 (20:70:10; *v/v/v*), flow rate: 1.0 mL/min, room temperature	UV (316 nm)	39
Reversed-phase LC–positively charged repulsion	A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, gradient: 10 to 60% B in 20 min, flow rate: 1 mL/min, 30 °C	UV (214 nm)	80
Reversed-phase LC–AEX–HILIC (dendritic polymer-modified porous silica)	5 mM ammonium formate, pH 4.8, acetonitrile/water (90:10, *v/v*), flow rate: 1 mL/min	UV (254 nm)	20
Reversed-phase LC–WAX	acetonitrile/0.2 M triethylammonium phosphate buffer, pH 7.5 (30:70, *v/v*), flow rate: 1 mL/min, 40 °C	UV (260 nm)	40
Reversed-phase LC–WAX	0.1% TFA in acetonitrile/water (70:30, *v/v*), flow rate: 1 mL/min, 25 °C	CAD	84
Reversed-phase LC–WAX	A: acetonitrile/water (30:70, *v/v*) + 20 mM acetic acid, pH 6.45, B: acetonitrile/water (80:20, *v/v*) + 20 mM acetic acid, pH 7.45, gradient: 100% A to 100% B in 10 min, plateau at 100% B until 22.5 min, flow rate: 1.0 mL/min, 25 °C	ESI-MS/MS	88

as peptides or proteins. As a result, this technique is particularly used in pharmaceutical analysis for impurity profiling of APIs, counterions, drugs, vitamins, biopharmaceuticals, and much more. The high MS compatibility of mobile phases used with mixed-mode chromatography also permits the analysis of complex mixtures in metabolomics and lipidomics. For two-dimensional separations, one mixed-mode column can advantageously replace the dual columns used in classical 2D-LC.

Thus, the entire 2D-LC operation may be accomplished on one single column in off-line or on-line modes, by adjusting the analytical conditions in the two dimensions. Compared to classical reversed-phase C18 phases, higher efficiency, peak capacity, and resolution were described. With the high flexibility offered by mixed- mode columns, a large number of molecules can be analyzed, making the possibilities of applications and the method development almost infinite.

References

(1) R. Nageswara Rao and V. Nagaraju, *J. Pharm. Biomed. Anal.* **33**, 335–377 (2003) doi:10.1016/S0731-7085(03)00293-0.

(2) S. Fekete, I. Molnár, and D. Guillarme, J. *Pharm. Biomed. Anal.* **137**, 60–69 (2017) doi:10.1016/j.jpba.2017.01.013.

(3) K. Petritis, S. Brussaux, S. Guenu, C. Elfakir, and M. Dreux, *J. Chromatogr. A* **957**, 173–185 (2002) doi:10.1016/S0021-9673(02)00372-2.

(4) A.J. Alpert, *J. Chromatogr. A* **499**, 177–196 (1990) doi:10.1016/S0021-9673(00)96972-3.

(5) D. Guillarme and J.-L. Veuthey, *American Pharmaceutical Review* **20**, (2017).

(6) B. Buszewski and S. Noga, *Anal. Bioanal. Chem.* **402**, 231–247 (2012) doi:10.1007/s00216-011-5308-5.

(7) A. Periat, J. Boccard, J.-L. Veuthey, S. Rudaz, and D. Guillarme, *J. Chromatogr. A* **1312**, 49–57 (2013) doi:10.1016/j.chroma.2013.08.097.

(8) J. Ruta, S. Rudaz, D.V. McCalley, J.-L. Veuthey, and D. Guillarme, *J. Chromatogr. A* **1217**, 8230–8240 (2010) doi:10.1016/j.chroma.2010.10.106.

(9) B. Chauve, D. Guillarme, P. Cléon, and J.-L. Veuthey, *J. Sep. Sci.* **33**, 752–764 (2010) doi:10.1002/jssc.200900749.

(10) P. Cummins, K. Rochfort, and B. O'Connor, in *Protein Chromatogr.,* D. Walls and S.T. Loughran, Eds. (Springer, New York, 2017) pp. 209–223. doi:10.1007/978-1-4939-6412-3_11.

(11) A. Grand-Guillaume Perrenoud, J.-L. Veuthey, and D. Guillarme, *J. Chromatogr. A* **1266**, 158–167 (2012) doi:10.1016/j.chroma.2012.10.005.

(12) E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, and C. West, *J. Chromatogr. A* **1408**, 217–226 (2015) doi:10.1016/j.chroma.2015.07.037.

(13) E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, and C. West, *J. Chromatogr. A* **1408**, 227–235 (2015) doi:10.1016/j.chroma.2015.07.035.

(14) E. Lesellier and C. West, *J. Chromatogr. A* **1382**, 2–46 (2015) doi:10.1016/j.chroma.2014.12.083.

(15) A.P. Halfpenny and P.R. Brown, *Chromatographia* **21**, 317–320 (1986) doi:10.1007/BF02311602.

(16) L.A. Kennedy, W. Kopaciewicz, and F.E. Regnier, *J. Chromatogr.* **359**, 73–84 (1986).

(17) Q. Wang, M. Ye, L. Xu, and Z. Shi, *Anal. Chim. Acta.* **888**, 182–190 (2015) doi:10.1016/j.aca.2015.06.058.

(18) H. Qiu, M. Zhang, T. Gu, M. Takafuji, and H. Ihara, *Chem.– Eur. J.* **19**, 18004–18010 (2013) doi:10.1002/chem.201302746.

(19) Y. Liu, Q. Du, B. Yang, F. Zhang, C. Chu, X. Liang, *Analyst* **137**, 1624–1628 (2012) doi:10.1039/C2AN16277F.

(20) Y. Li, J. Yang, J. Jin, X. Sun, L. Wang, and J. Chen, *J. Chromatogr. A* **1337**, 133–139 (2014) doi:10.1016/j.chroma.2014.02.044.

(21) H. Qiu, A.K. Mallik, M. Takafuji, S. Jiang, and H. Ihara, *Analyst* **137**, 2553–2555 (2012) doi:10.1039/C2AN35348B.

(22) L. Zhang, Q. Dai, X. Qiao, C. Yu, X. Qin, and H. Yan, *TrAC Trends Anal. Chem.* **82**, 143–163 (2016) doi:10.1016/j.trac.2016.05.011.

(23) L. Wang, W. Wei, Z. Xia, X. Jie, and Z.Z. Xia, *TrAC Trends Anal. Chem.* **80**, 495–506 (2016) doi:10.1016/j.trac.2016.04.001.

(24) B.-Y. Zhu, C.T. Mant, and R.S. Hodges, *J. Chromatogr. A* **548**, 13–24 (1991) doi:10.1016/S0021-9673(01)88590-3.

(25) X. Liu and C. Pohl, *J. Chromatogr. A* **1191**, 83–89 (2008) doi:10.1016/j.chroma.2007.12.012.

(26) T. Aral, H. Aral, B. Ziyadanoğulları, R. Ziyadanoğulları, *Talanta* **131**, 64–73 (2015) doi:10.1016/j.talanta.2014.07.060.

(27) Y. Li, Z. Xu, Y. Feng, X. Liu, T. Chen, and H. Zhang, *Chromatographia* **74**, 523 (2011) doi:10.1007/s10337-011-2120-5.

(28) S. Ray, M. Takafuji, and H. Ihara, *J. Chromatogr. A* **1266**, 43–52 (2012) doi:10.1016/j.chroma.2012.10.004.

(29) K. Ohyama, Y. Inoue, N. Kishikawa, and N. Kuroda, *J. Chromatogr. A* **1371**, 257–260 (2014) doi:10.1016/j.chroma.2014.10.073.

(30) X. Qiao, L. Zhang, N. Zhang, X. Wang, X. Qin, H. Yan, and H. Liu, *J. Chromatogr. A* **1400**, 107–116 (2015) doi:10.1016/j.chroma.2015.04.060.

(31) S. Liu, H. Xu, J. Yu, D. Li, M. Li, X. Qiao, X. Qin, and H. Yan, *Anal. Bioanal. Chem.* **407**, 8989–8997 (2015) doi:10.1007/s00216-015-9064-9.

(32) Y. Li, Y. Feng, T. Chen, and H. Zhang, *J. Chromatogr. A* **1218**, 5987–5994 (2011) doi:10.1016/j.chroma.2011.04.023.

(33) L. Qiao, X. Shi, X. Lu, and G. Xu, *J. Chromatogr. A* **1396**, 62–71 (2015) doi:10.1016/j.chroma.2015.03.081.

(34) X. Cai, Z. Guo, X. Xue, J. Xu, X. Zhang, and X. Liang, *J. Chromatogr. A* **1228**, 242–249 (2012) doi:10.1016/j.chroma.2011.06.042.

(35) Y. Zhang and P.W. Carr, *J. Chromatogr. A* **1218**, 763–777 (2011) doi:10.1016/j.chroma.2010.11.009.

(36) A. Abbood, C. Smadja, C. Herrenknecht, X. Alahmad, A. Tchapla, and M. Taverna, *J. Chromatogr. A* 1216, 3244–3251 (2009) doi:10.1016/j.chroma.2009.02.031.

(37) J. Wei, Z. Guo, P. Zhang, F. Zhang, B. Yang, and X. Liang, *J. Chromatogr. A* **1246**, 129–136 (2012) doi:10.1016/j.chroma.2012.03.047.

(38) M. Lämmerhofer, R. Nogueira, and W. Lindner, *Anal. Bioanal. Chem.* **400**, 2517–2530 (2011) doi:10.1007/s00216-011-4755-3.

(39) R. Nogueira, M. Lämmerhofer, and W. Lindner, *J. Chromatogr. A* **1089**, 158–169 (2005) doi:10.1016/j.chroma.2005.06.093.

(40) A. Zimmermann, R. Greco, I. Walker, J. Horak, A. Cavazzini, and M. Lämmerhofer, *J. Chromatogr. A* **1354**, 43–55 (2014) doi:10.1016/j.chroma.2014.05.048.

(41) A. Zimmermann, J. Horak, O.L. Sánchez-Muñoz, and M. Lämmerhofer, *J. Chromatogr. A* **1409**, 189–200 (2015) doi:10.1016/j.chroma.2015.07.036.

(42) Y. Shan, L. Qiao, X. Shi, and G. Xu, *J. Chromatogr. A* **1375**, 101–109 (2015) doi:10.1016/j.chroma.2014.11.084.

(43) M. Sun, H. Qiu, L. Wang, X. Liu, and S. Jiang, *J. Chromatogr. A* **1216**, 3904–3909 (2009) doi:10.1016/j.chroma.2009.02.089.

(44) M. Sun, J. Feng, C. Luo, X. Liu, and S. Jiang, *Talanta* **105**, 135–141 (2013) doi:10.1016/j.talanta.2012.11.077.

(45) M. Sun, J. Feng, X. Wang, H. Duan, L. Li, and C. Luo, *J. Sep. Sci.* **37**, 2153–2159 (2014) doi:10.1002/jssc.201400176.

(46) G. Shen, F. Zhang, B. Yang, C. Chu, and X. Liang, *Talanta* **115**, 129–132 (2013) doi:10.1016/j.talanta.2013.04.046.

(47) L. Qiao, S. Wang, H. Li, Y. Shan, A. Dou, X. Shi, and G. Xu, *J. Chromatogr. A* **1360**, 240–247 (2014) doi:10.1016/j.chroma.2014.07.096.

C. Bo, X. Wang, C. Wang, and Y. Wei, *J. Chromatogr. A* **1487**, 201–210 (2017) doi:10 016/j.chroma.2017.01.061.

P. Zhang, J. Chen, and L. Jia, *J. Chromatogr. A* **1218**, 3459–3465 (2011) doi:10.1016/j.chroma.2011.03.062.

(50) H. Huang, Z. Lin, Y. Lin, X. Sun, Y. Xie, L. Zhang, and G. Chen, *J. Chromatogr. A* **1251**, 82–90 (2012) doi:10.1016/j.chroma.2012.06.032.

(51) Z. Lin, X. Tan, R. Yu, J. Lin, X. Yin, L. Zhang, and H. Yang, *J. Chromatogr. A* **1355**, 228–237 (2014) doi:10.1016/j.chroma.2014.06.023.

(52) P.G. Stevenson, J.N. Fairchild, and G. Guiochon, *J. Chromatogr. A* **1218**, (2011) 1822–1827. doi:10.1016/j.chroma.2011.01.078.

(53) M. Biba, E. Jiang, B. Mao, D. Zewge, J.P. Foley, and C.J. Welch, *J. Chromatogr. A* **1304**, 69–77 (2013) doi:10.1016/j.chroma.2013.06.050.

(54) A.A. Kazarian, P.N. Nesterenko, P. Soisungnoen, R. Burakham, S. Srijaranai, and B. Paull, *J. Sep. Sci.* **37**, 2138–2144 (2014) doi:10.1002/jssc.201400411.

(55) H. Choi, S. Kim, S. Ahn, H. Chang, S. Lee, and Y. Lee, *Forensic Sci. Int.* **259**, 69–76 (2016) doi:10.1016/j.forsciint.2015.12.016.

(56) X. Liu and C.A. Pohl, *J. Chromatogr. A* **1232**, 190–195 (2012) doi:10.1016/j.chroma.2011.12.009.

(57) X. Liu, M. Tracy, U. Aich, and C. Pohl, "Exploring Mixed-Mode Chromatography: Column Chemistry, Properties, and Applications", (2014). https://tools.thermofisher.com/content/sfs/posters/PN-20947-Mixed-Mode-Chromatography-HPLC-2014-PN20947-EN.pdf (accessed 5 April 2017).

(58) L. Qiao, X. Zhou, Y. Zhang, A. Yu, K. Hu, S. Zhang, and Y. Wu, *Talanta* **153**, 8–16 (2016) doi:10.1016/j.talanta.2016.02.055.

(59) A. Shen, X. Li, X. Dong, J. Wei, Z. Guo, and X. Liang, *J. Chromatogr. A*

1314, 63–69 (2013) doi:10.1016/j.chroma.2013.09.002.
(60) Q. Wang, Y. Long, L. Yao, M. Ye, and L. Xu, *Phytochem. Anal.* (2017) doi:10.1002/pca.2680.
(61) F. Ianni, R. Sardella, A. Carotti, B. Natalini, W. Lindner, and M. Lämmerhofer, *Chirality* **28**, 5–16 (2016) doi:10.1002/chir.22541.
(62) C.V. Hoffmann, R. Reischl, N.M. Maier, M. Lämmerhofer, and W. Lindner, *J. Chromatogr. A* **1216**, 1147–1156 (2009) doi:10.1016/j.chroma.2008.12.045.
(63) K. Zhang, L. Dai, N.P. Chetwyn, *J. Chromatogr. A* **1217**, 5776–5784 (2010) doi:10.1016/j.chroma.2010.07.035.
(64) D. Ilko, C.J. Nap, U. Holzgrabe, and S. Almeling, *Pharmeuropa Bio Sci. Notes* **2014**, 81–91 (2014).
(65) J. Li, S. Shao, M.S. Jaworsky, and P.T. Kurtulik, *J. Chromatogr. A* **1185**, 185–193 (2008) doi:10.1016/j.chroma.2008.01.083.
(66) B.A. Olsen, *J. Chromatogr. A* **913**, 113–122 (2001) doi:10.1016/S0021-9673(00)01063-3.
(67) M.A. Strege, S. Stevenson, and S.M. Lawrence, *Anal. Chem.* **72**, 4629–4633 (2000).
(68) Q. Wang, Y. Long, L. Yao, L. Xu, Z.-G. Shi, and L. Xu, *Talanta* **146**, 442–451 (2016) doi:10.1016/j.talanta.2015.09.009.
(69) Q. Wang, L. Xu, and Y. Xue, *J. Liq. Chromatogr. Relat. Technol.* **39**, 598–606 (2016) doi:10.1080/10826076.2016.1213174.
(70) B. Yang, H. Liu, J. Chen, M. Guan, and H. Qiu, *J. Chromatogr. A* **1468**, 79–85 (2016) doi:10.1016/j.chroma.2016.09.021.
(71) X. Zhou, X. Li, A. Cao, Q. Lijun, A. Yu, S. Zhang, and Y. Wu, *Talanta* **144**, 1044–1051 (2015) doi:10.1016/j.talanta.2015.07.063.
(72) C.-H. Hung, J. Zukowski, D.S. Jensen, A.J. Miles, C. Sulak, A.E. Dadson, and M.R. Linford, *J. Sep. Sci.* **38**, 2968–2974 (2015) doi:10.1002/jssc.201500156.
(73) D.W. Later, B.E. Richter, D.E. Knowles, and M.R. Andersen, *J. Chromatogr. Sci.* **24**, 249–253 (1986) doi:10.1093/chromsci/24.6.249.
(74) M. Lämmerhofer, M. Richter, J. Wu, R. Nogueira, W. Bicker, and W. Lindner, *J. Sep. Sci.* **31**, 2572–2588 (2008) doi:10.1002/jssc.200800178.
(75) H. Wang, L. Zhang, T. Ma, L. Zhang, and X. Qiao, *J. Sep. Sci.* **39**, 3498–3504 (2016) doi:10.1002/jssc.201600448.
(76) R. Dabre, N. Azad, A. Schwämmle, M. Lämmerhofer, and W. Lindner, *J. Sep. Sci.* **34**, 761–772 (2011) doi:10.1002/jssc.201000793.
(77) X.-K. Liu, J.B. Fang, N. Cauchon, and P. Zhou, *J. Pharm. Biomed. Anal.* **46**, 639–644 (2008) doi:10.1016/j.jpba.2007.11.041.
(78) K. Fosgerau and T. Hoffmann, *Drug Discov. Today* **20**, 122–128 (2015) doi:10.1016/j.drudis.2014.10.003.
(79) Y.-X. Wang, K.-L. Zhao, F. Yang, L. Tian, Y. Yang, and Q. Bai, *Chin. Chem. Lett.* **26**, 988–992 (2015) doi:10.1016/j.cclet.2015.05.001.
(80) L. Ding, Z. Guo, Z. Hu, and X. Liang, *J. Pharm. Biomed. Anal.* **138**, 63–69 (2017) doi:10.1016/j.jpba.2017.01.004.
(81) S. Wernisch, R. Pell, and W. Lindner, *J. Sep. Sci.* **35**, 1560–1572 (2012) doi:10.1002/jssc.201200103.
(82) A.A. Ammann and M.J.-F. Suter, *J. Chromatogr. A.* **1456**, 145–151 (2016) doi:10.1016/j.chroma.2016.06.001.
(83) H. Eastwood, F. Xia, M.-C. Lo, J. Zhou, J.B. Jordan, J. McCarter, W.W. Barnhart, and K.-H. Gahm, *J. Pharm. Biomed. Anal.* **115**, 402–409 (2015) doi:10.1016/j.jpba.2015.08.001.
(84) H. Hinterwirth, M. Lämmerhofer, B. Preinerstorfer, A. Gargano, R. Reischl, W. Bicker, O. Trapp, L. Brecker, and W. Lindner, *J. Sep. Sci.* **33**, 3273–3282 (2010) doi:10.1002/jssc.201000412.
(85) S. Granafei, P. Azzone, V.A. Spinelli, I. Losito, F. Palmisano, and T.R.I. Cataldi, *J. Chromatogr. A* **1477**, 47–55 (2016) doi:10.1016/j.chroma.2016.11.048.
(86) E. Apfelthaler, W. Bicker, M. Lämmerhofer, M. Sulyok, R. Krska, W. Lindner, and R. Schuhmacher, *J. Chromatogr. A* **1191**, 171–181 (2008) doi:10.1016/j.chroma.2007.12.067.
(87) W. Bicker, M. Lämmerhofer, and W. Lindner, *Anal. Bioanal. Chem.* **390**, 263–266 (2008) doi:10.1007/s00216-007-1637-9.
(88) W. Bicker, M. Lämmerhofer, and W. Lindner, *J. Chromatogr. B* **822**, 160–169 (2005) doi:10.1016/j.jchromb.2005.06.003.
(89) M. Gilar, Y.-Q. Yu, J. Ahn, J. Fournier, and J.C. Gebler, *J. Chromatogr. A* **1191**, 162–170 (2008) doi:10.1016/j.chroma.2008.01.061.
(90) Q. Wang, L. Tong, L. Yao, P. Zhang, and L. Xu, *J. Pharm. Biomed. Anal.* **125**, 205–211 (2016) doi:10.1016/j.jpba.2016.03.033.
(91) H. Li, X. Zhang, L. Zhang, X. Wang, F. Kong, D. Fan, L. Li, and W. Wang, *Anal. Chim. Acta.* **962**, 104–113 (2017) doi:10.1016/j.aca.2017.01.028.
(92) D. Li, R. Dück, and O.J. Schmitz, *J. Chromatogr. A* **1358**, 128–135 (2014) doi:10.1016/j.chroma.2014.06.086.
(93) Y. Li, D. Hewitt, Y.K. Lentz, J.A. Ji, T.Y. Zhang, and K. Zhang, *Anal. Chem.* **86**, 5150–5157 (2014) doi:10.1021/ac5009628.

Elise Lemasson is a PhD student at the University of Orleans, France.
Her research focuses on developing generic methods in HPLC and SFC for impurity profiling of drug candidates.
Sophie Bertin is a senior scientist at Servier Research Institute, specializing in separation science and mass spectrometry.
Philippe Hennig is the head of the analytical department of Servier Research Institute.
Eric Lesellier is an Associate Professor in analytical chemistry, teaching at Paris-Orsay University and carrying out his research at the University of Orleans. He is a recognized expert in chromatographic stationary phases for HPLC and SFC. He has contributed significantly to developing fundamental knowledge and applications of SFC.
Caroline West is an Associate Professor in analytical chemistry at the University of Orleans. Her scientific interests lie in fundamentals of chromatographic selectivity, both in the achiral and chiral modes, mainly in SFC but also in HPLC. In 2015, she received the *LCGC* award for "Emerging Leader in Chromatography"

IV. La SFC

1) Histoire

La chromatographie en phase supercritique a vu le jour en 1962, lorsque Klesper *et al.* [97] ont démontré que le travail à une pression supérieure au point critique de la phase mobile permettait de travailler à des températures plus basses qu'en chromatographie en phase gazeuse (GC) et garantissait ainsi une meilleure stabilité des composés étudiés. A cette époque, le nom de HPGC (high pressure gas chromatography) a été proposé. Dans les années 60, d'autres groupes se sont intéressés à l'HPGC et ont démontré la supériorité de cette technique pour séparer les composés thermiquement instables en GC [98,99]. Cependant la SFC était uniquement vue comme une variante de la GC et non comme une nouvelle méthode de séparation innovante. Cette technique n'a donc pas réussi à s'imposer au profit de la GC, malgré la commercialisation d'un premier appareil de SFC dans les années 1970. Il s'agissait en fait d'un système de chromatographie liquide (LC) équipé d'un régulateur de pression [100]. La possibilité d'ajuster la pression avec ce système a permis de séparer des composés aromatiques polycycliques en utilisant un gradient de pression et en un temps plus court qu'en chromatographie liquide. Pour la première fois, la SFC devenait compétitive avec la GC.

Les années 1980 marquent à la fois la commercialisation des premiers instruments SFC mais aussi l'apparition des colonnes capillaires de longues dimensions similaires à celles utilisées en GC [101,102]. L'appareillage est lui aussi similaire à celui utilisé en GC, mais le manque de robustesse de ces appareils par rapport à la GC a entrainé un déclin rapide de la SFC sur colonnes capillaires. La SFC, considérée alors comme une approche intermédiaire entre GC et LC depuis ses débuts, ne réussit pas à s'imposer comme méthode chromatographique à part entière. Parallèlement à cela, Gere *et al.* [103] développent un système dédié à la SFC, qui reprend en grande partie les éléments d'un système LC, à l'exception d'un régulateur de contre-pression utilisé pour maintenir le fluide à l'état supercritique et permettait d'utiliser une phase mobile binaire constituée de CO_2 et de co-solvant en faible quantité. Ce système, commercialisé par Hewlett-Packard, utilise pour la première fois des colonnes remplies pour une application en SFC. On parle alors de pSFC (« packed column SFC »). Gere *et al.* ont montré qu'une augmentation de l'efficacité est obtenue en diminuant la taille des particules des colonnes utilisées (3, 5 et 10 µm). Ils ont également montré qu'à taille de particules identiques, la SFC et la LC offrent une efficacité similaire mais avec une vitesse linéaire optimale plus importante pour la SFC, ce qui signifie que de hautes efficacités sont obtenues en SFC même à hauts

débits. Malgré ces avancées, la pSFC reste à la fin des années 80 une méthode peu utilisée en raison des instabilités instrumentales, au niveau de la régulation de la contre-pression notamment, qui rendent cette technique peu fiable, sensible et robuste aux yeux des chromatographistes.

Dans les années 90, peu de laboratoires ont continué à croire en la SFC. A cette époque, Berger, considéré par beaucoup de chromatographistes comme le père de la SFC moderne, a notamment beaucoup travaillé sur l'analyse de composés polaires en pSFC [104] et l'emploi d'additif acides et basiques dans la phase mobile pour améliorer la forme des pics [105,106]. En 1995, employé par Hewlett-Packard, il fonde Berger Instruments Inc avec quelques-uns de ses collègues et entreprend alors de modifier le premier instrument commercialisé par Hewlett-Packard pour le rendre compatible avec l'utilisation de colonnes remplies [107]. Ce système a été principalement utilisé dans l'industrie pharmaceutique.

Pendant encore deux décennies, l'utilisation de la SFC à l'échelle analytique est restée très faible et la technique n'a survécu que grâce aux avantages reconnus pour la purification d'énantiomères à l'échelle préparative. Aujourd'hui, c'est la pSFC qui domine, une minorité d'applications se faisant encore sur colonne capillaire, typiquement dans le domaine pétrolier, et le terme générique de SFC est employé pour parler de la technique. La SFC fait figure de référence dans le domaine de la séparation chirale [108], tant à l'échelle analytique que préparative, et de nombreux laboratoires pharmaceutiques l'ont aujourd'hui adopté au profit ou en complément de l'HPLC [17,109–112]. En effet, la SFC présente de nombreux avantages par rapport à l'HPLC pour la séparation et la purification de composés chiraux : (i) les séparations menées sont rapides, (i) le CO_2 utilisé couramment comme fluide supercritique est non-toxique, (ii) l'évaporation du solvant à l'échelle préparative est facilitée par le retour à l'état gazeux du CO_2 lorsqu'il n'est plus maintenu à l'état supercritique ainsi que par la faible teneur en solvant organique des fractions collectées, ce qui représente un avantage économique non négligeable. De nombreux travaux traitent de la séparation et de la purification des composés chiraux en SFC dans la littérature [113–115] et ne seront que brièvement abordés dans la suite de ce manuscrit (partie IV-3).

La SFC s'est enfin imposée comme méthode séparative à part entière suite aux efforts des constructeurs et des fabricants de colonnes pour fournir du matériel robuste, notamment grâce à un contrôle précis de la contre-pression, et des colonnes dédiées (colonnes totalement poreuses sub-2 µm [116–119] ou superficiellement poreuses sub-3

µm [120,121]). Parmi les systèmes actuellement disponibles, on citera l'ACQUITY UPC², appareil dédié à la SFC commercialisé par Waters Corporation en 2012. En 2011, la société Agilent a commercialisé un système hydride LC/SFC, appelé 1260 Infinity SFC System, permettant à la fois de réaliser des analyses en UHPLC et de basculer en SFC grâce à l'ajout d'un système de pompage du CO_2 et d'un régulateur de contre-pression. Ces instruments ont été conçus de façon à réduire les volumes morts et le délai de gradient et ainsi améliorer les performances analytiques, comme pour les systèmes UHPLC. Le terme UHPSFC (pour « Ultra-high performance supercritical fluid chromatography ») fait alors son apparition [20,119], et fait référence à l'utilisation combinée d'un système SFC optimisé et de colonnes sub-2 µm. On notera également la commercialisation par Shimadzu d'un système hybride SFE/SFC/MS, le système Shimadzu UC Nexera, permettant de réaliser une extraction sous fluide supercritique (SFE) suivie d'une analyse SFC et d'une détection en masse.

Longtemps cantonnée essentiellement à l'analyse de produits pharmaceutiques, le champ d'applications de la SFC s'est aujourd'hui largement étendu. La SFC a ainsi montré son efficacité pour l'analyse de composés divers comme les lipides [122], les produits naturels comme les huiles végétales [123], les vitamines [124] ou les huiles essentielles [125], les produits cosmétiques [126,127], les produits pétroliers [128,129], les produits agroalimentaires [130]. Cette technique reste cependant largement employée dans le domaine pharmaceutique où les applications, nombreuses, seront détaillées dans la partie IV-3. On notera également l'apparition des analyses concernant la médecine traditionnelle chinoise [131].

2) Les fluides supercritiques

2.1. Définitions

Chaque composé, quand il est pur, peut exister sous trois états différents : solide, liquide ou gazeux. L'état dans lequel se trouve le composé est fonction de la pression et de la température (Figure 1.4). Les domaines de ces différents états sont délimités par les courbes de changements d'état, traduisant des transitions de phases (fusion pour le passage de l'état solide à liquide, vaporisation pour le passage de l'état liquide à gazeux et sublimation pour le passage de l'état solide à gazeux). Chaque corps pur existera donc sous un état de la matière, sauf sur les courbes de changement d'état où les deux états coexistent et au point triple où les trois états de la matière sont en équilibre.

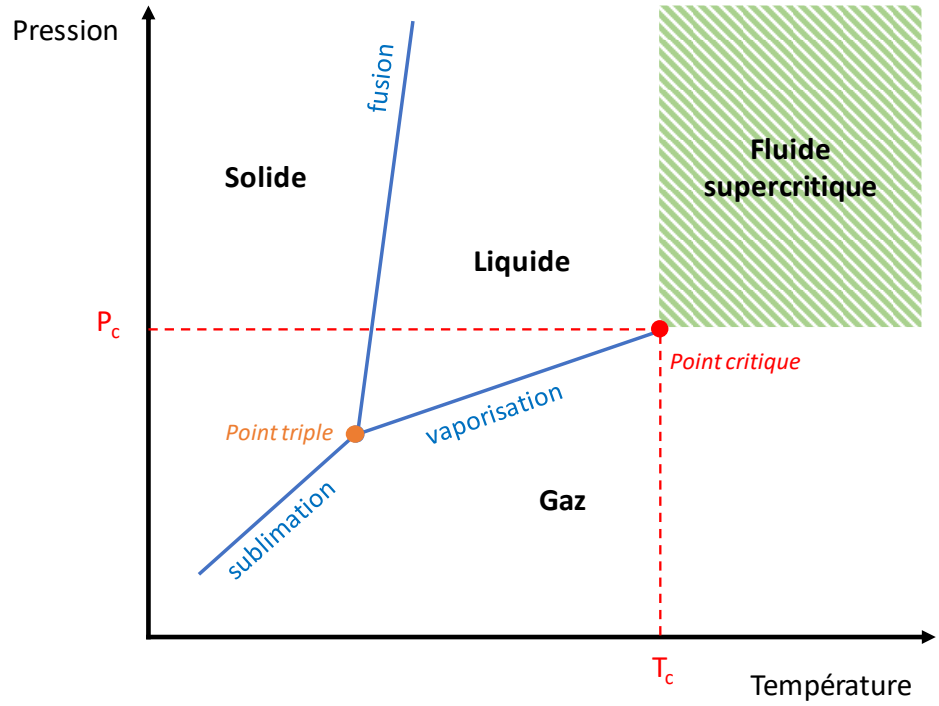
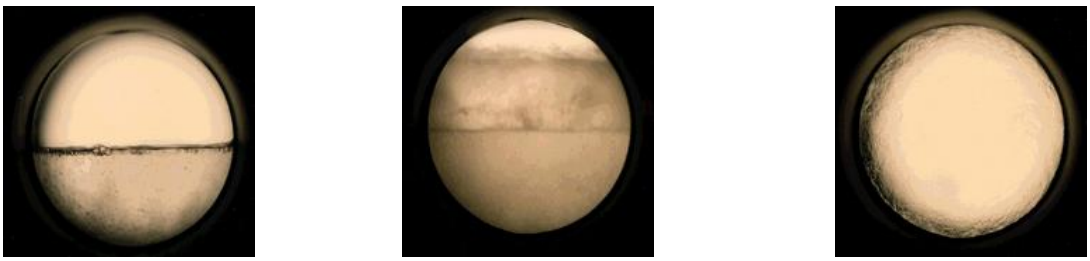


Figure 1.4 - Diagramme d'état d'un corps pur

La courbe de vaporisation s'interrompt au point critique, de coordonnées (T_c, P_c). L'état supercritique est obtenu lorsqu'un corps pur est soumis à une pression et une température supérieures à la pression critique (P_C) et la température critique (T_C). A l'interface liquide/gaz, sur la courbe de vaporisation, la séparation des deux phases est bien nette (Figure 1.5). Au point critique, la densité des phases liquide et gazeuse tend vers un équilibre et la courbe de changement d'état s'interrompt, ce qui garantit un continuum des propriétés physico-chimiques lors du passage à l'état supercritique. Il est alors plus difficile de distinguer la séparation des deux phases. Lorsqu'on est à l'état supercritique, les deux phases se confondent. Cette phase homogène est celle d'un fluide supercritique.



liquide/gaz | point critique | fluide supercritique

Figure 1.5 - Changement d'état de l'interface liquide gaz avec l'augmentation de P et T jusqu'au point critique et au passage à l'état supercritique (adapté d'après http://www1.chem.leeds.ac.uk//People/CMR/criticalpics.html)

2.2. Propriétés physico-chimiques du CO_2 supercritique

Les fluides supercritiques possèdent des propriétés physiques intermédiaires entre les liquides et les gaz (Tableau 1.I). La viscosité des fluides supercritiques est proche de celle d'un gaz, leur masse volumique est proche de celle d'un liquide et le coefficient de diffusion est à une valeur intermédiaire entre liquide et gaz.

Tableau 1.I - Propriétés physiques des liquides, des gaz et des fluides supercritiques [132]

	Viscosité η (cPoise)	**Coefficient diffusion D_m (m^2/s)**	**Masse volumique ρ (g/cm^3)**
Liquides *(1 atm, 15-30°C)*	0.2 - 3	(0.2 - 2) x 10^{-9}	0.6 - 1.6
Fluides supercritiques *(T_C, P_C)*	(1 - 3) x 10^{-2}	0.7 x 10^{-7}	0.2 - 1.2
Gaz *(1 atm, 15-30°C)*	(1 - 3) x 10^{-2}	(1 - 4) x 10^{-5}	(0.6 - 2) x 10^{-3}

Grâce à la faible viscosité des fluides supercritiques, il est possible de travailler à hauts débits tout en gardant une efficacité élevée et sans générer une pression excessive. Ajoutées à la faible viscosité, la valeur intermédiaire du coefficient de diffusion et la masse volumique proche de celle d'un liquide permettent d'avoir une haute diffusivité (garantissant une haute efficacité) et une bonne solubilisation des analytes.

2.3. Les différents fluides et leurs utilisations

Plusieurs types de fluides ont été utilisés à l'état supercritique en chromatographie [133], en fonction de l'application souhaitée : réaction, purification, extraction, chromatographie (Tableau 1.II).

Tableau 1.II - P_C et T_C de certains fluides [133]

Composé	**T_C (°C)**	**P_C (bar)**
Dioxyde de carbone	31	74
Oxyde nitreux	37	73
Fréon 22	96	49
Propane	97	43
Ammoniaque	132	113
Méthanol	240	80
Eau	374	221

Parmi ces fluides, certains ne sont pas utilisés en chromatographie car il est difficile d'atteindre les conditions de P_C et T_C. C'est le cas de l'eau (T_C = 374°C et P_C = 221°C), du méthanol (T_C = 240°C) et de l'ammoniaque (T_C et P_C élevées) qui est également extrêmement corrosif [134]. L'utilisation du méthanol, et de l'éthanol, supercritiques trouve cependant des applications dans la synthèse de biocarburants [135]. L'extraction par eau supercritique, eau chaude sous haute pression, est quant à elle utilisée pour l'extraction de plantes. Le romarin a ainsi été particulièrement étudié pour ses propriétés antioxydantes [136]. Ce procédé a également été utilisé pour extraire les huiles essentielles [137] ou végétales [138] et les microalgues [139]. Comparée à d'autres techniques, l'extraction par eau supercritique possède l'avantage d'être une méthode peu chère et rapide, mais elle reste corrosive. L'eau supercritique est également utilisée dans le traitement des déchets nucléaires [140]. L'oxyde nitreux, ou protoxyde d'azote, possède quant à lui des T_C et P_C plus accessibles et a été utilisé un temps pour extraire des composés polaires, mais c'est un oxydant fort et les risques d'explosion sont importants avec ce fluide [141]. Différents fluorocarbones, comme le Fréon 22, ont été utilisés pour extraire des composés polaires tels que les stéroïdes [142] et les phénols [143]. Cependant, ils sont aujourd'hui peu utilisés en raison de leur forte contribution au réchauffement climatique. Enfin, le CO_2 s'est très vite imposé comme fluide supercritique de choix en chromatographie de par ses nombreux avantages.

2.4. Intérêt du CO_2 supercritique pour la chromatographie

Au cours du développement de la SFC des années 60 à aujourd'hui, le CO_2 est devenu le fluide de référence et est actuellement utilisé dans une majorité des laboratoires. Cela s'explique par ses nombreux avantages :

- D'un point de vue pratique, les T_C et P_C (31°C et 74 bar) de ce fluide sont facilement atteignables. Dans ces conditions, on peut travailler à basse température et minimiser les risques de dégradation des composés thermolabiles. Les conditions sont également douces pour le matériel (instrument et colonnes). De plus, le CO_2 est non corrosif.

- D'un point de vue chromatographique, le CO_2 présente un avantage non négligeable lors de son utilisation en chromatographie préparative ou pour l'extraction. En effet, dans ces deux cas de figure les extraits ou fractions collectés seront hautement concentrés en raison de la décompression du CO_2 à la fin de

l'analyse et de son retour à l'état gazeux. Cela représente un avantage économique important, car l'évaporation des fractions pour obtenir des extraits secs en sera grandement facilitée.

- D'un point de vue écologique et économique, le CO_2 est non toxique et non inflammable. Il est également disponible en grande quantité et naturel, car c'est un sous-produit industriel (lors de la fabrication du ciment ou de l'ammoniac par exemple). Il peut également être recyclé. Enfin, il est peu coûteux et est disponible à des taux de pureté élevés (environ 1€30/kg pour du CO_2 de haute pureté).

- En termes de performances chromatographiques, l'emploi d'une phase mobile constituée majoritairement de CO_2 permet d'obtenir de très hautes performances chromatographiques. Grand-Guillaume Perrenoud *et al.* [13] ont montré qu'à taille de particules identique, la vitesse linéaire optimale (u) obtenue est plus haute en SFC qu'en LC (Figure 1.6). En raison de la faible viscosité du CO_2, les coefficients de diffusion sont plus élevés, ce qui permet de travailler à hauts débits sans perdre en efficacité mais en réduisant le temps d'analyse. Cependant, compte-tenu de la pression limite des systèmes UHPSFC actuels (400 bar), cette vitesse linéaire optimale ne peut être atteinte.

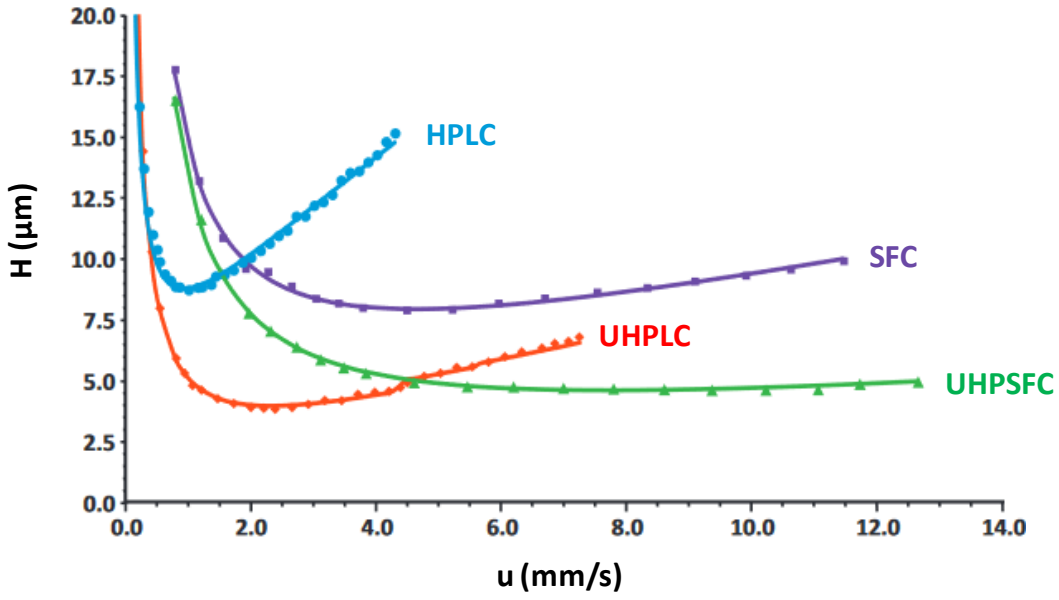


Figure 1.6 - Courbes de Van Deemter obtenues pour le butylparabène sur un système LC et un système SFC avec des colonnes différentes et deux tailles de particules : 1.7 µm et 3.5 µm. Colonnes XTerra RP18 (50 mm × 4.6 mm, 3.5 µm) (points bleus), Acquity Shield C18 (50 mm × 2.1 mm, 1.7 µm) (losanges rouges) testées en conditions LC : H_2O/ACN (60/40, v/v), 30 °C, 1 µL injecté, 254 nm. Colonnes Acquity UPC² BEH 2-EP (100 mm × 3.0 mm) en 3.5 µm (carrés violets) et 1.7 µm (triangles verts) testées en conditions SFC : CO_2/MeOH (96/4, v/v), 40 °C, 150 bar, 1 µL injecté, 254 nm. Adapté de [13]

3) Utilisation de la SFC pour l'analyse et la purification de produits pharmaceutiques

La review suivante traite de l'utilisation de la SFC chirale et achirale pour l'analyse et la purification de composés pharmaceutiques :

E. Lemasson, S. Bertin, C. West

Use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification

Journal of separation science, Volume 39, (2016), 212-233

J. Sep. Sci. 2016, *39*, 212–233

Elise Lemasson[1]
Sophie Bertin[2]
Caroline West[1]

[1]Institut de Chimie Organique et Analytique (ICOA), Univ Orleans, CNRS, Orléans cedex, France
[2]Institut de Recherches Servier, Suresnes, France

Received September 21, 2015
Revised October 21, 2015
Accepted October 21, 2015

Review Article

Use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification

The interest of pharmaceutical companies for complementary high-performance chromatographic tools to assess a product's purity or enhance this purity is on the rise. The high-throughput capability and economic benefits of supercritical fluid chromatography, but also the "green" aspect of CO_2 as the principal solvent, render supercritical fluid chromatography very attractive for a wide range of pharmaceutical applications. The recent reintroduction of new robust instruments dedicated to supercritical fluid chromatography and the progress in stationary phase technology have also greatly benefited supercritical fluid chromatography. Additionally, it was shown several times that supercritical fluid chromatography could be orthogonal to reversed-phase high-performance liquid chromatography and could efficiently compete with it. Supercritical fluid chromatography is an adequate tool for small molecules of pharmaceutical interest: synthetic intermediates, active pharmaceutical ingredients, impurities, or degradation products. In this review, we first discuss about general chromatographic conditions for supercritical fluid chromatography analysis to better suit compounds of pharmaceutical interest. We also discuss about the use of achiral and chiral supercritical fluid chromatography for analytical purposes and the recent applications in these areas. The use of preparative supercritical fluid chromatography by pharmaceutical companies is also covered.

Keywords: Enantioseparation / Impurity profiling / Pharmaceutical analysis / Purification / Supercritical fluid chromatography
DOI 10.1002/jssc.201501062

1 Introduction

Chromatographers in pharmaceutical companies encounter many different tasks that may be carried out at the analytical or preparative scale. Identification and impurity profiling of molecular entities is the key task, as the identity and proportion of impurities must be strictly controlled to guarantee the efficacy and limit toxicity of the active pharmaceutical ingredient (API) [1]. For this purpose, it is necessary to have complementary high-performance analytical tools. Preparative-scale chromatography is used at the early development of drug candidates. It is essentially concerned with enhancing the purity of synthetic intermediates before further chemical reaction, and purifying possible drug candidates before bioactivity testing. In this event, the purification of enantiomers is a special case. At this stage, a fast and economic method is preferred. At both scales, analytical or preparative, HPLC remains the preferred method in many cases, but SFC is also a favorite in many companies, and is gaining ground in others.

SFC makes use of supercritical or liquid mobile phases comprising a significant portion of pressurized carbon dioxide, usually mixed with another solvent (most often an alcohol such as methanol) [2]. CO_2 has major advantages over more conventional chromatographic solvents, as it has a low viscosity allowing for high diffusivities of the analytes (hence high efficiencies) and limited pressure drop over packed columns, even with columns packed with small particles. As a result, high flow rates can be used without strongly affecting efficiency.

With the emergence of capillary SFC [3, 4] in the 1970s and 1980s, SFC was reserved for the analysis of nonpolar compounds, due to the low eluting strength of neat CO_2, which was considered equivalent to that of pentane. Most pharmaceutical compounds being rather polar and therefore poorly soluble in pure CO_2, the interest of pharmaceutical companies for capillary SFC thus remained very limited. However, with the appearance of packed-column SFC and the use of mixed mobile phases comprising a cosolvent, the range of compounds amenable to the technique significantly increased. The introduction of a cosolvent greatly increases solubility for both polar and nonpolar analytes. Nevertheless, in the 1990s SFC did not manage to settle efficiently as a chromatographic

Correspondence: Caroline West, Institut de Chimie Organique et Analytique (ICOA), Univ Orleans, CNRS, UMR 7311, B.P. 6759, rue de Chartres, F-45067 Orléans cedex 2, France
E-mail: caroline.west@univ-orleans.fr

Abbreviations: APCI, atmospheric pressure chemical ionization; **API**, active pharmaceutical ingredient; **CSP**, chiral stationary phase; **NP**, normal phase

technique of choice for the pharmaceutical industry. The only field of application where SFC was usually recognized for its improved capabilities compared to HPLC was that of enantioseparations, particularly at the preparative scale [5]. In this field, the economic and ecological advantages of CO_2 were usually a significant motivation.

A limiting factor of SFC development in pharmaceutical companies was undoubtedly the lack of UV detection sensitivity compared to HPLC, specifically for the profiling and quantification of impurities present in very low concentrations. The recent introduction of improved SFC instruments with better UV sensitivity and the democratized use of mass spectrometric detection (MS) now render this issue much less a concern than in the past.

Indeed, new robust instruments dedicated to packed column SFC were recently introduced by several manufacturers (Agilent, Shimadzu, and Waters), while other systems had long been available from other manufacturers (Jasco, Novasep, Pic Solution, Sepiatec, and Waters again). Besides, the progresses in stationary phase technology (sub-2 μm particles [6,7], superficially porous particles [8]) intended for HPLC have also greatly benefited to SFC. The high-throughput capability and economic benefits of the method employing only small portions of organic solvents, but also the "green" aspect of the principal mobile phase component (CO_2 is a nontoxic recycled material and causes no waste disposal issues) together render SFC very attractive for a wide range of applications, whenever a replacement or complement to HPLC is desired [7–13]. As will be further developed below, it was shown in numerous occasions that SFC or SFC–MS is an adequate tool for small molecules of pharmaceutical interest: synthetic intermediates, drug candidates, active pharmaceutical ingredients, impurities, or degradation products.

In this review, we first discuss about general chromatographic conditions for SFC analysis to better suit compounds of pharmaceutical interest. We also discuss about the use of achiral and chiral SFC for analytical purposes and the recent applications in these areas. The use of preparative SFC in pharmaceutical companies is also covered. It was not our intention to present a comprehensive review of the literature, that may be found in other interesting reviews [14–16], but rather to point at significant features of SFC as they apply in the field of drug analysis.

2 Chromatographic conditions for SFC analysis of drug molecules

Two key factors must be considered for method development in SFC: first, a stationary phase to ensure good resolution and second, a mobile phase and operating conditions (temperature, pressure, and flow rate) to ensure good solubility of the analytes and modulate resolution.

2.1 Choice of stationary phases

2.1.1 Stationary phases for achiral SFC applications

Because SFC can be practiced with both polar and nonpolar stationary phases, all columns that are marketed for HPLC whether for RP, normal-phase (NP), HILIC or ion-exchange modes [17–23] can be used in SFC. The chemical diversity of the available stationary phases is currently significantly improving, benefiting from the diversity of phases produced for HPLC (particularly the recent development of many HILIC phases with original bonding chemistries) but also of the rising interest of the column manufacturers and research groups to produce original phases dedicated to SFC use [24–26]. Moreover, while different operating modes in HPLC require different mobile phase compositions (for instance, hydro/organic in RP, alkane/alcohol in NP), in SFC the same CO_2/cosolvent mobile phase may be used with all sorts of stationary phases. As a result, two stationary phases with different surface chemistry may be employed with SFC operating conditions and provide orthogonal selectivity [6, 7, 9–12, 17–21, 24–28] as will be further discussed below.

In the recent years, SFC chromatographers in the pharmaceutical industry have focused their attention essentially on polar stationary phases. Indeed, because pharmaceutical compounds usually comprise polar groups, some polar characteristic must be present in the stationary phase, either from silanol groups of a silica support, or in the bonded ligands, to ensure sufficient retentiveness [18]. Apart from purely lipophilic species like steroids, nonpolar phases (like octadecylsiloxane-bonded silica phases with hydrophobic end-capping groups) were rarely found to be useful. Among the phases that are familiar to the HPLC chromatographers, most often observed are bare silica [29–32], cyanopropyl [13,33–36], diol [27,37–40], aminopropyl [41,42], all sorts of HILIC columns [20, 40, 43], fluorophenyl phases, and non-endcapped C_{18}-bonded silica stationary phases [44]. However, more and more stationary phases are available, which were designed specifically for SFC use. The most famous stationary phase dedicated to SFC is 2-ethylpyridine- bonded (2EP) silica. The 2EP phase is known to offer good peak shapes for basic compounds, even without any additive in the mobile phase [45]. It was first introduced by Prince- ton Chromatography, more than 10 years ago, but its success prompted several manufacturers (Waters, ES Industries, Kromasil, Nacalai Tesque) to develop similar phases [13, 45–47]. Early on, Princeton Chromatography also had a range of other columns with original stationary phases designed for SFC, such as 4-ethylpyridine, diethylaminopropyl, dinitrophenyl, or benzenesulfonamide [27, 40]. Some academic and industry teams have also developed new stationary phase chemistries for SFC use with pharmaceutical compounds, based on original neutral or ionic liquid ligands [24–26, 48, 49]. Whether they will yield any commercial product is

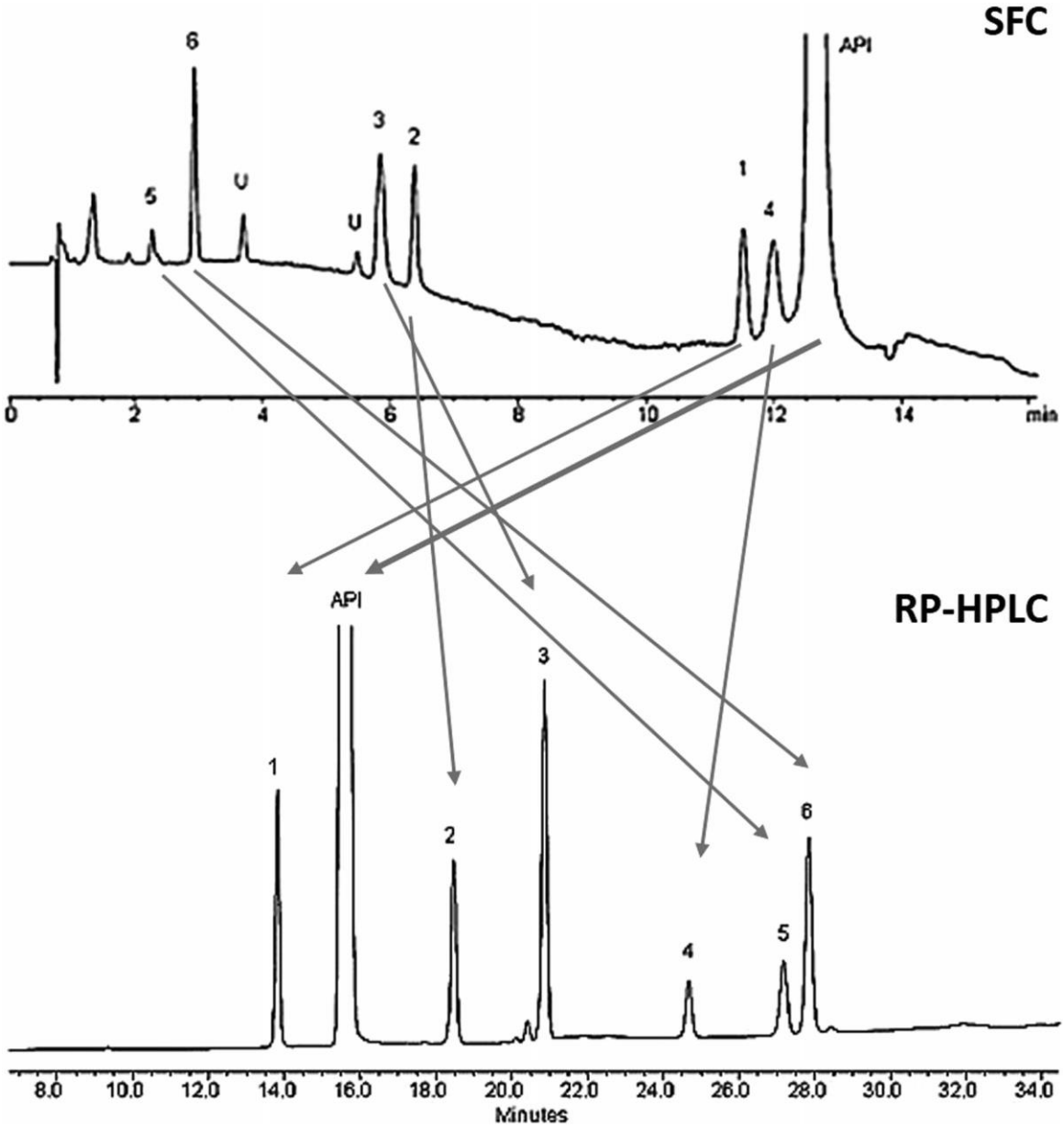


Figure 1. The orthogonal selectivity of SFC method versus RP-HPLC method. Separation of API plus six impurities at the 0.5% level. SFC conditions: DEAP column (4.6 × 150 mm × 5 µm). CO_2/MeOH with 0.1% water v/v, gradient elution 10–60%, 2 mL/min, 35°C, 150 bar BPR, UV detection 220 nm. RPLC conditions: Luna PFP (4.6 × 100 mm × 5 µm), water/acetonitrile with 0.05% TFA, gradient elution 10–50%, 1 mL/min, 30°C, UV detection 240 nm. Adapted from [43] with permission from Elsevier.

however unsure yet. Several manufacturers now propose "SFC stationary phases" that are simply NP-HPLC or HILIC stationary phases (silica, amino-propyl, propanediol, cyanopropyl etc.), although the packing procedure and hardware were sometimes adapted to take account of the specificities of SFC. Other manufacturers have followed in the footsteps of Princeton Chromatography to design stationary phases specifically for SFC use, like ES Industries with the GreenSep columns (for instance, 4-ethylpyridine, pyridyl amide, amino phenyl) and, more recently. Waters with the ACQUITY UPC^2 and Torus columns (e.g. 1-aminoanthracene and 2-picolylamine stationary phases). Waters also prompted the current trend of ultra-high performance columns similar to those employed in UHPLC (packed with sub-2 µm particles) to allow for higher efficiency, but with original stationary phases developed for SFC. Stationary phases based on sub-3 µm superficially porous particles also permit to reach high efficiency [7, 50]. Given the current upper pressure limit set by the available pumping systems (400–600 bars), columns packed with superficially porous particles may have the advantage of generating smaller pressure drops, especially at the end of a gradient program when the

fluid density is the largest, and whenever faster flow rates are desired.

Given the large number of possibilities, choosing a column to develop a method in achiral SFC is rather difficult. Current practice is to select a set of columns and to screen them with generic conditions (most often, a gradient elution program with fixed pressure and temperature conditions). Because gradient analyses may be very short [51] (even below 1 min on short columns [52]) and large diffusion coefficients together with large flow rate allow for very fast column equilibration, the process of screening for columns may be extremely fast. It is even faster with the apparatus equipped for parallel screening of columns that have been employed for several years in HPLC and SFC chiral screening strategies [53–56]. When the columns are adequately selected to provide orthogonal selectivity properties, this screening procedure generally allows identifying a good starting point for further optimization within a few minutes.

It was shown several times that the selectivity of a given column employed in HPLC or in SFC may be very different [57, 58]. To assist the users of achiral SFC in the selection of orthogonal columns, the classification of columns dedicated to SFC, developed by West and Lesellier [58–61], can be helpful. The physicochemical characteristics of stationary phases were studied with quantitative structure-retention relationships, to characterize more than 80 stationary phases [62, 63]. The properties of the phases were compared to understand their differences in terms of retention behaviors and selectivity properties [17–20, 64]. The classification is presented in a simple fashion, with a figure called a spider diagram. It can be of help to chromatographers, especially SFC beginners, willing to select stationary phases with diversity of selectivities in the initial screening procedure.

The possible need to transfer a method to preparative scale must also be considered when selecting a column. Naturally, those stationary phases that are available in larger dimensions and identical bonding chemistry should be privileged in that case.

2.1.2 Stationary phases for chiral SFC applications

The success of chiral chromatography is related to chiral stationary phases (CSP) innovation. Identically to the situation observed in chiral HPLC, the most commonly used CSPs in SFC are the ones based on polysaccharides [65], now available from several column manufacturers. These phases are based on amylose or cellulose derivatives that are coated [66] or immobilized [67] on a silica gel support. They ensure the resolution of a wide variety of enantiomers through complementarity and diversity of derivatives. The most commonly cited stationary phase is (*tris*-(3,5-dimethylphenylcarbamate) of amylose (Chiralpak AD from Daicel, in its first commercial version, but now copied by several other manufacturers). This CSP usually has the highest success rate of all columns with a variety of compounds. It is most often associated to three other polysaccharide CSPs (Chiralpak AS, Chiralcel OD, and Chiracel OJ from Daicel, or similar phases from other manufacturers) and were long known as "the Golden Four." Together, they reach a statistical success rate of more than 80% of the chiral drugs currently on the market. In the recent years, chlorinated polysaccharide CSPs were released by Phenomenex (for instance Lux Cellulose-2, Lux Cellulose-4, and Lux Amylose-2) and are now also available from a range of manufacturers. They have proven useful in providing complementary selectivity to the traditional non-halogenated polysaccharide CSPs [68]. Another recent development in polysaccharide CSPs is the availability of immobilized phases, while the original phases were all coated. With coated phases, solvents in which polysaccharides are highly soluble (like dichloromethane or dimethylsulfoxide) must be avoided otherwise the polysaccharide may be stripped from the silica surface and the column permanently damaged. In case of immobilized polysaccharide, any solvent may be used. This has two interesting consequences: (i) other solvents may allow for improved resolution through diversified enantioselectivity or improved efficiency; (ii) other solvents may improve analyte solubility, which is critical to high productivity at the preparative scale.

Aside the high success rate, one significant reason to explain the popularity of polysaccharide CSPs in SFC is their high loading capacity [69]. Indeed, when transferring a method to preparative scale, it is useful to have columns that are capable to adsorb large quantities of sample, to improve productivity. As preparative SFC really has been a driving force during the 1990s and the beginning of the 21st century, it is no surprise that the polysaccharide CSPs have emerged as the preferred separation media in most cases, while other stationary phases allowing for inferior loading capacity became less popular.

However, other stationary phases may still be employed for analytical chiral SFC. Brush-type stationary phases are some of the most ancient and most recent phases developed for enantioselective chromatography. Because they are based on a well-defined ligand with a chiral center of known R/S configuration, they are usually available in both configurations. This is advantageous for enantioresolution of chiral drugs, as it allows the reversal of the elution order of the enantiomers in changing the configuration of the stationary phase. Indeed, when measuring enantiopurity, it is preferable to have the minor enantiomer eluted first, to avoid drowning the minor peak in the tail of the major peak. On the contrary, when enantiopure compounds must be produced by preparative chromatography, it is best to have the preferred enantiomer eluted first, as the first analyte usually has the best final purity. The oldest brush-type CSPs were the phases initially developed by Pirkle [70], with the most famous one still available as WhelkO-1 from Regis Technologies. More recently, the zwitterionic phases developed by the group of Lindner and now trademarked as Chiralpak ZWIX by Daicel have proven to be promising supports for amino acid enantioseparation [71, 72]. We may also note that the fact that brush-type CSPs are bonded to the silica support make them capable to support cosolvents with high eluting strength, similarly to immobilized polysaccharides.

Table 1. Achiral pharmaceuticals applications

Families of compounds	Analytes	Stationary phase	Conditions	Detection mode	References
Pharmaceuticals	Seven component cocktail (a basic mono-chlorinated tricyclic heterocyclic API and six related mono-chlorinated components)	GS-diethyl amino propyl (150 mm × 4.6 mm, 5 μm)	MeOH containing 0.1 % water v/v, gradient: 10% –1 min hold, linear to 60% –14 min, 35°C, 150 bar, 2 mL/min	UV (220 nm)	[49]
	Nine component cocktail (aripiprazole plus eight related components)	GS amino Phenyl (150 mm × 4.6 mm, 5 μm)	MeOH containing 0.3% IPA v/v and 5% ACN v/v, gradient: 5 % –4 min hold, linear to 20% –20 min, 12°C, 160 bar, 3 mL/min	UV (254 nm)	
Antiretroviral drugs	Three antiretroviral drugs and their impurities	Princeton 2-ethylpyridine (150 mm × 4.6 mm, 3 μm)	MeOH containing 10 mM AA and 0.1% IPA, gradient: 0% –1 min hold, linear to 12% –12 min, 25°C, 160 bar, 2.5 mL/min	UV (260 nm)	[13]
β-Blockers	Acetamidophenol, metoprolol, oxprenolol, propranolol, nadolol, acebutolol, atenolol, pindolol	Lichrosphere cyanopropyl (125 mm × 4.0 mm, 5 μm)	MeOH containing 0.4% IBA, gradient: 5% no hold, 45%/min to 65% modifier, final hold of 0.25 min, 35°C, 100 bar, 5 mL/min	UV (260 nm)	[77]
Antipsychotics	Triflupromazine, carphenazine, methotrimeprazine, promazine, molindone, perphenazine, chlorprothixine, deserpidine, thiothixene, reserpine	Lichrosphere cyanopropyl (250 mm × 4.6 mm, 5 μm)	MeOH containing 0.5% IPA: 6%, 50°C, 200 bar, 3 mL/min	UV (254 nm)	[41]
Antidepressants	Amitriptyline, desipramine, imipramine, nortriptyline, protriptyline, buclizine, benactyzine, hydroxyzine, perphenazine, thioridazine	Lichrosphere cyanopropyl (250 mm × 4.6 mm, 5 μm)	MeOH containing 0.5% IPA: 10%, 50°C, 200 bar, 3 mL/min	UV (220 nm)	[116]
Stimulants	Amphetamine, methamphetamine, phenmetrazine, ephedrine, phenylephrine, hydroxyamphetamine, nylidrine, phenylpropanolamine, naphazoline	Lichrosphere cyanopropyl (250 mm × 4.6 mm, 5 μm)	MeOH containing 0.5% IPA: gradient 5%,linear to 20% (1.5%/min), 40°C, 200 bar, 2 mL/min	UV (220 nm)	[118]

(*Continued*)

Table 1. Continued

Families of compounds	Analytes	Stationary phase	Conditions	Detection mode	References
Bronchodilator (β-blocker)	Salbutamol and six related impurities	Lichrosphere diol (250 mm × 4.6 mm, 5 μm)	MeOH containing 0.5% n-PA: gradient 30% hold 9.5 min, linear to 45% (1.5%/min), 70°C, 300 bar, 1.5 mL/min	UV	[44]
Steroids	Cholestane, 5β-pregnane-3,20-dione, progesterone, 5β-pregnane-3α-ol,20-dione, pregnenolone, testosterone	Spherisorb silica (250 mm × 4.6 mm, 3 μm)	MeOH: 10%, 70°C, 210 bar, 4 mL/min	UV and ELSD	[36]
Antibiotics	Diloxanide furoate and metronidazole in solid dosage	JASCO-C-18 (250 mm × 4.0 mm, 10 μm)	MeOH: 26%, 40°C, 176 bar, 0.7 mL/min	UV (230 nm)	[112]
Anticonvulsants	Phenobarbitone, phenytoin sodium, phethenylate sodium, nitrazepam, clonazepam, carbamazepine, primidone	JASCO, RP-C18 (250 mm × 4.0 mm, 10 μm)	MeOH: 14.29%, 50°C, 98.1 bar, 3 mL/min	UV (215 nm)	[103]
Seventeen pharmaceutical compounds: steroids, xanthines, and nucleotides, nonsteroidal anti-inflammatory drugs (NSAIDs), sulfonamides	Cortisone, estradiol, progesterone, testosterone,17-methyltestosterone, caffeine, theophylline, thymine, uracil, fenoprofen, ketoprofen, naproxen, sulfadimethoxine, sulfamerazine sulfamethizole, sulfamethoxazole, sulfaquinoxaline	Zorbax SB-CN (250 mm × 4.6 mm, 5 μm) :1 or 5 coupled columns	ACN:MeOH 1:3 containing 0.5% TFA and DIPA, gradient: 5 % hold 0.2 min to 40% at 10%/min or 5% hold 1 min to 40% at 2%/min, 40°C, 100 bar, 2 mL/min	UV (254 nm)	[57]
Twenty-six pharmaceutical compounds: steroids, xanthines, purines, and pyrimidines, anti-inflammatory drugs (NSAIDs), sulfonamides	Cortisone, estradiol, estriol, estrone, hydrocortisone, progesterone, testosterone,17-Methyl-testosterone, adenine, caffeine, cytosine hypoxanthine, theobromine, theophylline thymine, uracil, flurbiprofen, ibuprofen, ketoprofen, naproxen, sulfadimethoxine, sulfaguanidine, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfaquinoxaline	Princeton 2-ethylpyridine (250 mm × 4.6 mm, 3 μm)	ACN: MeOH 35%: 65% containing FA and IPA (0.25%), gradient: 5% hold 1 min to 40% at 2%/min, 40°C, 100 bar, 2 mL/min	UV (254 nm)	[58]

(*Continued*)

Table 1. Continued

Families of compounds	Analytes	Stationary phase	Conditions	Detection mode	References
Nine pharmaceutical compounds: steroids, anti-inflammatory drugs (NSAIDs), sulfonamides	Theophylline, testosterone, cortisone, naproxen, sulfadimidine, sulfamerazine, sulfamethoxazole, sulfaquinoxaline, sulfamethizole	Princeton 2-ethylpyridine (250 mm × 4.6 mm, 3 μm)	MeOH, gradient: 10% for 1 min, increasing to 35% at 2%/min, 40°C, 100 bar, 2 mL/min	UV (254 nm), CAD	[102]
Sulfonamides	Sulfamethoxazole, sulfadimethoxine, sulfamethazine, sulfamerazine, sulfadiazine, sulfamethizole	Cyanopropyl column (250 mm × 4.6 mm, 5 μm)	MeOH, gradient: 10% for 5 min, increasing to 15% over the next 10 min (15 min total run time), 50°C, 180 bar, 2 mL/min	UV (230 nm), MS (APCI)	[97]
Purine nucleoside antimetabolite	Clofarabine, its imputrities and degradation products	ACQUITY BEH 2EP (100 mm × 3 mm, 1.7 μm)	MeOH: 30%, 50 °C, 152 bar, 0.7 mL/min	UV (254 nm)	[52]
Benzodiazepines	Prazepam, flunitrazepam, clorazepate, desmethylflunitrazepam, nitrazepam, clonazepam, midazolam, brotizolam, 7-minoflunitrazepam, alprazolam, triazolam	ACQUITY BEH (100 mm × 3 mm, 1.7 μm)	MeOH, gradient 2–17% at 2.5 mL/min in 5 min, 40 °C, 150 bar, 2 mL/min	UV (220 nm)	[7]
Steroids	17-Methyltestosterone, 4-androsen-3,17-dione, testosterone, boldenone, stanozolol	ACQUITY BEH (100 mm × 3 mm, 1.7 μm)	MeOH, gradient 10–12% at 3.25 mL/min in 1 min, 40 °C, 145 bar, 3.25 mL/min	UV (220 nm)	
Twenty-four pharmaceutical compounds	Androstendione, lovastatine, prazepam, flunitrazepam, caffeine, metyltestosterone, clorazepate, testosterone, nandrolone, methandienone, clonazepam, boldenone, theobromine, midazolam, sulfamethoxazole, indapamide, paracetamol, bendroflumethiazide, clopamide, betamethazone, brotizolam, alprazolam, triazolam, chlortalidone	(A) 4 ACQUITY BEH (100 mm × 3 mm, 3.5 μm) or (B) 2 ACQUITY BEH (100 mm × 3 mm, 1.7 μm) and 2 ACQUITY BEH 2EP (75 mm × 3 mm, 1.7 μm) coupled in series	MeOH: 10%, (A) 40°C, 150 bar, 2.7 mL/min; (B) 40°C, 150 bar, 0.95 mL/min	UV (220 nm)	[106]

(*Continued*)

Table 1. Continued

Families of compounds	Analytes	Stationary phase	Conditions	Detection mode	References
Varied basic drugs	Three mixtures: (1) prazepam, flunitrazepam, clorazepate, clonazepam, alprazolam, triazolam; (2) ketamine, cetirizine, noscapine, sulconazole, papaverine, quetiapine; (3) pethidine, buprenorphine, dextromethorphan, codeine, pholcodine, morphine	ACQUITY BEH (100 mm × 3 mm, 1.7 μm)	MeOH containing 20 mM NH_4OH, gradient: (1) and (2) 2 to 27% over 3 min (2.5 mL/min); (3) 15–40% over 6 min (1.25 mL/min), 40°C, 150 bar	UV (220 nm)	[53]
β-Blockers	Metoprolol and related amino-alcohols	Porous graphitic carbon, Hypercarb (100 mm × 4.6 mm, 5 μm)	MeOH containing amine additives, isocratic: 20–25%, 30–60°C, 150 bar, 2 mL/min	UV (273 nm)	[114]
Retinoids	Six retinoid (vitamin A) compounds	Pursuit XRs Diphenyl (250 mm × 2 mm, 3 μm)	EtOH containing 0.1% FA, gradient 3.5–20%, 55°C, 130 bar, 1.25 mL/min	UV (340 nm)	[71]
Imidazole derivatives	1-Vinylimidazole, 1-methylimidazole, 1,2-dimethylimidazole, 1-acetylimidazole, 4-methylimidazole, 2-ethylimidazole, 2-methylimidazole, 2-ethyl 4-methylimidazole.	Aminopropyl-bonded silica	MeOH containing methylamine (0.054%)-H_2O (0.14%): 10% (molar percentages), 36°C, 190 bar, 3 mL/min	UV (215 nm)	[121]

(*Continued*)

Table 1. Continued

Families of compounds	Analytes	Stationary phase	Conditions	Detection mode	References
Anti-psychotic dosage forms	Formulations containing a combination of: (1) haloperidol-trihexyphenidyl; (2) haloperidoltrifluoperazine; and (3) trifluoperazinetrihexyphenidyl	JASCO ODS (250 mm × 4.6 mm, 5 μm)	MeOH containing 0.8% IPA: 16.67%, 60°C, 294 bar, 3 mL/min	UV (210 nm)	[117]
Glucocorticoid anti-inflammatory agent	Mometasone furoate and eight impurities and degradation products	Kromasil Si (250 mm × 4.6 mm, 5 μm)	MeOH, gradient 5–15% in 15 min, 30°C, 100 bar, 4 mL/min	UV (245 nm)	[54]

Abbreviations:
MeOH Methanol
ACN Acetonitrile
AA Ammonium acetate
IPA Isopropylamine
IBA Isobutylamine
n-PA n-Propylamine
DIPA Diisopropylamine
TFA Trifluoroacetic acid
FA Formic acid
DEA Diethylamine
CAD Corona charged aerosol detector
ELSD Evaporative light-scattering detection

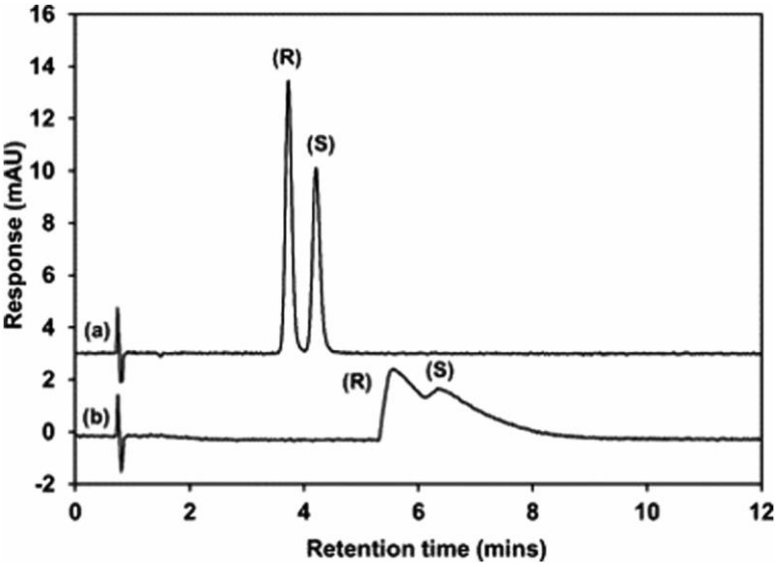


Figure 2. Separation of R–and S-timolol using a mobile phase with (A) and without (B) the presence of 0.1% v/v TEA. Column: Chiralcel OD-H (4.6 mm × 250 mm, 5 µm). Mobile phase: (93:7) CO_2 /MeOH with 0.1% TEA for chromatogram (A) and (93:7) CO_2 /MeOH for chromatogram (B). A 4.0 mL/min, 40°C, 130 bar, UV detection 297 nm. Reproduced from [163] with permission from Elsevier.

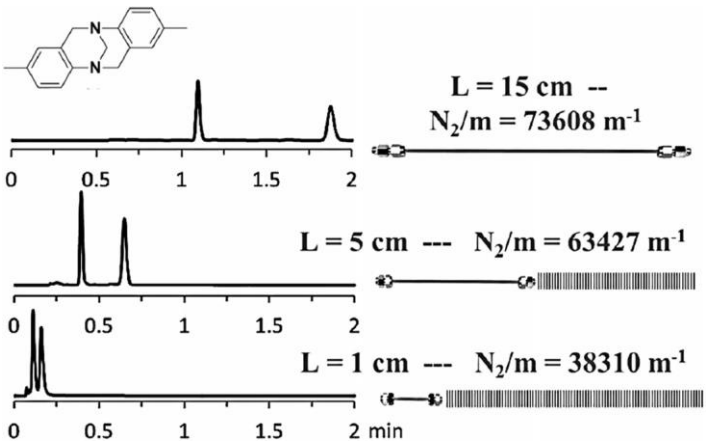


Figure 3. Example of ultra-fast enantioseparation of Tröger's base using very short columns. Chiralpak AD-H columns with different lengths (15, 5, and 1 cm). Mobile phase: 25 mM IBA in MeOH-CO_2, isocratic 40%. 40°C, 200 bar, UV detection 210 nm. Flow rate 3 mL/min or 2.3 mL/min so as to keep the same linear velocity on all columns. Adapted from [52] with permission from Wiley.

Macrocyclic glycopeptides have also been cited in some occasions [73], especially for the enantioseparation of amino acids. Synthetic polymers [74], cyclodextrins [75], and cyclofructans [76] may also be of use but are rarely mentioned.

Even more than in achiral SFC, prevision of the right stationary phase before chiral analysis is hazardous. The selection of a column is thus achieved through a systematic screening process, in a similar manner to the usual practice in chiral HPLC. Nowadays, the most common screening strategies are based on the use of a group of CSPs comprising several polysaccharide CPSs, while some of the chlorinated polysaccharides more recently introduced now tend to enter the first round of screening. De Klerck et al. [77] proposed a short selection of stationary phases to achieve a high success rate in a minimum of experiments, without the need for a complete screening of the selected CSP and mobile phases. Beside polysaccharide phases, the screening strategy may include a brush-type phase (the most frequent cited being WhelkO-1), one or more macrocyclic glycopeptides (Chirobiotic phases from Sigma–Aldrich), one or two polymer-type (like Kromasil CHI-TBB or DMB, or Supelcosil P-CAP) or a cyclodextrin-type CSP.

Again the possible need to transfer the method to preparative scale (very frequent in chiral SFC) must be taken into account to favor the columns that are available in larger dimensions. The loading capacity of the stationary phase and the possibility to use "exotic" solvents to improve solubility are also key points to consider to achieve high productivity of the purification process.

Tandem column (coupling two different stationary phases with complementary selectivities) is sometimes observed to be an interesting option, particularly when more than one chiral center is present [78]. The chances of success improve when some identification of the enantiomers is possible (through optical rotation detection) to ensure that the second column will not undo the work of the first column.

Finally, a word may be said about particle technology. The trend toward smaller particles of silica support is also appearing now for chiral stationary phases, but it is not yet as prominent as in the achiral field. Unlike bonded-silica

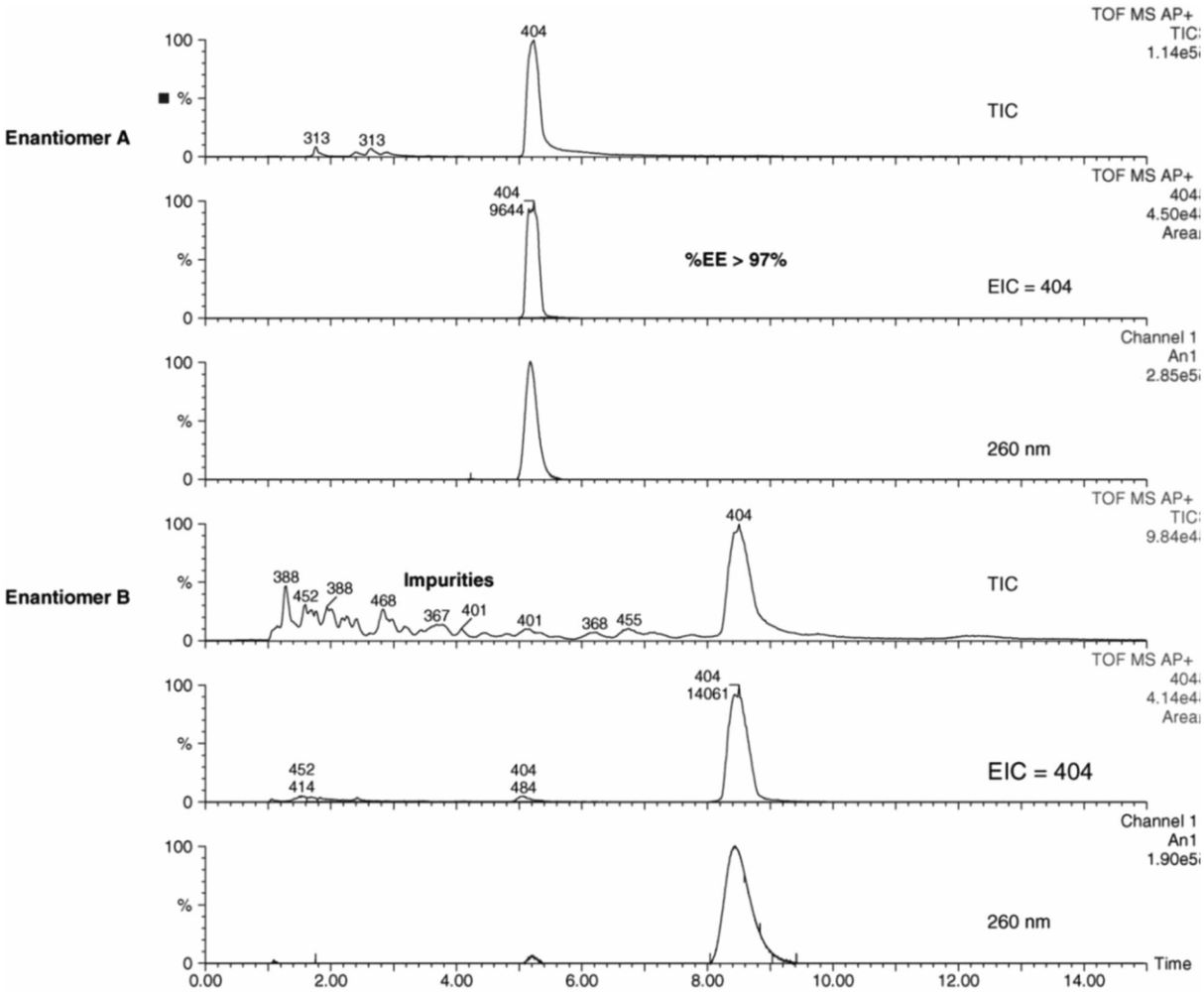


Figure 4. SFC-MS analysis of two SFC-purified enantiomers (404 *m/z*). Enantiomer A was very pure but enantiomer B was shown to have impurities that were detected only by MS. Adapted from [134] with permission from Elsevier.

stationary phases, the difficulty to produce polysaccharide phases with small particles is a key point, explaining why the first sub-2 μm particle CSP described was not a polymer but a brush-type phase [79]. Commercially available polysaccharide CSP currently remain limited to 2.5 μm (Waters Trefoil stationary phases) or 3 μm (Daicel, Phenomenex, Regis Technologies, Dr. Maisch, YMC, and Kromasil). However, here again the benefits of improved efficiency to achieve improved enantioresolution [79,80] will certainly prompt further developments in this field in the near future.

2.2 Mobile phase composition

2.2.1 Cosolvent

Because neat CO_2 is not polar enough for most pharmaceutical compounds, virtually all analyses are now performed with a mixture of pressurized CO_2 and a cosolvent (often called modifier). Cosolvent addition is necessary to improve analyte solubility but has other side-effects, namely increasing mobile phase density and modulating selectivity. CO_2 is miscible with all organic solvents that are commonly employed in LC (except water, which has a very low solubility), which is an advantage for the possible modulation of selectivity through mobile phase composition. To analyze complex pharmaceutical mixtures, gradient elution program is usually preferred, typically from 5 to 40% of cosolvent. The addition of a cosolvent changes the critical values of the mobile phase (pressure and temperature) [2]. Consequently, the analyses are usually performed in subcritical (thus liquid), not supercritical conditions. This should be of no concern, as all the advantageous properties of supercritical CO_2 remain available. In a general way, an increase of cosolvent percentage in the mobile phase, and therefore of the elution strength, causes a decrease of retention. The cosolvent can also improve peak shape by reducing the undesirable interactions between analytes and residual silanol groups [81]. The silanol groups

Table 2. Chiral pharmaceuticals applications

Drug families	Analytes	Stationary phase	Operating conditions	Detection mode	Reference
β-Blockers	Standards of timolol and impurities	Chiralcel OD-H (250 mm × 4.6 mm, 5 μm)	MeOH containing 0.1% TEA, gradient: 0–50% modifier, 40°C, 130 bar, 4 mL/min	UV (297 nm)	[163]
Stimulants	Ephedrine enantiomers	Chiralpak AS (250 mm × 4.6 mm, 10 μm)	EtOH containing 0.5% IPA: 15%, 30°C, 100 bar, 1.5 mL/min	UV (220 nm)	[83]
Propionic acid	2-(6-ethoxy-2-naphthyl) propionic acid	(S,S)-Whelk-O-1 (3.0 mm, 5 μm different column lengths: 150, 50, or 20 mm)	MeOH containing 25 mM IBA: 40 %, 40°C, 200 bar, 3 mL/min	UV (210 nm)	[52]
Sulfonamides	Racemic chlorthalidone	Chiralpak AD (250 mm × 4.6 mm, 5 μm)	MeOH: 50%, 40°C, 120 bar, 4 mL/min	UV (254 nm)	[172]
Benzodiazepines and derivatives	Camazepam and derivatives	Chiralcel OD-H (250 mm × 4.6 mm, 5 μm)	EtOH, gradient: 10–18% modifier, 30°C, 200 bar, 2 mL/min	UV	[139]
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Fenoprofen	Chiralpak AD (250 mm × 4.6 mm, 10 μm)	IPOH containing 0.1% IPA: 20%, 30°C, 200 bar, 3 mL/min	UV (220 nm)	[83]
Barbiturates	Butabarbital, pentobarbital, secobarbital, hexobarbital, mephobarbital	Chiralcel OJ (250 mm × 4.6 mm, 10 μm)	MEOH containing 0.1% TFA and TEA (each at 0.5% v/v), gradient 5–30% at 5%/min, 30°C, 200 bar, 2 mL/min	UV (220 nm)	[166]
Chiral sulfoxides	Racemic omeprazole, lansoprazole, pantoprazole, rabeprazole, ricobendazole, oxfendazol	Chiralpak AD (250 mm × 4.6 mm, 10 μm)	MeOH: 30%, 35°C, 200 bar, 2 mL/min	UV (280 and 225 nm)	[173]

Table 2. Continued

Drug families	Analytes	Stationary phase	Operating conditions	Detection mode	Reference
Antihistamines	1-(chlorobenzhydryl)-piperazine	Chiralpak AD (250 mm × 4.6 mm, 10 μm)	IPOH: 40%, 40°C, 100 bar, 2.5 mL/min	UV	[176]
Anticoagulants	Warfarin and hydroxylated isomers	Chiralcel OD-H (150 mm × 4.6 mm, 3 μm)	ACN containing 25 mM IBA, gradient: 12% for 4 min, then ramp at 40% in 6 min, 40°C, 200 bar, 3 mL/min	UV (210 nm)	[178]
Native amino acids	Alanine (a), Serine (b), Asparagine (c), Glutamine (d), Methionine (e), Tryptophan (f), Phenylalanine (g), Threonine (h), Lysine (i), Arginine (j), Norvaline (k)	Chirobiotic T (250 mm × 4.6 mm, 10 μm)	(a) & (k): MeOH containing 0.1% TEA, TFA, 2% H_2O, 0.3% glycérol: 48%. (b): MeOH containing 0.1% TEA, TFA, 3.5% H_2O: 46.5% MeOH. (c), (d), (j): MeOH containing 0.15% TEA, TFA, 2.4% H_2O: 57.6% (e), (f), (g), (h), (k): MeOH containing 0.1% TEA, TFA, 2% H_2O, 0.3% glycerol: 48% (i): MeOH containing 0.15% TEA, TFA, 2.8% H_2O: 67.2%. 31°C, 4 mL/min, 100 bar	UV (254, 220, and 214 nm)	[73]

Abbreviations:
MeOH Methanol
ACN Acetonitrile
H_2O Water
IPA Isopropylamine
IBA Isobutylamine
TEA Triethylamine
TFA Trifluoroacetic acid
DEA Diethylamine

have H-bond acceptor character and to avoid these interactions, modifiers with H-bond donor character like alcohols (methanol, ethanol, isopropanol) are preferred. Among these alcohols, methanol is most often employed because of its high polarity that permits a good solubility of the analytes in the mobile phase. Its low viscosity and boiling point are also important for preparative analysis or MS detection. Ethanol and isopropanol, less polar solvents, are also used but often yield increased retention compared to methanol, especially for the most polar compounds [82].

Concerning chiral separations, the choice of alcohol cosolvent has other consequences. Indeed, enantiomers could coelute on one column with one solvent, while changing the cosolvent may provide baseline resolution [83]. However, enantioselectivity varies in an unpredictable way between two different separations. For this reason, several mobile phase compositions comprising different cosolvents or mixtures of cosolvents are usually employed in systematic screening strategies.

Contrary to the general observations in reversed-phase HPLC, the use of acetonitrile as a sole cosolvent often results in poor efficiency and deteriorated peak shapes because of the nonprotic character of this solvent, resulting in a very low recovery rate of silanol groups. Compared to methanol, an increase of retention is also usually observed. It may however be of use for small molecules that may react with alcohol modifiers or are subject to hydrolysis (like boronic ester compounds). Also, in combination with methanol, acetonitrile permits to further modulate selectivity. Brunelli et al. have demonstrated the possibility to take advantage of this change in selectivity without losing chromatographic performance by mixing varying proportions of acetonitrile and methanol [84].

Because all other solvents that are common to the HPLC chromatographers can also be mixed with pressurized CO_2, it is also possible to use other solvents, like dichloromethane, dimethylsulfoxide, or tetrahydrofuran [85]. This is especially useful in the case of preparative separations, when one needs to increase solubility to further increase productivity. Such strong solvents are usually not used alone, but rather in combination with methanol (typically 10–20% strong solvent in methanol).

Some comment should be added here about the supposed "greenness" of SFC mobile phases, compared to HPLC mobile phases. Green chemistry is indeed a growing concern in pharmaceutical companies. Comparing analytical reversed-phase HPLC to analytical SFC, the advantage of the SFC mobile phase composition is not tremendous, especially when one considers the small volumes of solvent consumption with modern HPLC systems (especially ultra-high performance systems). The green advantage of SFC is however more significant in the field of NP-HPLC and chiral separations, which are most often conducted in the normal-phase mode in HPLC, with ecologically unfriendly solvents. When transferring the method to the preparative scale, litters of hexane, and dichloromethane are exchanged for smaller volumes of methanol. At the preparative scale, the advantage of SFC is still significant over reversed-phase preparative HPLC. Moving for even greener alternatives, as most pharmaceutical companies are now required to do, should depend on selecting other cosolvents. First, ethanol may be preferred over methanol when a "green" solvent is favored, as it is significantly less toxic than methanol and produced from renewable resources. When mixed with pressurized CO_2, none of the usual problems observed in HPLC and associated to the high viscosity of ethanol are encountered. However, the boiling point of ethanol is significantly higher than that of methanol, increasing the energy cost to evaporate it from purified fractions, thus the advantage at the preparative scale may not be so true. Alternative "green" solvents have been tested such as 2-methyl-tetrahydrofuran or cyclopentylmethyl ether [85].

The acidity of the CO_2-based mobile phase must also be mentioned. Because CO_2 and alcohols react to form alkylcarbonic acid [86, 87], SFC mobile phases are somewhat acidic [88]. This is a significant point as acidic species may then be deprotonated, while basic species most often encountered in active pharmaceutical ingredients may be protonated.

2.2.2 Additives

Nowadays, additives (acids, bases, or salts) are commonly used as a third component in the mobile phase. Additives usually favor the elution of polar analytes with good peak shapes, especially for ionizable species like most active pharmaceutical ingredients. Additives are introduced at lower concentrations than the cosolvent, typically 0.1–1% in the cosolvent, resulting in an overall concentration of 0.005–0.5% depending on cosolvent percentage.

First, it should be said that a large portion of analytes (typically about 50% [15]) can be eluted satisfactorily from many good columns without the need for an additive. It is also important to note that compounds which exhibit satisfactory elution in the absence of an additive are most often unaffected by the presence of an additive [30,89]. An additive is desirable when peak shape is not satisfactory (fronting, tailing, and distorted peaks), which may result either from poor solubility of the analyte in the CO_2/cosolvent mobile phase, or from strong interactions with the stationary phase.

The contributions of additives to the elution and separation mechanisms are still rather unclear. Because basic pharmaceutical compounds can be protonated in the acidic CO_2/alcohol mobile phase [2, 88], unwanted interactions between deprotonated silanols and protonated basic compounds may take place. The additive adsorbs on polar sites of the stationary phase thus may cancel these unwanted interactions. Moreover, additives can also form ion pairs with ionic analytes of opposite charge. Ion-pairing mechanism helps the elution of the analytes by improving their solubility [30, 90–94]. An additive with strong acid or base character may also work as an ion suppressor toward a weaker analyte [95, 96] (strong acid toward less acidic analyte or strong base toward less basic analyte). In chiral SFC, the role of additives is even stronger than in achiral SFC, as it may also significantly influence enantioselectivity.

Basic additives are often used for pharmaceutical applications because a large majority of drugs have basic functions. Isopropylamine is most commonly cited [96], but also diethylamine and trimethylamine [97]. Acidic additives, less cited than bases, prove efficient for the analysis of acidic compounds (e.g. TFA [82], acetic acid, formic acid [98], ethanesulfonic acid [99], or citric acid [93, 100]). While some works reported the use of both acid and base [84, 101–103], the current trend is rather to use volatile salts. Such salts permit the elution of both acidic and basic compounds and ensure MS and preparative-scale compatibility. Ammonium formate [38, 104, 105] and ammonium acetate [13, 30, 89, 106] are frequently cited. However, their high UV absorption may be a problem in the case of gradient elution when low level impurities must be identified. Alternatively, ammonium hydroxide and ammonia have been recommended in many recent reports [89,107,108], as they have an additional benefit of limited UV baseline drift when gradient elution is employed.

Some studies report the use of a small proportion of water [109–112] (typically 1–5%, because of the limited miscibility of water and CO_2) as an additive in alcohol cosolvent to improve peak shape and help the elution of compounds with very high polarities. It seems however that the joint action of water and a volatile salt is even more efficient to obtain good peak shapes for very polar species.

The additive concentration differs depending on the type of analyte and stationary phases and should be optimized during method development. It was shown several times that even with a very low concentration of additive, significant improvements in peak shapes and retention times happened [30, 89, 113]. However, it is advisable to employ higher concentrations of additives, when the peak shapes and MS response are more stable, to ensure an improved robustness of the analytical method [89].

2.3 Other operating parameters (pressure, temperature, flow rate)

Mobile phase density influences selectivity [114]. The effects of pressure changes on density are certainly the easiest to understand. When the pressure increases, the density of the fluid, thus the elution strength increases causing a decrease of retention time. The range of retention variation depends on mobile phase composition. For neat CO_2, the density varies from 0.2 to 1.1 g/mL. The change of density influences solubility and can dramatically modify retention factors [115–118]. With the addition of high modifier proportion, the pressure variation has a lower impact on density and thus on retention. In chiral SFC, it was shown that pressure changes in the 10–20 MPa range have a very limited effect on enantioselectivity with binary mobile phases of CO_2 and methanol [119, 120].

The effect of temperature depends on the fluid density. At low pressure and low cosolvent proportions, i.e. low fluid density, the increase in temperature favors the solubility, and the retention factor increases, which is opposite to the behavior that is familiar to HPLC chromatographers. At high pressure and high cosolvent proportions, i.e. high fluid density, the increase in temperature increases solubility (equivalent to the behavior of liquids [121]) and decreases the retention factor. Changing temperature in the course of the optimization of an achiral separation may then have positive or negative effects and causes more or less significant changes in selectivity [43]. For enantioselective separations, temperature has additional effects as it may contribute to changes in the rigidity of the stationary phase and analytes, thereby affecting the analyte fit into chiral cavities in a positive or negative fashion [122]. Also the way of controlling and measuring temperature varies between systems produced by different manufacturers, sometimes resulting in temperature differences as large as 15°C.

The influence of temperature and pressure can be difficult to predict [123], otherwise by testing. Fortunately, the pressure and temperature are not the most influential parameters on retention and can be used in a second step in the method development. In a detailed review on SFC, Leselier, and West have demonstrated many reasons to prefer subcritical rather than supercritical conditions [2] and proposed a starting point: moderate temperature of 25–30°C and pressure of 150 bar.

With the low viscosity of CO_2 it is possible to use high flow rates while pressure drop still remains manageable with current SFC systems. In a general way, increasing the flow rate results in shorter analysis time while maintaining reasonable column efficiency thanks to high diffusivity of the solutes. However, in SFC, flow rate changes do not affect only efficiency but also retention and selectivity. Indeed, as the fluid is compressible, changing its linear velocity in the column affects its density, therefore affects its eluting strength. For instance, lowering flow rate with polysaccharide stationary phases generally results in improved enantioresolution. Flow rate is then also an optimization parameter to adjust resolution.

3 Use of achiral SFC for analytical purposes

The chemical impurities that may be found in a sample of pharmaceutical interest (intermediate, drug candidate, or final product) can have multiple origins (synthesis, purification, or storage) and include lots of species (reagents, degradation products, intermediates from synthesis) [124], and may cause unwanted effects. That is why the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) imposes a precise control of these impurities (i.e. identification and quantification), for safety reasons. A general screening method for impurity profiling of drug candidates should naturally allow the resolution of a maximum of species. In addition, while detection is most often carried out with a UV detector, mass spectrometric detection is desirable to confirm peak identity and support peak purity.

RP-HPLC has always been considered as the technique of choice for impurity profiling [125]. The versatility provided by achiral SFC, with an extremely wide choice of stationary phases and possibilities of operating parameters, altogether providing an extended range of selectivities, is now raising interest. The recent rise of achiral SFC for analytical purposes is noticeable, especially in recent years. The use of achiral SFC is actually more common in the pharmaceutical industry than the number of publications might suggest, because many industry users do not communicate their results.

An interesting feature of SFC is that, in addition to possibly providing an orthogonal method to a RP-HPLC one, it can also be orthogonal to itself, when stationary phases are adequately selected: two columns with different stationary phase chemistry can be employed with the same operating conditions and provide orthogonal selectivity [89].

Several studies compared impurity profiling between SFC and RP-HPLC. Wang et al. [47] developed a method for the determination of eight impurities and degradation products of mometasone furoate at 0.05% of API area. Compared to HPLC, SFC provided higher efficiency and faster analyses. Moreover, the SFC and HPLC methods provided orthogonal selectivity. Nevertheless, this study highlighted the lesser sensitivity of the SFC method. Xu et al. [32] proposed a fast separation with good selectivities between bromosulfone and seven impurities, which are instable in water. The absence of water in SFC eliminates the risk of bromosulfone degradation during the analysis, which was a major concern with RP-HPLC. Moreover, the method was sensitive enough to detect the impurities at 0.5 mg/mL level. Two studies from Alexander et al. compared HPLC and SFC for quantification aspects. First, the authors studied two API and their impurities mixtures. They showed that the recent material used, an Agilent 1100 LC system when converted to a supercritical fluid chromatograph by addition of an Aurora Fusion F5 SFC module, provided reproducible retention time and better sensitivity (12-fold) over previous reports. It proved that SFC can be used for impurity profiling and quantification with drug loading concentration of 2 mg/mL [43]. The second study compared impurity profiling of three antiretroviral drugs and their impurities with SFC and HPLC [13]. Even if the selectivity was slightly lower with SFC (total separation of all species with HPLC, against one coeluting pair with SFC), SFC had many advantages over HPLC: better distribution of peaks across the separation space, no baseline drift and easier method development. In both studies, high orthogonality was obtained between the two methods, as exemplified in Fig. 1. The new generation of SFC instrumentation was found to exhibit the required sensitivity for successful quantification of potential impurities/degradation products at the 0.05–0.1% area level. These applications also undermined the lack of sensitivity in UV usually found, and lack of reproducibility of the analysis. Sensitivity is crucial to quantify very low concentrations of impurities with good enough S/N [126,127].

The possible coupling between SFC and mass spectrometers can overcome limited UV sensitivity. It was already proven that SFC–MS could compete with LC–MS for the purpose of pharmaceuticals analysis [35, 40, 128–130]. Atmospheric pressure chemical ionization (APCI) [27, 35, 40, 102, 128–131] and ESI [106–108, 132, 133] sources are both commonly used. The use of atmospheric pressure photoionization was also reported [134]. As in the case of LC–MS, the choice of the source depends on compound ionization capability. The SFC–MS interface is often used with an additional solvent, called make-up solvent, which avoids the precipitation of analytes that may be caused by the cool depressurization of CO_2, but also promotes analyte ionization. For instance, the use of methanol as make-up flow with a flow rate of 0.2 mL/min was selected to improve the S/N with negative APCI [129]. A larger proportion of CO_2 than methanol in the mobile phase, from 50 to 70%, was found to improve the APCI-MS response for pharmaceutical products. A higher LOD in SFC–MS (four to tenfold increase) than LC–MS was reported by Grand–Guillaume Perrenoud et al. for ESI based on the analysis of six drugs [108]. Based on the analysis of 110 doping agents in urine samples, 32% of the compounds were found to exhibit higher ESI-MS response with SFC than with HPLC [104]. However, in another paper, the same authors [135] achieved opposite conclusions. These differences were attributed to the use of different mass spectrometers.

The families of compounds typically observed in SFC achiral analysis of drug-like compounds are varied: steroids [13,27,29,82,84,132,136], benzodiazepines, and their derivatives [108,137–139], alkaloids [7,27,31,38,40,92,102,106,108, 140], nonsteroidal anti-inflammatory drugs [7, 30, 38, 82, 84, 106,140–142], sulfonamides [30,84,131,132,136,143], antibiotics [3, 42, 144–146], β-blockers [25, 30, 41, 93, 101, 103, 147–149], anti-depressants [150], anti-psychotics [34,107,130,151], stimulants [152], barbiturates [153,154], imidazoles [155,156], taxanes [157, 158], cannabinoids [3], retinoids [98, 159], antihistamines [36], and antiretroviral drugs [13,160,161]. Typical analytical conditions (stationary and mobile phases, operating conditions) reported for the separation of generic families of drug compounds are detailed in Table 1.

4 Use of chiral SFC for analytical purposes

Because guidelines regulate the development of chiral products, the stereoisomers must be analyzed separately for their potential differences in toxicity and activity. Consequently, the production of individual enantiomers has become a priority for pharmaceutical companies [162]. While chiral synthesis is clearly desirable from an economic point of view, early developments require the production of all stereo-isomers to identify the bioactivity of each of them. HPLC and SFC are both commonly used for this enantioresolution, but SFC clearly is a favorite in this field. For a long time, analytical chiral SFC was thus employed essentially in the purpose of transferring the analytical method to preparative scale (see next section). The recent progress in stationary phase technology, and most importantly the improvement in apparatus allowing for more reliable and sensitive quantitation now

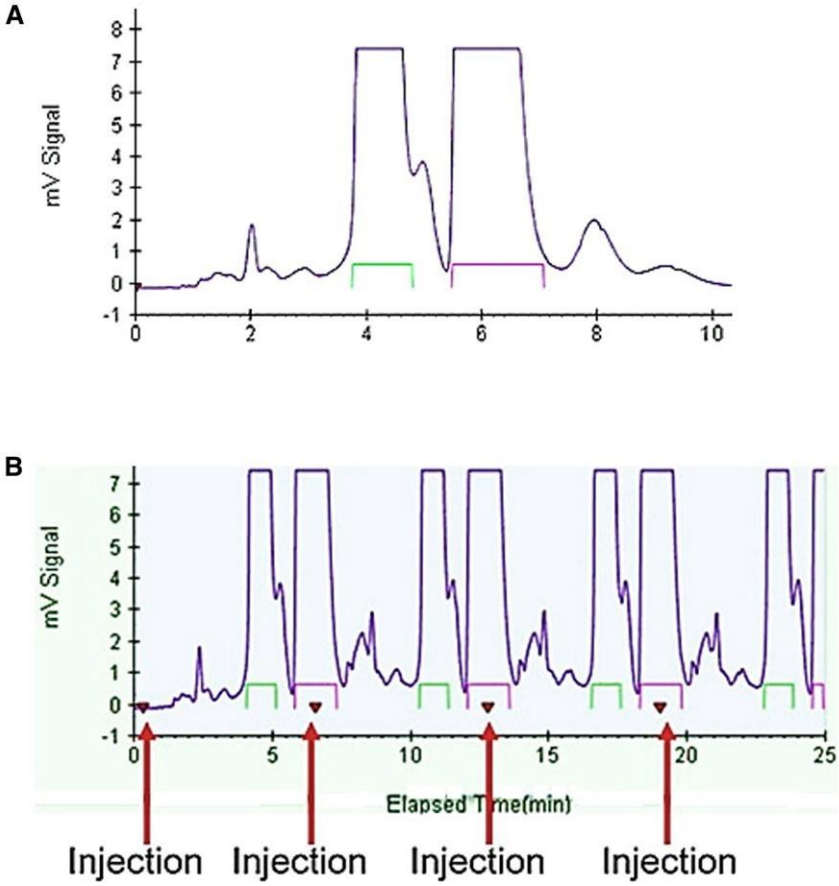


Figure 5. Illustration of stacked injections in preparative enantioresolution. (A) Single injection to identify collection windows. (B) Stacked injections to improve productivity. Adapted from [184] with permission from Elsevier.

make it a favorite for analytical scale enantiopurity assessment as well.

Several studies compared the performances of chiral SFC and chiral HPLC. However, as pointed out above, NP-HPLC is most common in the case of chiral separations. Marley et al. [163] developed a separation method of timolol maleate enantiomers on a Chiralcel OD-H stationary phase within 5 min, which was three times shorter and 11 times consume less as the NP-HPLC reference method. The authors also studied the importance of the mobile phase composition and particularly the impact of additives use: for these compounds, the simple addition of 0.1% v/v of TEA significantly improves peak shape (Fig. 2).

Combinatorial chemistry has rendered the development of high-throughput enantioselective methods particularly necessary. Recently, with the improvements in apparatus and column technologies, Regalado et al. [52] were able to achieve ultrafast enantioseparations of pharmaceutically relevant compounds on the order of seconds with a 1 cm column length (Fig. 3).

Mass detection can be particularly helpful for enantiopurifications [164]. Alexander et al. [165] point out that MS detection can be essential to differentiate the enantiomers of interest from other achiral impurities. Bolaños et al. [134] point out that accurately analyzing chiral compounds by SFC–MS is vital for properly assessing the potency of test ligands in biological assays. Indeed, they illustrated this point with an SFC–MS analysis of two SFC-purified enantiomers. Although the purity of enantiomer A assessed by both MS and UV was very good, enantiomer B was shown to have impurities that were detected only by MS (Fig. 4).

The families of compounds typically observed in SFC chiral analysis are similar to those encountered in achiral SFC: benzodiazepines and their derivatives [139, 166], nonsteroidal anti-inflammatory drugs [167–169], antibiotics, β-blockers [139, 163, 168, 170], sulfonamides [171, 172], stimulants [83], barbiturates [166], chiral sulfoxides (benzimidazoles, imidazoles) [162, 173–175], antihistamines [83, 176], anticoagulants [177, 178], and amino acids [73]. Typical analytical conditions (stationary and mobile phases, operating conditions) reported for the separation of generic families of chiral drug compounds are detailed in Table 2.

5 Use of preparative SFC in pharmaceutical companies

Both chiral and achiral SFC purifications are performed on samples with quantities varying from a few milligrams to several kilograms [107]. Preparative SFC provides some benefits relative to preparative HPLC. As mentioned above, the low viscosity and high diffusivity of the mobile phase permit the use

of high flow rate without sacrificing so much in efficiency. For preparative-scale separation, a direct consequence is an improvement of productivity with SFC purification as compared to HPLC. Another important feature is that the use of a majority of CO_2 in the mobile phase provides significant reduction in organic solvent consumption, and consequent reduction in energy and time required to evaporate the fractions [179], which support the "green aspect" of SFC. A significant gain of evaporation time was reported in many studies [180, 181]: for instance, a gain of 7 h compared to the HPLC purification method was reported by McClain et al. [182]. The fast process and reduced consumption of solvent are further enhanced by the use of "stacking" mode injection, which permits to improve the productivity by a factor three, compared to successive injections [183]. Stacked injections have been in use for a long time for the purification of enantiomers [184], but can be applied to achiral purification as well, and are especially useful when a single compound needs to be retrieved. An example of a stacked injections chromatogram is given in Fig. 5.

Chiral SFC has been routinely used in many companies for over two decades. The goal of chiral purification is to generate individual enantiomers, lots of blockbusters being chiral species formulated with an enantiopure form [185]. For most pharmaceutical companies [184], the technique has replaced HPLC as first intention method for enantioseparation [186]. As mentioned in Section 2.1, a majority of chiral purifications are performed with polysaccharide phases, selected for their high success rate and high loading capacity [107, 162, 186–189].

Recently, there has been an increasing interest for achiral purification as well [27, 134, 179, 180, 190, 191]. In addition to the advantages previously mentioned, the alternative selectivity provided by SFC compared to HPLC provides an interesting separation tool for achiral high-throughput purification [180, 181]. Differences in selectivity can be helpful for certain separation cases, like isomeric species. Similarly to cases described at the analytical scale, the absence of water in SFC mobile phase overcomes the problems of instability of water-sensitive compounds. For some basic drugs, the use of additives is necessary to achieve efficient purification. Ventura et al. [107] drew attention to the choice of additives. In principle if the basic additive is more volatile than the mobile phase, it can be expected to be removed in the solvent evaporation step following purification. Again the authors advised the use of ammonium hydroxide rather than common basic additives like isopropylamine or diethylamine.

6 Conclusion and perspectives

Despite the obvious benefits of SFC as efficient analytical tool, it still does not hold the first place in many laboratories and HPLC remains the preferred technique. Nevertheless, many pharmaceutical companies have taken advantage of SFC and its presence is increasing today: first for preparative enantioresolution but also more recently for achiral applications at the analytical and preparative scales.

The versatility provided by achiral SFC, with an extremely wide choice of stationary phases and possibilities of operating parameters, providing an extended range of selectivities, is a significant strength of the technique compared to HPLC.

At the preparative scale, the excellent kinetic performance and high possible flow rates permit an improvement of productivity for SFC purification compared to HPLC. Moreover, the fractions are collected with only a small percentage of organic solvent, supporting the "green aspect" of SFC.

The recent marketing of new robust instruments dedicated to analytical SFC and the progresses in HPLC stationary phase technology have both greatly benefited to SFC. While the knowledge on the technique is still limited, SFC is now technologically fully mature and should find a place on every bench in pharmaceutical companies.

The authors of this paper have declared no conflict of interest.

7 References

[1] Abbott, E., Veenstra, T. D., Issaq, H. J., *J. Sep. Sci.* 2008, *31*, 1223–1230.

[2] Lesellier, E., West, C., *J. Chromatogr. A* 2015, *1382*, 2–46.

[3] Later, D. W., Richter, B. E., Knowles, D. E., Andersen, M. R., *J. Chromatogr. Sci.* 1986, *24*, 249–253.

[4] Springston, S. R., Novotny, M., *Chromatographia* 1981, *14*, 679–684.

[5] Cudiamat, G., *The Column* 2013, *9*, 8–9.

[6] Khater, S., West, C., Lesellier, E., *J. Chromatogr. A* 2013, *1319*, 148–159.

[7] Grand-Guillaume Perrenoud, A., Veuthey, J.-L., Guillarme, D., *J. Chromatogr. A* 2012, *1266*, 158–167.

[8] Grand-Guillaume Perrenoud, A., Veuthey, J.-L., Guillarme, D., *TrAC Trends Anal. Chem.* 2014, *63*, 44–54.

[9] Li, K., Fu, Q., Xin, H., Ke, Y., Jin, Y., Liang, X., *Analyst* 2014, *139*, 3577–3587.

[10] François, I., Sandra, P., *J. Chromatogr. A* 2009, *1216*, 4005–4012.

[11] Stevenson, P. G., Tarafder, A., Guiochon, G., *J. Chromatogr. A* 2012, *1220*, 175–178.

[12] Periat, A., Grand-Guillaume Perrenoud, A., Guillarme, D., *J. Sep. Sci.* 2013, *36*, 3141–3151.

[13] Alexander, A. J., Zhang, L., Hooker, T. F., Tomasella, F. P., *J. Pharm. Biomed. Anal.* 2013, *78–79*, 243–251.

[14] Desfontaine, V., Guillarme, D., Francotte, E., Nová ková , L., *J. Pharm. Biomed. Anal.* n.d., DOI 10.1016/j.jpba.2015.03.007.

[15] West, C., *Curr. Anal. Chem.* 2014, *10*, 99–120.

[16] Płotka, J. M., Biziuk, M., Morrison, C., Namieśnik, J., *TrAC Trends Anal. Chem.* 2014, *56*, 74–89.

[17] West, C., Lesellier, E., *J. Chromatogr. A* 2006, *1110*, 181–190.

[18] West, C., Lesellier, E., *J. Chromatogr. A* 2006, *1110*, 200–213.

[19] West, C., Lesellier, E., *J. Chromatogr. A* 2006, *1115*, 233–245.

[20] West, C., Khater, S., Lesellier, E., *J. Chromatogr. A* 2012, *1250*, 182–195.

[21] Poole, C. F., *J. Chromatogr. A* 2012, *1250*, 157–171.

[22] Lesellier, E., *J. Chromatogr. A* 2009, *1216*, 1881–1890.

[23] Lesellier, E., *J. Sep. Sci.* 2008, *31*, 1238–1251.

[24] McClain, R., Hyun, M. H., Li, Y., Welch, C. J., *J. Chromatogr. A* 2013, *1302*, 163–173.

[25] Smuts, J., Wanigasekara, E., Armstrong, D. W., *Anal. Bioanal. Chem.* 2011, *400*, 435–447.

[26] Chou, F.-M., Wang, W.-T., Wei, G.-T., *J. Chromatogr. A* 2009, *1216*, 3594–3599.

[27] de la Puente, M. L., López Soto-Yarritu, P., Burnett, J., *J. Chromatogr. A* 2011, *1218*, 8551–8560.

[28] West, C., Lesellier, E., *J. Chromatogr. A* 2008, *1203*, 105–113.

[29] Berry, A. J., Ramsey, E. D., Newby, M., Games, D. E., *J. Chromatogr. Sci.* 1996, *34*, 245–253.

[30] Cazenave-Gassiot, A., Boughtflower, R., Caldwell, J., Hitzel, L., Holyoak, C., Lane, S., Oakley, P., Pullen, F., Richardson, S., Langley, G. J., *J. Chromatogr. A* 2009, *1216*, 6441–6450.

[31] Crowther, J. B., Henion, J. D., *Anal. Chem.* 1985, *57*, 2711–2716.

[32] Xu, J., Thompson, R., Li, B., Ge, Z., *J. Liq. Chromatogr. Amp. Relat. Technol.* 2002, *25*, 1007–1018.

[33] Dost, K., Davidson, G., *Analyst* 2003, *128*, 1037–1042.

[34] Berger, T. A., Wilson, W. H., *J. Pharm. Sci.* 1994, *83*, 281–286.

[35] Pinkston, J. D., Wen, D., Morand, K. L., Tirey, D. A., Stanton, D. T., *Anal. Chem.* 2006, *78*, 7467–7472.

[36] Smith, M. S., Oxford, J., Evans, M. B., *J. Chromatogr. A* 1994, *683*, 402–406.

[37] Bernal, J. L., Nozal, M. J., Toribio, L., Serna, M. L., Borrull, F., Marcé, R. M., Pocurull, E., *Chromatographia* 1997, *46*, 295–300.

[38] Grand-Guillaume Perrenoud, A. G.-G., Farrell, W. P., Aurigemma, C. M., Aurigemma, N. C., Fekete, S., Guillarme, D., *J. Chromatogr. A* 2014, *1360*, 275–287.

[39] Gyllenhaal, O., Hulthe, J., *J. Pharm. Biomed. Anal.* 2002, *29*, 381–386.

[40] de la Puente, M. L., Soto-Yarritu, P. L., Anta, C., *J. Chromatogr. A* 2012, *1250*, 172–181.

[41] Lundgren, J., Salomonsson, J., Gyllenhaal, O., Johansson, E., *J. Chromatogr. A* 2007, *1154*, 360–367.

[42] Ramsey, E. D., Berry, A. J., Lawrence, S. D., Games, D. E., Startin, J. R., *Rapid Commun. Mass Spectrom.* 1995, *9*, 712–716.

[43] Alexander, A. J., Hooker, T. F., Tomasella, F. P., *J. Pharm. Biomed. Anal.* 2012, *70*, 77–86.

[44] Lemasson, E., Bertin, S., Hennig, P., Boiteux, H., Lesellier, E., West, C., *J. Chromatogr. A* 2015, *1408*, 227–235.

[45] Grand-Guillaume Perrenoud, A., Boccard, J., Veuthey, J.-L., Guillarme, D., *J. Chromatogr. A* 2012, *1262*, 205–213.

[46] Ganipisetty, V. N. R., D, J., G, G. dev, Bandari, R., P, M., D, D., R, V. N., *Anal. Methods* 2013, *5*, 7008–7013.

[47] Wang, Z., Zhang, H., Liu, O., Donovan, B., *J. Chromatogr. A* 2011, *1218*, 2311–2319.

[48] Dunkle, M., West, C., Pereira, A., Plas, S. V. der, Madder, A., Farrell, W., Lesellier, E., Lynen, F., Pat, S., *Sci. Chromatogr.* 2014, *6*, 85–103.

[49] da Silva, C. G. A., Collins, C. H., Lesellier, E., West, C., *J. Chromatogr. A* 2013, *1315*, 176–187.

[50] Khater, S., West, C., Lesellier, E., *J. Chromatogr. A* 2013, *1319*, 148–159.

[51] Hamman, C., Wong, M., Aliagas, I., Ortwine, D. F., Pease, J., Schmidt Jr., D. E., Victorino, J., *J. Chromatogr. A* 2013, *1305*, 310–319.

[52] Regalado, E. L., Welch, C. J., *J. Sep. Sci.* 2015, *38*, 2826–2832.

[53] Zeng, L., Xu, R., Laskar, D. B., Kassel, D. B., *J. Chromatogr. A* 2007, *1169*, 193–204.

[54] Laskar, D. B., Zeng, L., Xu, R., Kassel, D. B., *Chirality* 2008, *20*, 885–895.

[55] Zhang, Y., Watts, W., Nogle, L., McConnell, O., *J. Chromatogr. A* 2004, *1049*, 75–84.

[56] Wetli, H. A., Francotte, E., *J. Sep. Sci.* 2007, *30*, 1255–1261.

[57] Vera, C. M., Shock, D., Dennis, G. R., Samuelsson, J., Enmark, M., Fornstedt, T., Shalliker, R. A., *Microchem. J.* 2015, *119*, 40–43.

[58] West, C., Lesellier, E., *J. Chromatogr. A* 2008, *1191*, 21–39.

[59] West, C., Lesellier, E., in: Grushka, E., Grinberg, N. (Eds.), *Advances in Chromatography,* CRC Press, Boca Raton 2010, pp. 195–203.

[60] West, C., Lesellier, E., *J. Chemom.* 2012, *26*, 52–65. [61] West, C., Khater, S., Khalikova, M., Lesellier, E., *The Column* 2014, *10*, 2–8.

[62] Abraham, M. H., Ibrahim, A., Zissimos, A. M., *J. Chromatogr. A* 2004, *1037*, 29–47.

[63] Vitha, M., Carr, P. W., *J. Chromatogr. A* 2006, *1126*, 143–194.

[64] West, C., Lesellier, E., Tchapla, A., *J. Chromatogr. A* 2004, *1048*, 99–109.

[65] Felix, G., Berthod, A., Piras, P., Roussel, C., *Sep. Purif. Rev.* 2008, *37*, 229–301.

[66] Okamoto, Y., Ikai, T., *Chem. Soc. Rev.* 2008, *37*, 2593.

[67] Francotte, E., Huynh, D., *J. Pharm. Biomed. Anal.* 2002, *27*, 421–429.

[68] Pirzada, Z., Personick, M., Biba, M., Gong, X., Zhou, L., Schafer, W., Roussel, C., Welch, C. J., *J. Chromatogr. A* 2010, *1217*, 1134–1138.

[69] Francotte, E. R., *J. Chromatogr. A* 2001, *906*, 379–397.

[70] Pirkle, W. H., Finn, J. M., Schreiner, J. L., Hamper, B. C., *J. Am. Chem. Soc.* 1981, *103*, 3964–3966.

[71] Hoffmann, C. V., Reischl, R., Maier, N. M., Lämmerhofer, M., Lindner, W., *J. Chromatogr. A* 2009, *1216*, 1157–1166.

[72] Lajkó, G., Ilisz, I., Tóth, G., Fülöp, F., Lind- ner, W., Pé ter, A., *J. Chromatogr. A* 2015, DOI 10.1016/j.chroma.2015.08.058.

[73] Liu, Y., Berthod, A., Mitchell, C. R., Xiao, T. L., Zhang, B., Armstrong, D. W., *J. Chromatogr. A* 2002, *978*, 185–204.

[74] Han, X., Berthod, A., Wang, C., Huang, K., Armstrong, D. W., *Chromatographia* 2007, *65*, 381–400.

[75] Williams, K. L., Sander, L. C., Wise, S. A., *J. Chromatogr. A* 1996, *746*, 91–101.

[76] Vozka, J., Kalíková , K., Roussel, C., Armstrong, D. W., Tesařová, E., *J. Sep. Sci.* 2013, *36*, 1711–1719.

[77] De Klerck, K., Vander Heyden, Y., Mangelings, D., *J. Chromatogr. A* 2014, *1363*, 311–322.

[78] Wang, C., Tymiak, A. A., Zhang, Y., *Anal. Chem.* 2014, *86*, 4033–4040.

[79] Sciascera, L., Ismail, O., Ciogli, A., Kotoni, D., Cavazzini, A., Botta, L., Szczerba, T., Kocergin, J., Villani, C., Gasparrini, F., *J. Chromatogr. A* 2015, *1383*, 160–168.

[80] Biba, M., Regalado, E. L., Wu, N., Welch, C. J., *J. Chromatogr. A* 2014, *1363*, 250–256.

[81] Janssen, H.-G., Schoenmakers, P. J., Cramers, C. A., *J. Chromatogr. A* 1991, *552*, 527–537.

[82] Brunelli, C., Zhao, Y., Brown, M.-H., Sandra, P., *J. Chromatogr. A* 2008, *1185*, 263–272.

[83] Maftouh, M., Granier-Loyaux, C., Chavana, E., Marini, J., Pradines, A., Heyden, Y. V., Picard, C., *J. Chromatogr. A* 2005, *1088*, 67–81.

[84] Brunelli, C., Zhao, Y., Brown, M.-H., Sandra, P., *J. Sep. Sci.* 2008, *31*, 1299–1306.

[85] DaSilva, J. O., Coes, B., Frey, L., Mergelsberg, I., McClain, R., Nogle, L., Welch, C. J., *J. Chromatogr. A* 2014, *1328*, 98–103.

[86] Gohres, J. L., Marin, A. T., Lu, J., Liotta, C. L., Eckert, C. A., *Ind. Eng. Chem. Res.* 2009, *48*, 1302–1306.

[87] Weikel, R. R., Hallett, J. P., Liotta, C. L., Eckert, C. A., *Ind. Eng. Chem. Res.* 2007, *46*, 5252–5257.

[88] Wen, D., Olesik, S. V., *Anal. Chem.* 2000, *72*, 475–480.

[89] Lemasson, E., Bertin, S., Hennig, P., Boiteux, H., Lesellier, E., West, C., *J. Chromatogr. A* 2015, *1408*, 217–226.

[90] Gyllenhaal, O., Karlsson, A., *J. Biochem. Biophys. Methods* 2000, *43*, 135–146.

[91] Zheng, J., Taylor, L. T., Pinkston, J. D., *Chromatographia* 2006, *63*, 267–276.

[92] Suto, K., Kakinuma, S., Ito, Y., Sagara, K., Iwasaki, H., Itokawa, H., *J. Chromatogr. A* 1997, *786*, 371–376.

[93] Steuer, W., Baumann, J., Erni, F., *J. Chromatogr. A* 1990, *500*, 469–479.

[94] Ashraf-Khorassani, M., Fessahaie, M. G., Taylor, L. T., Berger, T. A., Deye, J. F., *J. High Resolut. Chromatogr.* 1988, *11*, 352–353.

[95] Berger, T. A., Deye, J. F., *J. Chromatogr. A* 1991, *547*, 377–392.

[96] Berger, T. A., Deye, J. F., *J. Chromatogr. Sci.* 1991, *29*, 310–317.

[97] Gyllenhaal, O., Vessman, J., *J. Chromatogr. A* 1993, *628*, 275–281.

[98] Mé jean, M., Vollmer, M., Brunelle, A., Touboul, D., *Chromatographia* 2013, *76*, 1097–1105.

[99] Stringham, R. W., *J. Chromatogr. A* 2005, *1070*, 163–170.

[100] Berger, T. A., Deye, J. F., *J. Chromatogr. Sci.* 1991, *29*, 141–146.

[101] Berger, T. A., Wilson, W. H., *J. Biochem. Biophys. Methods* 2000, *43*, 77–85.

[102] Dost, K., Davidson, G., *J. Biochem. Biophys. Methods* 2000, *43*, 125–134.

[103] Jones, D. C., Dost, K., Davidson, G., George, M. W., *Analyst* 1999, *124*, 827–831.

[104] Nová ková , L., Rentsch, M., Grand-Guillaume Perrenoud, A., Nicoli, R., Saugy, M., Veuthey, J., Guillarme, D., *Anal. Chim. Acta* 2015, *853*, 647–659.

[105] Pereira, A., David, F., Vanhoenacker, G., Brunelli, C., Sandra, P., *LC–GC N. Am.* 2011, *29*.

[106] Pinkston, J. D., Stanton, D. T., Wen, D., *J. Sep. Sci.* 2004, *27*, 115–123.

[107] Ventura, M., Murphy, B., Goetzinger, W., *J. Chromatogr. A* 2012, *1220*, 147–155.

[108] Grand-Guillaume Perrenoud, A., Veuthey, J.-L., Guillarme, D., *J. Chromatogr. A* 2014, *1339*, 174–184.

[109] Pyo, D., Lee, S., *Bull. Korean Chem. Soc.* 1999, *20*, 405.

[110] Wright, B. W., Smith, R. D., *J. Chromatogr. A* 1986, *355*, 367–373.

[111] Liu, J., Regalado, E. L., Mergelsberg, I., Welch, C. J., *Org. Biomol. Chem.* 2013, *11*, 4925–4929.

[112] Ashraf-Khorassani, M., Taylor, L. T., *J. Sep. Sci.* 2010, *33*, 1682–1691.

[113] Samimi, R., Xu, W. Z., Alsharari, Q., Charpentier, P. A., *J. Supercrit. Fluids* 2014, *86*, 115–123.

[114] Lou, X., Janssen, H.-G., Cramers, C. A., *J. Chromatogr. A* 1997, *785*, 57–64.

[115] Berger, T. A., *Chromatographia* 1993, *37*, 645–652.

[116] Tarafder, A., Kaczmarski, K., Ranger, M., Poe, D. P., Guiochon, G., *J. Chromatogr. A* 2012, *1238*, 132–145.

[117] Tarafder, A., Kaczmarski, K., Poe, D. P., Guiochon, G., *J. Chromatogr. A* 2012, *1258*, 136–151.

[118] Schmitz, F. P., Klesper, E., *J. High Resolut. Chromatogr.* 1987, *10*, 519–521.

[119] Wang, C., Zhang, Y., *J. Chromatogr. A* 2013, *1281*, 127–134.

[120] Åsberg, D., Enmark, M., Samuelsson, J., Fornstedt, T., *J. Chromatogr. A* 2014, *1374*, 254–260.

[121] Shah, S., Taylor, L. T., *Chromatographia* 1990, *29*, 453–458.

[122] West, C., Bouet, A., Routier, S., Lesellier, E., *J. Chromatogr. A* 2012, *1269*, 325–335.

[123] Tyteca, E., Desfontaine, V., Desmet, G., Guillarme, D., *J. Chromatogr. A* 2015, *1381*, 219–228.

[124] European Medicines Agency—ICH Topic Q3B (R2)—Impurities in New Drug Substances—Note for guidance on impurities in new drugs products, 2006.

[125] Nageswara Rao, R., Nagaraju, V., *J. Pharm. Biomed. Anal.* 2003, *33*, 335–377.

[126] Berger, T. A., Berger, B. K., *J. Chromatogr. A* 2011, *1218*, 2320–2326.

[127] Berger, T. A., Fogleman, K., *Peak E-Zine* 2009, *15*.

[128] Ventura, M. C., Farrell, W. P., Aurigemma, C. M., Greig, M. J., *Anal. Chem.* 1999, *71*, 2410–2416.

[129] Ventura, M. C., Farrell, W. P., Aurigemma, C. M., Greig, M. J., *Anal. Chem.* 1999, *71*, 4223–4231.

[130] Hsieh, Y., Favreau, L., Schwerdt, J., Cheng, K.-C., *J. Pharm. Biomed. Anal.* 2006, *40*, 799–804.

[131] Dost, K., Jones, D. C., Davidson, G., *Analyst* 2000, *125*, 1243–1247.

[132] Berry, A. J., Games, D. E., Perkins, J. R., *J. Chromatogr. A* 1986, *363*, 147–158.

[133] Schou-Pedersen, A. M. V., Østergaard, J., Johansson, M., Dubant, S., Frederiksen, R. B., Hansen, S. H., *J. Pharm. Biomed. Anal.* 2014, *88*, 256–261.

[134] Bolaños, B., Greig, M., Ventura, M., Farrell, W., Aurigemma, C. M., Li, H., Quenzer, T. L., Tivel, K., Bylund, J. M. R., Tran, P., Pham, C., Phillipson, D., *Int. J. Mass Spectrom.* 2004, *238*, 85–97.

[135] Spaggiari, D., Mehl, F., Desfontaine, V., Grand-Guillaume Perrenoud, A., Fekete, S., Rudaz, S., Guillarme, D., *J. Chromatogr. A* 2014, *1371*, 244–256.

[136] Brunelli, C., Górecki, T., Zhao, Y., Sandra, P., *Anal. Chem.* 2007, *79*, 2472–2482.

[137] Bhoir, I. C., Patil, S. T., Sundaresan, M., *Talanta* 1999, *48*, 1179–1189.

[138] Salvador, A., Jaime, M. A., Guardia, M. de la, *Anal. Proc. Anal. Commun.* 1995, *32*, 463–465.

[139] Wang, M. Z., Klee, M. S., Yang, S. K., *J. Chromatogr. B. Biomed. Sci. App.* 1995, *665*, 139–146.

[140] Grand-Guillaume Perrenoud, A., Hamman, C., Goel, M., Veuthey, J.-L., Guillarme, D., Fekete, S., *J. Chromatogr. A* 2013, *1314*, 288–297.

[141] Patil, S. T., Bhoir, I. C., Sundaresan, M., *Anal. Chim. Acta* 1999, *384*, 143–150.

[142] Simmons, B. R., Jagota, N. K., Stewart, J. T., *J. Pharm. Biomed. Anal.* 1995, *13*, 59–64.

[143] Combs, M. T., Ashraf-Khorassani, M., Taylor, L. T., *J. Chromatogr. Sci.* 1997, *35*, 176–180.

[144] Ramsey, E. D., Lawrence, S. D., Games, D. E., *Anal. Proc. Anal. Commun.* 1995, *32*, 455–457.

[145] Bari, V. R., Dhorda, U. J., Sundaresan, M., *Anal. Chim. Acta* 1998, *376*, 221–225.

[146] Bhoir, I. C., Raman, B., Sundaresan, M., Bhagwat, A. M., *Anal. Chim. Acta* 1997, *354*, 123–128.

[147] Gyllenhaal, O., Edström, L., Persson, B.-A., *J. Chromatogr. A* 2006, *1134*, 305–310.

[148] Gyllenhaal, O., Karlsson, A., *Chromatographia* 2010, *71*, 7–13.

[149] Gyllenhaal, O., Vessman, J., *J. Chromatogr. A* 1999, *839*, 141–148.

[150] Berger, T. A., Wilson, W. H., *J. Pharm. Sci.* 1994, *83*, 287–290.

[151] Patela, Y. P., Dhorda, U. J., Sundaresan, M., *Talanta* 1998, *47*, 625–630.

[152] Berger, T. A., Wilson, W. H., *J. Pharm. Sci.* 1995, *84*, 489–492.

[153] Smith, R. M., Sanagi, M. M., *J. Chromatogr. A* 1989, *481*, 63–69.

[154] Patil, S. T., Sundaresan, M., Bhoir, I. C., Bhagwat, A. M., *Talanta* 1998, *47*, 3–10.

[155] Parlier, D., Thiébaut, D., Caude, M., Rosset, R., *Chromatographia* 1991, *31*, 293–296.

[156] Patel, Y., Dhorda, U. J., Sundaresan, M., Bhagwat, A. M., *Anal. Chim. Acta* 1998, *362*, 271–277.

[157] Heaton, D. M., Bartle, K. D., Rayner, C. M., Clifford, A. A., *J. High Resolut. Chromatogr.* 1993, *16*, 666–670.

[158] Jagota, N. K., Nair, J. B., Frazer, R., Klee, M., Wang, M. Z., *J. Chromatogr. A* 1996, *721*, 315–322.

[159] François, I., dos Santos Pereira, A., Lynen, F., Sandra, P., *J. Sep. Sci.* 2008, *31*, 3473–3478.

[160] Kaul, N., Agrawal, H., Paradkar, A. R., Mahadik, K. R., *J. Biochem. Biophys. Methods* 2005, *64*, 121–141.

[161] Kaul, N., Agrawal, H., Paradkar, A. R., Mahadik, K. R., *J. Pharm. Biomed. Anal.* 2007, *43*, 471–480.

[162] Toribio, L., del Nozal, M. J., Bernal, Y. L., Alonso, C., Jiménez, J. J., *J. Sep. Sci.* 2008, *31*, 1307–1313.

[163] Marley, A., Connolly, D., *J. Chromatogr. A* 2014, *1325*, 213–220.

[164] Garzotti, M., Hamdan, M., *J. Chromatogr. B* 2002, *770*, 53–61.

[165] Alexander, A. J., Staab, A., *Anal. Chem.* 2006, *78*, 3835–3838.

[166] Van Overbeke, A., Sandra, P., Medvedovici, A., Baeyens, W., Aboul-Enein, H. Y., *Chirality* 1997, *9*, 126–132.

[167] Gyllenhaal, O., Stefansson, M., *Chirality* 2005, *17*, 257–265.

[168] Ren-Qi, W., Teng-Teng, O., Siu-Choon, N., Weihua, T., *TrAC Trends Anal. Chem.* 2012, *37*, 83–100.

[169] Hoke, S. H., Pinkston, J. D., Bailey, R. E., Tanguay, S. L., Eichhold, T. H., *Anal. Chem.* 2000, *72*, 4235–4241.

[170] Svensson, S., Karlsson, A., Gyllenhaal, O., Vessman, J., *Chromatographia* 2000, *51*, 283–293.

[171] Wang, R.-Q., Ong, T.-T., Ng, S.-C., *J. Chromatogr. A* 2012, *1224*, 97–103.

[172] Vozka, J., Kalíková, K., Tesařová, E., *Anal. Lett.* 2013, *46*, 2860–2869.

[173] Toribio, L., Alonso, C., del Nozal, M. J., Bernal, J. L., Jiménez, J. J., *J. Sep. Sci.* 2006, *29*, 1363–1372.

[174] Toribio, L., del Nozal, M. J., Bernal, J. L., Alonso, C., Jiménez, J. J., *J. Chromatogr. A* 2007, *1144*, 255–261.

[175] Toribio, L., Alonso, C., del Nozal, M. J., Bernal, J. L., Martín, M. T., *J. Chromatogr. A* 2006, *1137*, 30–35.

[176] Kraml, C. M., Zhou, D., Byrne, N., McConnell, O., *J. Chromatogr. A* 2005, *1100*, 108–115.

[177] Coe, R. A., Rathe, J. O., Lee, J. W., *J. Pharm. Biomed. Anal.* 2006, *42*, 573–580.

[178] Regalado, E. L., Schafer, W., McClain, R., Welch, C. J., *J. Chromatogr. A* 2013, *1314*, 266–275.

[179] Lindskog, M. A., Nelander, H., Jonson, A. C., Halvarsson, T., *Drug Discov. Today* 2014, *19*, 1607–1612.

[180] White, C., Burnett, J., *J. Chromatogr. A* 2005, *1074*, 175–185.

[181] Weller, H. N., Ebinger, K., Bullock, W., Edinger, K. J., Hermsmeier, M. A., Hoffman, S. L., Nirschl, D. S., Swann, T., Zhao, J., Kiplinger, J., Lefebvre, P., *J. Comb. Chem.* 2010, *12*, 877–882.

[182] McClain, R. T., Dudkina, A., Barrow, J., Hartman, G., Welch, C. J., *J. Liq. Chromatogr. Amp Relat. Technol.* 2009, *32*, 483–499.

[183] Pradines, 6th International Conference on Packed Column SFC, A., 2012.

[184] Miller, L., *J. Chromatogr. A* 2012, *1250*, 250–255.

[185] Piras, P., Roussel, C., *J. Pharm. Biomed. Anal.* 2008, *46*, 839–847.

[186] White, C., *J. Chromatogr. A* 2005, *1074*, 163–173.

[187] Miller, L., Potter, M., *J. Chromatogr. B* 2008, *875*, 230–236.

[188] Mukherjee, P.S., *J. Pharm. Biomed. Anal.* 2007, *43*, 464–470.

[189] Mukherjee, P. S., Cook, S. E., *J. Pharm. Biomed. Anal*. 2006, *41*, 1287–1292.

[190] Hettiarachchi, K., Kong, M., Yun, A., Jacobsen, J. R., Xue, Q., *J. Sep. Sci.* 2014, *37*, 775–781.

[191] Berger, T. A., Fogleman, K., Staats, T., Bente, P., Crocket, I., Farrell, W., Osonubi, M., *J. Biochem. Biophys. Meth- ods* 2000, *43*, 87–111.

4) Innovations récentes en SFC

La précédente publication datant de 2016, les plus récentes applications et innovations en SFC n'y figurent pas. Parmi elles, on notera les travaux de Dispas *et al.* sur l'utilisation du concept de « Quality by Design » pour optimiser et développer des méthodes analytiques robustes en SFC. Le concept de QbD est largement utilisé dans le développement pharmaceutique et est défini comme « une approche systématique du développement qui commence avec des objectifs prédéfinis et souligne la nécessité de comprendre les produits et de maitriser les procédés, basée sur la science et la gestion des risques qualité » selon la réglementation ICH Q8 R2 de 2009 [144]. Dispas *et al.* ont ainsi développé et validé une méthode SFC-UV pour la détermination et la quantification d'impuretés dans un principe actif, le salbutamol sulfate [145], et dans la vitamine D3 [146].

Desfontaine *et al.* se sont intéressés à l'influence de différents paramètres d'injection (solvant de dilution, solvant de rinçage de l'aiguille, volume d'injection, concentration de l'analyte, pourcentage initial de co-solvant dans la phase mobile) sur la forme des pics chromatographiques [147]. Cette étude a été réalisée en gradient, en utilisant 3 colonnes sub-2 µm et sur un set de 11 composés pharmaceutiques. Après une étude individuelle de chacun des paramètres, il apparait que les paramètres qui influencent le plus la forme des pics sont la nature du solvant d'injection et le volume d'injection. Les auteurs proposent un choix de solvants permettant d'injecter de larges volumes en SFC sans compromettre la forme des pics : MTBE (méthyl tert-butyl éther), DCM (dichlorométhane), ACN et CPME (méthoxycyclopentane) sont indiqués de préférence au MeOH, considéré comme la pire alternative. Cependant, il apparait que la nature de l'analyte et de la phase stationnaire influence également la forme des pics. Le solvant d'injection idéal en SFC reste donc difficile à prévoir. Les auteurs conseillent donc d'inclure dans le développement de méthode le choix du solvant d'injection.

V. Conclusions générales

En raison de la synthèse multi étapes des produits pharmaceutiques développés, augmentant de surcroit le nombre potentiel d'impuretés présentes, et des législations de plus en plus restrictives, il est nécessaire de disposer de méthodes analytiques performantes pour assurer l'efficacité du principe actif. Bien que largement privilégiée dans un grand nombre de laboratoires pharmaceutiques, la RPLC sur phase C18 est adaptée à une gamme limitée de composés ($-1 < \log P < 7$). Afin d'obtenir des sélectivités

variées, les chromatographistes s'orientent vers des méthodes alternatives les plus orthogonales possibles à la RPLC C18.

Dans un souci de simplicité, la stratégie la plus simple vise à utiliser une colonne avec un greffon différent du greffon C18 en RPLC. Ainsi, avec un minimum de changement et en conservant des conditions de travail proches, on peut changer la sélectivité.

Une autre stratégie vise à multiplier les possibilités d'interactions entre le soluté et la phase stationnaire. C'est le cas des colonnes mixed-mode, qui contiennent des greffons complexes susceptibles d'interagir de multiples façons avec l'analyte.

Malgré un démarrage difficile, la SFC tend de plus en plus à s'imposer comme méthode chromatographique de premier plan pour l'analyse de candidats médicaments. Cette technique, orthogonale à la RPLC, offre des avantages économiques importants pour des aspects préparatifs. Elle concurrence également l'HPLC en termes d'efficacité.

Ces trois stratégies (RPLC sur des phases stationnaires autres que C18, HPLC mixed-mode et SFC) ont été mises en œuvre dans le cadre de cette thèse.

Chapitre 2
Développement de méthodes HPLC pour le profilage d'impuretés

I. Introduction

Comme nous l'avons vu au premier chapitre de cette thèse, la RPLC sur phase stationnaire C18 est encore aujourd'hui la méthode de premier choix pour le profilage d'impuretés de produits pharmaceutiques. Bien que les laboratoires de contrôle-qualité soient majoritairement équipés de systèmes HPLC, l'usage de l'UHPLC s'est démocratisé dans les laboratoires de recherche et développement.

A l'Institut de Recherches Servier, ce sont deux méthodes UHPLC complémentaires en phase inverse (RPLC) sur colonne C18 qui sont utilisées. Ces méthodes sont utilisées en routine au laboratoire depuis de nombreuses années. Bien que très performantes, il arrive que ces méthodes échouent, notamment quand le PA ou les impuretés présentes dans l'échantillon ne sont pas suffisamment retenus sur la colonne. C'est le cas des composés polaires. Il est alors difficile voire impossible d'évaluer la pureté relative du PA et dénombrer et quantifier ses impuretés. Dans ce cas, il est essentiel de pouvoir se tourner vers des méthodes analytiques alternatives.

La première méthode alternative explorée dans ce chapitre est une méthode RPLC mais avec une phase stationnaire différente, une phase PFP (pentafluorophenyle). Ce type de phases est couramment utilisé comme alternative à la C18, en raison de la différence de sélectivité qu'elles offrent. Euerby *et al.* ont montré que des composés pharmaceutiques basiques (10 composés couvrant une large gamme de log P et pK_a) étaient davantage retenus sur des phases PFP. Les auteurs ont également montré que les séparations obtenues avec ces phases PFP étaient orthogonales à celles obtenues sur des phases C18 [6]. L'utilisation d'un fort pourcentage de solvant organique, l'absence d'agent d'appariement d'ion et les faibles pourcentages d'additif ajoutés à la phase mobile rendent le couplage à la masse aisé et permettent d'obtenir un gain de sensibilité par rapport à l'utilisation d'une phase C18 [148]. Regalado *et al.* ont également montré qu'une phase PFP fournissait les meilleures performances pour séparer des composés pharmaceutiques contenant notamment des halogènes (dont des atomes de fluor) [149,150]. D'un point de vue stratégique, cette méthode est la première alternative généralement mise en œuvre dans ce laboratoire Servier car elle est la plus rapide à mettre en place. En effet, seule la phase stationnaire change, les autres paramètres opératoires (appareillage et conditions analytiques) restant principalement les mêmes.

L'HPLC mixed-mode est également étudiée comme méthode alternative à la RPLC pour le profilage d'impuretés de candidats médicaments. Le principe de l'HPLC mixed-

mode (HPLC-MM) est d'utiliser plusieurs mécanismes de rétention dans une seule colonne (échange d'ion, phase inverse, HILIC). Une phase stationnaire bimodale (constituée d'une chaine C18 et d'un échangeur de cations faible) et une phase trimodale (modes RP, échangeur de cations fort, échangeur d'anions faible) ont été testées dans cette étude. Ainsi, des composés de polarités variées peuvent être séparés avec l'HPLC-MM grâce aux interactions multiples ayant lieu entre la phase stationnaire et les analytes [22].

Enfin, une comparaison des performances des méthodes HPLC développées pour le profilage d'impuretés est établie. Les méthodes alternatives développées doivent répondre à un objectif double : (i) être les plus orthogonales possible à la méthode de référence, (ii) augmenter la rétention des composés peu retenus sur C18.

II. Présentation des composés sondes sélectionnés pour l'étude

L'ensemble de composés sondes utilisé est constitué de 140 composés (sauf indication contraire). Ces produits sont des composés de « type S ». Ils sont tous de bas poids moléculaires (avec des masses molaires comprises entre 164.1 et 669.4). Parmi eux, une large majorité est basique (environ 75%). Leur gamme de polarité est assez variée, avec des log P compris entre -2 et 7.5 dont une large majorité de valeurs positives (Figure 2.7). Leurs structures restent confidentielles.

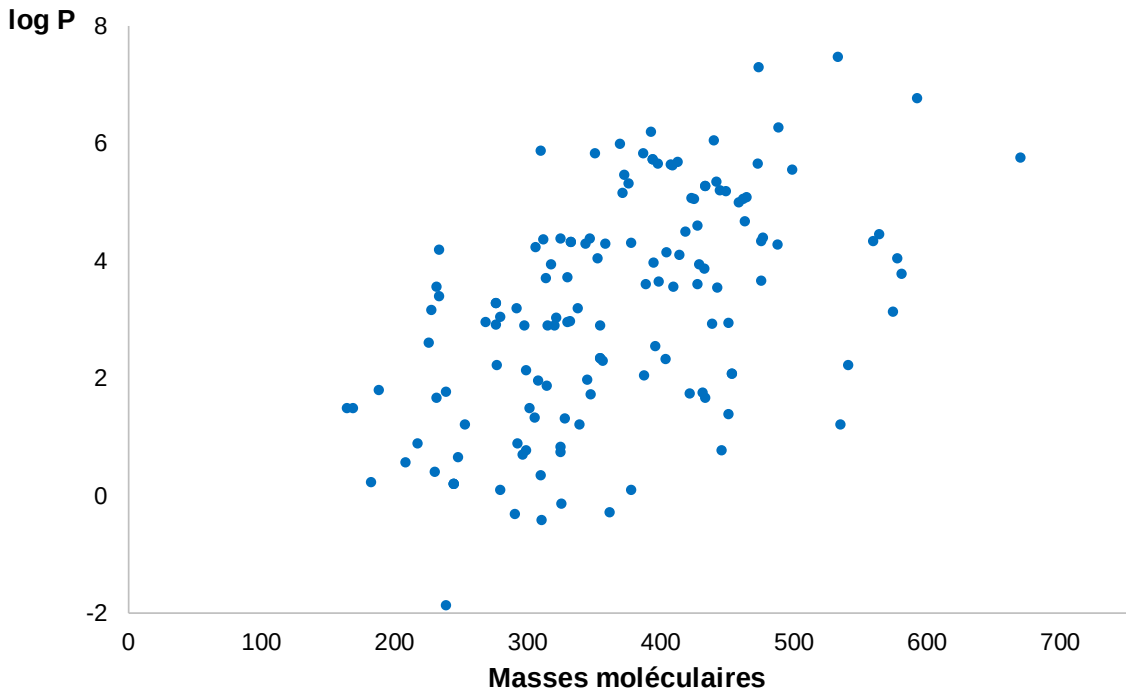


Figure 2.7 - Présentation des 140 composés Servier (log P et masses moléculaires)

III. Présentation des méthodes RPLC existantes à l'IdRS

1) Méthodes sur phase stationnaire C18

Les méthodes UHPLC utilisées comme méthodes de première intention pour le profilage d'impuretés à l'Institut de Recherches Servier ont été développées sur une seule et même colonne chromatographique, la colonne ACQUITY BEH C18 (colonne totalement poreuse de 50 x 2.1 mm, 1.7 µm commercialisée par Waters). Ces méthodes complémentaires ont été optimisées il y a plusieurs années pour permettre la séparation d'un maximum d'impuretés. La complémentarité de ces méthodes s'explique par l'utilisation de conditions analytiques différentes : acides (avec l'emploi d'acide méthane sulfonique) ou basiques (avec l'emploi de bicarbonate d'ammonium). La méthode utilisée en premier lieu est la méthode en conditions acides. Si cette méthode n°1 ne permet pas la séparation totale des impuretés et du PA, ou si le PA n'est pas correctement chromatographié ou suffisamment retenu sur la colonne ; la méthode n°2 en conditions basiques est utilisée. Deux systèmes UHPLC sont utilisés en parallèle, un pour chaque méthode. Les systèmes utilisés sont des systèmes ACQUITY UPLC® I-Class (Waters Corporation).

Les analyses sont effectuées dans les conditions suivantes :

(i) Conditions acides : utilisation d'acide méthane sulfonique à une concentration constante de 0.1% dans la phase mobile. La phase mobile est composée d'eau et d'acétonitrile (ACN). Le gradient utilisé passe de 2 à 98% d'ACN en 8 min, sans palier initial.

(ii) Conditions basiques : utilisation de bicarbonate d'ammonium à une concentration constante de 20 mM dans la phase mobile. La phase mobile est constituée d'eau et d'ACN. Le gradient varie de 2 à 80% d'ACN en 8 minutes, suivi par un palier de 2 minutes à 80% d'ACN.

Le débit est fixé à 0.4 mL/min et la température à 30°C.

Une double détection est permise avec ces systèmes : UV (détecteur UV-visible DAD, longueur d'onde extraite à 210 nm) et masse (ESI+/-). En conditions acides, un détecteur de masse simple quadripôle équipé d'une source électrospray est utilisé (détecteur ACQUITY QDa, Waters). La détection est effectuée en mode alterné positif et

négatif (m/z : 100-800). Le reste des paramètres est : balayage 10 points/s, voltage du capillaire 1kV en ESI+ et -0.8 kV en ESI-, voltage de cône 20 V, fréquence d'échantillonnage 8 Hz, température de la source 150°C, température du capillaire 600°C. Une pompe additionnelle est utilisée en amont de la MS, le débit de la pompe make-up est fixé à 0.4 mL/min (70% ACN / 30% H_2O avec 0.1% d'acide formique). En conditions basiques, le détecteur de masse utilisé est un détecteur simple quadripôle ACQUITY SQD® avec une source d'ionisation ESI. La détection est effectuée en mode positif et négatif (m/z : 100-1000), temps de balayage 0.3 s, voltage du capillaire 4 kV en mode positif et 3 kV en mode négatif, voltage de cône 20 V en positif et 30 V en négatif, température de la source 150°C, température de désolvatation 250°C (débit 500 L/h).

2) Méthode sur phase pentafluorophényle

A l'Institut de Recherches Servier, c'est l'utilisation de phases stationnaires pentafluorophenyle (PFP) qui a été privilégiée pour l'analyse de composés polaires peu retenus sur phase C18.

Pour cela, différentes phases PFP ont été testées au début des années 2000 de façon systématique sur les produits polaires analysés au laboratoire. C'est la colonne Discovery HSF5 (150 x 4.6 mm, 3.0 µm, Supelco) qui avait été retenue, en raison de sa capacité à retenir les composés polaires à hauteur de 80%. Cependant, une dégradation des résultats a été constatée en 2010 avec cette colonne (analyses non reproductibles d'une colonne à une autre) et la stratégie a dû être réévaluée. D'autres colonnes PFP ont alors été testées sur un set de 27 composés variés (acides, bases, neutres) avec des log P variés et représentatifs des composés analysés à l'IdRS, des composés polaires de synthèses issus de la Recherche ainsi que les produits analysés en routine au laboratoire afin de tester une diversité de structures importante. Parmi les 3 colonnes testées, la colonne XSELECT HSS PFP (150 x 4.6 mm 2.5 µm, Waters) a été retenue pour ses capacités rétentives et la reproductibilité des analyses au cours du temps et entre plusieurs colonnes provenant de lots différents.

L'ensemble des analyses est réalisé sur la XSELECT HSS PFP (150 x 4.6 mm 2.5 µm, Waters). Le système utilisé est le même que pour les méthodes UHPLC sur phase C18 (Chapitre 2, III, 1 : système ACQUITY UPLC® I-Class, équipé d'un détecteur UV DAD).

Les analyses sont réalisées en gradient, avec une phase mobile constituée de H_2O/ACN/TFA (100/1/0.1) pendant 3 minutes, puis un gradient d'élution avec une phase mobile commençant avec un mélange H_2O/ACN/TFA (100/1/0.1) et finissant avec un mélange H_2O/ACN/TFA (1/100/0.1) en 17 minutes suivi d'un palier de 5 minutes à la composition finale. Les analyses ont été réalisées à 0.9 mL/min et 30°C.

Le détecteur de masse est un triple quadripôle ACQUITY TQD® équipé d'une source d'ionisation électrospray. Les composés étudiés sont détectés en mode positif (m/z 100-1000), temps de scan 2s, voltage du capillaire 3kV, voltage de cône 15V, température de la source 150°C, température de désolvatation 600°C, débit du gaz de désolvatation 1000L/heure, débit cône 50L/heure.

IV. Utilisation de l'HPLC mixed-mode comme méthode alternative à la RPLC

1) Développement de méthode en HPLC mixed-mode sur une phase stationnaire bimodale

Afin de développer une méthode de profilage d'impuretés HPLC mixed-mode, nous avons dans un premier temps réalisé la caractérisation d'une colonne bimodale (Acclaim WCX-1 LC) afin de déterminer les conditions opératoires optimales. Ces travaux sont présentés dans l'article suivant :

E. Lemasson, Y. Richer, S. Bertin, P. Hennig, C. West

Characterization of retention mechanisms in mixed-mode HPLC with a bimodal reversed-phase / cation-exchange stationary phase

Chromatographia, 81, 3, (2018), 387-399

Chromatographia
https://doi.org/10.1007/s10337-018-3477-5

ORIGINAL

Characterization of Retention Mechanisms in Mixed-Mode HPLC with a Bimodal Reversed-Phase/Cation-Exchange Stationary Phase

Elise Lemasson[1] · Yoann Richer[1] · Sophie Bertin[2] · Philippe Hennig[2] · Caroline West[1]

Received: 13 November 2017 / Revised: 15 January 2018 / Accepted: 17 January 2018

Abstract
Mixed-mode HPLC (MM-HPLC), combining different interactions or retention modes in a single column, can be an interesting alternative to reversed-phase HPLC, notably to achieve the combined retention of polar and non-polar species. In the present fundamental study, we have selected one bimodal stationary phase allowing for both reversed-phase and weak cation-exchange retention modes (Acclaim mixed-mode WCX-1 LC). First, the mobile phase buffer composition (buffer pH ranging from 5 to 7 and concentration ranging from 20 to 100 mM) was explored with a small set of probe compounds (15 acids, bases and neutrals) to ensure adequate retention and peak shapes for the target compounds, and to evaluate the relative contributions of reversed-phase and ion-exchange mechanisms. Second, retention values measured for 63 probe compounds with various proportions of acetonitrile (ranging from 30 to 80%) served to establish linear solvation energy relationships based on (a) the usual and (b) a modified version of the solvation parameter model comprising additional descriptors to take account of interactions with ionizable species to bring some insights into the retention mechanisms. Finally, temperature effects at the low (30%) and high (60%) proportions of acetonitrile were observed between 20 and 40 °C (with 5 °C increments) and Van't Hoff plots were drawn to measure the changes in interactions energies when the mobile phase composition changed.

Keywords Reversed-phase liquid chromatography · Mixed-mode chromatography · Linear solvation energy relationships (LSER) · Solvation parameter model · Stationary phase characterization · Thermodynamic characterization

Introduction

Among the numerous separation modes available, reversed-phase liquid chromatography (RPLC) is one of the most frequently used to analyze small molecules in any application area. RPLC mode is particularly suited for the separation of compounds of low and moderate polarity but usually fails to retain polar neutral or ionic compounds. Different chromatographic modes must be envisaged as an alternative to RPLC for the analysis of such compounds: ion-pairing RPLC mode [1], hydrophilic interaction chromatography (HILIC) mode [2], ion-exchange chromatography (IEX) [3] or supercritical fluid chromatography (SFC) [4, 5]. However, because of the limited compatibility to mass spectrometric (MS) detection (for ion-pairing RPLC and IEX) or because of the apparent complexity of implementation involving other instruments or knowledge (HILIC and SFC), these methods are not widely accepted as viable alternatives to RPLC. It is therefore easier to propose methods that are compatible with, and most similar to RPLC systems to ensure their broadest applicability and adoption by the chromatographers.

Although it was certainly used in previous experiments but not formally recognized as such, the concept of mixed-mode liquid chromatography (MM-HPLC) was first described in 1984 by Bischoff and McLaughlin who used both alkyl chains and silanol groups to analyze nucleic acids on a C18-bonded stationary phase [6]. Some time later in 1986, Kennedy et al. [7] used the combination of RPLC and IEX modes in one column for the separation of proteins. The intent of MM-HPLC is to make use of several retention mechanisms in a single column to increase the separation power [8, 9]. Judging from the

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10337-018-3477-5) contains supplementary material, which is available to authorized users.

✉ Caroline West
caroline.west@univ-orleans.fr

[1] Institut de Chimie Organique et Analytique (ICOA), CNRS, UMR 7311, University of Orleans, B.P. 6759, Rue de Chartres, 45067 Orléans Cedex 2, France

[2] Institut de Recherches Servier, 11 Rue des Moulineaux, 92150 Suresnes, France

Published online: 25 January 2018

types of interactions combined, mixed-mode stationary phases can be classified into four different groups: reversed-phase/hydrophilic interaction (RPLC/HILIC), reversed-phase/ion-exchange (RPLC/IEX), hydrophilic interaction/ion-exchange (HILIC/IEX) and trimodal MM-HPLC (with several possible combinations). Thanks to the numerous combinations offered, MM-HPLC promises great flexibility and versatility to retain and separate both polar and non-polar compounds, owing to the multiple possibilities for interactions taking place between stationary phase and analytes [10]. Analyses of pharmaceutical compounds are the most popular applications of mixed-mode chromatography, as appears in several research papers [11–15]. These studies showed that the selectivity of mixed-mode stationary phases was highly orthogonal to RPLC with C18 phase. In addition, MM-HPLC is also highly compatible with MS or evaporative light-scattering detection (ELSD) (provided that volatile buffers are employed), unlike the majority of ion-exchange materials available today, usually requiring high concentrations of buffer salt that are not compatible with this kind of detectors.

In this research paper, we are interested in improving understanding of the retention mechanisms participating in MM-HPLC. For this purpose, we selected one bimodal stationary phase: a weak cation-exchange (WCX)/RPLC stationary phase. First, we explored the effects of buffer pH from 5 to 7 and ionic strength of the buffer, varied from 20 to 100 mM with a selection of 15 small molecules probes to find condi- tions ensuring both retention and good peak shapes. Second, we sought to understand the retention mechanisms involved in this column depending on mobile phase composition. In this aim, column characterization was achieved with quantitative structure–retention relationships (QSRRs). Using the retention data acquired for a set of 63 probe compounds with varied isocratic conditions (proportion of acetonitrile ranging from 30 to 80%), the interaction capabilities of the stationary phase were evaluated with linear solvation energy relationships (LSER) with the modified solvation parameter model using five Abraham descriptors [16] and two additional descriptors to take account of ionic interactions [17, 18], as described in the experimental section. These experiments served to determine the dominant retention mechanism prevailing in each mobile phase condition and to determine optimum conditions to retain analytes with diverse characteristics. Finally, temperature effects between 20 and 40 °C were also observed to measure the changes in enthalpy and entropy occurring when mobile phase composition is varied.

Materials and methods

Chemicals

The solvent used was HPLC grade acetonitrile (ACN) provided by VWR (Fontenay-sous-Bois, France). Ammonium acetate was provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France) and ultra-pure water was obtained from an Elga UHQ system from Veolia (Wissous, France). Ammonium acetate buffer was prepared according to the instructions of PhoEBus, an application program aid for buffer preparation (Analis, Namur, Belgium). For improved reproducibility, after preparation of the recommended mixture of ammonium acetate salt and acetic acid solution, the buffer ${}^{w}_{w}$pH was always verified and if necessary, adjusted. Then, the buffer-acetonitrile ${}^{S}_{w}$pH was measured, with the pH meter calibrated in aqueous buffers. For instance, when mixing the ${}^{w}_{w}$pH 5 buffer to 30% ACN the resulting ${}^{S}_{w}$pH was 5.7. When the percentage of ACN increased to 80%, the resulting ${}^{S}_{w}$pH was 7.0. In the calculations of analyte charges, D descriptors and log$D$ values, only the ${}^{S}_{w}$pH value was employed.

For the investigation of mobile phase effects, 63 test compounds (Table 1) were obtained from a range of suppliers. A subset of 15 compounds (marked with an asterisk in Table 1) served for preliminary investigations of buffer pH and concentration and for thermodynamic studies. The Abraham solute descriptors (E, S, A, B, V) used for QSRRs were extracted from an in-house database established from all the available literature on the solvation parameter model, and based on calculations with the Absolv module in ACD I-lab (https://www.ilab.acdlabs.com/iLab2/index.php). For the purpose of simplicity, the two additional descriptors (D^- and D^+) and log$D$ values were calculated based on apparent ${}^{S}_{w}$pH and aqueous pK_a values determined with Chemicalize (http://www.chemicalize.org/). This may seem a rough approximation, but retention prediction was not an objective, only some understanding of the retention mechanisms. The series of test compounds has been selected by observing the requirements of a good QSRR analysis [19]. The compounds were chosen so as to provide a uniform distribution of each descriptor within a wide enough space (see Figure S1 in supplementary material) and the absence of cross-correlation among the descriptors was checked. Some correlation was observed between E and S, as is usual when only aromatic species are present in the test set. No aliphatic analytes were introduced in this set to break the correlation, for detection reasons as only UV detection was available. Another typical (but moderate) correlation is that observed between the S and B descriptors. Diversity in the polarity range may also be appreciated through log$P$ values (Table 1) ranging from

Table 1 Chromatographic solutes and descriptors

No.	Compounds	*E*	*S*	*A*	*B*	*V*	D^- (5.7)	D^+ (5.7)	D^- (7.2)	D^+ (7.2)	Acid pK_a	pK_a	Log *P*
1	Naphthalene	1.340	0.92	0.00	0.20	1.0854	0.00	0.00	0.00	0.00			3.0
2	Anthracene	2.290	1.34	0.00	0.26	1.4540	0.00	0.00	0.00	0.00			4.0
3	Ethylbenzene	0.613	0.51	0.00	0.15	0.9982	0.00	0.00	0.00	0.00			2.9
4	Pentylbenzene	0.594	0.51	0.00	0.15	1.4209	0.00	0.00	0.00	0.00			4.7
5	Nitrobenzene	0.871	1.11	0.00	0.28	0.8906	0.00	0.00	0.00	0.00			1.9
6	Benzyl alcohol	0.803	0.87	0.39	0.56	0.9160	0.00	0.00	0.00	0.00	14.9		1.2
7	Uracil	0.810	1.00	0.44	1.00	0.7516	0.00	0.00	0.00	0.00	9.8; 13.8		− 0.9
8	Benzamide	0.990	1.50	0.49	0.67	0.9728	0.00	0.00	0.00	0.00	14.6	− 0.4	0.8
9	Benzenesulfonamide	1.130	1.55	0.55	0.80	1.0971	0.00	0.00	0.00	0.00	10.2		0.6
10	*p*-Toluenesulfonamide	1.100	1.55	0.55	0.87	1.2380	0.00	0.00	0.00	0.00	10.5		1.1
11	Phenylurea	1.110	1.40	0.77	0.77	1.0730	0.00	0.00	0.00	0.00	13.8		0.9
12	Carbazole	1.787	1.42	0.47	0.26	1.3150	0.00	0.00	0.00	0.00	15.0		3.1
13	Indazole	1.180	1.25	0.54	0.34	0.9050	0.00	0.00	0.00	0.00	12.2	1.2	1.3
14	Cytidine	2.090	2.21	0.87	2.62	1.6234	0.00	0.00	0.00	0.00	12.6; 13.9; 14.8	0.1	− 2.8
15	Cytosine	1.430	1.90	0.60	1.02	0.7927	0.00	0.00	0.00	0.00	10.0	2.4	− 1.2
16	Caffeine*	1.500	1.60	0.00	1.35	1.3630	0.00	0.00	0.00	0.00		− 0.9	− 0.6
17	Theobromine	1.500	1.60	0.50	1.38	1.2223	0.00	0.00	0.01	0.00	9.3		− 0.8
18	Theophylline	1.500	1.60	0.54	1.34	1.2223	0.01	0.00	0.19	0.00	7.8		− 0.8
19	Phenol*	0.805	0.89	0.60	0.30	0.7751	0.00	0.00	0.00	0.00	10.0		1.7
20	Resorcinol	0.980	1.00	1.10	0.58	0.8340	0.00	0.00	0.01	0.00	9.2; 10.7		1.4
21	α-Naphthol	1.520	1.05	0.61	0.37	1.1441	0.00	0.00	0.00	0.00	9.6		2.7
22	Eugenol	0.946	0.99	0.22	0.51	1.3540	0.00	0.00	0.00	0.00	9.9		2.6
23	Vanillin	1.040	1.04	0.32	0.67	1.1313	0.01	0.00	0.20	0.00	7.8		1.2
24	Paracetamol*	1.060	1.63	1.04	0.86	1.1724	0.00	0.00	0.01	0.00	9.5		0.9
25	Methylparaben*	0.900	1.37	0.69	0.45	1.1313	0.00	0.00	0.05	0.00	8.5		1.7
26	Ethylparaben*	0.860	1.35	0.69	0.45	1.2722	0.00	0.00	0.05	0.00	8.5		2.0
27	Propylparaben*	0.860	1.35	0.69	0.45	1.4131	0.00	0.00	0.05	0.00	8.5		2.5
28	Butylparaben*	0.860	1.33	0.71	0.46	1.5540	0.00	0.00	0.05	0.00	8.5		3.0
29	*o*-Nitrophenol	1.045	1.05	0.05	0.37	0.9490	0.11	0.00	0.79	0.00	6.6		1.6
30	Benzoic acid*	0.730	0.90	0.59	0.40	0.9317	0.98	0.00	1.00	0.00	4.1		1.6
31	2-Phenylpropionic acid	0.730	0.97	0.57	0.68	1.2135	0.93	0.00	1.00	0.00	4.6		2.2
32	2-Phenylbutyric acid	0.750	1.07	0.57	0.48	1.3544	0.91	0.00	1.00	0.00	4.7		2.6
33	1-Naphthylacetic acid	1.300	1.35	0.54	0.40	1.3007	0.90	0.00	1.00	0.00	4.8		2.6
34	2-Naphthylacetic acid	1.260	1.35	0.54	0.40	1.4416	0.89	0.00	1.00	0.00	4.8		2.6
35	2-Naphthoic acid	1.460	1.15	0.61	0.44	1.3007	0.98	0.00	1.00	0.00	4.0		2.6
36	2-Biphenylcarboxylic acid	1.536	1.49	0.59	0.59	1.5395	0.99	0.00	1.00	0.00	3.7		3.3

Table 1 (continued)

No.	Compounds	*E*	*S*	*A*	*B*	*V*	D^- (5.7)	D^+ (5.7)	D^- (7.2)	D^+ (7.2)	Acid pK_a	pK_a	Log *P*
37	4-Biphenylcarboxylic acid	1.536	1.49	0.59	0.59	1.5395	0.98	0.00	1.00	0.00	4.1		3.3
38	4-Biphenylacetic acid	1.480	1.35	0.59	0.58	1.4836	0.91	0.00	1.00	0.00	4.7		3.3
39	Diphenylacetic acid	1.317	1.38	0.59	0.67	1.6804	0.95	0.00	1.00	0.00	4.4		3.3
40	3,3-Diphenylpropionic acid	1.317	1.38	0.59	0.67	1.8213	0.91	0.00	1.00	0.00	4.7		3.5
41	Ferulic acid	1.110	1.46	0.85	0.87	1.4288	0.99	0.00	1.00	0.00	3.7		1.7
42	*o*-Coumaric acid	1.130	1.39	1.07	0.79	1.2292	0.98	0.00	1.00	0.00	4.0		1.8
43	*p*-Coumaric acid	1.130	1.39	1.07	0.79	1.2292	0.97	0.00	1.00	0.00	4.2; 9.5		1.8
44	Ibuprofen*	0.730	0.59	0.59	0.81	1.7771	0.86	0.00	1.00	0.00	4.9		3.8
45	Salicylic acid*	0.890	0.84	0.71	0.38	0.9904	1.00	0.00	1.00	0.00	2.8; 13.2		2.0
46	Barbituric acid	1.090	1.19	0.46	1.16	0.8103	0.99	0.00	1.20	0.00	3.9; 7.8; 11		− 1.3
47	Tryptophan*	1.620	1.80	1.09	1.26	1.5433	1.00	1.00	1.00	0.99	2.5	9.4	− 1.1
48	Tyrosine	1.180	1.60	1.28	1.29	1.3720	1.00	1.00	1.00	0.99	2; 9.8	9.2	− 1.5
49	2,2′-Bipyridyl	1.384	1.33	0.00	0.81	1.2420	0.00	0.00	0.00	0.00		3.3; 0.5	2.0
50	Aniline*	0.955	0.96	0.26	0.50	0.8162	0.00	0.07	0.00	0.00		4.6	1.1
51	*N*-Methylaniline	0.948	0.90	0.17	0.43	0.9570	0.00	0.09	0.00	0.00		4.7	1.5
52	*o*-Toluidine	0.966	0.92	0.23	0.45	0.9570	0.00	0.06	0.00	0.00		4.5	1.7
53	*m*-Toluidine	0.946	0.95	0.23	0.55	0.9570	0.00	0.14	0.00	0.00		4.9	1.7
54	*p*-Toluidine	0.923	0.95	0.23	0.52	0.9570	0.00	0.17	0.00	0.01		5.0	1.7
55	Pyridine	0.631	0.84	0.00	0.52	0.6753	0.00	0.20	0.00	0.01		5.1	0.8
56	2-Ethylpyridine	0.613	0.70	0.00	0.49	0.9570	0.00	0.44	0.00	0.02		5.6	1.6
57	2-Aminopyridine	0.980	1.10	0.32	0.63	0.7751	0.00	0.93	0.00	0.28		6.8	0.5
58	4-Aminopyridine	0.900	1.21	0.23	0.71	0.7751	0.00	1.00	0.00	0.98		9.0	− 0.1
59	1-Naphthylamine	1.670	1.26	0.20	0.57	1.1850	0.00	0.02	0.00	0.00		4.1	2.1
60	2-Naphthylamine	1.670	1.28	0.22	0.57	1.1850	0.00	0.03	0.00	0.00		4.3	2.1
61	Lidocaine*	1.010	1.50	0.12	1.21	2.0589	0.00	0.99	0.00	0.80		7.8	2.8
62	Procaine*	1.135	1.68	0.44	1.23	1.9767	0.00	1.00	0.00	0.98		2.7; 9	1.9
63	Nicotine*	1.050	1.09	0.00	1.11	1.3710	0.00	1.00	0.00	0.98		2.7; 8.9	1.2

E excess molar refraction, *S* dipolarity/polarizability, *A* hydrogen bond acidity, *B* hydrogen bond basicity, *V* McGowan's characteristic volume, D^- negative charge at pH 5,6 or 7, D^+ positive charge at pH 5, 6 or 7

Analytes marked with an asterisk are those included in the short selection for assessment of pH, salt concentration and temperature

– 2.8 to 4.7 or through log*D* values (Figure S1). Depending on the $^{S}_{w}$pH of the mobile phase, the estimated log*D* values then ranged from − 5.2 to 4.7 (mobile phase $^{S}_{w}$pH 5.7) or from − 6.7 to 4.7 (mobile phase $^{S}_{w}$pH 7.2).

Chromatographic System and Conditions

The stationary phase selected for this study was a bimodal phase, Acclaim mixed-mode WCX-1 LC (150 × 3.0 mm, 3.0 µm) from Thermo Fisher Scientifif (Villebon-sur-Yvette, France). This silica-based stationary phase features a hydrophobic alkyl chain with a carboxyl terminus that provides both hydrophobic reversed-phase and weak cation-exchange properties. The column can be used at pH values ranging from 2.5 to 7, with temperatures up to 50 °C and pressures up to 400 bar.

The chromatographic system was equipment manufactured by Agilent (Les Ulis, France). The HPLC system was equipped with a quaternary solvent delivery pump compatible with mobile phase flow rates up to 10 mL min^{-1} and pressures up to 400 bar (Agilent 1100 Series G1311A), an autosampler (Agilent 1100 Series G1313A ALS), a degasser pump (Agilent 1100 Series G1379A), a column compartment (Agilent 1100 Series G1316A) compatible with 150 mm length columns and temperatures from 10 to 80 °C and a diode array detector (Agilent 1100 Series G1315B). The detection wavelength was 210 nm for all probe compounds. Thermo Xcalibur software (V3.0.63) was used for system control, data acquisition and treatment. The mobile phase used in this study was always a mixture of acetonitrile and ammonium acetate buffer in various proportions. Elution conditions were isocratic in all cases. The flow rate was 0.5 mL min^{-1}. Injection volume was 5 µL for all compounds.

For pH studies, the salt concentration in the aqueous phase was 100 mM, ACN percentage was 30%, and the oven temperature was set at 30 °C. The $^{w}_{w}$pH was 5, 6 or 7, resulting in measured $^{S}_{w}$pH 5.7, 6.5 and 7.2, respectively.

For ionic strength studies, aqueous phase $^{w}_{w}$pH was 7, ACN percentage was 30%, and the oven temperature was set at 30 °C. The salt concentration was 20, 40, 60, 80 or 100 mM. The resulting measured $^{S}_{w}$pH was always 7.2.

For the ACN percentage studies, the aqueous phase $^{w}_{w}$pH was 5, the salt concentration in the aqueous phase 100 mM, and the oven temperature was set at 30 °C. The ACN percentage was 30, 50, 70 or 80%. The measured $^{S}_{w}$pH was 5.7, 6.0, 6.6 and 7.0, respectively.

Finally, for temperature studies, the aqueous phase $^{w}_{w}$pH was 7 (measured $^{S}_{w}$pH was 7.2), ACN percentage was 30%, and the salt concentration in the aqueous phase was 60 mM. The oven temperature was set at 20, 25, 30, 35 or 40 °C.

Data Analysis

Retention factors (*k*) were calculated based on the retention time t_R, determined using the peak maximum and on the hold-up time t_0 measured as the first baseline disturbance. Multilinear regressions were performed using XLStat2017 software, version 19.03.44850 (Addinsoft, New York, NY, SA). The quality of the QSRR fits was estimated using the adjusted determination coefficient (R^2_{adj}) and standard error in *the* estimate (SE). In certain conditions, the retention of some analytes was too low (elution in dead volume) thus they were excluded from the calculations. In all cases, a sufficient number of analytes with adequate diversity was retained in the fi set to ensure that meaningful models would be obtained. In addition, the coefficients must make chemical sense. Values of the system constants must be both large and significantly larger than their standard deviation.

QSRR models were established according to Eq. (1):

$$\log k = c + eE + sS + aA + bB + vV + d^{-}D^{-} + d^{+}D^{+}. \quad (1)$$

In this equation, capital letters represent the solute descriptors, related to particular interaction properties, while lower case letters represent the system constants, related to the complementary effect of the phases on these interactions. *c* is the model intercept term and is dominated by the phase ratio. *E* is the excess molar refraction (calculated from the refractive index of the molecule) and models polarizability contributions from *n* and *π* electrons; *S* is the solute dipolarity/polarizability; *A* and *B* are the solute overall hydrogen bond acidity and basicity, respectively; *V* is the McGowan characteristic volume; D^{-} represents the (partial or total) negative charge carried by anionic and zwitterionic species, and D^{+} represents the (partial or total) positive charge carried by cationic and zwitterionic species.

Other descriptors, named J^{-} and J^{+}, were developed by Abraham and Acree [20] to take account of ionic interactions. However, these are suitable to compounds in their fully ionized form, while in the present study, we aimed at varying operating conditions, which should result in intermediate ionization states.

The system constants (e, s, a, b, v, d^{-}, d^{+}), obtained through a multilinear regression of the retention data for a certain number of solutes with known descriptors, reflect the magnitude of difference for that particular property between the mobile and stationary phases. Thus, if a particular coefficient is numerically large, then any solute having the complementary property will interact strongly with either the mobile phase (if the coefficient is negative) or the stationary phase (if the coefficient is positive). The system constant is thus related to the interaction capabilities of the chromatographic systems controlling the retention and selectivity. Exhaustive information on the solvation parameter model

and its application to the characterization of chromatographic systems can be found elsewhere [19, 21].

Results and Discussion

Preliminary Experiments to Define Optimal Buffer pH

Because the bimodal stationary phase possesses a weak cation-exchange group (carboxylic function), the influence of mobile phase apparent ${}^{S}_{w}$pH was examined. To determine the optimum pH to obtain a sufficient retention of polar cationic species, i.e., the pH which makes possible to fully utilize the ionic interactions with the carboxylate groups, three different values of aqueous phase ${}^{w}_{w}$pH were tested: 5, 6 and 7. The measured ${}^{S}_{w}$pH values in the buffer-acetonitrile (70:30 v/v) mixtures were 5.7, 6.5 and 7.2, respectively. Note that, at ${}^{w}_{w}$pH 7, ammonium acetate is not acting as a buffer. However, we found it was preferable to maintain the same salt throughout all experiments, regardless of the buffer strength.

Naturally, it was anticipated that switching from a mobile phase ${}^{S}_{w}$pH 5.7–7.2 should allow for a greater deprotonation of the carboxylic acid groups to carboxylate groups. Anionic terminal functions favor the retention of protonated bases through coulombic attraction, thus the retention of basic compounds with moderate and high pK_a values should increase. The bases with the highest pK_a values (e.g., 4-aminopyridine, with an aqueous pK_a value of 9.3), expected to be fully protonated in the range of pH investigated, showed a continuous increase of retention (Fig. 1a), indicating that the carboxylic groups must be increasingly deprotonated. The bases with lower pK_a values (e.g., 2-aminopyridine with an aqueous pK_a value of 6.9) showed lower slopes when the apparent pH approached their pK_a values (Fig. 1a), as the basic analyte would be expected to deprotonate thus return to a neutral form.

On the contrary, anionic compounds such as deprotonated acids should experience electrostatic repulsion between their negative charges and the negative charges carried by carboxylate groups of the stationary phase, resulting in decreasing retention. Some of the acidic compounds in our set had aqueous pK_a values below 4 (for instance, salicylic acid with an aqueous pK_a value of 3.0 or barbituric acid with an aqueous pK_a value of 4.0), thus the acid function can reasonably be expected to be deprotonated in all conditions investigated. Indeed, only moderate retention decrease was observed (Fig. 1b). For other probe compounds, having pK_a values slightly larger (typically in the 4.5–5.0 range), the retention decreases between apparent pH 5.2 and 6.5 was sharp (Fig. 1b), as it must result from the combined effect of deprotonation of the analyte function and deprotonation of the carboxylate group. Consequently, the interaction between a neutral analyte and a neutral stationary phase is progressively changed for repulsion between two anionic groups. Further apparent pH increase (up to 7.2) caused less significant variation.

At the highest pH values, hydrophobic acids could still be sufficiently retained, but hydrophilic acids were now eluted even closer to dead time.

Naturally, neutral species (compounds with zero or near-zero charge in all conditions) showed no significant retention change.

From the above observations, we can conclude that larger (but moderate) pH values should favor the retention of basic compounds, but may be deleterious to the retention of hydrophilic acidic species on this stationary phase.

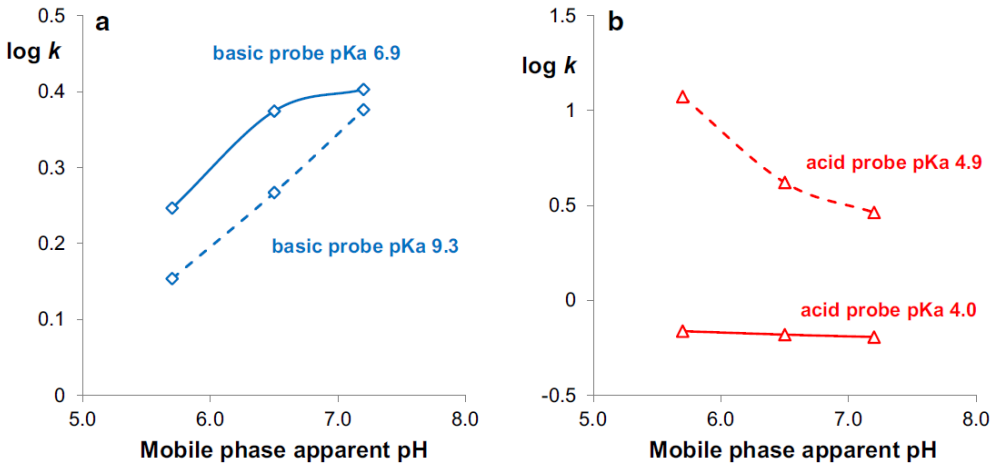


Fig. 1 Influence of mobile phase apparent pH on retention of different probe compounds: **a** basic compounds and **b** acidic compounds. Chromatographic conditions: acetonitrile/ammonium acetate buffer pH 5, 6 or 7: 30/70, 30 °C, 0.425 mL min^{-1}

Contribution of Ion-Exchange Mechanism with Different Salt Concentrations

Second, we investigated the influence of ionic strength. Five levels of ammonium acetate buff concentrations were tested, ranging from 20 to 100 mM. As the buffer was mixed to 30% acetonitrile, the overall concentration actually ranged from 14 to 70 mM. Judging from the previous observations, the buffer $^{w}_{w}$pH was adjusted to 7 (the measured mobile phase $^{S}_{w}$pH values were equal to 7.2 in all cases, whatever the buffer concentration). The effect on retention was predictable, but the peak shape was also of interest at this stage.

Examples of retention behaviors are provided in Fig. 2 where it appears that a weak base (aniline) that should not be ionized in these operating conditions and an ionizable molecule with charge-neutral state (tryptophan) are unaffected by salt concentration, while three strong bases that should be ionized (singly charged) at these operating conditions have decreased retention with increased concentration of counter-ion (ammonium). As expected, acidic species faced a retention increase. These observations are in accordance with previous reports [22], and with previous observations in HILIC chromatography on a zwitterionic stationary phase with terminal sulfonic group [17]. Additionally, the curves of retention factors (k) for basic species plotted against the inverse concentration of buff salt (between 20 and 80 mM) could be well fitted by linear trends, as is typical of ion-exchange retention mechanisms. The points at higher concentration of buff (100 mM) deviated from the trend line. According to the stoichiometric displacement model involving both the reversed-phase and ion-exchange mechanisms, for singly charged basic compounds, on a mixed-mode stationary phase combining hydrophobic ligands and ion-exchange groups, using a cationic displacer bearing a single charge at concentration C, the total retention factor (k_{total}) can be described as [15, 23]

$$k_{total} = k_{RP} + k_{IEX} = k_{RP} + \frac{B_{IEX}}{C}, \quad (2)$$

where k_{IEX} is the contribution of ion-exchange to total retention and the slope B_{IEX} is a measure of the strength of the ion-exchange interaction. k_{RP} is the contribution of reversed-phase to retention, and is measured at infinite concentration of the cationic displacer, thus in conditions where ion-exchange should not occur anymore. The values of k_{RP} and B_{IEX} can be determined from the linear regression equations in Fig. 2. From these values, the relative contributions of reversed-phase and ion-exchange mechanisms depending on buff salt concentration are calculated (Table 2). In these conditions, at the lowest concentration of ammonium tested (20 mM in aqueous phase, corresponding to 14 mM overall concentration), the contribution of ion-exchange to the retention of these three bases was about 70%. In the conditions of the average concentration tested (60 mM in aqueous phase,

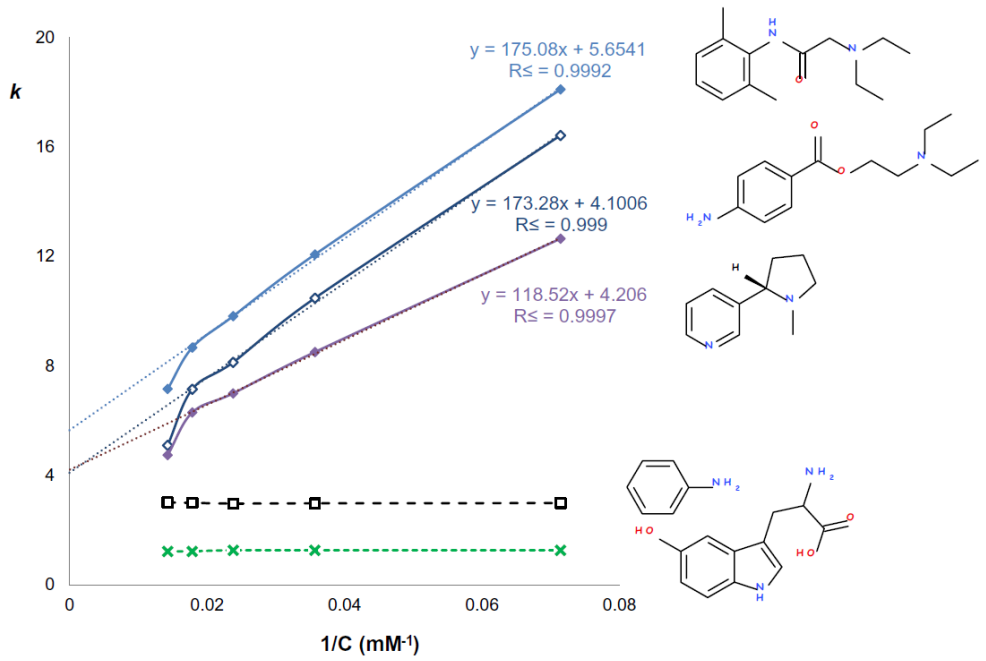


Fig. 2 Plots of retention factor (k) vs. the inverse concentration of ammonium acetate buffer in the mobile phase (overall concentration varying from 14 to 70 mM) for five species: a non-ionized weak base (aniline), an ionizable but charge-neutral molecule (tryptophan) and three ionized bases (lidocaine, procaine and nicotine, from top to bottom). Other chromatographic conditions: acetonitrile/ammonium acetate buffer pH 7: 30/70, 30 °C, 0.425 mL min^{-1}

Table 2 Relative contributions of reversed-phase (k_{RP}) and ion-exchange (k_{IEX}) mechanisms to the total retention (k_{total}) of strong bases as a function of ammonium concentration (C) according to Eq. (2)

	Lidocaine			Procaine			Nicotine		
k_{RP}	5.65			4.10			4.21		
B_{IEX}	175.08			173.28			118.52		
C (mM)	k_{total}	k_{IEX}	IEX %	k_{total}	k_{IEX}	IEX %	k_{total}	k_{IEX}	IEX %
70	7.16	1.51	21	5.10	1.00	20	4.75	0.54	11
56	8.67	3.02	35	7.15	3.05	43	6.30	2.09	33
42	9.82	4.17	42	8.13	4.03	50	7.00	2.79	40
28	12.08	6.42	53	10.48	6.38	61	8.51	4.30	51
14	18.10	12.45	69	16.42	12.32	75	12.65	8.44	67

corresponding to 42 mM overall concentration), the contribution of ion-exchange was still about 40–50%.

To conclude on this section, a low concentration of salts should then be favored to improve the retention of basic compounds, but again this may be detrimental to the retention of polar acidic compounds. Unfortunately, a low salt concentration (20 or 40 mM in buffer, corresponding to 14 or 28 mM overall concentration) was accompanied by a significant peak deformation. As a result, a compromise buffer concentration of 60 mM (42 mM overall concentration) could be advocated to maintain adequate retention of basic compounds without degrading too much the peak symmetry.

Variation of Retention Mechanisms with Different Proportions of Organic Solvent

Third, we investigated the influence of the mobile phase composition, with proportions of acetonitrile increasing from 30 to 80%. Our intention in this set of experiments was to observe the changes of interactions in the chromatographic system during a gradient elution, to experience whether the different retention modes (reversed-phase and ion-exchange) would be affected in the same manner. For this set of experiments, the buffer ${}^{w}_{w}$pH was adjusted to 5 and the buffer concentration was 100 mM. We reasoned that both (1) the mobile phase ${}^{S}_{w}$pH and (2) the concentration of buffer salt would vary when the proportion of acetonitrile is increased from 30 to 80%. Indeed, when the percentage of acetonitrile is 30%, the mobile phase ${}^{S}_{w}$pH will be 5.7, and the overall salt concentration 70 mM. When the percentage of acetonitrile is 80%, the mobile phase ${}^{S}_{w}$pH will be 7.0, and the overall salt concentration 20 mM. From the above observations, we can conclude that both parameters (pH increase and salt concentration decrease) will have the same effects: no changes on the retention of neutral species, increased retention of cations (basic compounds) and decreased retention of anions (acidic compounds).

Simply observing the retention curves (not shown) for the probe compounds was rather confusing as different trends were observed that could not be simply related to polarity or charge state of the analyte. Although extreme mobile phase compositions were not included (with acetonitrile percentage remaining between 30 and 80%), none of the curves could be adequately fitted with a linear trend line. However, most of them were very well fitted by a second order polynomial curve with convex function.

To gain some understanding of retention mechanisms, linear solvation energy relationships (LSER) were established based on Eq. (1), as described in experimental section. Previous works have described the use of LSER to characterize mixed-mode stationary phases combining hydrophobic ligands and ion-exchange groups [15, 24, 25], but the authors had used the classical equation based on fi e Abraham descriptors, thus could not characterize the ionic interactions. In the present case, two models were calculated for each condition. The first one was based on the usual equation with five Abraham descriptors (E, S, A, B and V) and the retention data for molecules that were neutral in all conditions (D^- and D^+ equal to zero in all four conditions). The second one was based on the augmented equation comprising seven descriptors (E, S, A, B, V, D^- and D^+) and the whole set of retention data.

The results of the solvation parameter models are presented in Fig. 3 and Table S1 in supplementary material. The fits were all of reasonable quality, R^2_{adj} ranging from 0.79 to 0.96 and standard error of estimate varying from 0.02 to 0.16. Although the quality of the fits is not very high and would be insufficient for retention prediction (which was not desired), we consider these results as good enough to allow for interpretation of retention mechanisms. Basically, a R^2 value equal to 0.80 indicates that 80% of the variance is explained by the model, which should be good enough to gain some understanding of the retention mechanisms. As it was never statistically significant, the a term will never be discussed in the following. Indeed, the standard deviation of this system constant was always larger than the value of the system constant itself.

First, it can be observed with the system maps on Fig. 3a, b that the reduced dataset (neutral species) or extended

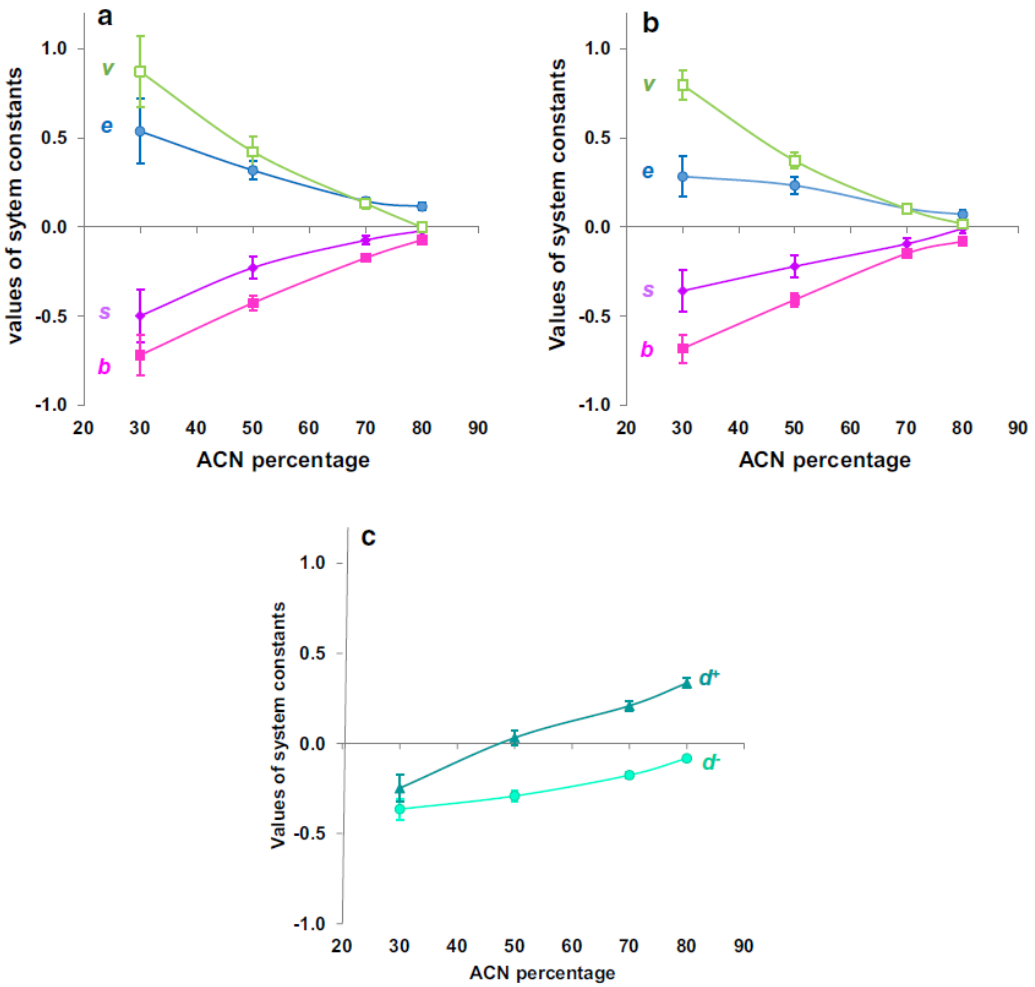


Fig. 3 System maps for normalized LSER models calculated with the retention data measured for the 63 species in Table 1. **a** System constants for charge-neutral species with only neutral species included in model calculation, **b** system constants for charge-neutral species with ionizable species included in model calculation, **c** additional system constants for ionizable species. Chromatographic conditions: acetonitrile/ammonium acetate buffer (100 mM) pH 5 30/70 (v/v), 50/50 (v/v), 70/30 (v/v) and 80/20 (v/v), 30 °C, 0.425 mL min^{-1}

dataset (neutral and ionizable species) result in identical trends for the system constants related to non-ionic interactions (*e*, *s*, *b*, *v*). The most significant difference is observed for the *e* term at 30% ACN, where the reduced dataset contained only 17 molecules, resulting in large error bars.

The variation of system constants (Table S1 and Fig. 3) over the range of mobile phase composition studied shows a clear change in retention mechanism. With a low percentage of acetonitrile in the mobile phase (30%), we mainly observe the reversed-phase retention mechanism, with a pattern that is quite characteristic of reversed-phase systems previously characterized with the solvation parameter model [21]. Indeed, the values for the system constants related to polar and ionic interactions (*s*, *b*, d^- and d^+) were negative while the system constants related to non-polar interactions (*e* and v were positive. The most significant terms were the large and positive *v* term (resulting from water cohesiveness and dispersive interactions with alkyl chains of the stationary phase) and the large and negative *b* term (resulting from hydrogen-bonding properties of water). It may be noted, however, that the absolute values of *b* and *v* are much lower than is usually observed in reversed-phase HPLC systems. This is probably due to the polarity of the stationary phase, resulting from the terminal carboxylic function of the alkyl chains. This is in line with previous models computed by Ali and Poole [26] on polar stationary phases (aminopropyl, cyanopropyl, propanediol) where the signs of system constants were in accordance with a reversed-phase mechanism but lower-than-usual values for *b* and *v* terms (typically 0.5–1.0 instead of 2.0–3.0).

When the percentage of acetonitrile increased, the *e* and *v* coefficients remained positive. The *e* term decreased moderately while the *v* term strongly decreased, down to a non-significant value, reflecting a lower retention of hydrophobic compounds. Conversely, the values of polar interactions system constants *s* and *b* increased but remained

negative. The changes observed for the system constants relating non-ionic interactions (*e*, *s*, *b* and *v*, Fig. 3a, b) are typically observed in system maps established in RPLC [21]. Actually, decreasing the proportion of aqueous buffer in the mobile phase always results in strong reduction of the mobile phase hydrogen-bonding capabilities (increase in the negative *b* term) and strong reduction of the mobile phase cohesiveness, resulting in lessened cavity energy (decrease in the positive *v* term).

Regarding interactions with ionic species (Fig. 3c), the d^+ term increased strongly, changing from negative to positive values. The d^- term also increased with a lower slope, and always remained negative. In other words, the absolute value of the d^- term decreased. Thus, polar neutral species were more and more retained when acetonitrile percentage increased, while polar ionic species faced a stronger retention increase. The anionic compounds remained moderately retained over the whole range of compositions tested (negative value for d^-).

With the highest percentage of acetonitrile (80%), the retention mechanism is rather far from typical reversed-phase, with a dominant d^+ term, indicating that ion-exchange mechanism was now the most significant. It is therefore in these conditions that we would expect the highest retention of basic compounds, which are not enough retained on classical C18 phases with hydrophobic end-capping groups. The change of retention trends can also be observed in Fig. 4, where the retention factors of all probe molecules were plotted against log*D* values at the mobile phase $^{S}_{w}$pH. On Fig. 4a, the reversed-phase elution conditions at 30% acetonitrile provided a clear trend relating retention and polarity, i.e., the most polar species (low log*D* values) eluted faster than the least polar species (high log*D* values). Apart from a few analytes of low retention that did not fit well in the main tendency (among which were a few basic analytes, possibly experiencing some ionic interactions), most analytes were well distributed over the whole retention range. On Fig. 4b, the pattern is clearly different. First, the retention range for neutral and acidic species (black squares and red triangles) was much smaller due to larger elution strength of the mobile phase, but the basic compounds (blue diamonds) strongly deviated from the main cluster of points, as they were much more retained (compared to other species in the same conditions), through ionic attraction to the carboxylate groups. At the highest proportion of ACN, mobile phase pH was higher (7.0) and acidic species should be in the deprotonated form, yielding lower log*D* values for this group of analytes than for the neutral species. On the contrary, the weak bases had higher log*D* values at the higher pH, as protonation should be lessened when pH increases.

In comparison, in HILIC chromatography, the most polar compounds are most retained, while the least polar compounds are eluted faster, thus a negative relation of log *k* and log*D* is usually observed [18]. It is interesting to note that no HILIC mechanism can be assumed here, as the tendency curve between retention factors and log*D* values was not the reverse of that in reversed-phase conditions, as only basic compounds were strongly retained, not all polar species. The retention mechanism is thus simply cation-exchange and not HILIC.

In conclusion, with this bimodal column there are two possible retention mechanisms: reversed-phase mechanism is dominant when the proportion of acetonitrile is low, while cation-exchange becomes dominant when the proportion of acetonitrile is high. However, it appears that these two mechanisms cannot be fully used simultaneously, as they depend on the percentage of organic solvent in the mobile phase.

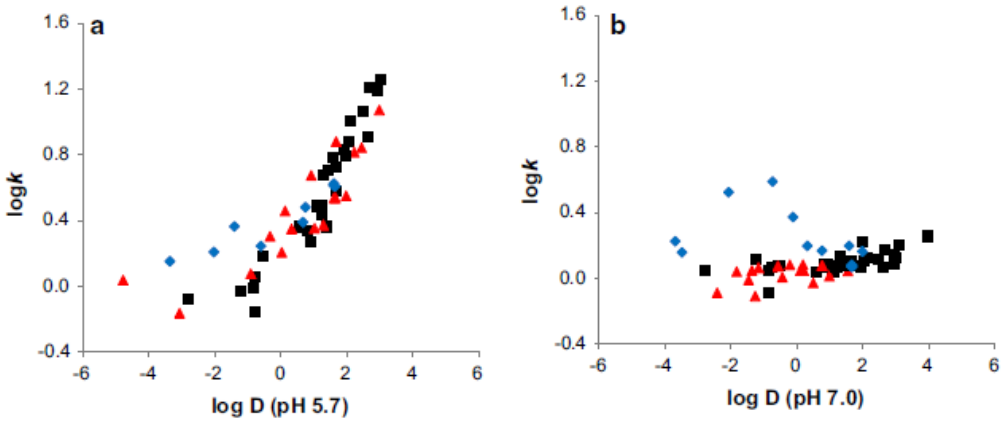


Fig. 4 Relation between chromatographic retention on the bimodal stationary phase and the octanol–water partition coefficient log*D* in two different mobile phase conditions. Chromatographic conditions: acetonitrile/ammonium acetate buffer (100 mM) pH 5 **a** 30/70 (v/v), and **b** 80/20 (v/v), 30 °C, 0.425 mL min^{-1}. The mobile phase pH was measured: 5.7 in conditions (**a**) and 7.0 in conditions (**b**). Identification of points: black squares are neutral species, red triangles are acids in the anionic form, blue diamonds are bases in the cationic form

Effects of Oven Temperature

The effects of temperature variation were investigated with isocratic conditions, buffer pH set at 7, buffer concentration 60 mM and acetonitrile percentage 30 and 60%. Five levels of temperature were tested, between 20 and 40 °C, with 5 °C increments. This temperature range may be considered as restricted, but further increases of temperature are not recommended on this stationary phase. Only the subset of 15 probe compounds (identified by an asterisk in Table 1) was used for these experiments. Van't Hoff equation relates retention to temperature according to Eq. (3)

$$\ln(k) = -\frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{RT} + \ln(1/\beta), \qquad (3)$$

where $\Delta H°$ and $\Delta S°$ are the standard molar enthalpy and molar entropy of transferring solute from mobile phase to stationary phase and β is the phase ratio. Thus, ln(k) plotted against 1/T should provide straight lines.

Van't Hoff plots were thus plotted for all 15 probes. Sample curves for fi e species are presented in Fig. 5. No curves could be plotted for acidic species at 60% acetonitrile because they were eluted in or very near the dead volume.

With both mobile phase compositions (30 and 60% acetonitrile), all curves could be correctly fitted by linear tendency curves, although the fits at 60% acetonitrile were of inferior quality, probably due to the more complex retention mechanism. Indeed, the retention mechanism may not be perfectly invariant over the range of temperature explored, with either reversed-phase or ion-exchange mechanism being more or less prominent. The slopes were positive, indicating that the enthalpy of association with the stationary phase was negative, as is usually observed in reversed-phase chromatographic systems [27]. In other words, the solute transfer from the mobile phase to the stationary phase was favorable to all analytes in both sets of conditions. This indicates that a moderate temperature increase (40 °C) would favor faster elution of all analytes. However, the slopes of the curves varied significantly between the two conditions in a different way depending on analyte charge state. For neutral species (example of ethylparaben and phenol, with zero or near-zero charges in these conditions), the absolute value of $\Delta H°$ decreased when increasing acetonitrile percentage, while the contrary was observed for basic (example of lidocaine) and zwitterionic (example of tryptophan) species. This seems logical and in accordance with above observations as interaction energy between cationic species and the stationary phase increased (exoergic process) when acetonitrile percentage increased, while the endoergic solvophobic effect repulsing the hydrophobic analytes from the mobile phase should be reduced when the proportion of water decreases.

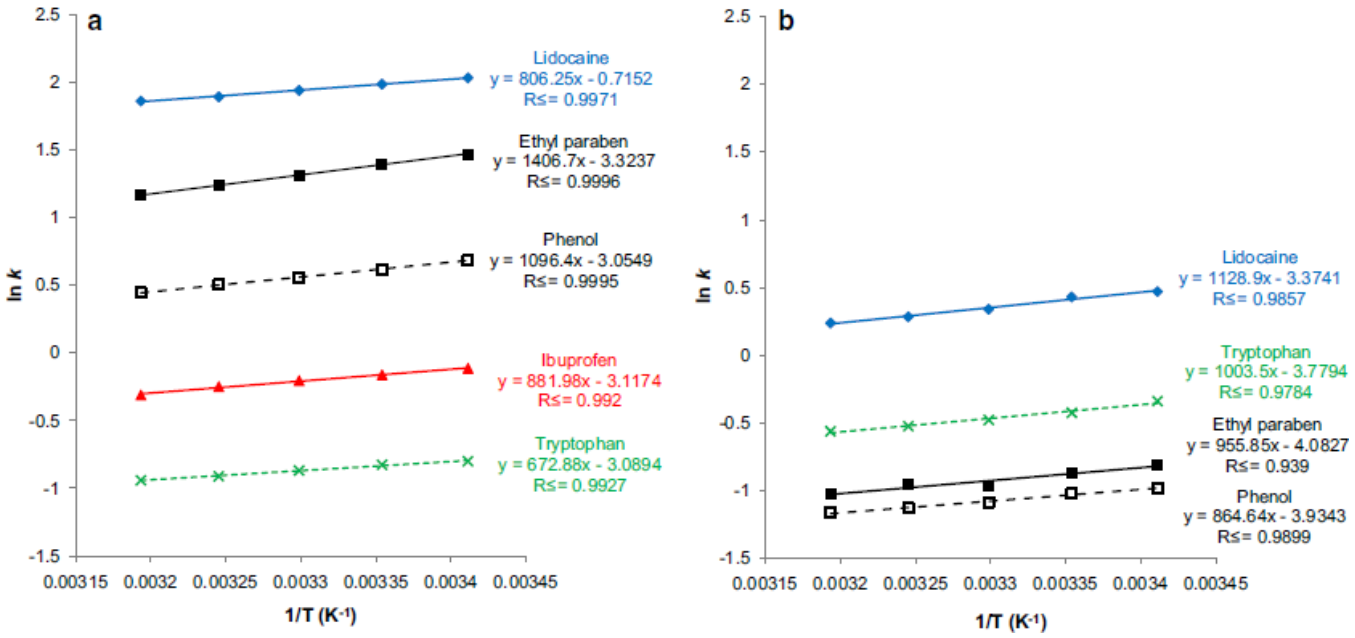


Fig. 5 Van't Hoff plots relating retention to temperature for five representative analytes: two neutral compounds (ethylparaben, black squares and phenol, open black squares), an acidic compound (ibuprofen, red triangles), a basic compound (lidocaine, blue diamonds) and a zwitterionic compound (tryptophan, green crosses). Chromatographic conditions: acetonitrile/ammonium acetate buffer (60 mM) pH 7 **a** 30/70 (v/v) or **b** 40/60 (v/v). Flow rate 0.425 mL min^{-1}

Conclusions

The characterization of the bimodal reversed-phase/ion-exchange stationary phase with extra-thermodynamic studies (modified LSER and Van't Hoff plots) together with assessment of the quantitative contributions of RP and IEX mechanisms provided some insights in the retention mechanisms involved in mixed-mode HPLC when mobile phase composition is changed. MM-HPLC provides unique flexibility because of the multiple retention mechanisms offered in one column. By adjusting the ratio of organic and aqueous phases and concentration of aqueous buffers, RPLC and ion-exchange modes can be successively used, with RPLC mechanism dominating the process at low percentage of acetonitrile, and ion-exchange being more prominent at high percentage of acetonitrile. Consequently, it appears that "mixed-mode" retention mechanism cannot be truly obtained in a single set of operating conditions. However, both modes may be encountered in the course of an elution gradient. Method development in MM-HPLC would then be improved when gradient elution is employed, to take full advantage of combined retention mechanisms.

The bimodal RP-WCX column allowed a greater retention of polar basic compounds which are poorly retained in RPLC on classical C18 stationary phases. However, this bimodal stationary phase did not retain the polar acidic compounds. Another column combining reversed-phase, anion-exchange and cation-exchange mechanisms may be beneficial in that case.

Acknowledgements Waters Corporation is warmly acknowledged for continuous support through the Centers of Innovation program. Warm thanks are also due to Thierry Domenger (Thermo Scientific) for the kind gift of columns. Miss Katarzyna Knobloch is acknowledged for technical assistance with HPLC experiments. CW acknowledges the support of the Institut Universitaire de France (IUF), of which she is a Junior member.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Petritis K, Brussaux S, Guenu S et al (2002) Ion-pair reversed-phase liquid chromatography–electrospray mass spectrometry for the analysis of underivatized small peptides. J Chromatogr A 957:173–185. https://doi.org/10.1016/S0021-9673(02)00372-2
2. Alpert AJ (1990) Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J Chromatogr A 499:177–196. https://doi.org/10.1016/S0021-9673(00)96972-3
3. Cummins P, Rochfort K, O'Connor B (2017) Ion-exchange chromatography: basic principles and application. In: Walls D, Loughran ST (eds) Protein chromatography. Springer, New York, pp 209–223
4. Grand-Guillaume Perrenoud A, Veuthey J-L, Guillarme D (2012) Comparison of ultra-high performance supercritical fl chromatography and ultra-high performance liquid chromatography for the analysis of pharmaceutical compounds. J Chromatogr A 1266:158–167. https://doi.org/10.1016/j.chroma.2012.10.005
5. Lemasson E, Bertin S, Hennig P et al (2016) Comparison of ultra-high performance methods in liquid and supercritical fluid chromatography coupled to electrospray ionization–mass spectrometry for impurity profiling of drug candidates. J Chromatogr A 1472:117–128. https://doi.org/10.1016/j.chroma.2016.10.045
6. Bischoff R, McLaughlin LW (1984) Nucleic acid resolution by mixed-mode chromatography. J Chromatogr A 296:329–337. https://doi.org/10.1016/S0021-9673(01)96427-1
7. Kennedy LA, Kopaciewicz W, Regnier FE (1986) Multimodal liquid chromatography columns for the separation of proteins in either the anion-exchange or hydrophobic-interaction mode. J Chromatogr 359:73–84
8. Halfpenny AP, Brown PR (1986) Mixed mode chromatography via column switching for the simultaneous HPLC analysis of lonic and non-lonic nucleic acid constituents. Chromatographia 21:317–320. https://doi.org/10.1007/BF02311602
9. Lemasson E, Bertin S, Hennig P et al (2017) Mixed-mode chromatography—a review. LC GC Eur 30:22–33
10. Qiu H, Mallik AK, Takafuji M et al (2012) New poly(ionic liquid)-grafted silica multi-mode stationary phase for anion-exchange/reversed-phase/hydrophilic interaction liquid chromatography. Analyst 137:2553–2555. https://doi.org/10.1039/C2AN35348B
11. Liu X, Pohl CA (2012) Comparison of reversed-phase/cation-exchange/anion-exchange trimodal stationary phases and their use in active pharmaceutical ingredient and counterion determinations. J Chromatogr A 1232:190–195. https://doi.org/10.1016/j.chroma.2011.12.009
12. Liu X, Tracy M, Aich U, Pohl C (2014) Exploring mixed-mode chromatography: column chemistry, properties, and applications. https://tools.thermofisher.com/content/sfs/posters/PN-20947-Mixed-Mode-Chromatography-HPLC-2014-PN20947-EN.pdf. Accessed 5 Apr 2017
13. Zhang K, Dai L, Chetwyn NP (2010) Simultaneous determination of positive and negative pharmaceutical counterions using mixed-mode chromatography coupled with charged aerosol detector. J Chromatogr A 1217:5776–5784. https://doi.org/10.1016/j.chroma.2010.07.035
14. Li J, Shao S, Jaworsky MS, Kurtulik PT (2008) Simultaneous determination of cations, zwitterions and neutral compounds using mixed-mode reversed-phase and cation-exchange high-performance liquid chromatography. J Chromatogr A 1185:185–193. https://doi.org/10.1016/j.chroma.2008.01.083
15. Zhang Y, Carr PW (2011) Novel ultra stable silica-based stationary phases for reversed phase liquid chromatography—study of a hydrophobically assisted weak acid cation exchange phase. J Chromatogr A 1218:763–777. https://doi.org/10.1016/j.chroma.2010.11.009
16. Abraham MH, Ibrahim A, Zissimos AM (2004) Determination of sets of solute descriptors from chromatographic measurements. J Chromatogr A 1037:29–47. https://doi.org/10.1016/j.chroma.2003.12.004
17. West C, Auroux E (2016) Deconvoluting the effects of buffer salt concentration in hydrophilic interaction chromatography on a zwitterionic stationary phase. J Chromatogr A 1461:92–97. https://doi.org/10.1016/j.chroma.2016.07.059

18. Chirita R-I, West C, Zubrzycki S et al (2011) Investigations on the chromatographic behaviour of zwitterionic stationary phases used in hydrophilic interaction chromatography. J Chromatogr A 1218:5939–5963. https://doi.org/10.1016/j.chroma.2011.04.002
19. Vitha M, Carr PW (2006) The chemical interpretation and practice of linear solvation energy relationships in chromatography. J Chromatogr A 1126:143–194. https://doi.org/10.1016/j.chroma.2006.06.074
20. Abraham MH, Acree WE (2016) Descriptors for ions and ion-pairs for use in linear free energy relationships. J Chromatogr A 1430:2–14. https://doi.org/10.1016/j.chroma.2015.07.023
21. Poole CF, Poole SK (2002) Column selectivity from the perspective of the solvation parameter model. J Chromatogr A 965:263–299. https://doi.org/10.1016/S0021-9673(01)01361-9
22. Liu X, Pohl C (2009) A weak cation-exchange, reversed-phase mixed-mode HPLC column and its applications. Am Lab 41:26–29
23. Bij KE, Horváth C, Melander WR, Nahum A (1981) Surface silanols in silica-bonded hydrocarbonaceous stationary phases: II. Irregular retention behavior and effect of silanol masking. J Chromatogr A 203:65–84. https://doi.org/10.1016/S0021-9673(00)80282-4
24. Lepont C, Poole CF (2002) Retention characteristics of an immobilized artificial membrane column in reversed-phase liquid chromatography. J Chromatogr A 946:107–124. https://doi.org/10.1016/S0021-9673(01)01579-5
25. Poole CF, Lenca N (2017) Applications of the solvation parameter model in reversed-phase liquid chromatography. J Chromatogr A 1486:2–19. https://doi.org/10.1016/j.chroma.2016.05.099
26. Ali Z, Poole CF (2004) Insights into the retention mechanism of neutral organic compounds on polar chemically bonded stationary phases in reversed-phase liquid chromatography. J Chromatogr A 1052:199–204. https://doi.org/10.1016/j.chroma.2004.08.109
27. Melander W, Campbell DE, Horváth C (1978) Enthalpy–entropy compensation in reversed-phase chromatography. J Chromatogr A 158:215–225. https://doi.org/10.1016/S0021-9673(00)89968-9

2) Présentation de la méthode HPLC mixed-mode développée sur une colonne trimodale

Dans l'article précédent, nous avons observé que la colonne bimodale ne permettait pas de retenir efficacement les composés polaires acides. Dans l'espoir d'améliorer ce point, une colonne trimodale a également été étudiée.

L'ensemble des analyses est réalisé sur la colonne Acclaim Trinity P1 (150 x 3.0 mm, 3 µm, Thermo Fisher Scientific). Le système utilisé est le même que pour les méthodes UHPLC sur phase C18 et PFP (Chapitre 2, III, 1 : système ACQUITY UPLC® I-Class, équipé d'un détecteur UV DAD).

Les analyses sont réalisées en gradient, avec une phase mobile constituée d'un tampon acétate d'ammonium ($_{w}^{w}pH$ 7, 60 mM) et d'acétonitrile. Le pourcentage d'acétonitrile passe de 30 à 60% en 7 minutes, suivi d'un plateau à 60% pendant 8 min. Les analyses ont été réalisées à 0.425 mL/min et 25°C.

Nous avions d'abord essayé de travailler avec le même additif acide que dans la méthode de référence RPLC sur phase C18 (AMS) car la colonne trimodale possède un groupement échangeur de cation fort. Cependant, cet additif s'est révélé inefficace pour l'analyse de composés pharmaceutiques sur cette colonne, avec un pourcentage de composés élués et de pics symétriques plus faible qu'avec l'AA. Cet additif acide n'a donc pas été retenu pour la suite des essais sur la colonne trimodale.

Le détecteur de masse est un simple quadripôle ACQUITY SQD® équipé d'une source d'ionisation électrospray. Les composés étudiés sont détectés en mode positif (m/z 100-1000), temps de scan 0.3 s, voltage du capillaire 3kV, voltage de cône 20V, température de la source 150°C, température de la sonde 600°C, débit du gaz de désolvatation 500L/heure.

Les performances des méthodes mixed-mode seront comparées dans le paragraphe suivant (Chapitre 2, V.).

V. Comparaison des méthodes HPLC pour le profilage d'impuretés de candidats médicaments

Cinq méthodes HPLC sont maintenant disponibles :

1. Une méthode RPLC avec phase stationnaire C18 et phase mobile acide, méthode de première intention qui servira de référence
2. Une méthode alternative RPLC avec phase stationnaire C18 et phase mobile basique
3. Une méthode alternative RPLC avec phase stationnaire PFP et phase mobile acide
4. Une méthode alternative MM-HPLC avec phase stationnaire bimodale RPLC/IEX et phase mobile neutre
5. Une méthode alternative MM-HPLC avec phase stationnaire trimodale RPLC/IEX et phase mobile neutre.

Ces cinq méthodes doivent être comparées en termes de performances chromatographiques, et plus précisément pour la tâche à laquelle elles doivent servir : le profilage d'impuretés. Cette comparaison est décrite dans l'article suivant.

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West

Impurity profiling of drug candidates: analytical strategies using reversed-phase and mixed-mode high-performance liquid chromatography method

Journal of Chromatography A, Volume 1535, (2018), 101-113

Journal of Chromatography A, 1535 (2018) 101–113

Contents lists available at ScienceDirect

Journal of Chromatography A

Impurity profiling of drug candidates: Analytical strategies using reversed-phase and mixed-mode high-performance liquid chromatography methods*

Elise Lemasson[a], Sophie Bertin[b], Philippe Hennig[b], Eric Lesellier[a], Caroline West[a,*]

[a] Univ Orléans, Institut de Chimie Organique et Analytique (ICOA), CNRS UMR 7311, Pôle de Chimie, rue de Chartres, B.P. 6759, 45067 Orléans, Cedex 2, France

[b] Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France

ARTICLE INFO

Article history:
Received 13 November 2017
Received in revised form 2 January 2018
Accepted 7 January 2018
Available online 8 January 2018

Keywords: Impurity profiling Mixed-mode HPLC Pentafluorophenyl phase Pharmaceutical ingredients Performance comparison Reversed-phase HPLC

ABSTRACT

The development of new active pharmaceutical ingredients (API) requires accurate impurity profiling. Nowadays, reversed-phase HPLC (RPLC) on C18 stationary phase is the method of first choice for this task and usually employed in generic screening methods. However, this method sometimes fails, especially when the target analyte is not sufficiently retained, making impurity analysis difficult or even impossible. In such cases, a second method must be available.

In the present paper, we compare the merits of RPLC on C18 phase to those of previously optimized alternative methods, based on the analysis of a large and diverse set of small-molecule drug candidates. Various strategies are considered: RPLC on C18 phase but with different mobile phase composition (acidic or basic), RPLC with a pentafluorophenyl stationary phase, or mixed-mode HPLC with both bimodal and trimodal stationary phases. First, method performances were compared in terms of response rate (proportion of compounds eluted) and peak shapes for a large set of synthetic drugs (140) with structural diversity and their orthogonality was evaluated. Then a subset of compounds (25) containing varied impurity profiles was used to compare the methods based on the capability to detect impurities and evaluate the relative purity of the API.

1. Introduction

Pharmaceutical manufacturers must guarantee the efficacy and limited toxicity of all synthesized products [1]. For this reason, the identification and quantification of residual solvents [2] and impurities (also called impurity profiling) requires strict control and high-performance chromatographic tools. For this task, while gas chromatography is useful to analyze volatile chemicals [3], reversed-phase high performance liquid chromatography (RPLC) with C18 stationary phase combined with UV and mass spectrometric (MS) detection modes is the gold standard in most companies [4–6], with about 90% of low-molecular weight pharmaceutical compounds carried out by RPLC [7]. A generic, universal method is usually desired in order to provide a fast response for any new product [8]. However, due to the complexity of some of the synthesized active pharmaceutical ingredients (API) causing a larger number of impurities and variety of polarities encountered, RPLC mode sometimes fails. Indeed, with C18 phases, one type of interaction between stationary phase and analytes dominates: the dispersion interactions. Besides these major interactions, secondary hydrophilic interactions can occur between residual silanols and analytes. These secondary interactions may cause increased retention of polar analytes, but they mostly cause peak shape deformation and loss of efficiency, especially for basic compounds. To avoid these problems, modern stationary phases include protection strategies against silanophilic interactions. As a result, RPLC mode is best suited for the separation of hydrophobic compounds but either fails to retain polar or charged compounds, or does retain them but with poor peak shape, yielding difficult or even impossible impurity profiling. It is then essential to turn to other analytical methods that offer different opportunities in terms of selectivity and specificity. Firstly, for ionizable species (acidic or basic API) with low polarity, the simplest and fastest method to implement is to adjust the pH of the mobile phase. When this

* Selected paper from 45th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2017), 18–22 June 2017, Prague, Czechia.

* Corresponding author.
E-mail address: caroline.west@univ-orleans.fr (C. West).

https://doi.org/10.1016/j.chroma.2018.01.014
0021-9673/

strategy is not sufficient, the second easiest method may be to replace the C18 phase with a different sort of stationary phase. In this regard, column manufacturers have developed a variety of stationary phases, promising different selectivities. In particular, in the recent years, fluorinated ligands have been established as an alternative to octadecyl ligands, because of the unique selectivity [9,10] and orthogonality [11] they offer. In particular, pentafluorophenyl (PFP) phases have been found to offer superior selectivity and peak efficiency, compared to perfluoroalkyl phases [12]. The unique selectivity can be explained by the different interactions taking place between the stationary phase and the analyte: π–π interactions (between the analyte and electron acceptor PFP group), strong dipole–dipole and ionic interactions (related to the negative partial charges on the fluorine atoms [13]). A large number of PFP columns are now commercially available with most column manufacturers proposing these phases in their portfolio. PFP phases are particularly powerful for the analysis of basic [9,14], polar [15,16] and halogenated compounds [17] or to separate halogenated from dehalogenated compounds [6]. PFP phases also proved their worth for impurity profiling [18] and the separation of isomers [19,20].

Alternatively, other retention modes can be considered. Hydrophilic interaction liquid chromatography (HILIC) is adequate for polar species [21], thus not the preferred method when a large chemical space must be explored [22]. While compatibility to MS detection is excellent [23], equilibration times are excessively long. Mixed-mode HPLC (MM-HPLC) [24] involves the combined use of two (or more) retention mechanisms in a single chromatographic system (reversed-phase, ion exchange or hydrophilic interaction chromatography for example). MM-HPLC shows great flexibility, versatility and high orthogonality with RPLC in the separation of various polar and non-polar pharmaceutical compounds [25–28] and retains good compatibility to MS detection [29,30]. MM-HPLC covers a wide range of applications in the pharmaceutical field (APIs, impurities, synthetic intermediates and degradation products) and has shown possible applicability for impurity profiling of small molecules for therapeutic use [31–33].

In this study, we compare the performance of RPLC with a C18 phase (in acidic or basic conditions) to RPLC with a PFP phase, and to two mixed-mode HPLC methods (with bimodal and trimodal stationary phases combining reversed-phase and ion-exchange mechanisms) coupled to UV and MS detection for impurity profiling of drug candidates. First, method performances were compared in terms of response rate and peak shapes for a large set of synthetic drugs (140), then a subset of compounds (25) containing varied impurity profiles was used to compare the methods based on the capability to detect impurities and evaluate the relative purity of the API. Because these methods are intended for a laboratory analyzing drug candidates at an early stage of drug development, accurate quantitation is not desired but it is expected that the most abundant impurities (above 0.04%) should be separated from the main compound and detected.

2. Material and methods

2.1. Chemicals and solvents

For the evaluation of chromatographic performance and orthogonality, the set of 140 drug candidates was obtained from Servier Research Laboratories (Suresnes, France). The structures are confidential, but they were previously described [34]. Briefly, molecular weights ranged from 150 to 750 g/mol, and log P values varied between −1.9 and 7.5, with a large majority of positive values. Moreover, as is usual in compounds of pharmaceutical interest, a large portion of them have basic functions (80%). 14 mixtures of 10 compounds each were prepared at 1 mg/mL in acetonitrile-water (1:2 v/v) for RPLC methods (C18 and PFP phases) or in a 50:50 mixture of acetonitrile-ammonium acetate buffer (pH 7, 60 mM in water) for mixed-mode HPLC methods.

For the sample applications of impurity profiling, a subset of 25 diverse drug candidates was selected from the initial set. In this subset, molecular weights ranged from 200 to 670 g/mol and log P values varied between 0 and 7.5. 74% of them had a basic function and 12% had an acidic function. Considering that the larger selection was representative of the diversity of structures normally encountered in this research laboratory, this subset adequately represents the same diversity. They were selected so as to reflect the diversity of samples to be processed every day at the laboratory: they included some compounds with high purity (above 95%) and others with lower purity and a large number of impurities. They were injected individually (dilution solvent as described above for 10-component mixtures) to evaluate the relative purity of the main compound and provide an estimated quantification of major impurities. Two of them have a pair of diastereoisomers as principal ingredient thus 27 substances were considered for purity evaluation but 25 substances were considered for the number of impurities.

Water was obtained from a Milli-Q Purification System from Millipore (Millipore SAS, France), HPLC-grade acetonitrile was purchased from Merck (VWR international SAS, France), methanesulfonic acid, trifluoroacetic acid and ammonium acetate were provided by Sigma-Aldrich (Sigma Aldrich Chimie, France).

2.2. Instruments

The UHPLC systems used in all cases were ACQUITY UPLC ® I- Class from Waters Corporation. They were equipped with a binary solvent delivery pump compatible with mobile phase flow rates up to 2 mL/min and pressures up to 827 bar, an autosampler that included partial loop volume injection system, 2-position column oven compatible with 150 mm length columns and a photodiode- array (PDA) detector. The extracted wavelength for UV detection was fixed at 210 nm. Frequency was set at 20 pts/s and resolution at 1.2 nm.

For analyses performed in RPLC on C18 phase and acidic conditions an ACQUITY QDa ® single-quadrupole mass detector (Waters Corporation) with electrospray ionization source was used. An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was then split by the on-board flow-splitter assembly. With this sys- tem only 1/10th of the column flow enters the MS. For analyses performed in all other conditions, an ACQUITY SQD ® single-quadrupole mass detector (Waters Corporation) with electrospray ionization source was used. MS operating conditions are specified below. Note that no comparison of the MS responses is used in the following, as the MS detection served only at identifying the peaks of API and major impurities.

2 μL of each sample (10-compounds mixture or single drug candidate) were injected with a 10 μL-loop and acetonitrile was used to rinse the system.

MassLynx ® software (V4.1) was used for system control and data acquisition. Empower ® 3 was used for integration of peaks for column efficiency measurements. Waters Data Converter (V2.1) was used to convert data from MassLynx to Empower.

2.3. Analytical methods

2.3.1. RPLC methods with C18 phase

At Servier Research laboratories, it is common practice to use two complementary reversed-phase UHPLC methods that were optimized several years ago to maximize the chances to identify and estimate correctly all impurities. Both methods make use of the

same chromatographic column (ACQUITY BEH C18, 50 × 2.1 mm, 1.7 μm fully porous silica, from Waters) but with two different mobile phase compositions: (i) acidic conditions with methanesulfonic acid and (ii) basic conditions with ammonium bicarbonate, thereby providing some complementarity. If the preferred method (acidic conditions) is not satisfactory (when the main compound elutes too close to dead volume, to the gradient end, or is incorrectly or not eluted, if co-elution with impurities is observed or to obtain better structural information), the second method (basic conditions) is used. Two UHPLC systems are used in a parallel fashion. These two methods have been routinely used for day-to-day impurity profiling of synthetic products for several years and constitute reference methods at Servier Research laboratories. The unusual acid employed (methanesulfonic acid) was selected after investigation of other, more common acidic additives (formic acid, acetic acid or trifluoroacetic acid). It was found that methanesulfonic acid offered excellent efficiency through ion-pairing with basic compounds, together with low UV absorbance. However, it is worth mentioning that the resulting low pH is not suited to all stationary phases and may be rather aggressive to certain parts of the instrument.

Analyses were performed at 0.4 mL/min, 30 °C, with a gradient elution program in the following conditions:

(1) For analyses performed with methanesulfonic acid (MSA) (constant concentration of 0.1% in both solvents), the mobile phase composition was water with 2% acetonitrile, increased to 98% in 8 min. The MS conditions were based on pre-optimized conditions recommended by the manufacturer: scan rate 10 pts/s, capillary voltage 0.8 kV, cone voltage 15 V, sampling frequency 8 Hz, ion source temperature 150 °C and probe temperature 600 °C. The analytes were detected in positive electrospray ionization mode (m/z 100–800). Make-up flow was 0.4 mL/min with 70% acetonitrile –30% water comprising 0.1% formic acid.

(2) For analyses performed with ammonium bicarbonate (BICAR) (constant concentration of 20 mM in both solvents), the mobile phase composition was water with 2% acetonitrile, increased to 80% in 10 min. The compounds were detected in positive and negative electrospray ionization mode (m/z 100–1000), scan time 0.3 s, capillary voltage 4 kV (ESI +) or – 3 kV (ESI -) cone voltage 20 V (ESI+) or 30 V (ESI–), ion source temperature 150 °C, desolvation temperature 250 °C, probe temperaturee 600 °C, gas flow desolvation 500 L/h and gas flow cone 0 L/h

2.3.2. RPLC methods with PFP phase

An alternative RPLC method to C18 phase was studied. The stationary phase used was a pentafluorophenyl-bonded silica (PFP): XSelect HSS PFP (150 × 4.6 mm, 2.5 μm) from Waters. The mobile phase composition was H_2O/ACN/TFA (100/1/0.1) for 3 min, then a gradient elution program from H_2O/ACN/TFA (100/1/0.1) to H_2O/ACN/TFA (1/100/0.1) in 22 min. The flow rate was 0.9 mL/min, and the oven temperature was set at 30 °C. Unlike the BEH C18 phase described above, this PFP phase cannot be used with mobile phases with very acidic pH, explaining why TFA was preferred over methanesulfonic acid in this case.

The compounds were detected with MS in positive electrospray ionization mode (m/z 100–1000), scan time 0.3 s, capillary voltage 3 kV (ESI+), cone voltage 20 V (ESI+), ion source temperature 150 °C, probe temperature 600 °C, gas flow desolvation 500 L/h and gas flow cone 0 L/h.

2.3.3. Mixed-mode HPLC methods with bimodal and trimodal phases

Finally, mixed-mode HPLC methods were developed, with both bimodal and trimodal stationary phases. The bimodal phase allowed for reversed-phase and weak cation-exchange (RP-WCX) retention modes: Acclaim WCX-1 column (150 × 3.0 mm, 3.0 μm). The trimodal phase allowed for reversed-phase, strong cation-exchange and weak anion-exchange (RP-SCX-WAX) retention modes: Acclaim Trinity P1 (150 × 3.0 mm, 3.0 μm) column. Both columns were provided by Thermo Fisher Scientific (Villebon-sur-Yvette, France). The mobile phase composition was ammonium acetate buffer (60 mM, pH 7) with 30–60% of acetonitrile in 15 min, 0.45 mL/min, 25 °C.

The compounds were detected in positive and negative electrospray ionization modes (m/z 100–800), scan time 0.3 s, capillary voltage 3 kV (ESI+) or −1.5 kV (ESI−), cone voltage 15 V (ESI+ and ESI−), ion source temperature 150 °C, probe temperature 600 °C, gas flow desolvation 500 L/h and gas flow cone 0 L/h.

2.4. Orthogonality measures

The degree of orthogonality between two chromatographic methods may be assessed with the determination coefficient (R^2) between the values of retention factors. A low value for R^2 indicates a high degree of orthogonality [35]. Unfortunately, the sole use of R^2 is limited to assess orthogonality because it is significantly affected by lever points, thus strongly depends on the analytes selected to evaluate the orthogonality. Furthermore, with the determination coefficient, no indications regarding the retention space covered by the two methods is provided.

For these reasons, we also use another method for the evaluation of orthogonality. This method, based on a geometric approach, was proposed by D'Attoma et al. [36] to determine the retention areas covered by two different methods. Because we wish to compare chromatographic methods based on different gradient time and limit values, the retention times obtained with each method must be transformed into elution composition (C_e) [37] using Eq. (1):

$$C_e = C_i + \frac{(C_f - C_i)}{t_G} * (t_R - t_D) \quad (1)$$

Where C_e corresponds to the percentage of ACN required to elute the analyte of interest; C_i and C_f are the initial and final compositions of the gradient, respectively; t_G is the gradient time; t_R is the retention time retention time of the compound and t_D is the system dwell time.

The dwell volume of the UHPLC system was measured according to the method described in [38] and the value found was 0.203 mL. The methods can then be compared two by two in terms of C_e, which allows visualizing the distribution of compounds in the retention space. Plotting the linear regression line, the retention space covered by the two methods is constructed by drawing a polygon. The two diagonal lines are parallel to the regression line and represent the confidence envelope at 95%.

3. Results and discussion

While the first method to be used for any new compounds should remain the RPLC method with C18 phase and acidic conditions, the objective of this work was to determine the best alternative analytical methods for:

(i) compounds exhibiting insufficient retention (22% of the compounds) with the preferred RPLC C18 method;
(ii) cases where a lack of selectivity is observed with the preferred method resulting in inaccurate impurity profiling.

The alternative methods should naturally be efficient for the task of impurity profiling but also be easy to set up. For instance, retaining the same dilution solvent would be considered ideal because the amount of sample available is not always sufficient to produce

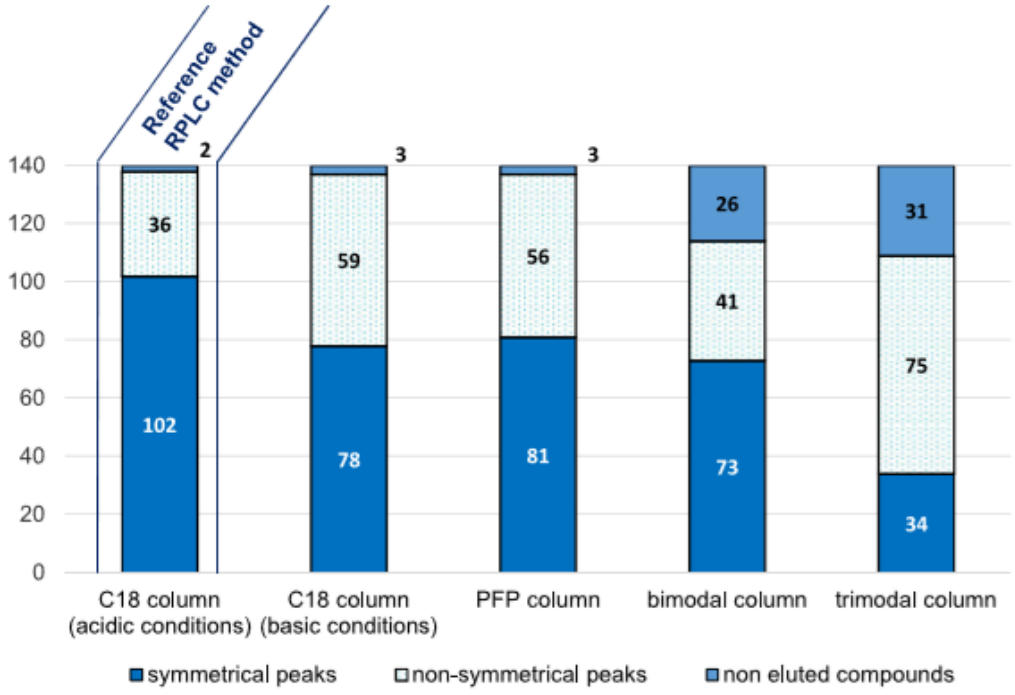


Fig. 1. Comparison of response rate, proportion of symmetrical and non-symmetrical peaks (tailing, fronting, shoulders), with an identical set of 140 compounds in all cases.

two solutions for chromatographic analyses. This would a priori preclude the use of HILIC mode because the dilution solvent should be carefully adjusted [39], with a composition that should be rather less polar than in RPLC. Note that, while compounds with low retention are usually polar species, the compounds typically analyzed in this laboratory mostly have positive log P values, thus HILIC would not be the preferred method anyway. Additionally, it is worth mentioning that instrument availability is not an issue in the target laboratory, thus different alternative methods may be useful for different cases (for instance: neutral, acidic or basic species).

3.1. Chromatographic performance evaluated with large set of drugs

First, we measured the response rate of each method, in other words the number of compounds observed with UV or MS detection (and in a large majority of cases with both of them). The objective is to have a maximum of compounds eluted with good peak shapes, to ensure the most probable detection, identification and quantification of impurities. Peak symmetry (measured on UV chromatograms) was assessed based on the measurement of asymmetry at 10% of peak height (As), according to the definition of the European Pharmacopoeia. For each compound eluted among the 140 compounds studied, we separated symmetrical peaks ($0.8 < As < 1.4$), from non-symmetrical peaks including fronting ($As < 0.8$), tailing ($As > 1.4$), distortions and shoulders. The results are summarized in Fig. 1. The highest response rate was obtained with the reference method (RPLC with the C18 phase in acidic conditions), with almost 99% (138 compounds) of eluted peaks. High response rates were also obtained with RPLC C18 column in basic conditions and PFP methods (98% in both cases). On the contrary, with mixed-mode HPLC methods, the results were worse: only 81% (114) of analytes were successfully eluted with bimodal column and 78% (109) with the trimodal one.

The highest proportion of symmetrical peaks was obtained with the reference RPLC C18 method (74% of symmetrical peaks). In that particular case, the methanesulfonic acid (MSA) additive probably forms ion pairs with basic analytes to favour their elution with good peak shapes. Although the mobile phase also contained a strong acid (TFA), only 59% of symmetrical peaks were observed with RPLC PFP method, not much better than the RPLC C18 method in basic conditions (57%). With mixed-mode HPLC method and bimodal column, it was 64%. In this case, the mobile phase pH was relatively high (buffer pH 7) to maintain the weak-cation exchanger in anionic form. For the trimodal column (with both strong-cation and weak-anion exchangers), we first tried to work with the same acid additive (MSA) as in the RPLC C18 method, but the response rate and proportion of symmetrical shapes were very low (unpublished results) thus this particular mobile phase composition was not retained. Surprisingly, for this trimodal column in the same conditions as the bimodal column, we obtained only 31% of symmetrical peaks.

We can thus observe that the RPLC C18 method provided both the highest response rate and the largest proportion of symmetrical peaks. This was a satisfying result because this method was and should remain the method of first choice. But a good alternative method is desirable.

Secondly, the retention space covered by each method was observed. In Fig. 2, the distribution of compounds in the retention space is shown for each of the five methods with the normalized values of C_e for all compounds detected. Comparing the retention of compounds obtained with the C18 column and basic mobile phase (Fig. 2b) with those obtained with the RPLC C18 reference method (Fig. 2a), the distribution of compounds is rather similar between the two RPLC C18 methods, although the average retention is slightly higher with the basic conditions than with the acidic conditions. The selectivity, meaning the dispersion of C_e values around the average C_e, is somewhat limited in the two methods (standard deviation = 16.1% with the reference RPLC method against 18.3% with the C18 phase in basic conditions).

With the RPLC PFP method (Fig. 2c), the average retention is higher but it appears that this method offers rather limited selectivity. Indeed, although the elution gradient was very large, the retention space covered is small (standard deviation = 9.9%), with a large retention space remaining unused at the beginning and end of the gradient. In this situation, even though polar compounds should be more retained than on the C18 phase, we may expect the selectivity to be unsatisfying with several co-elutions existing between API and impurities. The method could be further improved with a narrower range of acetonitrile gradient (20–80% instead of

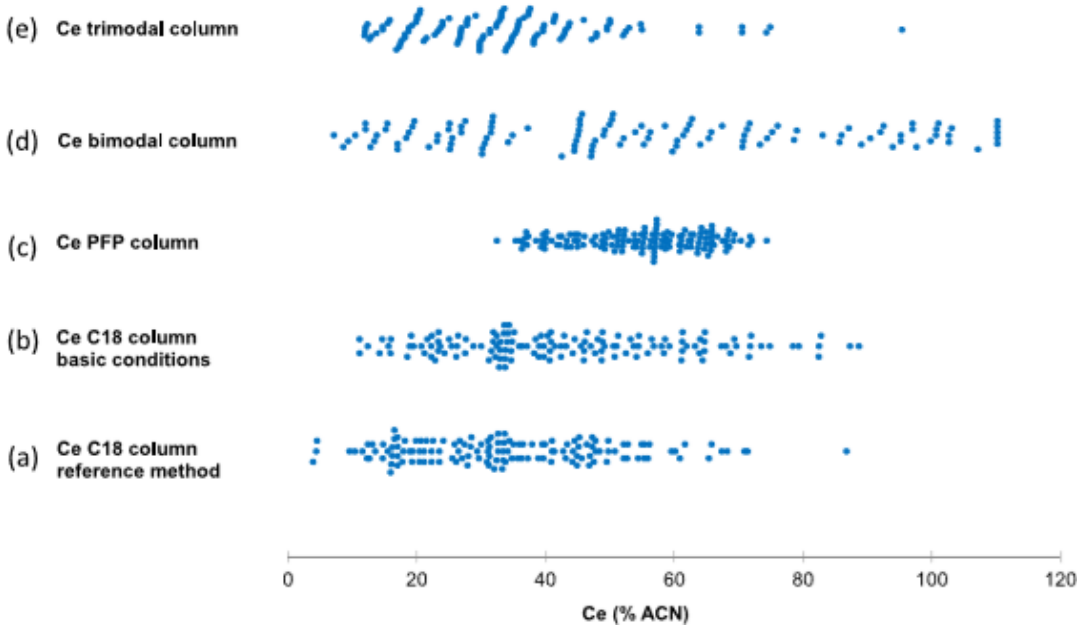


Fig. 2. Comparison of elution composition (normalized values) obtained with (a) the reference method (RPLC C18 method in acidic conditions), (b) RPLC C18 method in basic conditions, (c) RPLC PFP method, (d) HPLC mixed-mode method with bimodal and (e) trimodal columns, set of 140 drug candidates.

0–100%, for instance). This RPLC PFP method was the subject of an in-depth method development at Servier Research laboratories, where several PFP stationary phases had been tested (unpublished work) to achieve a method providing sufficient retention of polar compounds. The conclusions drawn here remain valid only for this stationary phase and these conditions of analysis and cannot be generalized to all PFP phases. Indeed, Euerby et al. in HPLC [9] and West et al. in supercritical fluid chromatography (SFC) [13] showed that significant differences in selectivity and retention exist between the different PFP phases from various manufacturers.

With the RP-WCX bimodal stationary phase, the distribution of retention was very large (Fig. 2d), as almost 100% of the retention space was covered (standard deviation = 30.1%). We can also notice that some compounds were eluted with C_e higher than 100%, which is theoretically impossible but results from Eq. (1) when the compound elutes during column re-equilibration between two successive injections. As observed from Fig. 1, the retention space should be even larger as a significant number of compounds remained non-eluted. This large retention space is encouraging in terms of selectivity.

With the RP-SCX-WAX trimodal stationary phase (Fig. 2e), the retention space covered was similar to that of the reference C18 method, with a majority of compounds eluted with $C_e < 60\%$, but again a significant number of compounds were missing thus a second cluster of points should appear at higher C_e values.

3.2. Orthogonality evaluated with large set of drugs

Apart from chromatographic performance, a good alternative method must fulfil several criteria. The first is to provide the widest orthogonality with the RPLC C18 method to ensure that lack of selectivity in the first method should be overcome in the second method. The second is to provide increased retention for some classes of compounds that are not sufficiently retained on C18 (polar compounds, or sub-categories of polar neutral, polar acidic or polar basic compounds). To compare these two criteria, the elution composition (C_e) values were plotted for each alternative method vs. the reference method with (i) all points considered to evaluate orthogonality (Fig. 3) or (ii) sub-categories of neutral, acidic and basic compounds to evaluate specific complementarity (Fig. 4).

We had previously observed [40] that, comparing the elution composition (C_e) obtained for the set of 140 compounds with the two RPLC C18 methods (acidic and basic conditions), the retention space covered by the combination of the two methods was not very large and the orthogonality limited (Fig. 3a), with a determination coefficient equal to 0.39. Quite logically, it is however observed that (i) neutral compounds are mostly unaffected by the change of conditions; (ii) acidic compounds are more retained in the acidic conditions and (iii) basic compounds are more retained in the basic conditions (Fig. 4a). Thus the C18 method with basic conditions may be a suitable alternative for polar basic compounds that are not sufficiently retained with the reference method. These observations are also in accordance with previous works from Molina-Martin et al. [41] and from Dong [8].

The comparison to the PFP method (Fig. 4b) is less favourable in term of orthogonality as the correlation is rather high ($R^2 = 0.61$). Both methods make use of acidic mobile phases thus the comparison here is mostly that of different stationary phases. Because of the narrow elution range on the PFP phase (as described above with Fig. 2), the retention space covered when combining the two methods is rather small. The objective of orthogonality is not achieved with the PFP phase. However, all compounds that were initially not sufficiently retained on the C18 phase ($C_e < 20\%$ ACN), whether they were neutral, acidic or basic, were now more retained, with an increase of C_e value ($32\% < C_e < 52\%$). These results are in accordance with the works of Euerby et al. [9] for basic species, but in contradiction with their conclusions on neutral species as they observed lower retention of neutral species. As pointed out in the previous section, lack of selectivity may however be expected with this PFP method.

In Figs. 3c and 4c, the reference RPLC method is compared to the bimodal RP-WCX column. In that case, the mobile phase conditions are also markedly different, as the reference method is in acidic conditions and the mixed-mode method in neutral conditions. In this configuration, the retention space covered by the two methods was very large and the determination coefficient very low ($R^2 = 0.002$). These methods were highly orthogonal. Similarly to what was observed with the C18 column in basic conditions when comparing classes of compounds, the basic compounds (Fig. 4c, blue diamonds) that were initially not enough retained on the C18 phase ($C_e < 20\%$ ACN) were now more retained [33]. Their elution composition was comprised between 25% and 95%, thus the retention increase was more significant to some analytes (high C_e values) and less significant to others (low C_e values). The compounds with lowest retention on the bimodal column were mostly acids (Fig. 4c, red triangles). Their low retention was expected because the mobile

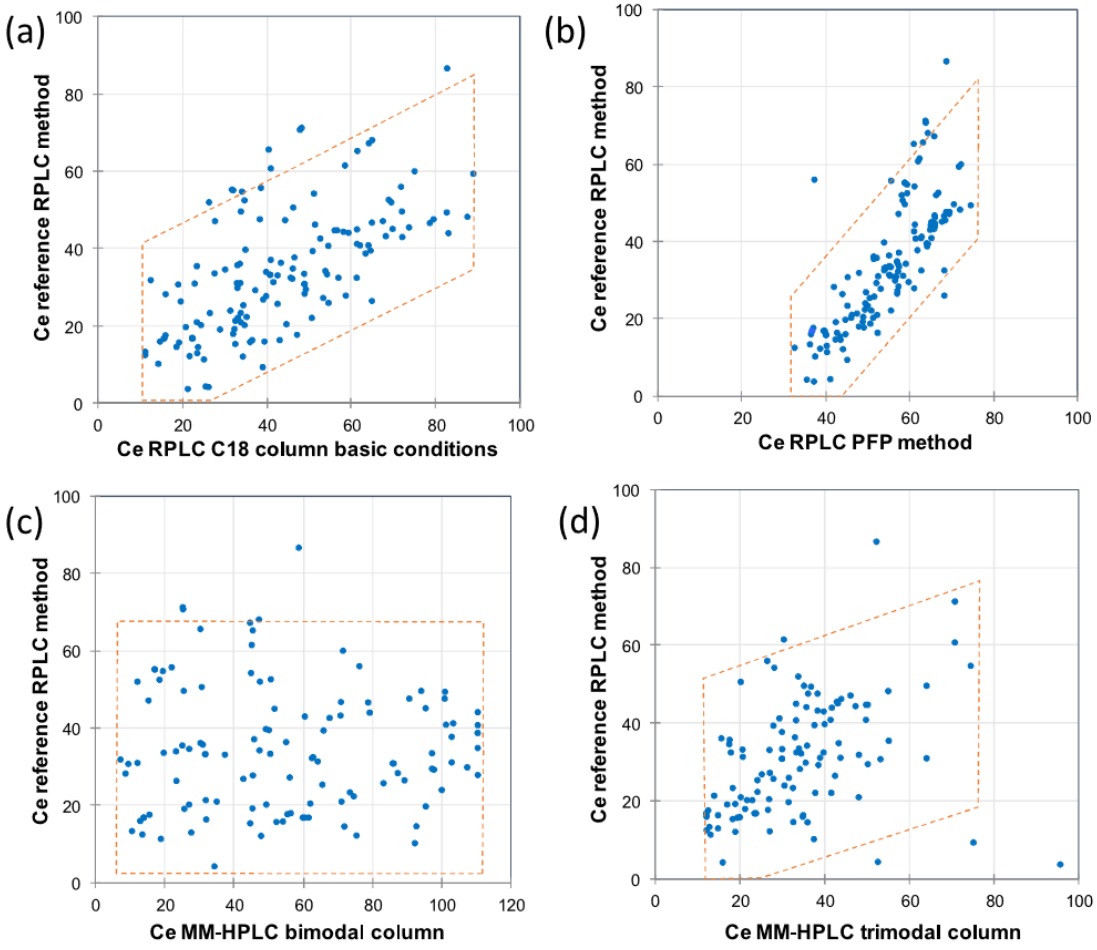


Fig. 3. Comparison of elution composition (% ACN, normalized values) obtained with reference RPLC method (C18 column with acidic conditions) and (a) RPLC C18 method with basic conditions, (b) RPLC PFP method, (c) mixed-mode HPLC bimodal column, (d) mixed-mode HPLC trimodal column, set of 140 drug candidates. The orange interrupted lines delimit the retention space covered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phase buffer pH was set at 7 to allow for full deprotonation of the carboxylate groups from the stationary phase, therefore improving the retention of protonated basic compounds but decreasing retention of deprotonated acidic compounds. However, most acidic compounds were sufficiently retained with the reference method, so this class of compounds should rarely require an alternative method. Some polar neutral compounds (Fig. 4c, black squares) were insufficiently retained with both methods (RPLC C18 and mixed-mode HPLC). As a result, the bimodal column could not be used as an alternative method to improve the retention of such compounds.

Expecting to retain also acidic species, we also investigated a trimodal stationary phase with both strong cation-exchange (vs. weak cation-exchange for the bimodal phase) and weak anion-exchange groups. The retention space covered with the combination of reference RPLC method and the MM-HPLC trimodal column is not so large as the one covered with bimodal phase (Fig. 2d), even if the determination coefficient was low ($R^2 = 0.14$). Unfortunately, it did not allow for combined improvement of the retention of bases and acids: acids (Fig. 4d, red triangles) were indeed more retained than on the C18 phase but bases (Fig. 4d, blue diamonds) were not significantly more retained.

These observations on mixed-mode phases are in accordance with previous reports or Périat et al. [22], who explored several different chromatographic modes for the analysis of natural products. In their study, the mixed-mode stationary phases combining reversed-phase and ion-exchange mechanisms were also found to provide interesting complementarity to RPLC systems, but the versatility was also observed to be limited, judging from the low proportion of analytes that could be suitably eluted.

To summarize these observations:

The only method providing an increase of retention for polar neutral species (Fig. 4, black squares), was the PFP phase. However, because of the very low selectivity provided with this phase, it may not be a good option when the reference method failed.

- For polar acidic species (Fig. 4, red triangles), an increase of retention was provided by the trimodal phase, although the chromatographic performance was rather poor.
- For polar basic species (Fig. 4, blue diamonds), the bimodal phase seemed to be the best alternative, as it provided both increased retention and high orthogonality with the reference method.

3.3. Capacity for impurity profiling evaluated with reduced set of drugs

At this early stage of API development and in the research unit where our methods should apply, impurity profiling methods must furnish only limited information. Analytical chemists are usually required to provide: (i) identity confirmation (based on MS information, and NMR experiments not discussed here) and purity level of the API (based on UV integrated chromatogram); (ii) proposed

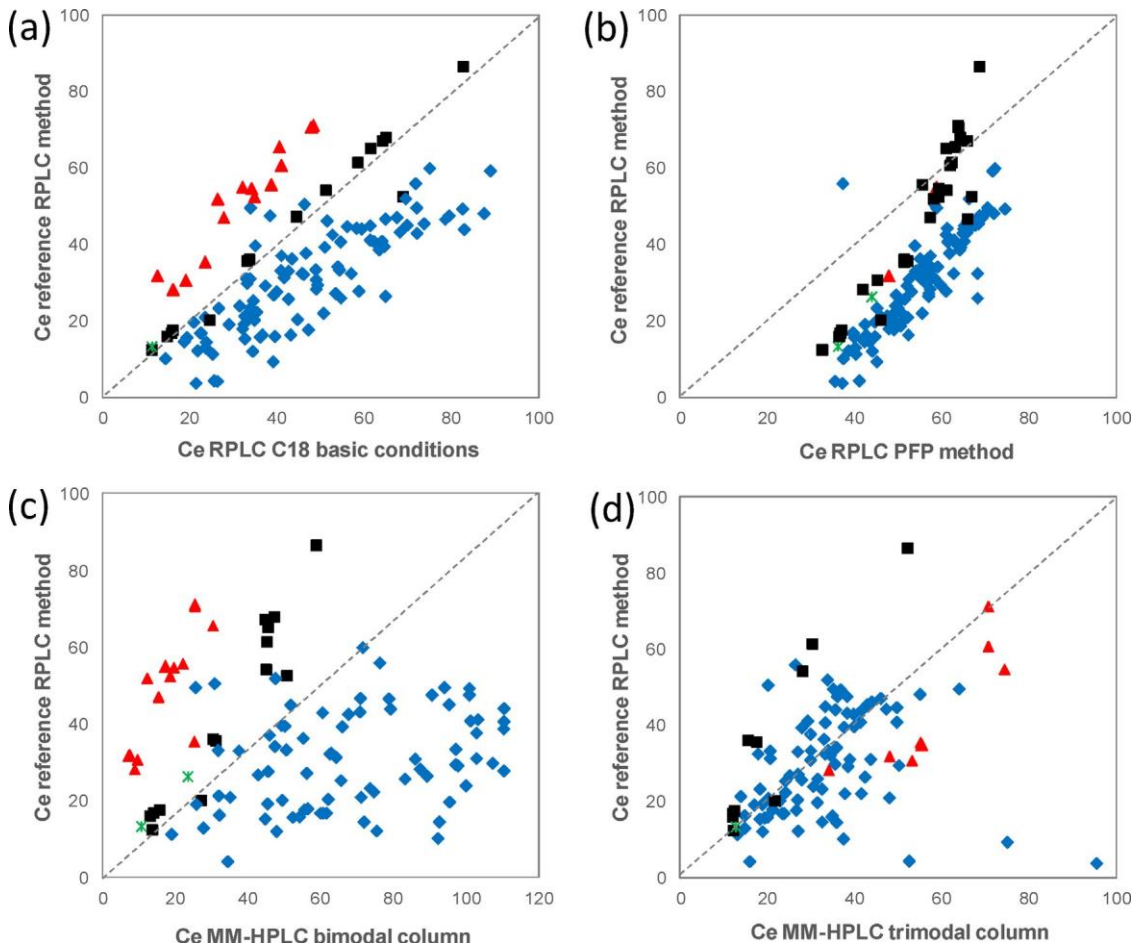


Fig. 4. Comparison of elution composition (normalized values) of neutrals (black squares), ionized acids (red triangles), ionized bases (blue diamonds) and zwitterionic species (green stars) from the set of 140 pharmaceutical compounds between the reference RPLC method (C18 column with acidic conditions) and (a) RPLC C18 method with basic conditions, (b) HPLC PFP phase, (c) mixed-mode bimodal phase, (d) mixed-mode trimodal phase. The interrupted grey line is the first bisector representing isoelution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure elucidation (based on MS information) of impurities with estimated concentration above 1% (based on UV response); (iii) indication of all impurities with estimated concentration above 0.04% (based on UV response).

In a previous study, we had already compared four different chromatographic systems (the two RPLC C18 methods presented in this paper and two SFC systems), considering their ability for impurity profiling [40]. From these studies, we had concluded that the RPLC C18 method with acidic mobile phase (serving here as reference method) appeared to be often the best. For the present comparison, we used the same subset of 25 compounds. Each of them was analyzed individually in the five different chromatographic systems.

For each compound and chromatographic system, we compared:

(i) the purity of API estimated with UV relative peak area
(ii) the number and relative concentrations of detected impurities, identified with their molecular mass when UV-estimated concentration was above 1%;
(iii) the elution orders and retention patterns.

A summary of results can be found in Supplementary information (Table S1).

Firstly, we compared the estimated purity of the API between the five methods. In that case, the method with highest performance should be able to separate a maximum of impurities from the API. Consequently, the best method should provide the lowest estimated purity for the API, i.e. the lowest peak area relative to the total area of all integrated peaks on the chromatogram. In this respect, we proceeded to rank the five methods.

In Fig. 5, the API relative purity between the reference method and each of the other method is compared. The orange stars falling below the first bisector indicate that the value for relative purity was lower with the reference RPLC method, while orange stars falling above the first bisector are related to cases when another method brought an improvement. The reference RPLC method appeared to be most often the best, or very near the best (less than 1% difference from the best) according to this criterion (48% cases). However, each of the other four methods sometimes brought an improvement for this criterion: the PFP method was the best with 37% cases (Fig. 5b); the C18 method in basic conditions brought an improvement in 30% cases (Fig. 5a); the two mixed-mode methods brought an improvement in about 20% cases (Fig. 5c and d).

Secondly, the number of impurities with estimated proportion >1%, or comprised between 0.04 and 1% were counted. The best method should be the one providing the largest number of separated and detected impurities.

In Fig. 5, the number of impurities was also plotted. When purple diamonds (representing the number of impurities with concentration >1%) and blue lines (number of impurities <1%) fall above the first bisector, it indicates that the reference RPLC method yielded a

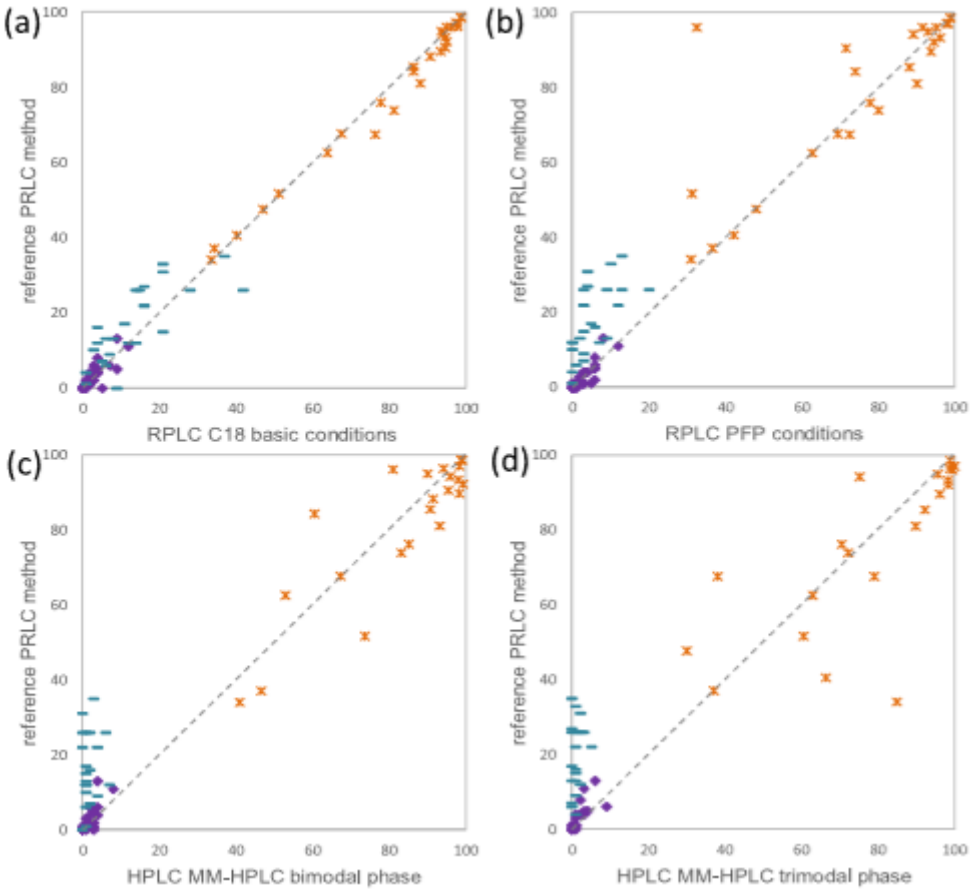


Fig. 5. Comparison of API relative purity (orange stars), number of impurities >1% (purple diamonds) and number of impurities >0.04% (blue lines) from the subset of 25 pharmaceutical compounds between the reference RPLC method (C18 with acidic conditions) and (a) RPLC C18 method with basic conditions, (b) HPLC PFP phase, (c) mixed-mode bimodal phase, (d) mixed-mode trimodal phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

larger number of impurities detected, while points falling below the first bisector indicate that an alternative method permitted to separate and detect more impurities. Considering the total number of impurities detected, the reference RPLC C18 method was most often superior to other methods (76% of cases). The second best method was the C18 phase with basic conditions, bringing an improvement in 24% cases. No other method improved this criterion.

Considering both criteria, the reference C18 method was clearly the best as it was most capable to separate and detect a large number of impurities from the API.

To get a better understanding of the reasons for such superior behaviour of the reference method, we compared signal-to-noise ratio (s/n) values measured in UV for the 25 compounds. It was observed that UV s/n was always the best with the reference method, followed by the PFP method, and the C18 phase in basic conditions. The mixed-mode methods were both greatly inferior to the first three. These differences must be essentially related to lower column efficiency causing (i) lower resolution of impurities from API and (ii) lower sensitivity, thus less impurities could be detected. First, the C18 column had the smallest particle size, which should provide the best efficiency for both methods using this column. Also, as mentioned in Section 3.1, the methanesulfonic acid and trifluoroacetic acid in the reference method and the PFP method respectively should contribute to form ion pairs with basic analytes, favoring their elution as narrow peaks, thereby improving UV detection. On the contrary, with the mixed-mode methods, the combination of retention mechanisms is causing less favourable kinetics and much wider peaks were observed, thus significantly lower UV responses. Baseline noise was also one order of magnitude larger with ammonium acetate mobile phases (mixed-mode methods) than with the other mobile phases, further contributing to lower s/n values.

Consequently, the superior performance of the reference method can be attributed for a large part to its better capability to resolve (through better efficiency) and detect (through better s/n values) small impurities.

The MS responses will not be compared, judging that different MS instruments were used between the reference method and the other four methods.

To exemplify this performance evaluation, three case studies are presented, illustrating the three scenarios observed: (1) some cases where the reference RPLC C18 method yielded a lower estimated purity for the API than the three other cases (Table 1), (2) some cases where the opposite occurred (a lower estimated purity for the API obtained with at least one alternative method) (Table 2) and

Table 1
Comparison of impurity profiling methods (Case 1).

RPLC reference method (C18 phase, acidic conditions)

	RT (min)	%	MS ([M+H]$^+$) peak 1	MS ([M+H]$^+$) peak 2
API	2.46	81.03	560.3	
Impurities >1%				
IMP 1	2.41	8.81	556.2	
IMP 2	3.08	3.28	574.3	
IMP 3	2.36	3.25	542.2	
IMP 4	3.26	1.12	548.4	
undetected				
undetected				
undetected				
Impurities <1%				
IMP A	1.11	0.08		
IMP B	1.18	0.04		
IMP C	1.23	0.19		
IMP D	1.93	0.04		
IMP E	1.96	0.09		
IMP F	2.00	0.08		
IMP G	2.02	0.05		
IMP H	2.07	0.40		
IMP I	2.15	0.04		
IMP J	2.89	0.54		
IMP K	3.06	0.12		
IMP L	3.14	0.22		
IMP M	3.20	0.06		
IMP N	3.22	0.28		
IMP O	3.28	0.28		
undetected				
undetected				
undetected				
undetected				
UV s/n	4.85E+03			

RPLC method (C18 phase, basic conditions)

	RT (min)	%	MS ([M+H]$^+$) peak 1	MS ([M+H]$^+$) peak 2
API	5.59	88.09	560.6	
Impurities >1%				
IMP 1	4.56	5.55	556.2	
undetected				
undetected				
undetected				
IMP 5	4.59	2.40	279.0	
undetected				
undetected				
Impurities <1%				
IMP A	1.48	0.16		
IMP B	1.55	0.08		
IMP C	3.64	0.09		
IMP D	4.13	0.16		
IMP E	4.23	0.34		
IMP F	4.34	0.52		
IMP G	4.85	0.39		
IMP H	5.01	0.11		
IMP I	5.38	0.50		
IMP J	5.47	0.09		
IMP K	5.76	0.19		
IMP L	5.86	0.39		
IMP M	5.90	0.06		
IMP N	5.97	0.35		
IMP O	6.02	0.10		
IMP P	6.09	0.11		
IMP Q	6.11	0.13		
IMP R	6.15	0.06		
IMP S	6.26	0.12		
UV s/n	1.45E+04			

Table 1 (*Continued*)

RPLC method (PFP phase, acidic conditions)

	RT (min)	%	MS ([M+H]+)	MS ([M2+H2]+)
API	14.44	90.23	280.7	560.2
Impurities >1%				
undetected				
undetected				
IMP 3	14.07	3.10	271.5	542.1
undetected				
undetected				
IMP 6	14.36	1.82	286.7	572.1
IMP 7	15.17	3.19	323.4	645.2
Impurities <1%				
IMP A	13.50	0.44		
IMP B	13.90	0.61		
IMP C	14.75	0.60		
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
UV s/n	2.26E+03			

mixed-mode HPLC (bimodal phase, basic conditions)

	RT (min)	%	MS ([M+H]$^+$) peak 1	MS ([M+H]$^+$) peak 2
API	7.78	93.34	560.2	
Impurities >1%				
IMP 1	8.49	5.32	556.2	
IMP 2	6.83	1.15	574.2	
undetected				
undetected				
undetected				
undetected				
undetected				
Impurities <1%				
IMP A	6.52	0.20		
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
UV s/n	1.37E+02			

undetected
IMP 3 14.07 3.10
undetected
undetected
IMP 6 14.36 1.82
IMP 7 15.17 3.19
Impurities <1%
IMP A 13.50 0.44
IMP B 13.90 0.61
IMP C 14.75 0.60
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
undetected
UV s/n 2.26E+03

Table 1 (*Continued*)

mixed-mode HPLC (trimodal phase, basic conditions)				
	RT (min)	%	MS ([M+H]$^+$) peak 1	MS ([M+H]$^+$) peak 2
API	4.29	89.81	560.2	
Impurities >1%				
IMP 1	4.07	7.41	556.2	
IMP 2	3.35	2.28	574.2	
undetected				
undetected				
undetected				
undetected				
undetected				
Impurities <1%				
IMP A	5.24	0.49		
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
undetected				
UV s/n	5.51E+02			

Table 2
Comparison of impurity profiling methods (Case 2). RPLC

reference method (C18 phase, acidic conditions)			
	RT (min)	%	MS ([M+H]$^+$)
API	2.40	95.03	344.2
Impurities >1%			
IMP 1	1.56	3.08	267.3
undetected			
undetected			
undetected			
Impurities <1%			
IMP A	1.46	0.06	
IMP B	1.80	0.13	
IMP C	2.06	0.05	
IMP D	2.12	0.11	
IMP E	2.36	0.40	
IMP F	2.67	0.05	
IMP G	2.94	0.06	
IMP H	3.46	0.37	
IMP I	3.72	0.66	
UV s/n	1.15E+04		

RPLC method (C18 phase, basic conditions)			
	RT (min)	%	MS ([M+H]$^+$)
API	6.77	93.68	344.3
Impurities >1%			
IMP 1	5.91	3.20	267.3
undetected			
undetected			
IMP 4	2.65	1.68	228.0
Impurities <1%			
IMP A	2.52	0.31	
IMP B	4.61	0.09	
IMP C	8.14	0.33	
IMP D	8.32	0.40	

Table 2 (*Continued*)

RPLC method (C18 phase, basic conditions)			
	RT (min)	%	MS ([M+H]$^+$)
IMP E	9.11	0.31	
undetected			
undetected			
undetected			
undetected			
UV s/n	2.33E+03		

RPLC method (PFP phase, acidic conditions)			
	RT (min)	%	MS ([M+H]$^+$)
API	14.44	93.16	344.2
Impurities >1%			
IMP 1	12.67	3.08	267.1
IMP 2	9.40	0.95	177.0
IMP 3	11.72	1.81	186.0
undetected			
Impurities <1%			
IMP A	11.87	0.10	
IMP B	17.06	0.23	
IMP C	17.51	0.66	
undetected			
undetected			
undetected			
undetected			
undetected			
undetected			
UV s/n	4.42E+03		

mixed-mode HPLC (bimodal phase, basic conditions)				
	RT (min)	%	MS ([M+H]$^+$) peak 1	MS ([M+H]$^+$) peak 2
API	12.41	90.01	344.2	
Impurities >1%				
IMP 1	13.77	8.18	267.2	344.2
undetected				
undetected				
undetected				
Impurities <1%				
IMP A	1.46	0.16		
IMP B	1.80	0.27		
IMP C	2.06	0.59		
IMP D	2.12	0.11		
undetected				
undetected				
undetected				
undetected				
undetected				
UV s/n	6.38E+01			

mixed-mode HPLC (trimodal phase, basic conditions)			
	RT (min)	%	MS ([M+H]$^+$) peak 1
API	6.57	95.54	344.2
Impurities >1%			
IMP 1	5.43	3.58	267.1
undetected			
undetected			
undetected			
Impurities <1%			
IMP A	3.14	0.88	
undetected			
undetected			
undetected			
undetected			
undetected			
undetected			
undetected			
undetected			
UV s/n	7.20E+02		

Table 3
Comparison of impurity profiling methods (Case 3).

Reference method (C18 phase, acidic conditions)

	RT (min)	%	MS ([M+H]$^+$)
API	4.31	98.51	413.3
Impurities >1%			
IMP 1	2.81	1.37	397.3
Impurities <1%			
IMP A	5.67	0.05	
IMP B	4.13	0.07	
UV s/n	3.92E+04		

RPLC method (C18 phase, neutral conditions)

	RT (min)	%	MS ([M+H]$^+$)
API	3.68	98.77	413.4
Impurities >1%			
IMP 1	7.12	1.23	unidentified
Impurities <1%			
undetected			
undetected			
UV s/n	2.53E+03		

RPLC method (PFP phase, acidic conditions)

	RT (min)	%	MS ([M+H]$^+$)
API	15.50	99.01	413.1
Impurities >1%			
IMP 1	17.32	0.99	397.1
Impurities <1%			
undetected			
undetected			
UV s/n	4.16E+03		

MM-HPLC (bimodal phase, neutral conditions)

	RT (min)	%	MS ([M+H]$^+$)
API	4.35	98.44	413.3
Impurities >1%			
IMP 1	2.63	1.11	397.3
Impurities <1%			
IMP A	5.87	0.05	
IMP B	6.92	0.40	
UV s/n	2.55E+04		

MM-HPLC (bimodal phase, neutral conditions)

	RT (min)	%	MS ([M+H]$^+$)
API	10.39	99.12	413.2
Impurities >1%			
undetected			
Impurities <1%			
IMP A	1.33	0.88	
undetected			
UV s/n	8.60E+01		

(3) close percentages for API and impurities between all methods (Table 3).

As indicated above, the situation (1), when more impurities were found with reference RPLC C18 method than with other methods, was the most frequent case. In Table 1, the API and all impurities with a concentration above 0.04% are listed for all four methods. Impurities with estimated concentration above 1% are aligned between the five methods according to m/z value. First, we can notice that important differences in API's purity existed: 81.0% for reference RPLC C18 method against 88.1%, 90.2%, 93.3% and 89.3% for RPLC C18 with basic conditions, RPLC PFP method and mixed-mode HPLC methods with bimodal and trimodal columns respectively. While an almost identical number of impurities with estimated proportion >1% was found with all methods (between 2 and 4 depending of the methods), we can notice that several impurities with lower proportions found in RPLC C18 method were undetected with the other three methods. Again, this is probably related to the superior chromatographic performance of this method (peak efficiency, peak symmetry, response rate), or because some impurities were co-eluted with the API with other methods.

In case (2), the four alternative methods provided lower purity than RPLC C18 method. However, the differences between purity levels were not so strong and varied between 90% (with bimodal phase) and 95% (with reference RPLC C18 method). In this example, a larger number of impurities with concentration above 1% was detected with three alternative methods (Table 2). Three impurities were detected with the RPLC PFP method, while two impurities were detected with RPLC C18 method with basic conditions and mixed-mode HPLC with the bimodal phase. On the contrary, only one impurity was detected with the reference RPLC C18 method and with the trimodal phase. This impurity (IMP 1 in Table 2) was identified with all methods, with a concentration close to 3% with all alternative methods (except with the bimodal phase, with IMP = 8.2%, where some other impurities must have co-eluted). Judging from the estimated purity of the API between reference RPLC method (95%), RPLC PFP method and RPLC C18 method with basic conditions (93.2% and 93.7% respectively), some impurities probably co-eluted with the API in RPLC C18 method.

Finally, in case (3), all methods yielded highly similar results for the API purity (about 99%) and impurities percentages (Table 3). One major impurity was identified with the two alternative RPLC methods and MM-HPLC with the bimodal phase, with very close percentage.

Finally, in all cases we observed different elution orders and selectivities for the API and impurities between the different methods. To illustrate this point, Fig. 6 is presenting a case where the reference method failed (purity of the API was not the lowest) because of low retention of the API and impurities, resulting in poor selectivity. In the case of this basic compound, it can be observed that the PFP phase (Fig. 6b), although retaining the analyte adequately, does not provide much selectivity (difference in the location of the points following the horizontal (for PFP) and vertical (for C18) axes). The RPLC C18 phase with basic conditions (Fig. 6a) and the MM-HPLC method with the bimodal phase (Fig. 6c) both better succeeded in separating the API and impurities, as expected from Fig. 4 showing the higher retention of basic compounds with this two methods. In this case, the MM-HPLC method with trimodal phase was most unsuccessful as only a small number of impurities could be detected. The chromatograms related to this case study can be observed in Supplementary information (Fig. S1).

4. Conclusions

The objective of this work was to find an alternative analytical method for impurity profiling of drug candidates when the first-place RPLC method with a C18 phase failed. This method was compared with different chromatographic systems: RPLC with the same C18 phase but with basic mobile phase composition, RPLC with a PFP phase and acidic conditions, mixed-mode HPLC with a bimodal and a trimodal phase. To compare these methods, different parameters were evaluated like peak shapes, response rate, orthogonality and capability for impurity profiling. It was shown that the reference C18-RPLC method with acidic conditions yielded largely superior chromatographic quality and ability for impurity profiling

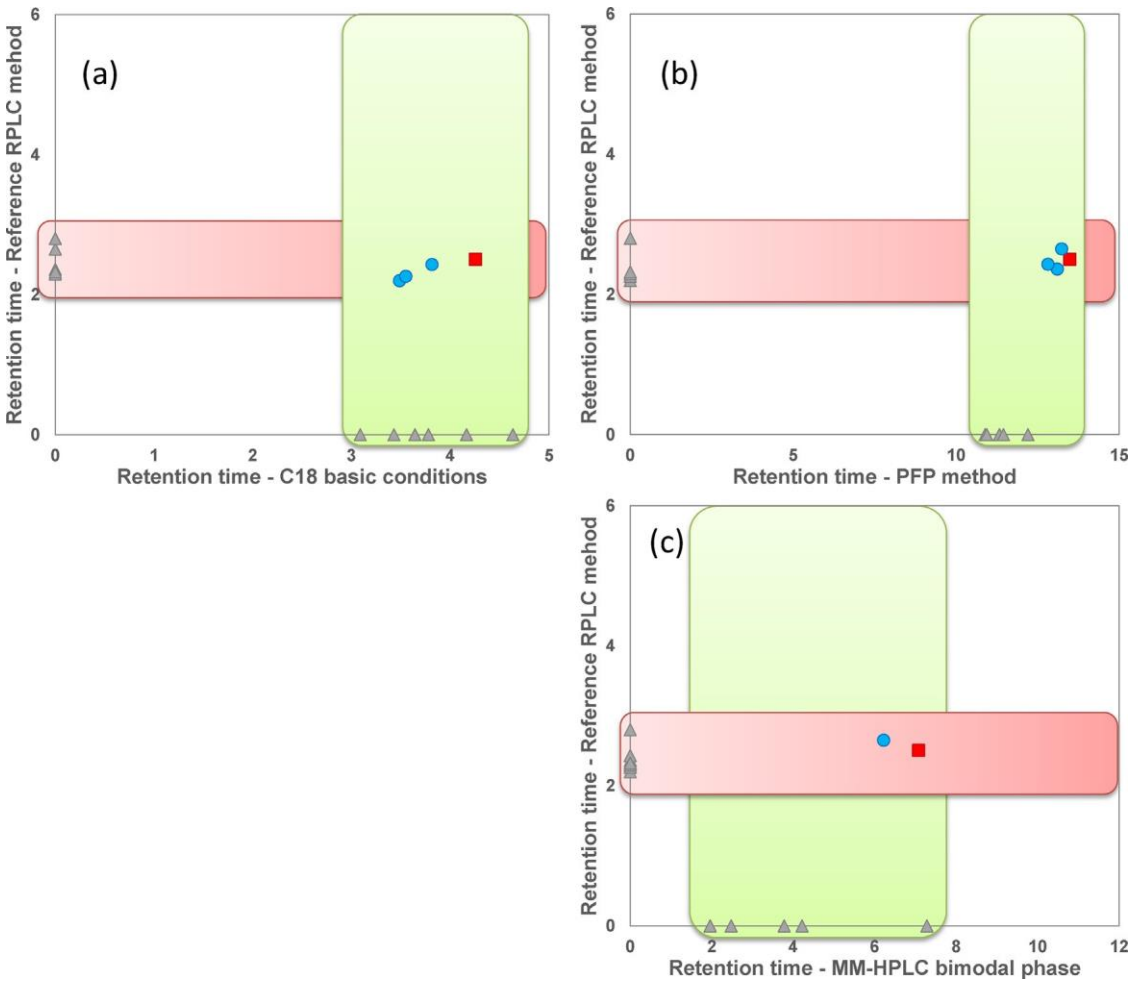


Fig. 6. Comparison of the retention measured for API (red square) and impurities with UV-estimated concentration above 1% (blue circles indicate impurities identified in both methods, grey triangles indicate impurities identified only with one method), between the reference RPLC method (C18 column with acidic conditions) and (a) RPLC C18 method with basic conditions, (b) HPLC PFP phase with acidic conditions, (c) MM-HPLC method with bimodal phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

than the other four methods. This was a positive observation as this method was confirmed in its position as first-choice method.

Because different instruments are available in the target laboratory, it was not our intention to find a single orthogonal method, but rather to know which alternative method should be proposed for any specific case depending on the attributes of the analyte.

After the analysis of all results, the strategy of analysis could be this one. A first analysis must be performed with the reference RPLC method. If the API was eluted with sufficient retention, the impurity profiling can be performed. HRMS and NMR analysis will confirm API and impurities identities.

On the other hand, if the API was not sufficiently retained with the reference method to ensure that impurities would be adequately separated, a second analysis must be performed with one alternative method.

Although orthogonality with the reference RPLC method was limited, the PFP phase should be the best option for polar neutral species.

Contrary to our expectations regarding acidic compounds, the mixed-mode method with trimodal column was unsatisfying, due to inadequate retention (two-mode retention behaviour with low retention for eluted compounds and a large portion of non-eluted compounds) and poor chromatographic performance (large and asymmetric peaks). Therefore, this method will not be kept for further experiments.

The C18 phase with basic conditions and the bimodal WCX-RP phase both allowed a greater retention of polar basic compounds which were poorly retained in RPLC on C18 column, with the additional benefit of excellent orthogonality observed for the mixed-mode column. In addition to its original selectivity, the bimodal column had other significant advantages over HILIC methods: a short column equilibration time and no effect of the injection solvent on retention and peak shape.

Acknowledgments

Waters Corporation is warmly acknowledged for continuous support through the Centers of Innovation program. We also thank Thierry Domenger (Thermo) for the kind gift of columns. CW acknowledges the support of the Institut Universitaire de France (IUF) of which she is a Junior Member.

AppendixA. Supplementarydata

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.01. 014.

References

[1] ICH, Harmonised Tripartite Guideline: Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/biological Entities) Q11, 2012 (Accessed 21 December 2017) http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.

[2] T. Nowak, G.C. Graffius, Y. Liu, N. Wu, X. Bu, X. Gong, C.J. Welch, E.L. Regalado, GC-FID method for high-throughput analysis of residual solvents in pharmaceutical drugs and intermediates, Green Chem. 18 (2016) 3732–3739, http://dx.doi.org/10.1039/C6GC01210H.

[3] G.C. Graffius, B.M. Jocher, D. Zewge, H.M. Halsey, G. Lee, F. Bernardoni, X. Bu, R. Hartman, E.L. Regalado, Generic gas chromatography-flame ionization detection method for quantitation of volatile amines in pharmaceutical drugs and synthetic intermediates, J. Chromatogr. A 1518 (2017) 70–77, http://dx.doi.org/10.1016/j.chroma.2017.08.048.

[4] R. Nageswara Rao, V. Nagaraju, An overview of the recent trends in development of HPLC methods for determination of impurities in drugs, J. Pharm. Biomed. Anal. 33 (2003) 335–377, http://dx.doi.org/10.1016/S0731-7085(03)00293-0.

[5] F.T. Mattrey, A.A. Makarov, E.L. Regalado, F. Bernardoni, M. Figus, M.B. Hicks, J. Zheng, L. Wang, W. Schafer, V. Antonucci, S.E. Hamilton, K. Zawatzky, C.J. Welch, Current challenges and future prospects in chromatographic method development for pharmaceutical research, TrAC Trends Anal. Chem. 95 (2017) 36–46, http://dx.doi.org/10.1016/j.trac.2017.07.021.

[6] E.L. Regalado, R.K. Dermenjian, L.A. Joyce, C.J. Welch, Detection of dehalogenation impurities in organohalogenated pharmaceuticals by UHPLC–DAD–HRESIMS, J. Pharm. Biomed. Anal. 92 (2014) 1–5, http://dx.doi.org/10.1016/j.jpba.2013.12.043.

[7] Y.V. Kazakevich, R. LoBrutto, HPLC for Pharmaceutical Scientists, John Wiley & Sons, 2007.

[8] M.W. Dong, A universal reversed-phase HPLC method for pharmaceutical analysis, LC-GC N. Am. 34 (2016) 408–419.

[9] M.R. Euerby, A.P. McKeown, P. Petersson, Chromatographic classification and comparison of commercially available perfluorinated stationary phases for reversed-phase liquid chromatography using principal component analysis, J. Sep. Sci. 26 (2003) 295–306, http://dx.doi.org/10.1002/jssc.200390035.

[10] M. Przybyciel, Fluorinated HPLC phases—looking beyond C18 for reversed-phase HPLC, LC-GC Eur. 19 (2006) 19–27.

[11] C. André, L. Ismaili, J. Millet, M. Thomassin, Y.C. Guillaume, Effect of sodium chloride on herbicide-fluorinated stationary phase association-evidence of a dual mechanism, Chromatographia 57 (2003) 771–776, http://dx.doi.org/10.1007/BF02491764.

[12] E.L. Regalado, A.A. Makarov, R. McClain, M. Przybyciel, C.J. Welch, Search for improved fluorinated stationary phases for separation of fluorine-containing pharmaceuticals from their desfluoro analogs, J. Chromatogr. A 1380 (2015) 45–54, http://dx.doi.org/10.1016/j.chroma.2014.12.025.

[13] C. West, E. Lemasson, S. Khater, E. Lesellier, An attempt to estimate ionic interactions with phenyl and pentafluorophenyl stationary phases in supercritical fluid chromatography, J. Chromatogr. A 2015 (1412) 126–138, http://dx.doi.org/10.1016/j.chroma.2015.08.009.

[14] S.R. Needham, P.R. Brown, K. Duff, D. Bell, Optimized stationary phases for the high-performance liquid chromatography–electrospray ionization mass spectrometric analysis of basic pharmaceuticals, J. Chromatogr. A 869 (2000) 159–170, http://dx.doi.org/10.1016/S0021-9673(99)00986-3.

[15] U.D. Neue, K. Van Tran, P.C. Iraneta, B.A. Alden, Characterization of HPLC packings, J. Sep. Sci. 26 (2003) 174–186, http://dx.doi.org/10.1002/jssc.200390025.

[16] E.L. Regalado, R. Helmy, M.D. Green, C.J. Welch, Chromatographic resolution of closely related species: drug metabolites and analogs, J. Sep. Sci. 37 (2014) 1094–1102, http://dx.doi.org/10.1002/jssc.201400038.

[17] A.B. Daley, R.D. Oleschuk, Development of fluorinated, monolithic columns for improved chromatographic separations of fluorous-tagged analytes, J. Chromatogr. A 1216 (2009) 772–780, http://dx.doi.org/10.1016/j.chroma.2008.11.078.

[18] A.J. Alexander, L. Zhang, T.F. Hooker, F.P. Tomasella, Comparison of supercritical fluid chromatography and reverse phase liquid chromatography for the impurity profiling of the antiretroviral drugs lamivudine/BMS-986001/efavirenz in a combination tablet, J. Pharm. Biomed. Anal. 78–79 (2013) 243–251, http://dx.doi.org/10.1016/j.jpba.2013.02.019.

[19] E.L. Regalado, P. Zhuang, Y. Chen, A.A. Makarov, W.A. Schafer, N. McGachy, C.J. Welch, Chromatographic resolution of closely related species in pharmaceutical chemistry: dehalogenation impurities and mixtures of halogen isomers, Anal. Chem. 86 (2014) 805–813, http://dx.doi.org/10.1021/ac403376h.

[20] E.L. Regalado, W. Schafer, R. McClain, C.J. Welch, Chromatographic resolution of closely related species: separation of warfarin and hydroxylated isomers, J. Chromatogr. A 1314 (2013) 266–275, http://dx.doi.org/10.1016/j.chroma.2013.07.092.

[21] B. Dejaegher, Y. Vander Heyden, HILIC methods in pharmaceutical analysis, J. Sep. Sci. 33 (2010) 698–715, http://dx.doi.org/10.1002/jssc.200900742.

[22] A. Periat, D. Guillarme, J.-L. Veuthey, J. Boccard, S. Moco, D. Barron, A. Grand-Guillaume Perrenoud, Optimized selection of liquid chromatography conditions for wide range analysis of natural compounds, J. Chromatogr. A 1504 (2017) 91–104, http://dx.doi.org/10.1016/j.chroma.2017.05.024.

[23] H.P. Nguyen, K.A. Schug, The advantages of ESI–MS detection in conjunction with HILIC mode separations: fundamentals and applications, J. Sep. Sci. 31 (2008) 1465–1480, http://dx.doi.org/10.1002/jssc.200700630.

[24] L.A. Kennedy, W. Kopaciewicz, F.E. Regnier, Multimodal liquid chromatography columns for the separation of proteins in either the anion-exchange or hydrophobic-interaction mode, J. Chromatogr. 359 (1986) 73–84.

[25] K. Zhang, L. Dai, N.P. Chetwyn, Simultaneous determination of positive and negative pharmaceutical counterions using mixed-mode chromatography coupled with charged aerosol detector, J. Chromatogr. A 1217 (2010) 5776–5784, http://dx.doi.org/10.1016/j.chroma.2010.07.035.

[26] X. Liu, C.A. Pohl, Comparison of reversed-phase/cation-exchange/anion-exchange trimodal stationary phases and their use in active pharmaceutical ingredient and counterion determinations, J. Chromatogr. A 1232 (2012) 190–195, http://dx.doi.org/10.1016/j.chroma.2011.12.009.

[27] S. Ray, M. Takafuji, H. Ihara, Chromatographic evaluation of a newly designed peptide-silica stationary phase in reverse phase liquid chromatography and hydrophilic interaction liquid chromatography: mixed mode behavior, J. Chromatogr. A 1266 (2012) 43–52, http://dx.doi.org/10.1016/j.chroma.2012.10.004.

[28] M. Lämmerhofer, M. Richter, J. Wu, R. Nogueira, W. Bicker, W. Lindner, Mixed-mode ion-exchangers and their comparative chromatographic characterization in reversed-phase and hydrophilic interaction chromatography elution modes, J. Sep. Sci. 31 (2008) 2572–2588, http://dx.doi.org/10.1002/jssc.200800178.

[29] D. Ilko, C.J. Nap, U. Holzgrabe, S. Almeling, Validation and application of an HPLC-CAD-TOF/MS method for identification and quantification of pharmaceutical counterions, Pharmeur. Bio Sci. Notes 2014 (2014) 81–91.

[30] M.A. Strege, S. Stevenson, S.M. Lawrence, Mixed-mode anion-cation exchange/hydrophilic interaction liquid chromatography–electrospray mass spectrometry as an alternative to reversed phase for small molecule drug discovery, Anal. Chem. 72 (2000) 4629–4633.

[31] J. Li, S. Shao, M.S. Jaworsky, P.T. Kurtulik, Simultaneous determination of cations, zwitterions and neutral compounds using mixed-mode reversed-phase and cation-exchange high-performance liquid chromatography, J. Chromatogr. A 1185 (2008) 185–193, http://dx.doi.org/10.1016/j.chroma.2008.01.083.

[32] B.A. Olsen, Hydrophilic interaction chromatography using amino and silica columns for the determination of polar pharmaceuticals and impurities, J. Chromatogr. A 913 (2001) 113–122, http://dx.doi.org/10.1016/S0021-9673(00)01063-3.

[33] Y. Zhang, P.W. Carr, Novel ultra stable silica-based stationary phases for reversed phase liquid chromatography—study of a hydrophobically assisted weak acid cation exchange phase, J. Chromatogr. A 1218 (2011) 763–777, http://dx.doi.org/10.1016/j.chroma.2010.11.009.

[34] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part I: optimization of mobile phase composition, J. Chromatogr. A 1408 (2015) 217–226, http://dx.doi.org/10.1016/j.chroma.2015.07.037.

[35] J. Pellett, P. Lukulay, Y. Mao, W. Bowen, R. Reed, M. Ma, R.C. Munger, J.W. Dolan, L. Wrisley, K. Medwid, N.P. Toltl, C.C. Chan, M. Skibic, K. Biswas, K.A. Wells, L.R. Snyder, Orthogonal separations for reversed-phase liquid chromatography, J. Chromatogr. A 1101 (2006) 122–135, http://dx.doi.org/10. 1016/j.chroma.2005.09.080.

[36] A. D'Attoma, C. Grivel, S. Heinisch, On-line comprehensive two-dimensional separations of charged compounds using reversed-phase high performance liquid chromatography and hydrophilic interaction chromatography. Part I: orthogonality and practical peak capacity considerations, J. Chromatogr. A 1262 (2012) 148–159, http://dx.doi.org/10.1016/j.chroma.2012.09.028.

[37] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography. Part I: salt gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 33–44, http://dx.doi.org/10.1016/j.jpba.2014.08.035.

[38] J.W. Dolan, Dwell volume revisited, LCGC N. Am. 24 (2006) 458–466. [39] J. Ruta, S. Rudaz, D.V. McCalley, J.-L. Veuthey, D. Guillarme, A systematic investigation of the effect of sample diluent on peak shape in hydrophilic interaction liquid chromatography, J. Chromatogr. A 1217 (2010) 8230–8240, http://dx.doi.org/10.1016/j.chroma.2010.10.106.

[40] E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West, Comparison of ultra-high performance methods in liquid and supercritical fluid chromatography coupled to electrospray ionization –mass spectrometry for impurity profiling of drug candidates, J. Chromatogr. A 2016 (1472) 117–128, http://dx.doi.org/10.1016/j.chroma.2016.10.045.

[41] M. Molina-Martin, A. Marin, A. Rivera-Sagredo, A. Espada, Liquid chromatography-mass spectrometry and related techniques for purity assessment in early drug discovery, J. Sep. Sci. 28 (2005) 1742–1750, http://dx.doi.org/10.1002/jssc.200500107.

VI. Conclusions

Dans ce chapitre, différentes méthodes HPLC ont été comparées à la méthode de référence RPLC C18 (en conditions acides) utilisée à l'Institut de Recherches Servier. L'objectif des méthodes développées (RPLC sur phase C18 en conditions basiques, RPLC sur phase PFP, HPLC sur phase mixed-mode bimodale et trimodale) est de fournir une alternative lorsque la méthode de référence échoue à retenir le PA ou lorsque des coélutions entre le PA et ses impuretés sont suspectées.

Un résumé des performances obtenues avec ces différentes méthodes est proposé ci-dessous (Tableau 2.III) :

Tableau 2.III - Résumé des performances chromatographiques obtenues avec différentes méthodes HPLC

Méthode	RPLC C18 conditions acides	RPLC C18 conditions basiques	RPLC PFP	HPLC-MM colonne bimodale	HPLC-MM colonne trimodale
Composés élués	98%	98%	98%	81%	78%
Pics symétriques	74%	57%	59%	64%	31%
Rétention par rapport à la méthode de référence	22% de composés peu retenus	Bases polaires un peu plus retenues	Rétention des composés neutres polaires	Rétention des bases polaires	Rétention des acides polaires
Orthogonalité		Moyenne	Faible	La plus forte	Forte
Optimisation		Achevée	Tester d'autres phases PFP Modifier le gradient	Tester d'autres phases MM	Modifier le gradient

Par rapport à la méthode de référence, il apparait que :

(i) l'utilisation de la méthode RPLC sur phase PFP permet une augmentation de la rétention des espèces polaires neutres, mais le manque de sélectivité obtenu avec cette phase n'en fait pas une alternative intéressante ;

(ii) la phase mixed-mode trimodale permet une augmentation de la rétention des composés acides polaires, mais les performances chromatographiques obtenues sont très faibles, notamment en termes de symétrie de pics. Le gradient appliqué pourrait être modifié afin de retenir davantage l'ensemble des composés (une majorité des composés étant élués avec une C_e<60%) ;

(iii) les méthodes RPLC C18 en conditions basiques et HPLC mixed-mode bimodale permettent une rétention plus importante des espèces polaires basiques. La phase bimodale semble cependant être la meilleure alternative en raison de la forte orthogonalité avec la méthode de référence.

Chapitre 3
Développement de méthodes SFC pour le profilage d'impuretés

I. Introduction

Ce chapitre concerne le développement de méthode en SFC pour le profilage d'impuretés de candidats médicaments.

Dans ce chapitre, une première partie traite de l'optimisation de la composition de la phase mobile. Les composés pharmaceutiques étudiés étant en majorité basiques, l'utilisation d'additifs dans la phase mobile en SFC est souvent essentielle pour garantir l'élution des composés avec une bonne symétrie [151], et peut également favoriser la détection par un spectromètre de masse. A l'issue de cette première étape, des conditions d'élution optimales ont donc été sélectionnées.

L'avantage de la SFC est de pouvoir utiliser l'ensemble des phases stationnaires utilisées en HPLC (de type phase inverse, phase normale, HILIC, échange d'ions), sans pour autant changer la nature de la phase mobile. En plus d'être orthogonale à l'HPLC [13], la SFC peut également être orthogonale à elle-même lorsque les phases stationnaires sont correctement choisies. Une seconde partie de ce chapitre traite de la sélection de phases stationnaires en SFC, afin de proposer une paire de colonnes orthogonales. L'idée étant de se rapprocher des conditions de travail utilisées au laboratoire Servier, où deux méthodes RPLC complémentaires sont utilisées pour le profilage d'impuretés. A l'issue de ce travail, deux colonnes complémentaires ont donc été sélectionnées.

Une fois les conditions de travail définies en SFC, la robustesse des méthodes développées est vérifiée à l'aide d'un second jeu de colonnes de phases stationnaires identiques à celles sélectionnées à l'étape précédente, mais d'un batch différent.

Pour une analyse de pureté, la stratégie employée au laboratoire Servier repose sur l'emploi d'une analyse générique en mode gradient, souvent suivie d'une analyse isocratique ou d'un gradient focus, pour s'assurer que des impuretés ne coéluent pas avec le PA lors du premier gradient. De la même façon, nous avons donc également étudié le transfert de la méthode générique SFC en mode gradient vers une méthode isocratique et une méthode gradient focus. La comparaison de ces trois méthodes est faite sur une série de produits sur la base des impuretés détectées selon chaque méthode.

Parfois, une seule phase stationnaire ne permet pas de résoudre totalement certains échantillons complexes. Dans ce cas, le couplage en série de deux phases stationnaires complémentaires peut apporter un gain de sélectivité. Pour vérifier cette hypothèse, le couplage des deux phases stationnaires précédemment sélectionnées a également été étudié.

Enfin, dans la dernière partie de ce chapitre, une comparaison des méthodes RPLC et SFC pour le profilage d'impuretés de médicaments potentiels est présentée et les performances des différentes méthodes sont comparées.

II. Développement de méthodes SFC : optimisation de la composition de la phase mobile

La composition de la phase mobile en SFC est un paramètre important à prendre en compte, en particulier lorsqu'on analyse des composés basiques. En effet, la phase mobile contenant du dioxyde de carbone est naturellement acide, donc les composés basiques peuvent y être sous forme protonée. Cette forme cationique peut être cause (i) de solubilité limitée et (ii) d'interactions indésirables avec la phase stationnaire (par exemple avec les silanols). Aujourd'hui, l'utilisation d'additifs dans la phase mobile en SFC est courante. La nature et la concentration de l'additif dans le co-solvant varient selon les analytes étudiés. Une base, un acide (ou un mélange de base et d'acide), un sel et même l'eau peuvent être ajoutés en faible quantité (généralement de 0.1% à 1% dans le co-solvant). L'article suivant traite de l'optimisation de la phase mobile en SFC-UV-MS pour l'analyse de composés pharmaceutiques.

E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West

Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part I: Optimization of mobile phase composition

Journal of Chromatography A, Volume 1408, (2015), 217-226

Journal of Chromatography A, 1408 (2015) 217–226

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part I: Optimization of mobile phase composition

Elise Lemasson[a], Sophie Bertin[b], Philippe Hennig[b], Hélène Boiteux[c], Eric Lesellier[a], Caroline West[a,*]

[a] Université d'Orléans, Institut de Chimie Organique et Analytique (ICOA), CNRS UMR 7311, Pôle de Chimie, rue de Chartres, B.P. 6759, 45067 Orléans Cedex 2, France
[b] Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France
[c] Waters Corporation, 5 rue Jacques Monod, 78280 Guyancourt, France

ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 3 July 2015
Accepted 8 July 2015
Available online 13 July 2015

Keywords:
Additives
Derringer desirability functions Mass detection
Mobile phase composition
Pharmaceutical ingredients
Supercritical fluid chromatography

ABSTRACT

Supercritical fluid chromatography (SFC) is a very useful tool in the purpose of impurity profiling of drug candidates, as an adequate selection of stationary phases can provide orthogonal separations so as to maximize the chances to see all impurities. The purpose of the present work is to develop a method for chemical purity assessment. The first part, presented here, focuses on mobile phase selection to ensure adequate elution and detection of drug-like molecules, while the second part focuses on stationary phase selection for optimal separation and orthogonality.

The use of additives in the carbon dioxide – solvent mobile phase in SFC is now commonplace, and enables in particular to increase the number of eluted compounds and to improve peak shapes. The objective of this first part was to test different additives (acids, bases, salts and water) for their chromatographic performance assessed in gradient elution with a diode-array detector, but also for the mass responses obtained with a single-quadrupole mass detector, equipped with an electrospray ionization source (Waters ACQUITY QDa).

In this project, we used a selection of one hundred and sixty compounds issued from Servier Research Laboratories to screen a set of columns and additives in SFC with a Waters ACQUITY UPC^2 system. The selected columns were all high-performance columns (1.7–1.8 μm with totally porous particles or 2.6–2.7 μm with superficially porous particles) with a variety of stationary phase chemistries.

Initially, eight additives dissolved in the methanol co-solvent were tested on a UPC^2 ACQUITY UPC^2 HSS C18 SB column. A Derringer desirability function was used to classify the additives according to selected criteria: elution capability, peak shapes, UV baseline drift, and UV and mass responses (signal-to-noise ratios). Following these tests, the two best additives (ammonium acetate and ammonium hydroxide) were tested on a larger number of columns (10) where the two additives appeared to provide very comparable overall scores. However, ammonium acetate was selected for slightly better chromatographic quality.

In a second step, we investigated the effects of ammonium acetate concentration (between 0 and 25 mM in the methanol co-solvent) on retention and peak efficiency. Two types of silica supports were tested by working with ACQUITY UPC^2 HSS C18 SB and BEH columns. 20 mM ammonium acetate in methanol with 2% water was finally selected as the best co-solvent composition.

1. Introduction

Impurity profiling of organic products that are synthesized as possible drug candidates is a significant concern. For this purpose, it is necessary to have complementary high-performance analytical methods to ensure that all impurities are identified. A general

* Corresponding author. Tel.: +33 238 49 47 78; fax: +33 238 41 72 81.
E-mail address: caroline.west@univ-orleans.fr (C. West).

http://dx.doi.org/10.1016/j.chroma.2015.07.037
0021-9673/

screening method for impurity profiling of drug candidates should naturally allow the elution of a maximum of species with good peak shapes. In addition, while detection is most often carried out with a UV detector, mass spectrometric (MS) detection is desirable to confirm peak identity and support peak purity. Some single-quadrupole mass spectrometers with a small footprint are now available at a rather low price. Some of these instruments allow for "push-button" operating mode with most parameters having been optimized to ensure reasonably good response to a large array of analytes. Such apparatus are bound to expand the number of routine methods with MS detection, especially as limited expertise is necessary to operate them.

SFC (usually expanded as Supercritical Fluid Chromatography, although the fluid employed is now rarely in the supercritical state) makes use of liquid mobile phases comprising a significant portion of pressurized carbon dioxide mixed to another solvent (most often an alcohol as methanol) [1]. The high-throughput capability and economic benefits of the method, but also the "green" aspect of a non-toxic solvent together render SFC very attractive for a wide range of applications, whenever a replacement or complement to HPLC is desired. Thus the recent introduction of improved analytical SFC systems that take full advantage of all these features is currently causing a revival of the technique.

It was shown in numerous occasions that SFC is an adequate tool for small molecules of pharmaceutical interest: active pharmaceutical ingredients, impurities or degradation products [2–6]. Additionally, it was proven already some 10 years ago that SFC-MS could efficiently compete with LC–MS for the purpose of screening libraries of pharmaceutical compounds [7].

Because active pharmaceutical ingredients are most often basic molecules, and the carbon dioxide – methanol mixture is acidic, adequate elution of such analytes is preferably achieved with an adjusted mobile phase composition comprising a small percentage (typically 0.1–1% in the co-solvent) of a polar additive [8]. The additive may be a base (like isopropylamine [9] or ammonium hydroxide [10,11]), an acid (formic acid [10], ethanesulfonic acid [12] or citric acid), a combination of an acid and a base [13,14], or a salt (most often ammonium formate or ammonium acetate [15,16]). Water is also increasingly cited as an additive to improve elution of polar analytes [17]. While the effect of additive nature and concentration in SFC was often discussed as regards chromatographic features (retention or peak shapes) [15,18] and is considered to cause most significant changes to SFC chromatograms than usually observed in RPLC [6], the impact on MS detection was rarely addressed [10,16,19].

The present study aims at developing a rapid screening method for impurity profiling of drug candidates with SFC-ESI-MS. The first part presented in this paper will focus on the selection of a versatile mobile phase composition to ensure elution of the largest proportion of drug-like compounds with good peak shape and the best possible ESI-MS response. Several additives introduced in the CO_2-methanol mobile phase were thus tested with a wide range of stationary phases to assess their capabilities for successful chromatography and MS detection. Because the method aims at direct applicability in a pharmaceutical company, a large selection (160) of drug candidates (further presented in Section 3.1) were evaluated.

The second part, presented in a subsequent paper, will focus on stationary phase selection to achieve orthogonal methods.

2. Materialandmethod

2.1. Chemicals, solvents and reagents

160 drug candidates were obtained from Servier Research Laboratories (Suresnes, France) whose structures are confidential, but they are further described in Section 3.1. For the additives: ammonium acetate, ammonium formate, diethylamine, diethanolamine and isopropylamine were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France); ammonium hydroxide solution was provided by Fisher Scientific (Illkirch, France); ultra-pure water was provided by an Elga UHQ system from Veolia (Wissous, France) and trifluoroacetic acid was obtained from VWR (Fontenay-sous-Bois, France). Solvents used were HPLC-grade methanol (MeOH) and ethanol provided by VWR (Fontenay-sous-Bois, France). Formic acid was obtained from VWR (Fontenay-sous-Bois, France). Carbon dioxide of industrial grade 99.5% was provided by Messer (Puteaux, France).

2.2. Stationary phases

For this study, eleven commercialized columns were compared. The known features of the stationary phase chemistries and dimensions are gathered in Table 1. The columns selected were all high efficiency phases (1.7 or 1.8 μm fully porous and 2.6 or 2.7 μm superficially porous particles) with a variety of stationary phase chemistries. The columns were kindly provided by Waters (Guyancourt, France), Phenomenex (Le Pecq, France), Thermo Fisher Scientific (Villebon, France) and Macherey-Nagel (Hoerdt, France).

2.3. Instrumentation

The supercritical fluid chromatography system was a Waters Corporation (Millford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®). It was equipped with a binary solvent delivery pump compatible with mobile phase flow rates up to 4 mL/min and pressures up to 414 bar, an autosampler that included partial loop volume injection system, a back pressure regulator, 4-position column oven compatible with 150 mm length columns and two detectors: a photodiode-array (PDA) detector and an ACQUITY QDa® single-quadrupole mass detector with electrospray ionization source. An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was then splitted by the on-board flow-splitter assembly. With this system, most of the column flow goes to the back-pressure regulator and only an unknown portion goes to the MS. MassLynx® software (V4.1) was used for system control and data acquisition. Empower® 3 was used for integration of peaks for column efficiency measurements. Waters Data Converter (V2.1) was used to convert data from MassLynx to Empower.

2.4. Chromatographic conditions

The screening of the different additives with the selection of stationary phases was performed in a gradient elution program in the following conditions:

(1) For columns with 100 × 3.0 mm dimensions (1.7–1.8 μm fully porous particles), the mobile phase composition was CO_2 with 5–50% MeOH (+additive) in 10 min, flow rate was fixed at 1 mL/min, temperature was set at 25 °C and the outlet pressure was maintained at 150 bar. Inlet pressure at the beginning and end of the gradient program varied from 215 to 330 bar respectively.
(2) For columns with 150 × 4.6 mm dimensions (2.6 μm superficially porous particles), the mobile phase composition was CO_2 with 5–50% MeOH (+additive) in 15 min, flow rate was fixed at 2.35 mL/min, temperature was set at 25 °C and the outlet pressure was maintained at 150 bar. Inlet pressure at the beginning

Table 1
11 columns used in this study.

Column name	Manufacturer	Support	Bonded ligand	Dimensions (mm)	Particle size (μm)
ACQUITY UPC^2 HSS C18 SB	Waters	Fully porous silica	Octadecyl, non endcapped	100 × 3.0	1.8
ACQUITY UPC^2 BEH	Waters	Fully porous hybrid silica	–	100 × 3.0	1.7
ACQUITY UPC^2 BEH 2-EP	Waters	Fully porous hybrid silica	2-Ethylpyridine	100 × 3.0	1.7
ACQUITY UPC^2 Torus 1-AA	Waters	Fully porous hybrid silica	1-Amino-anthracene	100 × 3.0	1.7
ACQUITY UPC^2 Torus 2-PIC	Waters	Fully porous hybrid silica	2-Picolyl-amine	100 × 3.0	1.7
ACQUITY UPC^2 Torus DEA	Waters	Fully porous hybrid silica	Diethylamine	100 × 3.0	1.7
ACQUITY UPC^2 Torus DIOL	Waters	Fully porous hybrid silica	Propanediol	100 × 3.0	1.7
Kinetex HILIC	Phenomenex	Superficially porous silica	–	150 × 4.6	2.6
Accucore HILIC	Thermo	Superficially porous silica	–	150 × 4.6	2.6
Accucore Phenyl-X	Thermo	Superficially porous silica	Phenyl-alkyl	150 × 4.6	2.6
Nucleoshell HILIC	Macherey-Nagel	Superficially porous silica	Sulfobetaine	150 × 3.0	2.7

and end of the gradient program varied from 180 to 215 bar respectively.

(3) For the single column having different dimensions from the first two groups (150 × 3.0 mm), Nucleoshell HILIC (2.7 μm superficially porous particles), the mobile phase composition was CO_2 with 5–50% MeOH (+additive) in 15 min, flow rate was fixed at 1 mL/min, temperature was set at 25 °C and the outlet pressure was maintained at 150 bar. Inlet pressure at the beginning and end of the gradient program varied from 190 to 270 bar respectively.

Thus comparable linear velocity and gradient steepness were used with all columns. Variations between the average column pressure values was not compensated with outlet pressure, as we aimed at a simple operating procedure. The differences resulting from different column dimensions and particle type and size should cause only limited differences in elution strength of the mobile phase, because the outlet pressure and oven temperature conditions selected result in a fluid of limited compressibility.

For salt-type additives with limited solubility in methanol, solutions were first prepared at 1 M in water and then diluted down to 20 mM in MeOH. The final composition of mobile phase co-solvent thus comprises 20 mM additive *and* 2% water. 16 mixtures comprising 10 compounds each, selected to avoid isobaric compounds being present in the same mixture, were prepared at 1 mg/mL in ethanol. 1 μL of each mixture was then injected with a 10 μL-loop and MeOH was used to rinse the system. The extracted wavelength for UV detection was fixed at 210 nm. Frequency was set at 20 pts/s and resolution at 1.2 nm.

The mass detector unit was pre-optimized by the manufacturer. The studied compounds were detected in positive and negative electrospray ionization mode (*m/z* 150–750), scan rate 10 pts/s, capillary voltage 0.8 kV, cone voltage 15 V, sampling frequency 8 Hz, ion source temperature 150 °C and probe temperature 600 °C. Nitrogen was used as nebulizing gas. Make-up flow was 0.45 mL/min with 90% methanol–10% water comprising 1% formic acid. As the pre-selected ionization parameters caused only little fragmentation, only the precursor ions were considered ($[M+H]^+$ in ESI^+ mode, $[M-H]^-$ in ESI^-).

3. Results and discussion

3.1. Description of the analyte set

(a) The analytes selected for this study were extracted from a library of drug candidates from Servier Research Laboratories. In designing this test set, the purpose was to have a set that would be as representative as possible of the diversity of chemical structures usually encountered in this pharmaceutical company. As a result, molecular weight varies between 150 and 750 while log *P* values also vary in a wide range, from −1.87 to 7.48 (Fig. 1). However, most analytes have positive log *P* values. A large portion of these compounds (about 80%) were basic. A significant portion of the analytes (about 60%) had acidic functions. Judging from the pK_a values, a large part of the basic analytes (Fig. 1a) are probably protonated in CO_2–methanol mobile phase, while only a small portion of the acidic functions may be deprotonated (Fig. 1b). While the structures of these analytes must remain confidential, the above significant numbers should be sufficient to translate the applicability of the present study to other compounds of pharmaceutical interest.

Applicability of a "diverse" set of analytes to impurity profiling may be questioned, because the structural diversity encountered in a synthetic mixture should be rather limited, with all compounds sharing some common skeleton and differing only in minor features. To verify this point, the set comprised families of compounds with a significant portion of common structural elements.

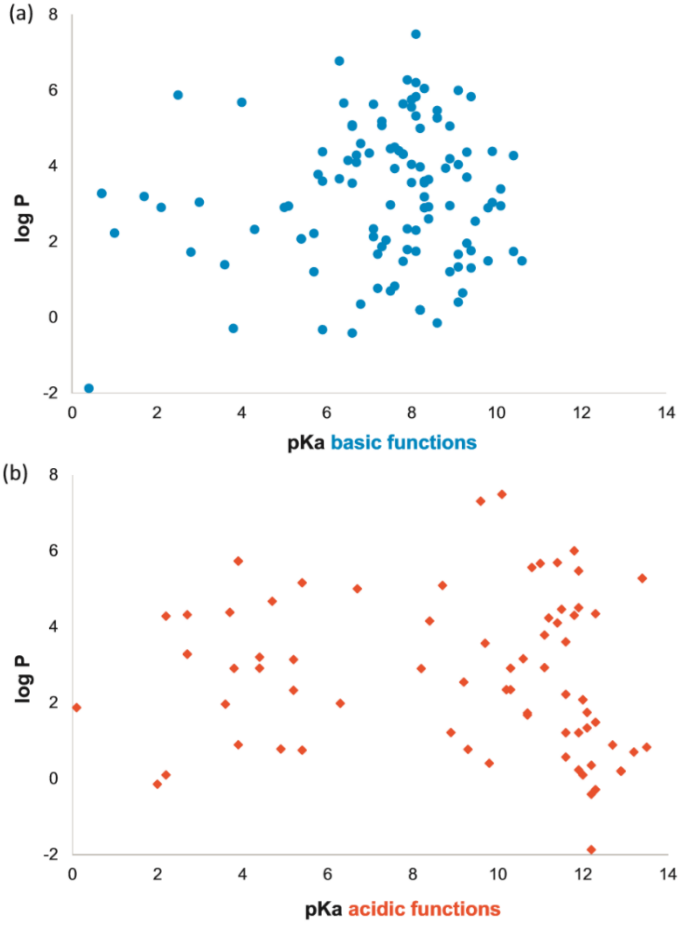


Fig. 1. Partition coefficient (log *P*) *versus* log of acid dissociation constant (aqueous pK_a) for (a) basic compounds and (b) acidic compounds.

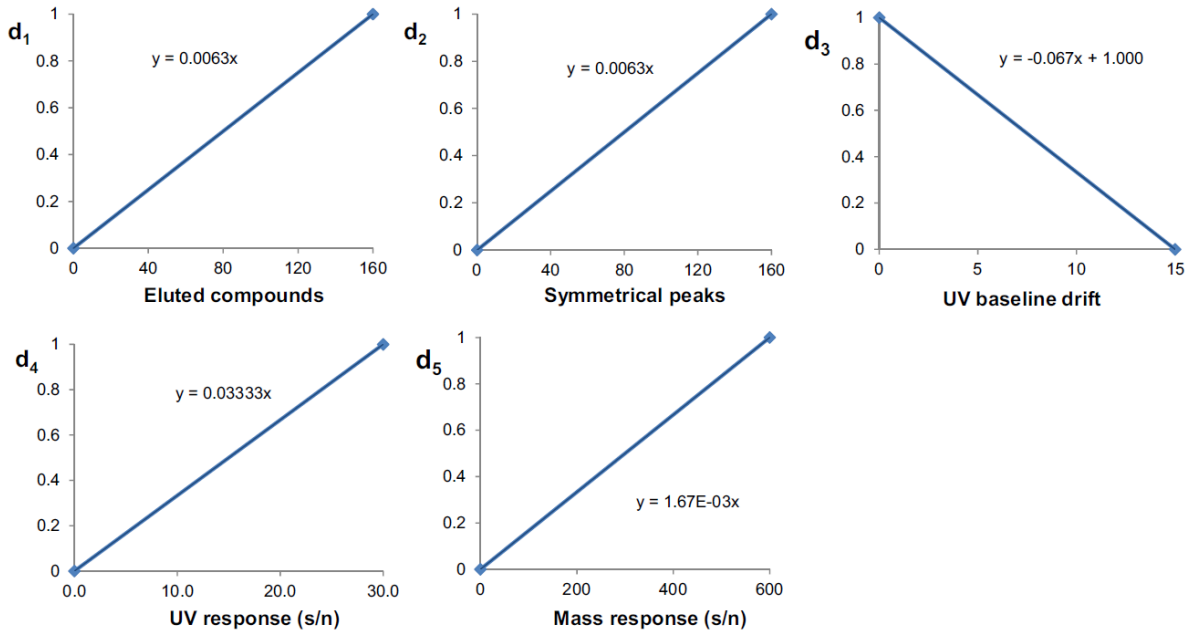


Fig. 2. Derringer desirability functions. (d_1) Based on the number of eluted compounds, (d_2) based on the number of symmetrical peaks, (d_3) based on UV baseline drift caused by the gradient elution, (d_4) based on the average UV responses measured for eluted compounds, and (d_5) based on the average mass responses measured for eluted compounds.

3.2. Definition of Derringer desirability functions

First of all, Derringer desirability functions [20] were defined to rank the performance of the selected additives as regards their benefits for chromatographic elution and detection. To implement Derringer functions, the first step is to choose the criteria that influence the quality of results, so the classification of additives studied. For the purpose of selecting the best mobile phase conditions, we chose to consider the influence of five different criteria. We thus created Derringer functions and calculated values of different Derringer parameters (d_i) for each additive (Fig. 2).

The first studied criterion was the number of eluted compounds. We created a d_1 function as follows: 160 compounds were injected so we expected the elution of 160 compounds at best. If the tested additive allowed the elution of 160 compounds, we assigned a score of 1 to this additive. In other words, a score of 1 is the maximum value for the d_1 coefficient, corresponding to a totally desirable response. On the contrary, if no compounds should be eluted, a value of 0 would be assigned to the additive. 0 is thus the minimum value for d_1 coefficient, corresponding to a totally undesirable response, a case that obviously never occurred. We counted the number of eluted compounds for each additive and assigned a value of d_1 between 0 and 1 according to the linear function that was defined (Fig. 2).

The procedure was the same for the number of symmetrical peaks and we can assign for each additive a value for the d_2 coefficient between 0 and 1 (Fig. 2). Although it may be expected that high d_2 values (large number of symmetrical peaks) can only be obtained with high d_1 values (large number of eluted peaks), it was verified that no correlation existed between the d_1 and d_2 criteria. Indeed, some of the additives selected appeared to ensure elution of a large portion of analytes but provided good peak shapes only for a small portion of them, thus were not versatile enough for the large set of compounds in this study.

The third studied criterion was the UV baseline drift measured between the beginning and end of the gradient. The lowest baseline drift expected is naturally zero, thus the d_3 value should be 1 in such case. The worst additives employed in this study had a baseline drift above 10 absorbance units. As it is not known whether other additives result in larger drifts, a d_3 value of 0 was defined for a baseline drift of 15. This d_3 criterion is obviously unrelated to the first two. However, because this criterion should have little significance when MS detection is available, it was given twice less weight than the other criteria in the final Derringer ranking.

The fourth studied criterion was based on UV response (peak height). The d_4 value was obtained by averaging the UV signal- to-noise ratio (s/n) values measured for all eluted compounds. Background noise was always measured at the end of the gradient, where its amplitude was the largest. An additive which provided the highest average s/n (close to 30) received a score of 1 for the d_4 coefficient, while a zero average s/n (never occurred in practice) was given a score of 0 for the d_4 coefficient (Fig. 2). Good UV response evaluated with peak height can be related to peak shapes and efficiency, but the noise level can vary significantly between additives in a manner that is unrelated to chromatographic performance, especially at the end of the elution gradient when additive concentration is the highest. Thus it was observed that the d_4 values had a limited correlation with d_2 and d_3 values.

The fifth and last studied criterion was the ESI-MS response (peak height), by averaging s/n for the mass responses of 160 compounds measured on the extracted ion chromatograms. An additive which provided the highest average s/n (near 600) received a score of 1 for the d_5 coefficient, while a zero average s/n (never observed) was given a d_5 score of 0 (Fig. 2). In calculating the average s/n, the responses measured in positive and negative ionization mode were considered together, because a large majority of analytes were observed in positive mode. Indeed, the proportion of analytes that provided better MS responses in the negative ionization mode was typically less than 5% of the analytes observed with MS detection. Also, it is worth mentioning that a very small portion of analytes (less than 5%) was observed with UV detection but not with ESI-MS. Because electrospray ionization involves specific effects that are totally different from the chromatographic features, the d_5 values were uncorrelated to those obtained with the other criteria. Mass response is an important criterion because of the need to ensure that the compound of interest is separated from its impurities.

Regarding the two detection criteria (d_4 and d_5), the question of representativeness of the whole data by the average value may be asked. Indeed, it may seem simplistic to translate the diversity of UV and MS responses into a single average value. This point will be further discussed in Section 3.4.

Once the coefficients d_1, d_2, d_3, d_4, d_5 were known for each additive, we calculated the value of total desirability, D. The total desirability was defined as the geometrical mean of the different functions (with less weight given to the d_3 criterion, as indicated above):

$$D=(d_1*d_1*d_2*d_2*d_3*d_4*d_4*d_5*d_5)^{1/9} \quad (1)$$

An additive that gets a high D value will be at the top of the ranking, conversely an additive that gets a low D value will be at the bottom of the ranking. With a product function, an additive that has a very low score for one criterion (close to zero) is strongly penalized, it is considered totally undesirable and thus obtains a bottom rank.

3.3. Preliminary study: screening of eight additives with one column

We started this study with the screening of eight additives with the ACQUITY UPC^2 HSS C18 SB column, which had in a preliminary study proven very successful for the analysis of the compounds of interest, with the analytical conditions described in Section 2.4. The different additives tested were bases (ammonium hydroxide (NH_4OH), diethylamine (DEA), diethanolamine (DEOA) and isopropylamine (IPA)), acids (trifluoroacetic acid (TFA) and water (H_2O)) or salts (ammonium acetate (AA) and ammonium formate (AF)). Pure methanol (no additive) was used as a reference. The composition with water is also a reference for certain additives because the salts (AA and AF) were first dissolved in water prior to dilution with methanol. It may have been foreseen that some of the selected additives were not going to provide uniformly good results for the analytes selected. However, we were willing to explore a variety of additives to possibly reveal particular behaviors toward "difficult" analytes, that shall be addressed in future works.

Once all data were acquired, Derringer desirability functions were used to rank the additives according to the selected criteria: number of eluted compounds (d_1), number of symmetrical peaks (d_2), UV baseline drift (d_3), UV response (d_4) and mass response (d_5). We calculated the value of total desirability D for each additive (Table 2).

Table 2
Ranking of additives on column ACQUITY UPC^2 HSS C18 SB (see text for details).

Additive	D value	Rank
NH_4OH	0.818	1
AA	0.637	2
IPA	0.605	3
DEOA	0.410	4
AF	0.405	5
DEA	0.332	6
None	0.289	7
TFA	0.279	8
H_2O	0.224	9

Looking at each criterion one by one (Fig. 3), it appears that, apart from water alone, any additive improves the number of analytes that were successfully eluted (d_1), as compared to the mobile phase comprising no additive. Ammonium hydroxide, ammonium acetate, isopropylamine and trifluoroacetic acid provided the most significant improvement in this respect with a respective increase in the number of analytes eluted of 19, 18, 17 and 14%. Also, all additives, including water, improved the number of symmetrical peaks (d_2). Ammonium hydroxide, ammonium acetate, diethanolamine and isopropylamine were the most favorable to obtain symmetrical peaks with a respective increase of 190, 175, 170 and 160% as compared to the mobile phase composition comprising no additive. The UV baseline drift (d_3) was strongly dependent on mobile phase composition. Water, isopropylamine and ammonium hydroxide were the best, with no or minor changes as compared to the mobile phase without an additive. Diethanolamine and the two salts (ammonium formate and ammonium acetate) were the worst. Detector response varied greatly with mobile phase composition: average UV response (d_4) was especially improved by the use of basic additives and salts, probably because the peak shapes of bases were improved; average MS response was particularly favored by ammonium hydroxide and ammonium acetate.

Considering overall D values (Table 2), three groups of additives could be defined: ammonium hydroxide, ammonium acetate and isopropylamine ranked at the top; diethanolamine, ammonium formate and diethylamine followed; the last three compositions, no additive, trifluoroacetic acid or water were the worst.

Ammonium acetate and ammonium hydroxide were equally good in terms of chromatographic performance. Ammonium hydroxide provided better MS response and less UV baseline drift. Diethanolamine and trifluoroacetic acid were penalized by low MS

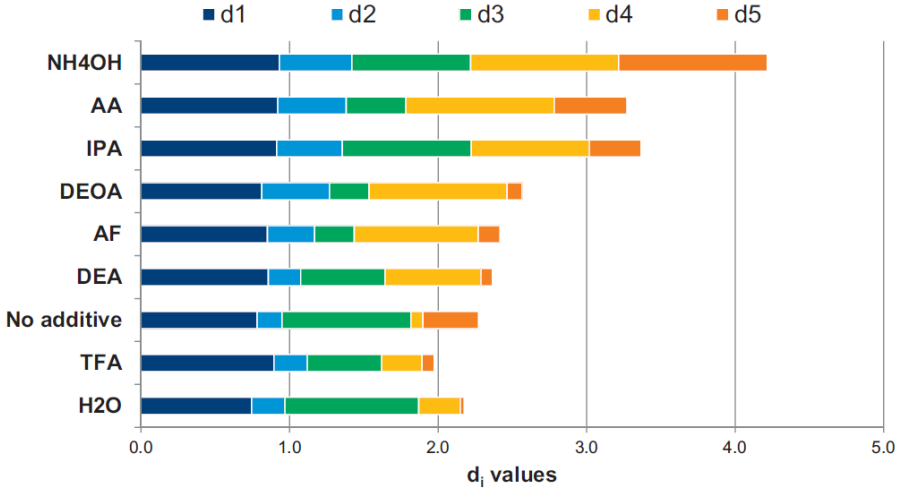


Fig. 3. Details of each criterion studied with Derringer's desirability functions on HSS C18 SB column. (d_1) Number of eluted compounds, (d_2) number of symmetrical peaks, (d_3) UV baseline drift, (d_4) average UV responses measured for eluted compounds, and (d_5) the average mass responses measured for eluted compounds.

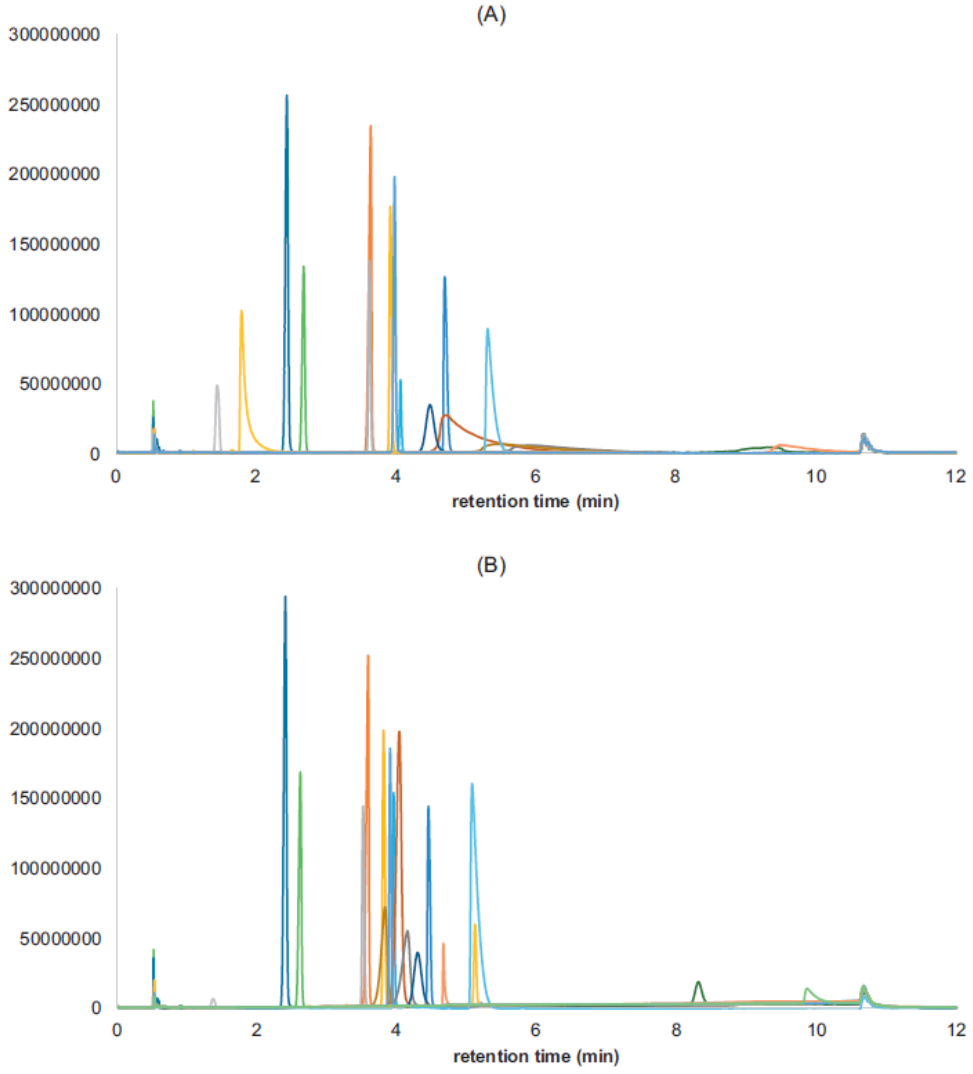


Fig. 4. Sample comparison of UV chromatograms for 20 compounds on ACQUITY UPC2 HSS C18 SB column: (a) without an additive in the mobile phase; (b) with 20 mM ammonium acetate in the methanol co-solvent (UV detection 210 nm).

response, which was not surprising as they have very poor volatility and cause ion suppression respectively.

The bottom four mobile phase compositions were impaired both by poor chromatographic performance (peak shapes represented by d_2) and poor detection (UV and MS s/n represented by d_4 and d_5).

From a general point of view, it was found that the use of the additives that ranked the highest brought significant improvements in the peak shapes of strong bases, as appears with larger d_2 values at the top of the figure.

It is worth noting that, in accordance with previous studies [15], the compounds that exhibited satisfactory elution in the absence of an additive were virtually unaffected by the presence of an additive. However, such compounds were a large minority as they represented about 20% of the compounds eluted in the absence of an additive. This point can be observed in Fig. 4, where the superimposed UV chromatograms of 20 individual analytes are compared with and without an additive (ammonium acetate). It is visible that the peaks that were already symmetrical without an additive are unchanged in the presence of ammonium acetate.

The poor MS response obtained with ammonium formate came as a surprise as it is a volatile salt and was often recommended in SFC-MS studies [19]. However, we must point out that some difficulties were encountered with the MS system. With the interface designed by the manufacturer, the portion of mobile phase entering the MS decompresses in the capillary and/or in the ion source. As this section is not heated up, plugging sometimes occurred that may have been caused either by CO_2 ice particles, or by salt additive precipitation, or by analyte precipitation, which are not adequately re-dissolved by the make-up solvent. The latter is the least likely as the observed plugging usually occurred upon re-equilibration of the system, thus when no analyte should have remained in this section and when the concentration of co-solvent in the mobile phase was the lowest. The MS system used here has an internal calibrant to control m/z values, but nothing indicates whether the MS response is normal. As a result, it is possible that partial plugging had occurred when the experiments with ammonium formate had been carried out. Frequent cleaning of the interface was thus considered necessary to ensure that the MS response observed would remain adequate. Some more robust interfacing is clearly necessary [21], which should be easily reached with additional moderate heating of the SFC-MS interface and would limit both ice formation and salt precipitation.

Additionally, not only MS response but overall chromatographic quality was limited with ammonium formate. This result differs from another recent study in SFC-MS where ammonium formate

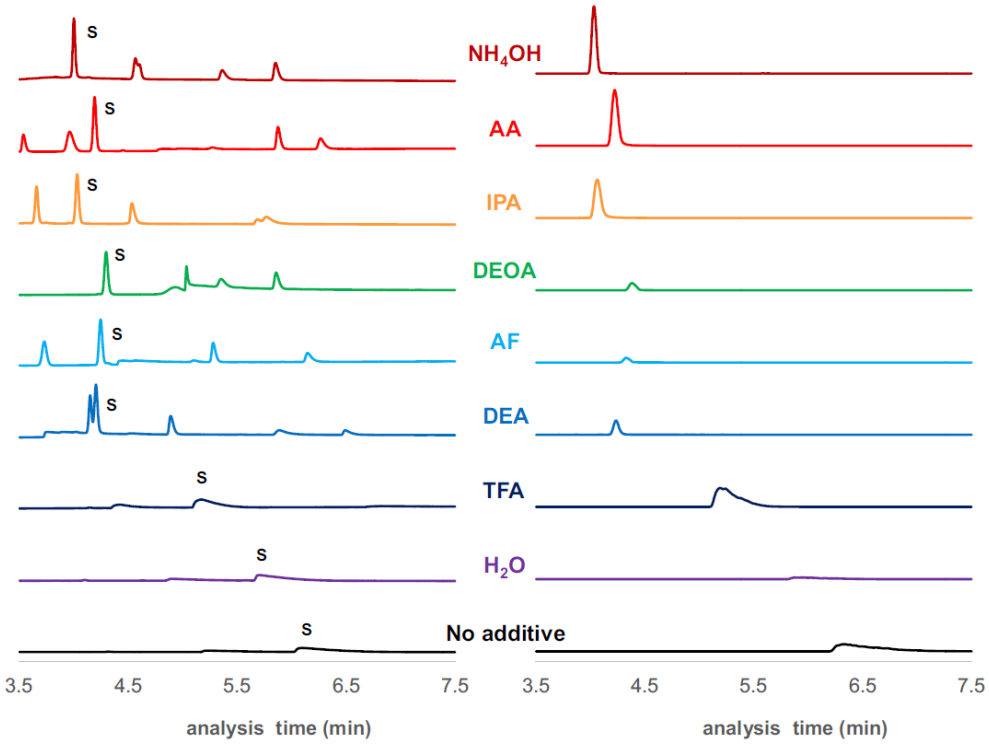


Fig. 5. Sample comparison of UV and MS responses for one compound (**S**) in a mixture of 10 on ACQUITY UPC^2 HSS C18 SB column. Left: UV chromatograms at 210 nm; right: corresponding mass chromatograms (m/z 473, detection ESI$^+$).

was preferred over ammonium acetate as it provided slightly improved peak shapes [19]. However, we may point out that different concentrations of ammonium formate were employed in this reference and the present work (10 mM *versus* 20 mM), while additive concentration appeared as a significant parameter for chromatographic performance as will be further discussed in the following section. Also other operating conditions (flow rate, temperature, make-up flow, analyte concentration), which were different between the two studies, are bound to influence the conclusions on MS response.

Fig. 5 illustrates the comments expressed above. In this example, a compound that was eluted (although demonstrating strong tailing) without an additive was selected. The nature of the additive influenced retention, but also impacted symmetry and efficiency as seen in the UV chromatograms. It had also a very significant impact on MS response. While the peaks observed in UV with the basic and salts additives (NH_4OH, AA, IPA, DEOA, AF and DEA) look very similar, the MS chromatograms are significantly different with ammonium hydroxide, ammonium acetate and isopropylamine providing the best chromatograms. This is in agreement with the respective values of d_5 criterion for these additives (Fig. 3).

3.4. Screening of the two best additives in a large set of columns

Because the next step of this method development, which will be developed in a second paper, is to develop orthogonal SFC-MS methods, thus making use of two or more columns, it was important to verify that the conclusions drawn from the first series of experiments were not dependent on a particular stationary phase but could be extended to other columns. We thus selected the two best additives from the previous step (ammonium acetate and ammonium hydroxide), but also retained pure methanol as a reference.

We thus tested again the 160 analytes with a large selection of columns having very different surface chemistries (Table 1): two other columns of ACQUITY UPC^2 set (BEH and BEH 2-EP), the four ACQUITY UPC^2 Torus columns (1-AA, 2-PIC, DEA, DIOL), and four columns with stationary phases based on superficially porous silica particles: Accucore HILIC and Accucore Phenyl-X, Nucleoshell HILIC, and Kinetex HILIC. The analytical conditions were the same as before, only the gradient and flow rate were adjusted to take account of different column dimensions, as detailed in the experimental section.

The same Derringer desirability functions were used again to rank the additives for each column.

The results are presented in Table 3. It can be seen that, in most cases, the use of an additive improves the overall score. Ammonium acetate and ammonium hydroxide result, on average, in comparable improvements. However, the improvement in chromatographic quality (number of analytes eluted with good peak shapes) was generally better with ammonium acetate.

Comparing only d_5 values related to MS responses, ammonium acetate ranked first in 7 cases out of 11 and ammonium hydroxide ranked first in 4 cases as well.

At this stage, we can get back to a question asked above, regarding the adequacy of the average s/n value to represent the

Table 3
D scores obtained based on Derringer desirability classification with 5 criteria on the 11 columns with Table 1 and for three different mobile phase compositions (see text for details).

Column name	No additive	AA	NH_4OH
ACQUITY UPC^2 HSS C18 SB	0.289	0.637	0.818
ACQUITY UPC^2 BEH	0.536	0.820	0.564
ACQUITY UPC^2 BEH 2-EP	0.538	0.716	0.494
ACQUITY UPC^2 Torus 1-AA	0.455	0.600	0.561
ACQUITY UPC^2 Torus 2-PIC	0.490	0.637	0.648
ACQUITY UPC^2 Torus DEA	0.474	0.564	0.578
ACQUITY UPC^2 Torus DIOL	0.441	0.627	0.645
Kinetex HILIC	0.218	0.438	0.382
Accucore HILIC	0.259	0.547	0.429
Accucore phenyl-X	0.319	0.558	0.511
Nucleoshell HILIC	0.276	0.727	0.573
Average *D* score	0.390	0.625	0.564

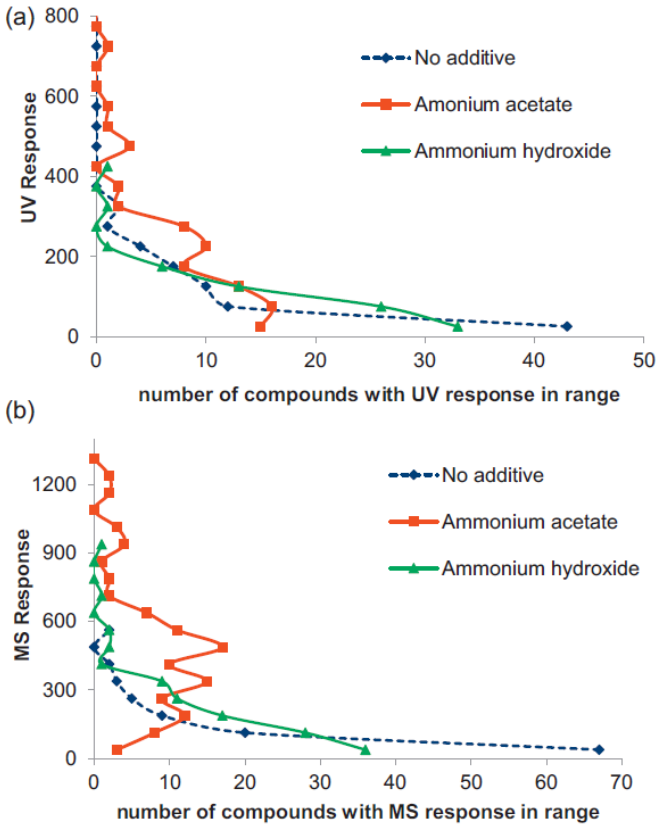


Fig. 6. Distribution of detector responses of all compounds eluted from the Kinetex HILIC column with no additive or with three different mobile phase compositions (pure methanol or 20 mM in methanol). (a) UV signal-to-noise at 210 nm (b) ESI-MS signal-to-noise. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

distribution of detector responses. We thus examined the distribution of the UV and MS response values measured for all compounds detected, on each column and with each mobile phase condition. For example, the results obtained with Kinetex HILIC are represented in Fig. 6. This figure represents the number of compounds observed in a given response range. For instance, in Fig. 6a, it appears that, in the mobile phase with no additive, more than 40 analytes were detected in UV with the lowest response values (blue diamond point at the bottom right of the figure). On the opposite, the number of analytes with high UV response in the same conditions were zero (blue diamond points on the upper part of the y axis). Looking at the distribution of UV responses for the different additives (Fig. 6a), we can see that ammonium acetate provided somewhat higher response values as the red curve is above the others for large response values: only a small portion of analytes provided low response values (red square points at the bottom of the figure), while high response values were often observed (red square points in the upper section of the figure). This is in good agreement with the ranking obtained by using Derringer desirability function based on the average value of UV response data (d_4 criterion). Secondly, looking at the distribution of MS responses (Fig. 6b), the curve for ammonium acetate is above ammonium hydroxide at large values of MS response (red square points in the upper section of the figure are farther to the right as compared to green triangles). They are both above the curve of pure methanol (no additive, blue diamonds). On the opposite, the curve for the "no additive" mobile phase is above all others at low MS responses (blue diamonds at the bottom right of the figure), indicating that a larger part of the analytes observed had only low MS response when no additive was present in the mobile phase. Again, this is in good agreement with the ranking obtained based on the average of value of MS s/n data (d_5 criterion). These elements indicate that the average value of detector response is an adequate criterion to summarize the effects of an additive on detector response for all compounds eluted.

We may conclude from the above observations that ammonium acetate and ammonium hydroxide are nearly equivalent regarding the improvement in chromatographic performance and detection. Ammonium acetate was only slightly better. The latter conclusion is in contrast with a recent SFC-MS study where ammonium hydroxide was considered inferior to ammonium acetate and ammonium formate [19], but this study had pointed out at the deficiencies of ammonium hydroxide for negative ionization mode while the present study comprised only a very small proportion of analytes with ESI^- MS response.

Ammonium acetate was thus selected for further studies.

3.5. *Effects of ammonium acetate concentration on retention and peak shapes*

As a starting point for the above comparisons, we had chosen to use a concentration of 20 mM additive in the mobile phase. At this stage, we were willing to verify whether this concentration was adequate and see how low it could possibly be reduced without degrading peak shape.

Two types of silica supports were tested, working with columns ACQUITY UPC^2 HSS C18 SB and BEH. The first was chosen because it had proven very successful in the preliminary study, and the second because BEH silica serves as a basis for the preparation of all other ACQUITY phases, which had also appeared as promising in the preliminary studies.

We identified nineteen compounds that were very poorly eluted (high retention and high asymmetry values) without any additive in the mobile phase, in the analytical conditions described in section 2.4. Different concentrations of ammonium acetate were tested: 0, 1, 2, 3, 4, 5, 10, 15, 20 and 25 mM in the methanol co-solvent.

The variation in retention time and peak width were plotted for the nineteen compounds according to the concentration of ammonium acetate. Because the analytes are eluted with a gradient elution, the actual concentration of ammonium acetate may differ from one analyte to the other, depending on the concentration of co-solvent required to elute them from the column. Thus for each compound, the overall concentration in the mobile phase, taking account of the elution time of each analyte, was considered. On both columns, the trends observed were very similar. A representative example with one analyte eluted from ACQUITY UPC^2 BEH column is provided in Fig. 7. Both concentrations (in methanol co-solvent and overall concentration in mobile phase) are represented in Fig. 7. As regards retention variation, a large majority of compounds exhibited a strong retention decrease between 0 and 1 mM ammonium acetate in methanol, and then retention further decreased with a lower slope until 15 mM in methanol. Between 15 and 25 mM, retention slightly increased again. This trend, represented in Fig. 7a, is in accordance with previously reported SFC analyses in the presence of ammonium acetate [15].

For the analyte selected in this example, the elution composition was 34% methanol (retention time above 7 min) when no additive was present, and about 18% methanol when ammonium acetate was present (retention time below 4 min). It is interesting to note that a total ammonium acetate concentration of only 0.2 mM was sufficient to divide retention time by a factor of 2. The most likely explanations are (i) the formation of an ion pair between acetate and the cationic analyte (protonated base), and/or that the additive adsorbs at the surface of the stationary phase and decreases analyte interactions, namely coulombic interactions

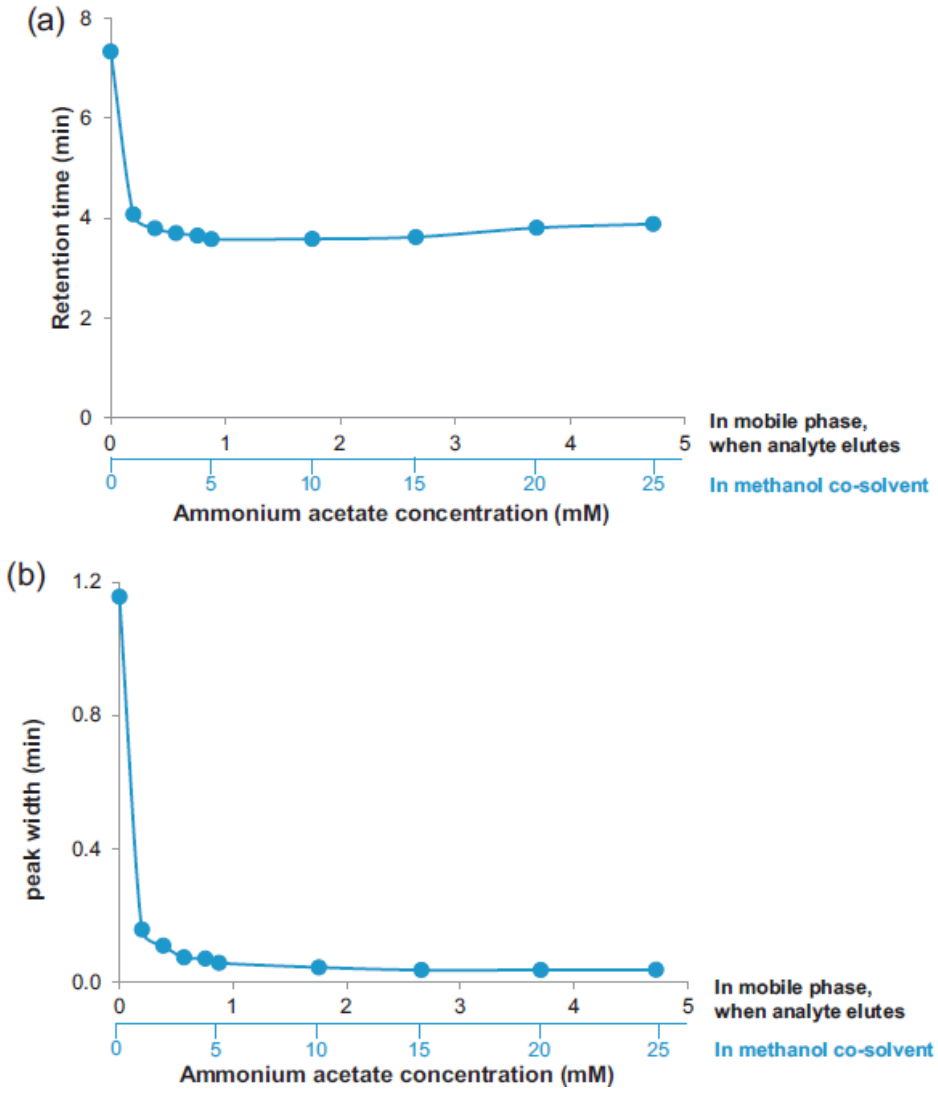


Fig. 7. Effect of the concentration of ammonium acetate in the mobile phase on ACQUITY UPC^2 BEH column for a compound that was poorly eluted without an additive. (a) Retention time variation and (b) peak width variation.

between silanol groups and protonated bases [18]. Further increase in additive concentration had a very small effect when compared to the first 0.2 mM.

As regards peak width variations, the behaviors of the selected analytes were less uniform than observed for retention variations, but the general trend seemed to be as follows (Fig. 7b): very large peaks were observed for the selected compounds without the use of additive in the mobile phase, and then peak width decreased gradually with the increase of ammonium acetate concentration until it reached a plateau at about 20 mM.

On the chromatograms, we could see that with no additive in the mobile phase, the peak shape is very bad (Fig. 8) and the retention time is long (about 7 min). As soon as we added ammonium acetate in the mobile phase, the retention time was shorter (3–4 min) and the peak shape improved significantly. It is then visible that peak width still improves with further addition of ammonium acetate in the methanol co-solvent, while retention slightly increases again.

As a result, our initial choice of 20 mM ammonium acetate in methanol was considered to be good, to ensure low retention and good peak shapes in a robust manner, thus keeping the concentration in an area where these features do not vary much. An additional benefit is that the preparation of mobile phase composition is particularly simple as a 1 M aqueous solution of ammonium acetate is simply dissolved to 2% with methanol. Easiness of mobile phase

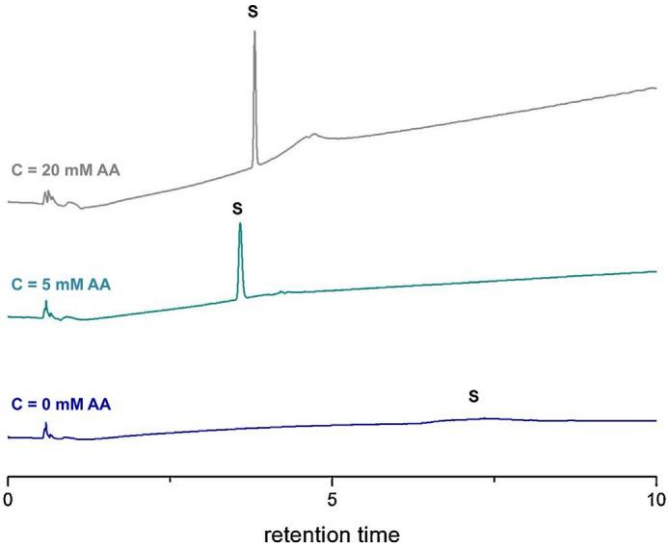


Fig. 8. Chromatograms obtained for one compound (**S**) on ACQUITY UPC^2 BEH column with different concentrations of ammonium acetate in the mobile phase

preparation is a significant point to ensure good routine use of a method and was considered an extra advantage.

4. Conclusion

In the purpose of developing an analysis method for drug candidates, the optimization of mobile phase composition included the study of different additives for their chromatographic performances but also for UV and MS responses. Some of them resulted in poor results (like DEA, TFA, or water), while others presented significant advantages. This was the case for ammonium acetate which was the best additive tested, both for chromatographic performances and detector responses. It was thus retained for all further studies. Another additive provided significant improvement compared to pure methanol: ammonium hydroxide. Although it was rather inferior to ammonium acetate for chromatographic performance, it provided good MS responses and low baseline drift with UV detection.

The investigation of ammonium acetate concentration on retention and efficiency has shown that a concentration of 20 mM ammonium acetate in the mobile phase is suitable for the development of a screening method in SFC-ESI-MS, with the selection of analytes that were employed here on a variety of stationary phases. Please note that the mobile phase selected in the end was not meant to be universal, and other analytes may yield other conclusions.

Derringer functions proved to be useful in comparing notably different criteria (quality of the chromatograms or detection issues). The approach proposed here should be applicable to develop methods with other types of analytes encountered in different application areas.

Acknowledgment

We warmly thank Waters Corporation for the ACQUITY UPC^2 system and ACQUITY QDa detector let at our disposal (Mark Baynham, Taraneh Kargar) and for the opportunity to test new stationary phases (Kevin Wyndham, Darryl Brousmiche, Jacob Fairchild, Steve Collier and team). We also thank Thierry Domenger (Thermo), Régis Guyon (Machery-Nagel), Magali Dupin and Marc Jacob (Phenomenex) for the kind gift of columns.

References

[1] E. Lesellier, C. West, The many faces of packed column supercritical fluid chromatography – a critical review, J. Chromatogr. A 1382 (2015) 2–46, http://dx.doi.org/10.1016/j.chroma.2014.12.083
[2] J.M. Płotka, M. Biziuk, C. Morrison, J. Namieśnik, Pharmaceutical and forensic drug applications of chiral supercritical fluid chromatography, Trends Anal. Chem. 56 (2014) 74–89, http://dx.doi.org/10.1016/j.trac.2013.12.012
[3] S. Singh, T. Handa, M. Narayanam, A. Sahu, M. Junwal, R.P. Shah, A critical review on the use of modern sophisticated hyphenated tools in the charac- terization of impurities and degradation products, J. Pharm. Biomed. Anal. 69 (2012) 148–173, http://dx.doi.org/10.1016/j.jpba.2012.03.044
[4] W. Ren-Qi, O. Teng-Teng, N. Siu-Choon, T. Weihua, Recent advances in pharmaceutical separations with supercritical fluid chromatography using chiral stationary phases, Trends Anal. Chem. 37 (2012) 83–100, http://dx.doi.org/10. 1016/j.trac.2012.02.012
[5] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Ultra high performance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents. I: Investigation of mobile phase and MS conditions, Anal. Chim. Acta 1266 (2012) 158–167, http://dx.doi.org/10.1016/j.chroma.2012.10.005
[6] A.J. Alexander, T.F. Hooker, F.P. Tomasella, Evaluation of mobile phase gradient supercritical fluid chromatography for impurity profiling of pharmaceutical compounds, J. Pharm. Biomed. Anal. 70 (2012) 77–86, http://dx.doi.org/10. 1016/j.jpba.2012.05.025
[7] J.D. Pinkston, D. Wen, K.L. Morand, D.A. Tirey, D.T. Stanton, Comparison of LC/MS and SFC/MS for screening of a large and diverse library of pharmaceutically relevant compounds, Anal. Chem. 78 (2006) 7467–7472, http://dx.doi.org/10. 1021/ac061033l
[8] T.A. Berger, W.H. Wilson, Separation of basic drugs by packed-column supercritical fluid chromatography 3. Stimulants, J. Pharm. Sci. 84 (1995) 489–492, http://dx.doi.org/10.1002/jps.2600840419
[9] K.W. Phinney, L.C. Sander, Additive concentration effects on enantioselective separations in supercritical fluid chromatography, Chirality 15 (2003) 287–294, http://dx.doi.org/10.1002/chir.10196
[10] A. Grand-Guillaume Perrenoud, J. Boccard, J.-L. Veuthey, D. Guillarme, Analysis of basic compounds by supercritical fluid chromatography: attempts to improve peak shape and maintain mass spectrometry compatibility, J. Chromatogr. A 1262 (2012) 205–213, http://dx.doi.org/10.1016/j.chroma.2012.08.091
[11] C. Hamman, D.E. Schmidt Jr., M. Wong, M. Hayes, The use of ammonium hydroxide as an additive in supercritical fluid chromatography for achiral and chiral separations and purifications of small, basic medicinal molecules, J. Chromatogr. A. 1218 (2011) 7886–7894, http://dx.doi.org/10.1016/j.chroma.2011.08.064
[12] R.W. Stringham, Chiral separation of amines in subcritical fluid chromatography using polysaccharide stationary phases and acidic additives, J. Chromatogr. A 1070 (2005) 163–170, http://dx.doi.org/10.1016/j.chroma.2005.02.044
[13] O. Gyllenhaal, J. Vessman, Potential of packed column supercritical fluid chromatography for the separation of metoprolol from closely related com- pounds, J. Chromatogr. A 839 (1999) 141–148, http://dx.doi.org/10.1016/ S0021-9673(99)00052-7
[14] K. De Klerck, Y. Vander Heyden, D. Mangelings, Generic chiral method development in supercritical fluid chromatography and ultra-performance supercritical fluid chromatography, J. Chromatogr. A 1363 (2014) 311–322, http://dx.doi.org/10.1016/j.chroma.2014.06.011
[15] A. Cazenave-Gassiot, R. Boughtflower, J. Caldwell, L. Hitzel, C. Holyoak, S. Lane, et al., Effect of increasing concentration of ammonium acetate as an additive in supercritical fluid chromatography using CO_2 –methanol mobile phase, J. Chromatogr. A 1216 (2009) 6441–6450, http://dx.doi.org/10.1016/j.chroma.2009.07.022
[16] J.D. Pinkston, D.T. Stanton, D. Wen, Elution and preliminary structure-retention modeling of polar and ionic substances in supercritical fluid chromatography using volatile ammonium salts as mobile phase additives, J. Sep. Sci. 27 (2004) 115–123, http://dx.doi.org/10.1002/jssc.200301672
[17] L.T. Taylor, Packed column supercritical fluid chromatography of hydrophilic analytes via water-rich modifiers, J. Chromatogr. A 1250 (2012) 196–204, http://dx.doi.org/10.1016/j.chroma.2012.02.037
[18] J.A. Blackwell, R.W. Stringham, J.D. Weckwerth, Effect of mobile phase additives in packed-column subcritical and supercritical fluid chromatography, Anal. Chem. 69 (1997) 409–415, http://dx.doi.org/10.1021/ac9608883
[19] L. Nováková, A. Grand-Guillaume Perrenoud, R. Nicoli, M. Saugy, J.-L. Veuthey, D. Guillarme, Ultra high performance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents. I: Investigation of mobile phase and MS conditions, Anal. Chim. Acta 853 (2015) 637–646, http://dx.doi.org/10.1016/j.aca.2014.10.004
[20] G. Derringer, R. Suich, Simultaneous optimization of several response variables, J. Qual. Technol. 12 (1980) 214.
[21] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Coupling state-of-the-art supercritical fluid chromatography and mass spectrometry: from hyphenation interface optimization to high-sensitivity analysis of pharmaceu- tical compounds, J. Chromatogr. A 1339 (2014) 174–184, http://dx.doi.org/10. 1016/j.chroma.2014.03.006.

III. Développement de méthodes SFC : choix de phases stationnaires

Ayant déterminé une composition de phase mobile qui permettra l'élution et la détection satisfaisante d'une majorité de nos analytes cibles, il nous faut maintenant sélectionner une (ou des) phase(s) stationnaire(s) qui permette(nt) d'obtenir la rétention, la sélectivité et l'efficacité adéquate pour le profilage d'impuretés. Le choix de la phase stationnaire est en effet un critère essentiel lors du développement de méthodes en SFC car elle impacte très fortement la séparation. La recherche de phases stationnaires pour l'analyse de composés pharmaceutiques est reportée dans l'article suivant :

E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West

Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates.

Part II: Selection of an orthogonal set of stationary phases

Journal of Chromatography A, Volume 1408, (2015), 227-235

Journal of Chromatography A, 1408 (2015) 227–235

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part II. Selection of an orthogonal set of stationary phases

Elise Lemasson[a], Sophie Bertin[b], Philippe Hennig[b], Hélène Boiteux[c], Eric Lesellier[a], Caroline West[a,*]

[a] Université d'Orléans et CNRS, Institut de Chimie Organique et Analytique (ICOA), UMR 7311, Pôle de Chimie, rue de Chartres, B.P. 6759, 45067 Orléans, Cedex 2, France
[b] Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France
[c] Waters Corporation, 5 rue Jacques Monod, 78280 Guyancourt, France

ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 3 July 2015
Accepted 8 July 2015
Available online 14 July 2015

Keywords:
Derringer desirability functions
Ranking
Mass detection
Pharmaceutical ingredients
Screening process
Supercritical fluid chromatography

ABSTRACT

Impurity profiling of organic products that are synthesized as possible drug candidates requires complementary analytical methods to ensure that all impurities are identified. Supercritical fluid chromatography (SFC) is a very useful tool to achieve this objective, as an adequate selection of stationary phases can provide orthogonal separations so as to maximize the chances to see all impurities.

In this series of papers, we have developed a method for achiral SFC-MS profiling of drug candidates, based on a selection of 160 analytes issued from Servier Research Laboratories.

In the first part of this study, focusing on mobile phase selection, a gradient elution with carbon dioxide and methanol comprising 2% water and 20 mM ammonium acetate proved to be the best in terms of chromatographic performance, while also providing good MS response [1].

The objective of this second part was the selection of an orthogonal set of ultra-high performance stationary phases, that was carried out in two steps. Firstly, a reduced set of analytes (20) was used to screen 23 columns. The columns selected were all 1.7–2.5 μm fully porous or 2.6–2.7 μm superficially porous particles, with a variety of stationary phase chemistries. Derringer desirability functions were used to rank the columns according to retention window, column efficiency evaluated with peak width of selected analytes, and the proportion of analytes successfully eluted with good peak shapes. The columns providing the worst performances were thus eliminated and a shorter selection of columns (11) was obtained. Secondly, based on 160 tested analytes, the 11 columns were ranked again. The retention data obtained on these columns were then compared to define a reduced set of the best columns providing the greatest orthogonality, to maximize the chances to see all impurities within a limited number of runs. Two high-performance columns were thus selected: ACQUITY UPC^2 HSS C18 SB and Nucleoshell HILIC.

1. Introduction

Impurity profiling of organic products that are synthesized as possible drug candidates is a significant concern. For this purpose, it is necessary to have complementary high-performance analytical methods to ensure that all impurities are identified. SFC (usually expanded as Supercritical Fluid Chromatography, although the fluid employed is now rarely in the supercritical state) is one such method. SFC makes use of liquid mobile phases comprising a significant portion of carbon dioxide mixed to a co-solvent [2]. CO_2 has major advantages over more conventional chromatographic solvents, as it has a low viscosity allowing for high diffusivities of the analytes (hence high efficiencies) and limited pressure drop over packed columns. As a result, high flow rates can be used without strongly affecting efficiency, and columns packed with sub-2 μm particles can be employed with relatively low- pressure pumping systems (400 bar) [3]. Consequently, the recent progresses in stationary phase technology (small particles [4,5], but also superficially porous particles [6]) has also benefited to SFC.

* Corresponding author. Tel.: +33 238 49 47 78; fax: +33 238 41 72 81.
E-mail addresses: caroline.west@univ-orleans.fr, west caroline@yahoo.fr (C. West).

http://dx.doi.org/10.1016/j.chroma.2015.07.035
0021-9673/

Table 1
23 columns used in this study.

Column Name	Manufacturer	Support	Bonded ligand	Dimensions (mm)	Particle size (μm)
ACQUITY UPC^2 HSS C18 SB	Waters	Fully porous silica	Octadecyl, non endcapped	100 × 3.0	1.8
ACQUITY UPC^2 BEH	Waters	Fully porous hybrid silica	–	100 × 3.0	1.7
ACQUITY UPLC BEH Shield RP18	Waters	Fully porous hybrid silica	Alkyl with embedded carbamate group	100 × 3.0	1.7
ACQUITY UPC^2 BEH 2-EP	Waters	Fully porous hybrid silica	2-ethylpyridine	100 × 3.0	1.7
ACQUITY UPC^2 CSH Fluorophenyl	Waters	Fully porous hybrid silica	Pentafluorophenyl	100 × 3.0	1.7
ACQUITY UPC^2 Torus 1-AA	Waters	Fully porous hybrid silica	1-Amino-anthracene	100 × 3.0	1.7
ACQUITY UPC^{2}Torus 2-PIC	Waters	Fully porous hybrid silica	2-Picolyl-amine	100 × 3.0	1.7
ACQUITY UPC^2 Torus DEA	Waters	Fully porous hybrid silica	Diethylamine	100 × 3.0	1.7
ACQUITY UPC^2 Torus DIOL	Waters	Fully porous hybrid silica	Propanediol	100 × 3.0	1.7
Synergi Polar RP	Phenomenex	Fully porous silica	Phenyl-oxypropyl	100 × 3.0	2.5
Kinetex HILIC	Phenomenex	Superficially porous silica	–	150 × 4.6	2.6
Kinetex PFP	Phenomenex	Superficially porous silica	Pentafluorophenyl	150 × 4.6	2.6
Kinetex Biphenyl	Phenomenex	Superficially porous silica	Biphenyl	150 × 4.6	2.6
Kinetex XB C18	Phenomenex	Superficially porous silica	Octadecyl, endcapped	150 × 4.6	2.6
Accucore HILIC	Thermo	Superficially porous silica	–	150 × 4.6	2.6
Accucore Phenyl-X	Thermo	Superficially porous silica	Phenyl-alkyl	150 × 4.6	2.6
Accucore Phenyl-hexyl	Thermo	Superficially porous silica	Phenyl-hexyl	150 × 4.6	2.6
Accucore C18	Thermo	Superficially porous silica	Octadecyl	150 × 4.6	2.6
Accucore PFP	Thermo	Superficially porous silica	Pentafluorophenyl	150 × 4.6	2.6
Ascentis Express OH5	Supelco	Superficially porous silica	Penta-hydroxyl	150 × 4.6	2.7
Ascentis Express F5	Supelco	Superficially porous silica	Pentafluorophenyl	150 × 4.6	2.7
Nucleoshell HILIC	Macherey-Nagel	Superficially porous silica	Sulfobetaine	150 × 3.0	2.7
Nucleoshell PFP	Macherey-Nagel	Superficially porous silica	Pentafluorophenyl	150 × 3.0	2.7

An interesting feature of SFC is that, in addition to possibly providing an orthogonal method to a reversed-phase HPLC one [5–10], it can also be orthogonal to itself, when stationary phases are adequately selected [11]. Indeed, all columns that are marketed for HPLC, whether for reversed-phase (RP), normal-phase (NP), hydrophilic interaction (HILIC) or ion-exchange modes, can also be used with mobile phases comprising carbon dioxide [12–16]. Chemical diversity of the available stationary phases is currently significantly improving, with rising interest of the column manufacturers and research groups to produce original phases dedicated to SFC use [17–19]. Moreover, while different operating modes in HPLC require different mobile phase composition (for instance, hydro-organic in RP, alkane-alcohol in NP), the same CO_2-co-solvent mobile phase may be used with all of them. As a result, two columns with different surface chemistry can be employed with the same operating conditions and provide orthogonal selectivity [4,20].

The present work aims at developing a rapid screening method for impurity profiling of drug candidates with SFC-ESI-MS. The first part presented in the previous paper focused on the selection of a versatile mobile phase composition to ensure elution of the largest proportion of drug-like compounds with good peak shape and the best possible UV and ESI-MS responses. Several additives introduced in the CO_2-methanol mobile phase were thus tested with a wide range of stationary phases to assess their capabilities for successful chromatography and MS detection. Because the method aims at direct applicability in a pharmaceutical company, a large selection (160) of drug candidates provided by Servier Research Laboratories was evaluated. We finally settled our choice on a gradient elution of methanol comprising 2% water and 20 mM ammonium acetate [1].

The second part, described in the present paper, will focus on stationary phase selection to achieve orthogonal methods.

2. Material and methods

2.1. Chemicals, solvents and reagents

160 drug candidates were obtained from Servier Research Laboratories (Suresnes, France) whose structures are confidential. More details about the compounds selected can be found in the first part of this study. Ammonium acetate was obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France) and ultra-pure water was provided by an Elga UHQ system from Veolia (Wissous, France). Solvents used were HPLC-grade methanol (MeOH) and ethanol provided by VWR (Fontenay-sous-Bois, France). Formic acid was obtained from VWR (Fontenay-sous-Bois, France). Carbon dioxide of industrial grade 99.5% was provided by Messer (Puteaux, France).

2.2. Stationary phases

For this study, 23 commercialized columns were compared. The known features of the stationary phase chemistries and dimensions are gathered in Table 1. The columns selected were all high efficiency phases (1.7, 1.8 or 2.5 μm fully porous and 2.6 or 2.7 μm superficially porous particles) with a variety of stationary phase chemistries. The columns were kindly provided by Waters, Phenomenex, Thermo, Supelco and Macherey-Nagel.

2.3. Instrumentation

The supercritical fluid chromatography system was a Waters Corporation (Millford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography™ (UPC^2®). It was equipped with a binary solvent delivery pump compatible with mobile phase flow rates up to 4 mL/min and pressures up to 414 bar, an autosampler that included partial loop volume injection system, a back-pressure regulator, 4-position column oven compatible with 150 mm length columns and two detectors: a photodiode-array (PDA) detector and an ACQUITY QDa® single-quadrupole mass detector with electrospray ionization source. An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was then splitted by the on-board flow-splitter assembly. With this system, most of the column flow goes to the back-pressure regulator and an unknown portion goes to the MS. MassLynx® software (V4.1) was used for system control and data acquisition. Empower® 3 was used for integration of peaks for peak width measurements. Waters Data

Converter (V2.1) was used to convert data from MassLynx to Empower.

2.4. Chromatographic conditions

The screening of the different columns was performed in a gradient elution program in the following conditions:

(1) For columns with 100 × 3.0 mm dimensions (1.7-2.5 μm fully porous particles), the mobile phase composition was CO_2 with 5 to 50% MeOH (+ ammonium acetate) in 10 min, flow rate was fixed at 1 mL/min, temperature was set at 25 °C and the outlet pressure was maintained at 150 bar. Inlet pressure at the beginning and end of the gradient program varied from 180 to 215 bar respectively.
(2) For columns with 150 × 4.6 mm dimensions (2.6 μm superficially porous particles), the mobile phase composition was CO_2 with 5 to 50% MeOH (+ ammonium acetate) in 15 min, flow rate was fixed at 2.35 mL/min, temperature was set at 25 °C and the outlet pressure was maintained at 150 bar. Inlet pressure at the beginning and end of the gradient program varied from 190 to 270 bar respectively. Note that this flow rate is close to the optimal point in van Deemter curves on this type of stationary phases [21].
(3) For the two columns having different dimensions from the first two groups (150 x 3.0 mm), Nucleoshell HILIC and PFP (2.7 μm superficially porous particles), the mobile phase composition was CO_2 with 5 to 50% MeOH (+ ammonium acetate) in 15 min, flow rate was fixed at 1 mL/min, temperature was set at 25 °C and the outlet pressure was maintained at 150 bar. Inlet pressure at the beginning and end of the gradient program varied from 200 to 270 bar respectively.
(4) Thus comparable linear speed and gradient conditions were used with all columns.

Ammonium acetate solution was first prepared at 1 M in water to achieve good solubility and then diluted down to 20 mM in MeOH. The final composition of mobile phase co-solvent thus comprises 20 mM ammonium acetate and 2% water.
16 mixtures comprising 10 compounds each were prepared at 1 mg/mL in ethanol. 2 μL of each mixture was then injected. The wavelength of UV detection was fixed at 210 nm. Frequency was set at 20 pts/s and resolution at 1.2 nm.
The mass detector unit was pre-optimized by the manufacturer. The studied compounds were detected in positive and negative electrospray ionization mode (m/z 150–750), scan rate 10 pts/s, capillary voltage 0.8 kV, cone voltage 15 V, sampling frequency 8 Hz, ion source temperature 150 °C and probe temperature 600 °C. Nitrogen was used as nebulizing gas. Make-up flow was 0.45 mL/min with 90% methanol–10% water comprising 1% formic acid. As the pre-selected ionization parameters cause only little fragmentation, the precursor ions only were considered ($[M+H]^+$ in ESI^+ mode, $[M-H]^-$ in ESI^-).

2.5. Data analysis

Hierarchical cluster analysis was carried out with XLStat 2014.3.02 software (Addinsoft, New York, NY). Ward's method was used to cluster the normalized data (retention data were centered and reduced for each column), and the Euclidean distance defined the distance between two clusters. Only the compounds that could be eluted on all 11 columns retained at this stage were used in the calculation (127 out of an initial set of 160).

3. Results and discussion

3.1. Description of the analyte set

The analytes selected for this study were extracted from a library of drug candidates from Servier Research Laboratories. In designing this test set, the purpose was to have a set that would be as representative as possible of the diversity of chemical structures usually encountered in this pharmaceutical company [1]. The resulting group of analytes comprises acids, bases and neutrals, with log P values in the range [−2; 8.0]. For the first screening of 23 columns, a short selection of 20 analytes was extracted so as to eliminate at an early stage those columns that would provide inadequate retention and peak shapes. In the short selection, log P values vary from −0.5 to 8.0, and acidic and basic pK_a values are scattered in a comparable manner to the pK_a values of the larger set of analytes.

3.2. Description of the column set

Initially, a large set of columns was selected (Table 1). Our intention was to try a large variety of stationary phase chemistries, to increase the probability to identify excellent and orthogonal columns. Different selectivities were expected based on previous characterization achieved with linear solvation energy relationships (LSER), resulting in a classification of columns, as appears in Fig. 1 [20,22]. It must be pointed out that the present classification has some significant differences from the analytes and operating conditions evaluated here, because it does not include ionic interactions (while the present set of analytes contains ionizable species) and because the mobile phase employed to classify the columns did not contain any ammonium acetate. We thus selected columns with polar stationary phases (silica or polar ligands bonded on silica, pink and red points respectively in Fig. 1), non-polar stationary phases (hydrophobic C18-bonded silica, dark blue points in Fig. 1) and different stationary phases with intermediate polarity (aromatic ligands, green points; fluoroaromatic ligands, purple points; alkyl ligands with embedded polar functions or no end-capping treatment, blue points in Fig. 1). Only columns with small particles were selected, to ensure optimal efficiency. Some of the most recently available columns are present in this set. It can be seen on Fig. 1 that the 23 columns selected were well scattered in the selectivity space defined by LSER classification.

3.3. Definition of Derringer desirability functions

First of all, Derringer desirability functions [23] were defined to rank the performance of the selected columns as regards retention, retention window and peak shapes. To implement Derringer functions, the first step is to choose the criteria that influence the quality of results, and will thus influence the ranking of the columns. For the purpose of selecting the best column, we chose to consider the influence of six different criteria. We thus created Derringer functions and calculated values of different Derringer parameters (d_i) for each column (Fig. 2).

The first studied criterion studied was the number of eluted compounds. We created a d_1 function as follows: 20 (first ranking) or 160 (second ranking) compounds were injected so we expected the elution of 20 or 160 compounds at best. If the chosen gradient composition allowed the elution of 20 or 160 compounds, we assigned a score of 1 to this column. In other words, a score of 1 is the maximum value for the d_1 coefficient, corresponding to a totally desirable response, and never occurred in practice. On the contrary, if no compounds should be eluted, a value of 0 would be assigned to the column. 0 is thus the minimum value for d_1 coefficient, corresponding to a totally undesirable response, a case that naturally never occurred neither. Practically, based on UV and MS

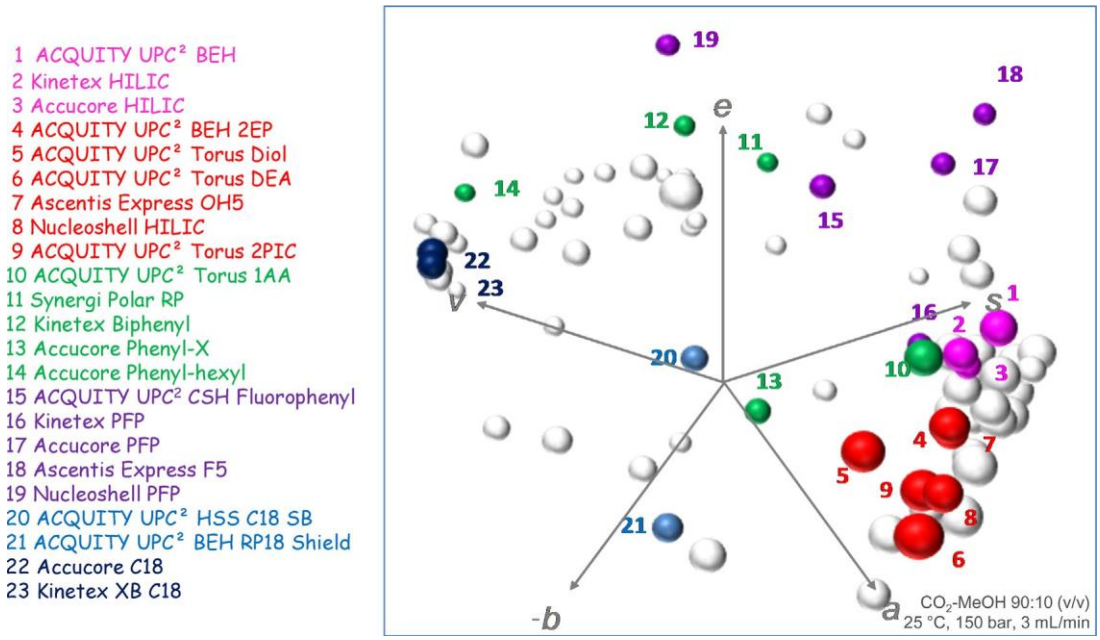


Fig. 1. LSER classification of columns evidencing the 23 tested columns.

detections, we counted the number of eluted compounds for each column and assigned a value of d_1 between 0 and 1 according to the defined linear function (Fig. 2).

The procedure was the same for the d_2 coefficient, but this time counting the number of symmetrical peaks. We could thus assign to each column a value for the d_2 coefficient between 0 and 1 (Fig. 2). Although it was expected that high d_2 values (large number of symmetrical peaks) could only be obtained with high d_1 values (large number of eluted peaks), it was verified that no correlation existed between the d_1 and d_2 criteria. Indeed, on some of the selected

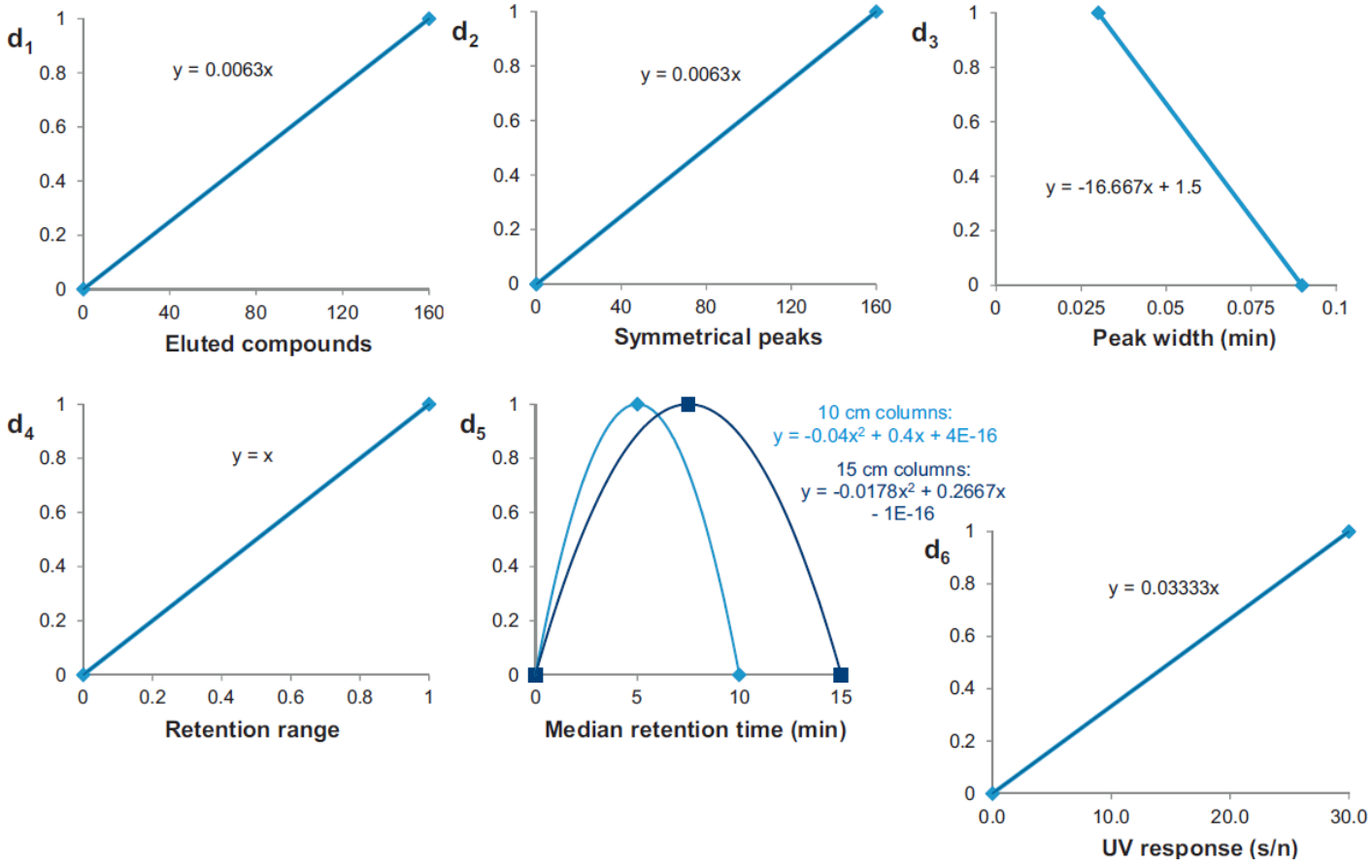


Fig. 2. Derringer desirability functions based on (d_1) the number of eluted compounds, (d_2) the number of symmetrical peaks, (d_3) peak width measured on 11 compounds, (d_4) retention range, (d_5) average retention time, and (d_6) the average UV responses measured for eluted compounds.

columns, the elution of a large portion of analytes was observed but good peak shapes only for a small portion of them, as will be further discussed later.

The third studied criterion studied was the efficiency, evaluated with peak width of selected analytes. In the first ranking (20 analytes), only one compound that could be eluted on all columns with reasonably good peak shape served to evaluate column efficiency. In the second ranking (160 analytes), an average peak width value was calculated for a set of eleven compounds that were eluted on all columns with reasonably good peak shapes. We attributed to the column which provided the lowest average peak width a score of 1 for the d_3 coefficient, and a score of near 0 for the column which provided the largest average peak width (Fig. 2). It was found important to select compounds with good peak shapes in this case, otherwise we may penalize one column twice for the same defect (redundancy with d_2 criterion). To confirm this, it was verified that no correlation existed between d_2 and d_3. It is thus believed that the d_2 and d_3 criteria evaluate two different things: provided that the mobile phase composition ensures good solubility of the analytes (which should be the case, based on the mobile phase optimization study [1]), non-symmetrical peaks (d_2) may still exist when analytes interact with strong adsorption sites on the stationary phase, while peak width measured on symmetrical peaks (d_3) results of many other factors as good column packing, particle size etc.

The fourth studied criterion was the retention range: we wanted the retention range to be as large as possible on the total duration of the gradient. Indeed, a large retention window indicates adequate selectivity towards this selection of compounds, thus a better chance that the target compound will be separated from its impurities. For each tested column, we calculated the difference between the maximal retention time and minimal retention time (Δt_R), obtained with the set of 20 or 160 compounds. This value was simply divided by the gradient time (10 or 15 min, depending on column dimensions), to obtain a value between 0 and 1 (Fig. 2).

The fifth studied criterion is the median retention time. Ideally, it should be superimposed with the middle of the gradient, thus at about 5 or 7.5 min for a column length of 100 or 150 mm, with gradient time of 10 and 15 min respectively. If a column had a median retention time at 5 or 7.5 min, a value of 1 was assigned for d_5. On the contrary, if the median retention time was very large or very low, the value of d_5 was equal to 0. Then, because some deviation from this ideal position should not be a strong handicap, we preferred a parabolic function rather than two linear (increasing and decreasing) functions (Fig. 2).

Median retention time is an interesting criterion because it indicates the overall retentiveness of the column towards this selection of analytes. When average retention is too low, it cannot be expected that adequate selectivity will be obtained. When retention is too high, there is a risk that some analytes may be strongly retained in the column and would not elute during the gradient time. It may however be argued that average retention depends on the selected gradient conditions: some columns may be less retentive than others and would reach a better score with lower proportions of co-solvent. However, preliminary tests had shown that, below 5% methanol in the mobile phase, poor solubility observed for some analytes caused serious peak distortion. Conversely, highly retentive columns may require elution conditions with higher elution strength. To be totally fair, the gradient features could have been adapted to the stationary phase retentiveness, but this seemed unrealistic to achieve fast column selection.

It was not expected that the retention criteria d_4 and d_5 could be correlated to the previous ones (d_1, d_2 and d_3), and indeed nothing of that sort was observed. However, some level of correlation, with a logarithmic relationship, existed between d_4 and d_5. Our intention with these two criteria was to differentiate the columns that would provide a narrow elution window close to the dead volume (inadequate retention), and the columns that would provide a narrow elution window close to the end of the gradient (inadequate retention), from the columns that would provide a narrow elution window close to the middle of the gradient (adequate retention but inadequate selectivity). In the latter case, the gradient slope could be changed to enlarge the retention window. However, it appears that in the selected set of columns, most columns providing small retention window also provided low retention. We may also take it as a proof that our compound set was adequately selected as it reflected nicely the selectivity capabilities. The interest of maintaining both d_4 and d_5 criteria may then be questioned and will be further discussed later.

For the largest selection of compounds, a sixth and last criterion was calculated based on UV response (signal-to-noise ratio). The d_6 value was obtained by averaging the UV response values (s/n) measured for all compounds that were eluted from the column. The column which provided the highest average UV response received a score of 1 for the d_6 coefficient, and the column which provided the lowest average UV response received a score of near 0 for the d_6 coefficient (Fig. 2). As discussed in the first part of this study [1], good UV response evaluated with peak height could be related to chromatographic features (peak symmetry and peak width). It was observed that the d_6 values were not correlated to d_2 and d_3 values. Once the coefficients d_1, d_2, d_3, d_4, d_5 and d_6 were known for each column, we calculated the value of total desirability, D. The total desirability was initially defined as the geometric mean of all six different functions:

$$D = (d_1 * d_2 * d_3 * d_4 * d_5 * d_6)^{1/6} \quad (1)$$

3.4. Pre-selection of adequate columns based on 20 compounds

We started this study with the screening of the 23 columns in Table 1 and a reduced set of 20 compounds, with the analytical conditions described in section 2.4.

Once all data were acquired, Derringer desirability functions were used to rank the columns according to the selected criteria: number of eluted compounds (d_1), number of symmetrical peaks (d_2), peak width (d_3), retention range (d_4) and median retention time (d_5). The values can be observed in Fig. 3.

It appeared that the first criterion, based on the number of eluted compounds, brought very little discrimination between the different columns. Indeed, in the worst case, 16 compounds out of 20 were eluted, while 20 compounds were observed in the best case. This is simply the result of a successful first-step optimization: because we have previously selected the best mobile phase conditions [1], ensuring good solubility for a majority of analytes, there remains little variations between the columns. As a result, we decided not to retain the d_1 value for the calculation of D.

The number of symmetrical peaks was much more discriminating as it varied between 10 and 19 depending on the column. The d_2 criterion was thus found to be a good selector.

Peak width measured on one analyte that was always eluted with reasonably good peak shape also varied to a great extent, thus the d_3 criterion was maintained.

The retention range varied greatly between the columns. In the worst case, less than 10% of the gradient time was covered, while in the best case, more than 90% of the gradient time was covered. We concluded that the d_4 criterion was also highly selective.

Finally, the median retention time also varied greatly. However, as indicated above, a significant logarithmic correlation was observed between the d_4 and d_5 criteria. Consequently, maintaining the two of them in the calculation of D made little sense. It was observed that using the d_5 criterion or not had very little effect on the ranking of the columns.

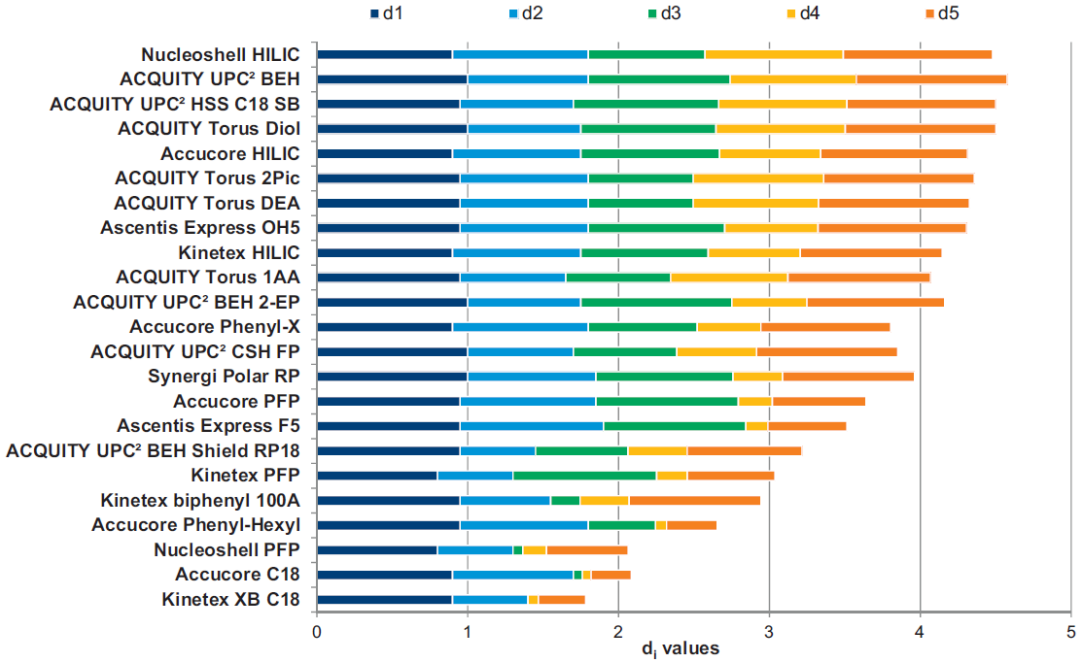


Fig.3. Details of each criterion studied with Derringer's desirability functions on 23 different columns, based on 20 analytes. (d_1) Number of eluted compounds, (d_2) number of symmetrical peaks, (d_3) peak width measured on 1 compound, (d_4) retention range, (d_5) median retention time.

Thus we proceeded to calculate the value of total desirability D for each column using the following formula:

$$D(20\,\text{analytes}) = (d_2 * d_3 * d_4)^{1/3} \quad (2)$$

The D values and resulting ranking can be seen in Table 2. Looking at the top of the ranking (Fig. 3 and Table 2), we can see that there are many different stationary phase chemistries with diverse particle types. We can also note that, for columns at the top of the ranking, all Derringer criteria were high. In other words, a good column performs well for many reasons. On the opposite, at the bottom of the ranking we found the less polar columns (Kinetex XB C18, Accucore C18, Nucleoshell PFP, Accucore Phenyl- Hexyl, Kinetex biphenyl and Kinetex PFP) whose D values were very low, while individual d_i values were not equally bad. The major reason for this low ranking was the low retentiveness observed for our set of compounds with these columns, which were therefore strongly penalized by the d_4 criterion (retention range). However, some of them provided thin peaks with satisfying peak shapes. For such columns, we must admit that the gradient conditions we had selected may have been responsible for the poor scores they obtained, as they forbade them to demonstrate their full potential. A lower gradient slope may have revealed them as better columns than they appear here.

Table 2
Ranking of 23 columns based on 20 analytes and criteria d_2, d_3 and d_4.

Column	*D* value	Rank
Nucleoshell HILIC	0.861	1
ACQUITY UPC^2 BEH	0.857	2
ACQUITY UPC^2 HSS C18 SB	0.850	3
ACQUITY UPC^2 Torus DIOL	0.832	4
Accucore HILIC	0.806	5
ACQUITY UPC^2 Torus 2-PIC	0.799	6
ACQUITY UPC^2 Torus DEA	0.789	7
Ascentis Express OH5	0.780	8
Kinetex HILIC	0.760	9
ACQUITY UPC^{2}Torus 1-AA	0.723	10
ACQUITY UPC^2 BEH 2-EP	0.721	11
Accucore Phenyl-X	0.649	12
ACQUITY UPC^2 CSH FP	0.634	13
Synergi Polar RP	0.633	14
Accucore PFP	0.576	15
Ascentis Express F5	0.507	16
ACQUITY UPLC BEH Shield RP18	0.493	17
Kinetex PFP	0.459	18
Kinetex biphenyl 100A	0.336	19
Accucore Phenyl-Hexyl	0.301	20
Nucleoshell PFP	0.172	21
Accucore C18	0.140	22
Kinetex XB C18	0.008	23

Considering overall D values (Table 2), we can separate the columns into two distinct groups: the first 11 columns have high D values (>0.7) and all Derringer criteria are high; from the 12th column (Accucore Phenyl-X) D values decrease down to a value that is very close to zero. We thus considered eliminating all the columns that were judged inadequate in terms of retention window and peak shapes.

Thus a selection of 11 columns was retained for further tests. It is interesting to note that this set comprises a majority of columns packed with sub-2 μm fully porous particles (seven columns), which were polar phases especially designed for SFC use, and a minority of superficially porous particles (four of them), which were HPLC columns designed for the HILIC mode (bare silica, diol and sulfoalkylbetaine).

3.5. Refining the selection of columns based on 160 analytes on the 11 best columns

The aim being to select an orthogonal set of stationary phases, it was important to expand the tested set of compounds tested in order to confirm the initial findings. We thus analyzed the 160 analytes with the best columns. The analytical conditions were the same as before, as detailed section 2.4.

Once all data were acquired, Derringer desirability functions were used to rank again the columns according to the selected criteria: number of eluted compounds (d_1), number of symmetrical peaks (d_2), peak width (d_3), retention range (d_4), median retention time (d_5) and average UV response (d_6). The results are presented in Fig. 4.

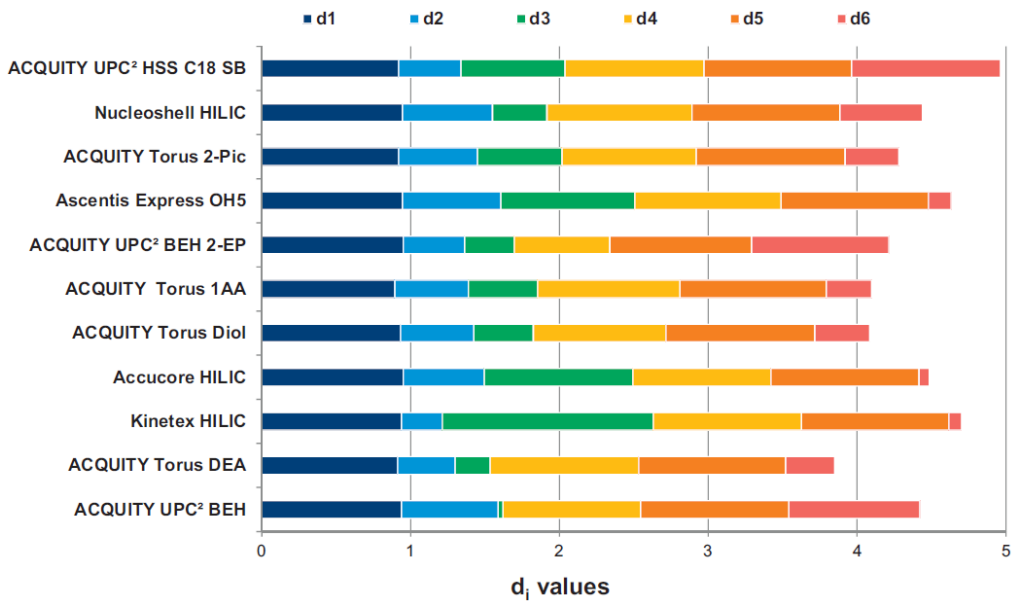


Fig.4. Details of each criterion studied with Derringer's desirability functions on 11 different columns based on 160 analytes. (d_1) Number of eluted compounds, (d_2) number of symmetrical peaks, (d_3) peak width measured on 11 compounds, (d_4) retention range, (d_5) median retention time, (d_6) average UV responses.

Again, d_1 provided little discrimination between the columns as the number of compounds that were successfully eluted varied between 143 and 152, out of 160 injected analytes. Incidentally, it shows that an adequate mobile phase allowed the elution of 89–94% of analytes. This criterion was thus eliminated.

The number of symmetrical peaks (d_2) is much more discriminating, varying between 62 and 106.

Average peak width (d_3) was still a good selector, although the worst columns had been eliminated at the previous step.

Since only the best columns from the first ranking had been retained, the discrimination provided by the retention window (d_4) was not as strong as described above: the worst column still covered more than 60% of the gradient time, while the best ones were close to 100%.

Again the median retention time (d_5) was correlated with retention window (d_4) and was thus not considered necessary in the final ranking.

Finally, the average UV response (d_6) varied to a large extent between the columns.

Then we calculated the value of total desirability D for each column according to the following formula:

$$D(160\,\text{analytes}) = (d_2 * d_3 * d_4 * d6)^{1/4} \quad (3)$$

The final ranking is presented in Table 3 and Fig. 4. The classification somewhat differs from the previous one, thus the effect of testing a very large group of analytes is significant.

Table 3
Ranking of 11 columns based on 160 analytes and criteria d_2, d_3, d_4 and d_6.

Column	*D* value	Rank
ACQUITY UPC2 HSS C18 SB	0.723	1
Nucleoshell HILIC	0.589	2
ACQUITY UPC^2 Torus 2-PIC	0.560	3
Ascentis Express OH5	0.546	4
ACQUITY UPC^2 BEH 2-EP	0.535	5
ACQUITY UPC^2 Torus 1-AA	0.509	6
ACQUITY UPC^2 Torus DIOL	0.505	7
Accucore HILIC	0.432	8
ACQUITY UPC^2 Torus DEA	0.416	9
Kinetex HILIC	0.384	10
ACQUITY UPC^2 BEH	0.364	11

It is interesting to note that the column with the highest D value and therefore best rank is a non-endcapped C18-bonded silica stationary phase. The second one is a sulfobetaine-bonded silica phase dedicated to HILIC. Thus two very different stationary phase chemistries rank at the top of this classification. The following ones are also very diverse, and it appears that fully porous sub-2 μm particles and superficially porous particles are intermixed.

As a note of caution, we must point out that the columns included in this second and final ranking all appeared to be good columns (obviously, as they were the best selected ones from the first ranking), but the purpose was to select the ones that would behave the best towards our set of analytes. Other sets of analytes may yield a somewhat different ranking.

3.6. Orthogonal selection of columns

The previous classification does not take into account the possible correlations between multiple columns. But many columns provided similar results in terms of retention time and elution order. To identify the most orthogonal columns we calculated hierarchical cluster analyses based on normalized retention time. Retention times were thus centered and reduced. Normalization was necessary because column dimensions were different, resulting in different gradient times. Only the 127 compounds that could be eluted on all eleven columns were retained for the calculation. Fig. 5 presents the classification obtained with hierarchical cluster analysis.

The number of groups issuing from such a classification depends on the position of the cutting line, as decided by the analyst, based on experience and previous knowledge of the items classified. For instance, the columns can be divided into four clusters:

(i) The first cluster comprises 1-aminoanthracene and 2-ethylpyridine phases.
(ii) The second cluster comprises diol-type phases and the sole sulfobetaine phase.
(iii) The third cluster comprises diethylamine and 2-picolylamine phases.
(iv) Finally, the fourth cluster, which is most different from the other three, comprises the three bare silica phases, and the C18-bonded phase. This group may seem surprising, but we

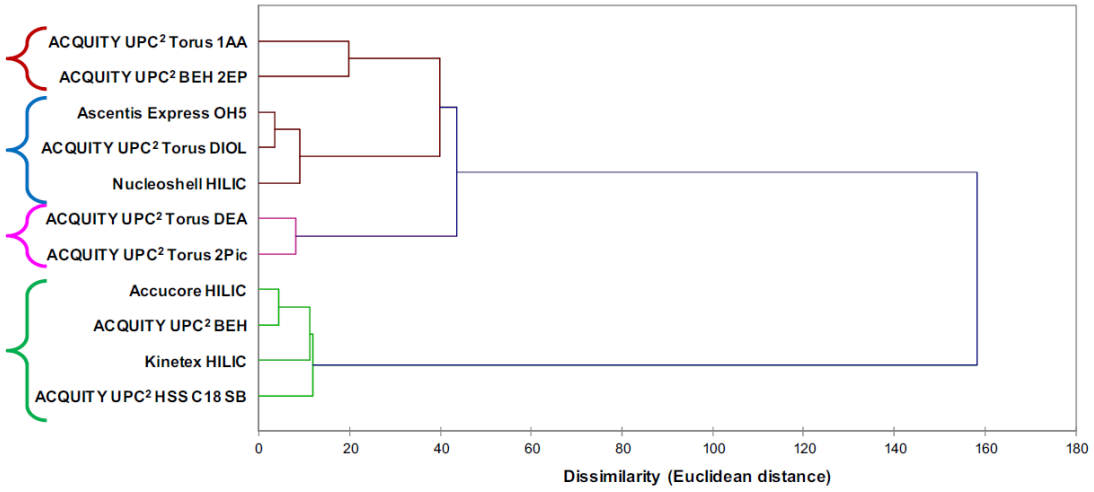


Fig. 5. Hierarchical cluster analysis based on the normalized retention times measured on the large set of analytes (127 compounds) on the 11 columns retained for the second ranking (see text for details).

have had occasions in the past to point out the high polarity of the HSS C18 SB phase, which is not endcapped [4].

The two top-ranked columns (Fig. 4) were ACQUITY UPC^2 HSS C18 SB and Nucleoshell HILIC. In Fig. 5, they appear in two separate clusters, with significant dissimilarity. To confirm that these columns were orthogonal, we compared the retention times measured on the ACQUITY UPC^2 HSS C18 SB column with those measured on the Nucleoshell HILIC column (Fig. 6). It is clear from this figure that there is no correlation between them (determination coefficient was R^2 = 0.41). For instance, it is visible that the analytes eluted at the very end of the gradient on the C18 phase are well scattered in the middle of the gradient on the sulfobetaine phase. Conversely, the analytes that are eluted near dead volume with the sulfobetaine phase are well separated with the C18 phase. Additionally, it can be noted that the retention space is well covered by each of these columns with points being scattered in the 10 and 15 minutes gradient times. Although it cannot be seen on this figure, we had also noted that those compounds that resulted in poor peak shapes on the first column often eluted nicely on the

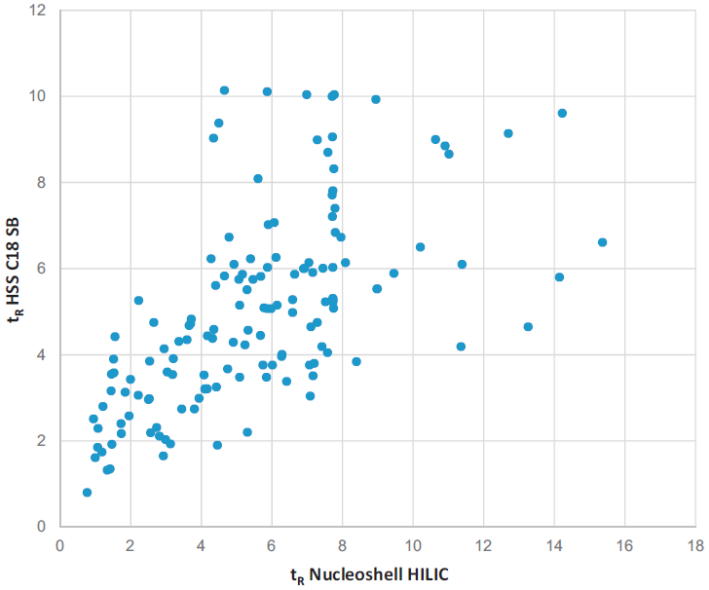


Fig.6. Comparison between retentions times obtained with ACQUITY UPC^2 HSS C18 SB and Nucleoshell HILIC columns.

second one, or vice-versa. These first two columns could thus be selected to define a duet of orthogonal systems, to maximize the chances to see all impurities within two runs.

Note that any other selection of columns from different clusters defined in Fig. 5 would have yielded similar conclusions.

4. Conclusions

In the aim of providing an orthogonal set of stationary phases, a selection of 11 efficient columns (out of an initial set of 23) with a variety of stationary phase chemistries were first selected based on the analysis of 20 analytes. Increasing the number of tested analytes to 160 with a thorough evaluation of chromatographic results permitted a ranking of the 11 best columns towards this particular selection of analytes. These 11 columns essentially comprised polar stationary phases and phases with mixed polarity (comprising both polar and non-polar features). Finally, taking into account the orthogonality of the columns in terms of retention times permitted to refine the selection of columns and propose a simple duet: ACQUITY UPC^2 HSS C18 SB (a non-endcapped C18-bonded phase) and Nucleoshell HILIC (a sulfoalkylbetaine-bonded phase).

Further optimization of the proposed method should include increasing gradient slope and, when the upper pressure limit of the pumping system permits it, possibly increasing flow rate to achieve faster methods.

Acknowledgments

We warmly thank Waters Corporation for the ACQUITY UPC^2 system and ACQUITY QDa detector let at our disposal (Mark Baynham, Taraneh Kargar) and for the opportunity to test new stationary phases (Darryl Brousmiche, Jacob Fairchild, Kevin Wyndham, Steve Collier and team). We also thank Thierry Domenger (Thermo), Régis Guyon (Machery-Nagel), Magali Dupin and Marc Jacob (Phenomenex) for the kind gift of columns.

References

[1] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates, J. Chromatogr. A 1408 (2015) 217–226.

[2] E. Lesellier, C. West, The many faces of packed column supercritical fluid chromatography - A critical review, J. Chromatogr. A 1382 (2015) 2–46, http://dx.doi.org/10.1016/j.chroma.2014.12.083

[3] L. Nováková, A. Grand-Guillaume Perrenoud, I. Francois, C. West, E. Lesel- lier, D. Guillarme, Modern analytical supercritical fluid chromatography using columns packed with sub-2 μm particles: A tutorial, Anal. Chim. Acta 824 (2014) 18–35, http://dx.doi.org/10.1016/j.aca.2014.03.034

[4] S. Khater, C. West, E. Lesellier, Characterization of five chemistries three particle sizes of stationary phases used in supercritical fluid chromatography, J. Chromatogr. A 1319 (2013) 148–159, http://dx.doi.org/10.1016/j.chroma.2013.10.037

[5] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Comparison of ultra-high performance supercritical fluid chromatography ultra-high performance liquid chromatography for the analysis of pharmaceutical compounds, J. Chromatogr. A 1266 (2012) 158–167, http://dx.doi.org/10.1016/j.chroma.2012.10.005

[6] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, The use of columns packed with sub-2μm particles in supercritical fluid chromatography, TrAC Trends Anal. Chem. 63 (2014) 44–54, http://dx.doi.org/10.1016/j.trac.2014.06.023

[7] K. Li, Q. Fu, H. Xin, Y. Ke, Y. Jin, X. Liang, Alkaloids analysis using off-line two-dimensional supercritical fluid chromatography × ultra-high performance liquid chromatography, Analyst 139 (2014) 3577–3587, http://dx.doi.org/10.1039/C4AN00438H

[8] P.G. Stevenson, A. Tarafder, G. Guiochon, Comprehensive two-dimensional chromatography with coupling of reversed phase high performance liquid chromatography supercritical fluid chromatography, J. Chromatogr. A 1220 (2012) 175–178, http://dx.doi.org/10.1016/j.chroma.2011.11.020

[9] I. Franç ois, A. dos Santos Pereira, F. Lynen, P. Sandra, Construction of a new interface for comprehensive supercritical fluid chromatography×reversed phase liquid chromatography (SFC×RPLC), J. Sep. Sci. 31 (2008) 3473–3478, http://dx.doi.org/10.1002/jssc.200800267

[10] A. Periat, A. Grand-Guillaume Perrenoud, D. Guillarme, Evaluation of various chromatographic approaches for the retention of hydrophilic compounds and MS compatibility, J. Sep. Sci. 36 (2013) 3141–3151, http://dx.doi.org/10.1002/jssc.201300567

[11] M.L. de la Puente, P. López Soto-Yarritu, J. Burnett, Supercritical fluid chromatography in research laboratories: Design development implementation of an efficient generic screening for exploiting this technique in the achiral environment, J. Chromatogr. A 1218 (2011) 8551–8560, http://dx.doi.org/10.1016/j.chroma.2011.09.029

[12] C. West, E. Lesellier, Characterization of stationary phases in subcritical fluid chromatography by the solvation parameter model: I. Alkylsiloxane-bonded stationary phases, J. Chromatogr. A 1110 (2006) 181–190, http://dx.doi.org/10.1016/j.chroma.2006.01.125

[13] C. West, E. Lesellier, Characterisation of stationary phases in subcritical fluid chromatography with the solvation parameter model: III. Polar stationary phases, J. Chromatogr. A 1110 (2006) 200–213, http://dx.doi.org/10.1016/j. chroma.2006.01.109

[14] C. West, E. Lesellier, Characterisation of stationary phases in subcritical fluid chromatography with the solvation parameter model IV: Aromatic stationary phases, J. Chromatogr. A 1115 (2006) 233–245, http://dx.doi.org/10.1016/ j.chroma.2006.02.050

[15] C. West, S. Khater, E. Lesellier, Characterization use of hydrophilic interaction liquid chromatography type stationary phases in supercritical fluid chromatography, J. Chromatogr. A 1250 (2012) 182–195, http://dx.doi.org/10.1016/j. chroma.2012.05.008

[16] C.F. Poole, Stationary phases for packed-column supercritical fluid chromatography, J. Chromatogr. A 1250 (2012) 157–171, http://dx.doi.org/10.1016/j. chroma.2011.12.040

[17] R. McClain, M.H. Hyun, Y. Li, C.J. Welch, Design, synthesis, and evaluation of stationary phases for improved achiral supercritical fluid chromatography separations, J. Chromatogr. A 1302 (2013) 163–173, http://dx.doi.org/10.1016/j.chroma.2013.06.038

[18] J. Smuts, E. Wanigasekara, D.W. Armstrong, Comparison of stationary phases for packed column supercritical fluid chromatography based upon ionic liquid motifs: a study of cation and anion effects, Anal. Bioanal. Chem. 400 (2011) 435–447, http://dx.doi.org/10.1007/s00216-011-4767-z

[19] F.-M. Chou, W.-T. Wang, G.-T. Wei, Using subcritical/supercritical fluid chromatography to separate acidic, basic, and neutral compounds over an ionic liquid-functionalized stationary phase, J. Chromatogr. A 1216 (2009) 3594–3599, http://dx.doi.org/10.1016/j.chroma.2009.02.057

[20] C. West, E. Lesellier, Orthogonal screening system of columns for supercritical fluid chromatography, J. Chromatogr. A 1203 (2008) 105–113, http://dx.doi.org/10.1016/j.chroma.2008.07.016

[21] E. Lesellier, Efficiency in supercritical fluid chromatography with different superficially porous and fully porous particles ODS bonded phases, J. Chromatogr. A 1228 (2012) 89–98, http://dx.doi.org/10.1016/j.chroma.2011.11.058

[22] C. West, S. Khater, M. Khalikova, E. Lesellier, A classification of columns for SFC use, The Column 10 (2014) 2–8.

[23] G. Derringer, R. Suich, Simultaneous optimization of several response variables, J. Qual. Technol. 12 (1980) 214.

IV. Robustesse des méthodes SFC développées

Après le développement de méthodes SFC réalisé précédemment ayant désigné une composition de phase mobile et deux phases stationnaires préférentielles, on souhaite s'assurer de la robustesse de ces méthodes. Pour cela, un second jeu de colonnes est utilisé. Pour la colonne ACQUITY HSS C18 SB, une colonne de dimensions identiques (100 x 3.0, 1.8 µm) provenant d'un lot différent a été achetée. Pour la colonne Nucleoshell HILIC, une colonne de dimensions différentes a été achetée (100 x 3.0, 2.7 µm). En effet, lorsque les premières expériences ont débuté, seule une colonne de 15 cm était disponible au laboratoire et c'est avec cette colonne que nous avions commencé les tests de criblage. Lors du rachat d'une seconde colonne pour les tests de robustesse, la question de la dimension de colonne s'est posée. Le choix s'est alors porté sur une colonne de 10 cm, afin de travailler avec des colonnes de dimensions identiques pour notre paire de deux colonnes orthogonales en SFC. Ainsi, le temps de gradient passe de 15 à 10 min sur cette colonne, le reste des paramètres restant identique (débit, pression, température). Dans la suite de ce travail et par soucis de simplicité, on parlera de la colonne C18 pour la colonne ACQUITY HSS C18 SB et de la colonne HILIC pour la Nucleoshell HILIC.

Nos précédents travaux (Chapitre 3, II et III) nous ont amené à conclure que l'utilisation d'acétate d'ammonium ou d'ammoniaque comme additif dans le co-solvant permettait d'obtenir les meilleures performances chromatographiques et la meilleure détection UV et MS. Bien qu'il ait fourni des performances supérieures à l'hydroxyde d'ammonium lors de nos tests, l'acétate d'ammonium absorbe fortement en UV. Cela crée une dérive importante de la ligne de base en gradient et peut gêner pour le profilage d'impuretés. Pour la suite des travaux, nous avons donc privilégié l'hydroxyde d'ammonium, à une concentration de 20 mM dans le MeOH (et 2% H_2O).

Afin d'évaluer la robustesse des méthodes en SFC, le même ensemble de 140 composés pharmaceutiques que précédemment a été utilisé. Ce jeu de composés a été analysé sur les deux paires de colonnes. Pour simplifier, les premières colonnes utilisées seront notées n°1 et les colonnes du second jeu (batch différent) seront notées n°2. Les conditions d'analyses n'étant pas parfaitement identiques (un gradient de 15 min pour la colonne Nucleoshell HILIC n°1 contre des gradients de 10 min pour chacune des colonnes HSS C18 SB et pour la colonne Nucleoshell HILIC n°2), on transformera les t_R obtenus en composition à l'élution C_e (Eq. 5) pour la comparaison des deux jeux de colonnes.

$$C_e = C_i + \frac{(C_f - C_i)}{t_G} * (t_R - t_D) \quad \text{Eq. 5}$$

Où C_e correspond au pourcentage de MeOH nécessaire à l'élution du PA ; C_i et C_f correspondent aux compositions initiale et finale du gradient (5 et 50% respectivement) ; t_G est le temps de gradient ; t_R est le temps de rétention du composé et t_D est le délai de gradient (0.46 min).

Sur la Figure 3.8a, on compare les C_e obtenues pour les 140 composés Servier entre les deux colonnes HSS C18 SB. Les composés semblent être davantage retenus avec la colonne HSS C18 SB n°2. Il faut noter cependant que la colonne n°1 n'était pas neuve au moment où nous avions débuté les tests. Cependant, le coefficient de détermination reste élevé (R^2 = 0.951), indiquant une bonne corrélation des valeurs de Ce entre les deux colonnes. Sur la Figure 3.8b, les C_e sont comparées entre les deux colonnes Nucleoshell HILIC. Cette fois, la corrélation entre les points est encore meilleure (R^2 = 0.988) avec une droite de régression très proche de la première bissectrice et très peu de points déviant significativement de la droite de régression.

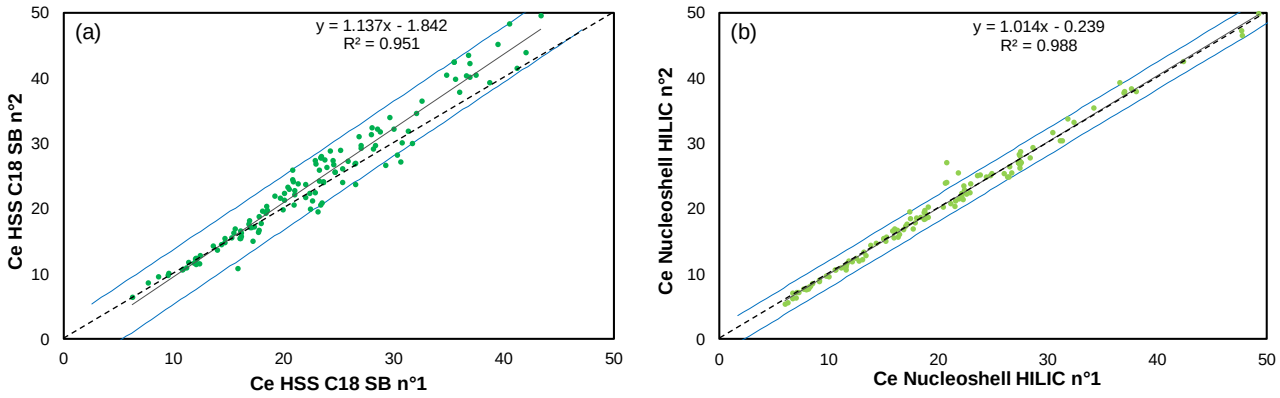


Figure 3.8 - Comparaison des Ce obtenues entre (a) les colonnes HSS C18 SB, (b) les colonnes Nucleoshell HILIC, set de 140 composés pharmaceutiques, les courbes de l'intervalle de confiance à 95% sont représentées en bleu

Nous sommes donc confiants quant à la reproductibilité des méthodes SFC développées d'une colonne à une autre mais également d'une taille de colonne à une autre (colonnes de 15 et 10 cm pour la Nucleoshell HILIC).

V. Développement de méthodes isocratique et gradient focus

Aujourd'hui pour chaque composé de type S ou I analysé au laboratoire Servier, une analyse en gradient est d'abord effectuée (méthode de première intention RPLC sur phase C18 en conditions acide, cf. chapitre II). A l'Institut de Recherches Servier, une analyse en conditions isocratiques est systématiquement réalisée à la suite de cette

analyse en gradient. Dans un autre centre de Recherche Servier, c'est un gradient focus (c'est-à-dire un gradient resserré autour de la C_e du PA) qui est privilégié. Dans les deux cas, l'objectif principal de cette analyse supplémentaire est de déceler ou de dégager les impuretés susceptibles d'être coéluées sous le pic principal ou celles qui sont proches de celui-ci, afin de pouvoir estimer la pureté relative aussi précisément que possible.

Le but de ce travail étant de développer des méthodes SFC pouvant être appliquées en routine au laboratoire, il nous paraissait essentiel de nous rapprocher le plus possible des procédures employées au laboratoire Servier. C'est pourquoi, après avoir développé des méthodes SFC en gradient, des méthodes SFC en mode isocratique et gradient focus ont également été développées et comparées au gradient générique.

1) Conditions analytiques

Pour cette étude, les colonnes de 10 cm provenant du lot n°2 ont été utilisées. Pour la méthode isocratique, on commence par calculer C_e, la composition à l'élution obtenue en gradient (Eq. 5). On applique ensuite la formule suivante (Eq. 6) pour déterminer C_{iso}, la composition à appliquer en mode isocratique :

$$C_{iso} = \frac{(C_e - 5)}{2} + 5 \qquad \text{Eq. 6}$$

C_{iso} est la composition moyenne avec laquelle le composé a été élué (entre la composition initiale du gradient (5%) et la composition au moment où le composé a été détecté (C_e)) pour chaque composé.

Pour la méthode en gradient focus, on utilise les conditions suivantes (Tableau 3.IV) :

Tableau 3.IV - Conditions analytiques du gradient focus

t (min)	% co-solvant
0	5
1	C_e - 5
11	C_e + 5
11.01	50
13	50

Principalement utilisé pour le transfert de l'échelle analytique à l'échelle préparative, l'utilisation d'un gradient focus permet un meilleur isolement du principe actif ciblé [152]. L'objectif est de séparer rapidement le composé d'intérêt de ses impuretés les plus

proches, avec une consommation moindre en solvant et un meilleur rendement de purification [153–155]. En effet, le gradient est ciblé autour de la composition à l'élution du principe actif, et non autour de l'échantillon complet.

Dans notre cas, le gradient focus est ciblé autour de la composition à l'élution du PA, plus ou moins 5%. Le gradient commence et se termine toujours à 5 et 50%, afin de s'assurer que l'ensemble des impuretés est élué.

À l'échelle analytique, l'objectif visé par l'utilisation de ces deux méthodes reste de s'assurer que l'ensemble des impuretés est identifié et qu'il n'y a pas de coélutions avec le principe actif. Les autres paramètres restent identiques à ceux utilisés pour le gradient générique : débit de 1 mL/min, pression de sortie de 150 bar et température de 25°C. Le co-solvant utilisé est le méthanol en présence de 20 mM d'ammoniaque et 2% H_2O.

2) Comparaison des méthodes gradient, gradient focus et isocratique

Afin de comparer les résultats obtenus avec les trois méthodes développées, on utilise un sous-ensemble de 24 composés. Le jeu de composés a été sélectionné afin de refléter la diversité des échantillons analysés quotidiennement au laboratoire, avec des masses molaires comprises entre 200 et 700 et des log P compris entre 0 et 8. Il est constitué de 14 composés basiques, 6 neutres, 2 acides et 2 zwitterions. Ce jeu de composés est identique à celui utilisé pour la comparaison des méthodes HPLC pour le profilage d'impuretés dans le Chapitre 2., à l'exclusion d'un composé jugé trop instable.

Notons que deux de ces produits contiennent une paire de diastéréoisomères, donc 26 pics majoritaires sont utilisés pour les mesures de pureté relative, mais 24 mélanges sont utilisés pour le comptage des impuretés. Sur chacune des deux colonnes sélectionnées en SFC et chaque composé analysé seul en solution, on compare :

(i) la pureté relative du PA, donnée en pourcentage ;

(ii) le nombre et la concentration relative des impuretés.

Avec la méthode de première intention sur phase C18 en SFC, tous les composés sont élués dans les trois conditions, et les paires de diastéréoisomères sont séparées. Pour la méthode SFC de seconde intention, sur la colonne Nucleoshell HILIC, deux composés n'ont jamais été élués et une paire de diastéréoisomères n'est pas séparée. L'ensemble des données est présenté en Annexe 1.

D'après nos critères, la meilleure méthode est celle qui permet la détection et la séparation d'un maximum d'impuretés et qui donne la pureté relative du PA la plus basse. Pour juger de l'intérêt d'une seconde méthode (gradient focus ou isocratique), on combine les deux critères. Pour chaque PA, on identifie :

(i) la méthode qui donne la pureté relative la plus basse ;

(ii) la méthode pour laquelle le nombre d'impuretés est le plus haut (toutes les impuretés sont comptabilisées : impuretés >1% et impuretés comprises entre 0.04 et 1%).

On considère une méthode comme étant la meilleure uniquement si les deux critères sont remplis. Pour la méthode de première intention SFC sur colonne C18, la méthode gradient générique (méthode de criblage de 5 à 50% de co-solvant) est la meilleure dans 11 cas sur 24 et l'utilisation d'une seconde méthode (gradient focus ou isocratique) n'apporte pas d'information supplémentaire. L'utilisation de la méthode gradient focus présente un intérêt par rapport à la méthode gradient dans 4 cas sur 24 (pureté relative du PA plus basse et nombre d'impuretés détectées plus élevé). Comparée à la méthode gradient générique, la méthode isocratique se révèle utile comme méthode secondaire dans 7 cas sur 24. Dans les deux cas restants, les trois méthodes fournissent les mêmes informations en termes de pureté et de nombre d'impuretés. Pour la méthode de seconde intention SFC (colonne Nucleoshell HILIC), la méthode gradient générique est la meilleure sur les critères de pureté et de nombre d'impuretés dans 10 cas sur les 22 composés élués dans ces conditions. Par rapport à cette méthode gradient, l'utilisation de la méthode gradient focus est avantageuse dans 4 cas sur 22 et la méthode isocratique dans 6 cas sur 22. Dans les deux cas restants, aucune méthode ne remplit les deux critères simultanément : la pureté relative du PA la plus basse est obtenue avec la méthode gradient mais le nombre d'impuretés détectées est plus haut avec la méthode isocratique.

Dans le tableau ci-dessous, on calcule la moyenne des largeurs à mi-hauteur sur le jeu de 24 composés étudié (Tableau 3.V) :

Tableau 3.V - Largeur à mi-hauteur mesurée sur 25 composés

Colonne	Gradient	Gradient Focus	Isocratique
C18	6.82E-02	8.42E-02	2.08E-01
HILIC	7.66E-02	1.15E-01	1.15E-01

La plus haute efficacité est obtenue avec la méthode gradient générique, sur les deux colonnes. La méthode gradient focus est ensuite la plus efficace. Sans surprise, avec la méthode isocratique on perd en efficacité, principalement car les pics sont moins symétriques (de nombreux cas de tailing sont observés) et les pics élargis.

Parmi les deux méthodes secondaires développées (gradient focus et isocratique), c'est la méthode gradient focus qui se révèle être la plus performante dans l'optique de séparer d'éventuelles impuretés coéluées avec le PA. Cependant, c'est la méthode isocratique qui permet, en moyenne, la détection du nombre d'impuretés le plus haut (Figure 3.9, majorité de points situés en dessous de la première bissectrice), quand ce nombre n'est pas équivalent ou très proche entre les deux méthodes.

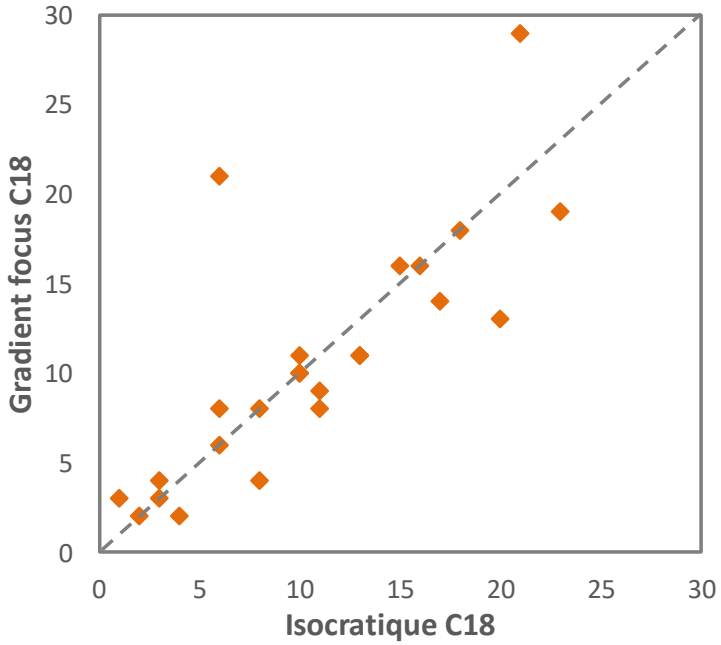


Figure 3.9 - Nombre total d'impuretés détectées avec les méthodes gradient focus et isocratique (colonne C18)

La Figure 3.10 illustre ce point, avec un composé S analysé sur la colonne C18. Dans cet exemple, on peut raisonnablement penser qu'une impureté co-élue avec le PA en gradient (Figure 3.10a, zoom sur le pic du PA, zone grise). Sur le chromatogramme suivant, une impureté (t_R = 4,12 min) est dégagée du PA en gradient focus (Figure 3.10b, zoom sur le pic du PA, zone grise). Enfin, sur le chromatogramme obtenu en mode isocratique, on observe une coélution entre le PA et au moins une impureté (t_R = 5.75 min) (Figure 3.10c, zoom sur le pic du PA, zone grise).

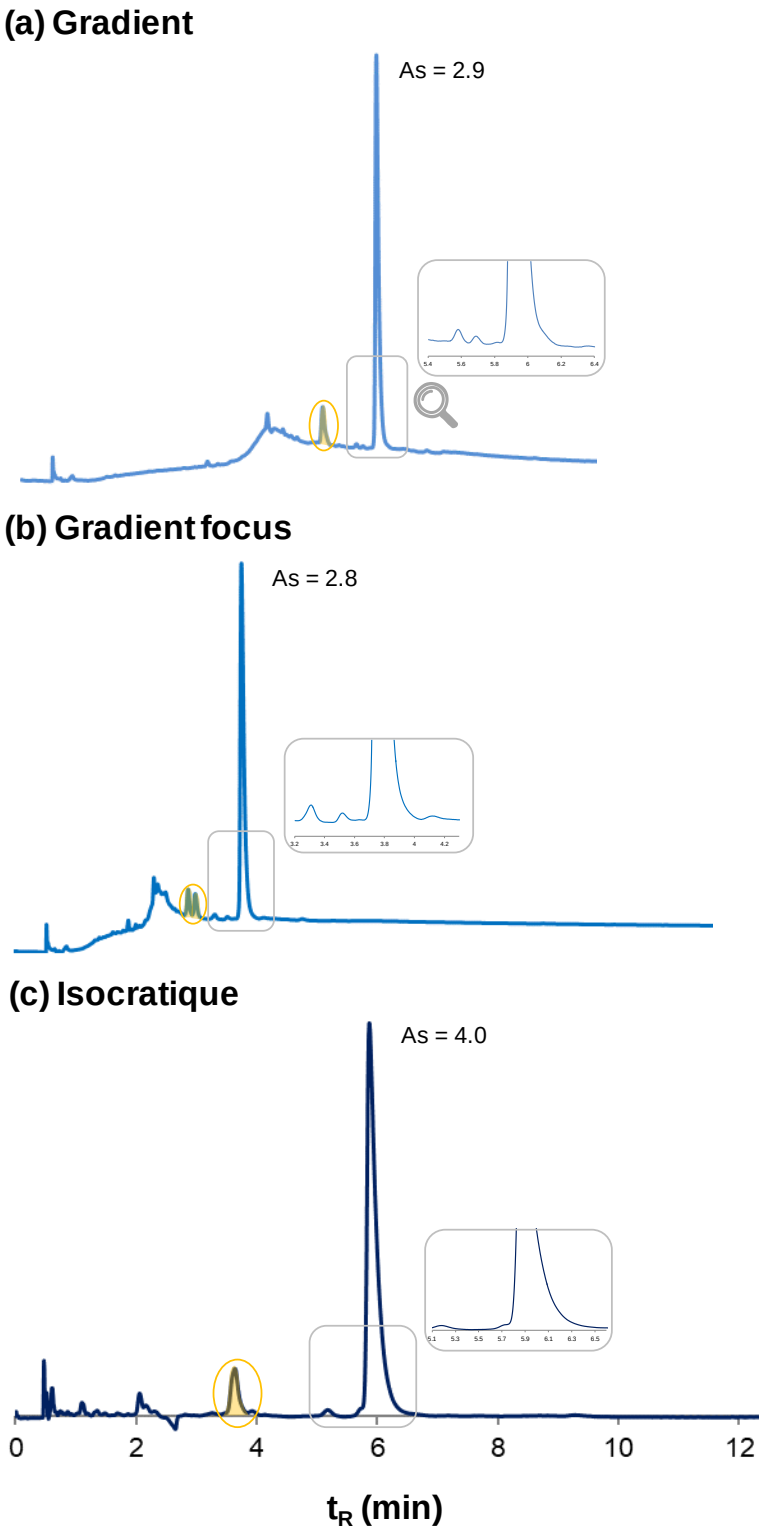


Figure 3.10 - Exemples de chromatogrammes obtenus pour un composé S en gradient (a), gradient focus (b) et isocratique (c) (colonne C18)

Sur cette figure, on peut également noter que l'utilisation du gradient focus a permis de séparer deux impuretés majoritaires, qui étaient identifiées comme une seule et même impureté avec le gradient générique (Figure 3.10a, pic identifié en jaune). Dans le Tableau 3.VI, cette impureté est identifiée comme l'impureté majoritaire 6 (IMP 6), avec m/z de 555.3. En gradient générique, le pourcentage estimé de cette impureté est de 8.09%. En isocratique l'IMP 6 est également identifiée, avec un pourcentage de 8.10%. En gradient

focus, on retrouve cette IMP 6 mais avec un pourcentage de 4.14%. Une impureté majoritaire supplémentaire est détectée (IMP 7) à 4.16%. Sur le chromatogramme du gradient focus, ces deux impuretés sont séparées avec retour à la ligne de base (Figure 3.10b, pics jaunes). L'utilisation du gradient focus a donc permis de séparer et d'identifier davantage d'impuretés majoritaires : 6 impuretés en gradient focus, contre seulement 3 en gradient générique. En isocratique, 5 impuretés sont détectées dont les IMP 1 et 2, qui sont identifiées seulement avec cette méthode. On peut néanmoins supposer que ces impuretés sont en fait le résultat de co-élutions entre plusieurs impuretés minoritaires et non de réelles impuretés majoritaires. En effet, on peut constater sur le chromatogramme (Figure 3.10c) que les pics obtenus en isocratique sont larges et proches en rétention, ce qui complique leur intégration.

Tableau 3.VI - Comparaison des profilages d'impuretés obtenus entre les différentes méthodes pour le même composé S (colonne C18)

(a) Gradient

	t_R (min)	%	MS ($[M+H]^+$)
PA	**5.91**	**83.11**	**541.33**
Impuretés > 1%			
non détectée			
non détectée			
non détectée			
non détectée			
IMP 5	4.10	1.82	556.36
IMP 6	5.02	8.09	555.29
non détectée			
non détectée			
IMP 9	9.80	2.55	514.25
Impuretés < 1%			
IMP A	1.56	0.12	
IMP B	2.49	0.05	
IMP C	2.71	0.07	
IMP D	2.89	0.07	
IMP E	2.96	0.04	
IMP F	3.10	0.66	
IMP G	3.28	0.09	
IMP H	3.48	0.12	
IMP I	3.50	0.07	
IMP J	4.36	0.42	
IMP K	4.42	0.09	
IMP L	4.49	0.26	
IMP M	4.59	0.31	
IMP N	4.86	0.13	
IMP O	4.93	0.17	
IMP P	5.19	0.08	
IMP Q	5.29	0.19	
IMP R	5.36	0.11	
IMP S	5.58	0.58	
IMP T	5.69	0.33	
IMP U	5.82	0.08	
IMP V	6.76	0.40	
non détectée			
UV s/n	7.00E+03		
MS s/n	1.88E+02		

(b) Gradient focus

	t_R (min)	%	MS ($[M+H]^+$)
PA	**3.75**	**79.47**	**541.32**
Impuretés > 1%			
non détectée			
non détectée			
IMP 3	2.30	1.63	530.30
IMP 4	2.36	1.00	481.30
non détectée			
IMP 6	2.87	4.14	555.31
IMP 7	2.99	4.16	542.31
IMP 8	3.31	1.03	557.33
IMP 9	11.84	3.74	514.26
Impuretés < 1%			
IMP A	1.32	0.07	
IMP B	1.40	0.10	
IMP C	1.62	0.28	
IMP D	1.70	0.16	
IMP E	1.79	0.16	
IMP F	1.84	0.05	
IMP G	1.88	0.87	
IMP H	1.92	0.10	
IMP I	1.97	0.05	
IMP J	2.00	0.31	
IMP K	2.03	0.06	
IMP L	2.06	0.16	
IMP M	2.16	0.12	
IMP N	2.40	0.17	
IMP O	2.50	0.51	
IMP P	2.65	0.12	
IMP Q	3.52	0.51	
IMP R	3.63	0.09	
IMP S	4.12	0.27	
IMP T	4.50	0.07	
IMP U	4.75	0.41	
IMP V	5.28	0.11	
IMP W	5.43	0.06	
UV s/n	6.37E+03		
MS s/n	2.27E+02		

(c) Isocratique

	t_R (min)	%	MS ($[M+H]^+$)
PA	**5.87**	**82.34**	**541.37**
Impuretés > 1%			
IMP 1	1.10	1.00	242.26
IMP 2	2.06	1.85	242.26
non détectée			
non détectée			
IMP 5	2.17	1.20	556.39
IMP 6	3.64	8.10	555.33
non détectée			
IMP 8	5.18	1.12	557.37
non détectée			
Impuretés < 1%			
IMP A	0.87	0.12	
IMP B	1.03	0.14	
IMP C	1.19	0.14	
IMP D	1.33	0.11	
IMP E	1.36	0.26	
IMP F	1.49	0.10	
IMP G	1.69	0.18	
IMP H	1.87	0.05	
IMP I	2.30	0.67	
IMP J	2.82	0.06	
IMP K	3.26	0.58	
IMP L	3.49	0.22	
IMP M	3.93	0.74	
IMP N	4.14	0.09	
IMP O	5.75	0.63	
IMP P	9.27	0.30	
non détectée			
non détectée			
non détectée			
non détectée			
non détectée			
non détectée			
non détectée			
UV s/n	2.33E+03		
MS s/n	1.20E+02		

La symétrie a été évaluée sur ce composé S, en se basant sur la mesure de l'asymétrie à 10% de la hauteur du pic (As). Les résultats sont identiques entre les méthodes gradient (As = 2.9) et gradient focus (As = 2.8) et indiquent une asymétrie du pic de type tailing. Pour la méthode isocratique, le pic est davantage déformé (As = 4). De plus, on mesure la largeur du pic à mi-hauteur (W_{50}), l'efficacité ne pouvant être calculée dans le cas de mesures en gradient. Là encore, les valeurs obtenues sont équivalentes

entre les méthodes gradient (W_{50} = 5.51E^{-2} min) et gradient focus (W_{50} = 5.90E^{-2} min). Pour la méthode isocratique, on perd naturellement en efficacité (W_{50} = 1.50E^{-1} min).

En conclusion, l'utilisation d'une seconde méthode gradient focus ou isocratique en complément de la méthode gradient générique n'apporte que peu d'informations supplémentaires. La méthode gradient générique fournit à la fois la pureté la plus basse et le nombre d'impuretés le plus haut dans une majorité de cas. Même si l'objectif principal d'une analyse complémentaire en isocratique ou gradient focus n'est pas d'identifier et de séparer l'ensemble des impuretés présentes dans l'échantillon mais de mettre en évidence les impuretés ayant une C_e proche de celle du PA, cela semble difficilement réalisable surtout à l'aide de la méthode isocratique, car les pics sont élargis et non symétriques. Sur la Figure 3.10c, on voit que le pic du PA est large (As = 4), rendant la détection d'impuretés à proximité du PA compliquée. Si on souhaite s'assurer qu'il n'y a pas de co-élutions entre le PA et des impuretés, l'utilisation d'une méthode orthogonale parait plus adaptée. On peut envisager de travailler avec la méthode de seconde intention SFC sur la colonne HILIC en gradient. Puisque l'efficacité la plus haute est obtenue en gradient, on peut également envisager de coupler en série les deux colonnes C18 et HILIC afin d'augmenter davantage l'efficacité et le pouvoir de séparation. Ce point va être abordé dans le paragraphe suivant (Chapitre 3, VI.).

VI. Couplage de phases stationnaires : intérêt du couplage

Dans l'optique de séparer des mélanges complexes de composés pour lesquels une phase stationnaire unique ne suffit pas à séparer le PA de ses impuretés, le couplage de deux colonnes SFC (HILIC et C18) a été envisagé et sera développé dans l'article suivant :

C. West, E. Lemasson, S. Bertin, P. Hennig, E. Lesellier

Interest of achiral-achiral tandem columns for impurity profiling of synthetic drugs with supercritical fluid chromatography

Journal of Chromatography A, Volume 1534, (2018), 161-169

Journal of Chromatography A, 1534 (2018) 161–169

Contents lists available at ScienceDirect

Journal of Chromatography A

Interest of achiral-achiral tandem columns for impurity profiling of synthetic drugs with supercritical fluid chromatography

Caroline West[a,*], Elise Lemasson[a], Sophie Bertin[b], Philippe Hennig[b], Eric Lesellier[a]

[a] Univ Orléans, Institut de Chimie Organique et Analytique (ICOA), CNRS UMR 7311, Pôle de Chimie, rue de Chartres, B.P. 6759, 45067 Orléans, Cedex 2, France

[b] Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France

ARTICLE INFO

Article history:
Received 13 November 2017
Received in revised form
21 December 2017
Accepted 22 December 2017
Available online 26 December 2017

Keywords:
High-resolution separations
Impurity profiling
Orthogonal methods
Performance comparison
Pharmaceutical ingredients
Tandem columns

ABSTRACT

To achieve the most complete impurity profiling of synthetic drugs with a single chromatographic technique, high resolution is required, which may be gained with a combination of high efficiency and versatile selectivity, allowing to separate most similar analytes. Compared to a single-column chromatographic method, coupling complementary stationary phases promises both an increase in efficiency and an increase in selectivity possibilities. With supercritical fluid chromatography (SFC), the use of long columns is facilitated by the low viscosity of the mobile phase. In this paper, we investigate the interest of coupling two achiral stationary phases (Acquity UPC² HSS C18 SB and Nucleoshell HILIC) that were previously observed to have excellent complementarity in SFC to carry out impurity profiling on 25 individual drug substances containing varied numbers and amounts of impurities. The single-column gradient methods are compared to tandem-column gradient methods with the two possible ordering of columns (C18 phase in first or second position) based on selectivity, peak capacity, sensitivity, UV-estimated purity of the active pharmaceutical ingredient and number of impurities detected with UV-estimated concentration >0.04%. It appears that it could be more beneficial to have two columns coupled in a single analysis than two consecutive methods with the single columns. The overall analysis time are nearly the same, but with more informative chromatograms in about 35% cases.

1. Introduction

Impurity profiling of synthetic drugs requires high-resolution methods to ensure that all impurities are separated from the main compound, detected and quantified. High resolution may be obtained through two different processes: either with high efficiency, or with versatile selectivity. Coupling two columns with complementary selectivities is an interesting and economic solution to achieve both high efficiency (in doubling column length) and versatile selectivity (in combining the different selectivities offered by two stationary phases), without the technical constraints of two-dimensional chromatography [1]. It is an interesting option when the sample complexity (in terms of number of compounds) is not too high, which is typically the case in synthetic drug products.

In HPLC, the concept of coupling stationary phases to achieve the desired efficiency and/or selectivity is somewhat restricted by the pressure drop caused by long columns, and sometimes because the appropriate mobile phase composition in the two columns is too different. In supercritical fluid chromatography (SFC), it is easier to increase column length because the mobile phase (most often a mixture of carbon dioxide and a co-solvent) has a low viscosity, as compared to a liquid, thus causes lower pressure drop along the column length. As a result, long columns have been used in several occasions to achieve very high efficiency. The first of such examples was presented in a seminal paper by Berger and Wilson published in 1993 [2], where they showed that a 2.2 m-long column packed with 5-µm fully porous particles allowed achieving 220,000 plates, generating no more than 16 MPa pressure drop and yielding very nice separations for several samples comprising lemon essential oil, chimney extract, gasoline or polynuclear aromatic hydrocarbons. This concept was later used by Gaudin et al. [3] to achieve a high-resolution separation of skin ceramides with 125 cm of Kromasil C18 columns. Roston et al. [4] employed a 2 m-long cyanopropyl-bonded stationary phase for impurity profiling of drugs. Some years later, Brunelli et al. [5] also employed this strategy to demonstrate

* Corresponding author.
E-mail address: caroline.west@univ-orleans.fr (C. West).

https://doi.org/10.1016/j.chroma.2017.12.061
0021-9673/

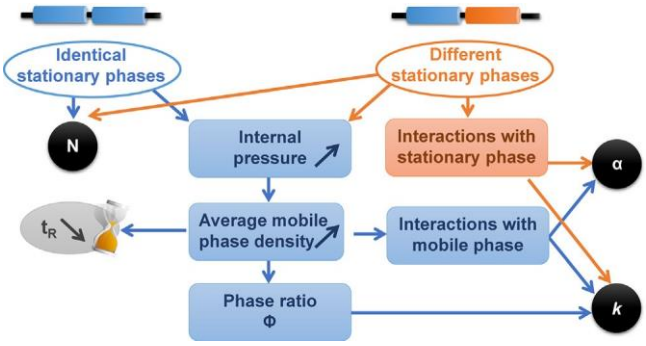


Fig. 1. Theoretical consequences of coupling columns in supercritical fluid chromatography.

the interest for the analysis of pharmaceuticals with a 125-cm long cyanopropyl-bonded stationary phase.

Similarly to the evolution of HPLC, most recent developments in SFC have been based on smaller particles, typically sub–2 μm fully porous particles. While the pressure drop on such columns is still much lower in ultra-high performance SFC (UHPSFC) than in ultra-high performance liquid chromatography (UHPLC), such small particles still cause significant pressure drops, making long column length incompatible with most pumping systems available for SFC (where the upper pressure limit is currently 40–60 MPa). Another solution is to use sub–3 μm superficially porous particles, which generate efficiencies comparable to those of sub–2 μm fully porous particles, but cause less pressure drop. Recently, Lesellier and co-workers [6,7] employed 75 cm of C18 columns packed with 2.7 μm superficially porous particles to achieve 120,000 plates in the separation of vegetable oils that could be qualified as "ultra-high resolution SFC".

While the expected benefit of increasing column length is to increase efficiency, it is not the only consequence (Fig. 1). A normal consequence observed also in HPLC is that analysis time will increase. But in the case of SFC, because the fluid is compressible, increasing column length while maintaining all other parameters identical will also cause an increase in internal pressure, which in turn is causing increased mobile phase density, which is affecting both the solvent strength and the phase ratio [8]. When solvent strength increases, the retention time resulting from increased column length is usually lower than what could be expected based on the number of columns. In other words, when column length is multiplied by two, analysis time increases by a factor less than two. This may be seen as an advantage, but also as a complication because it could be difficult to predict the outcome of the separation, as the separation occurring in the column placed in first position will be different when a second column is causing increased pressure. Lesellier et al. [9] showed how this problem could be overcome with the use of monolithic columns in the second position, as monolithic stationary phases generate much lower pressure drop than particle stationary phases, and it was then easy to predict the appropriate monolithic column length to achieve the desired selectivity for a sample of carotenoid pigments. Another option would be to have the mobile phase in each of the two columns independently controlled (either with different pumping systems or different controls of temperature), but that would of course require a much more complex chromatographic system.

Also interesting is the coupling of complementary stationary phases. This is facilitated in SFC by the fact that the same mobile phase can be used with a great variety of stationary phases (achiral or chiral, polar or non-polar). Naturally, all the previous comments regarding increased efficiency, increased mobile phase density and relatively decreased analysis time remain true when different stationary phases are combined (Fig. 1), but with the additional benefit of complementary selectivities, offering more opportunities to fine-tune a separation. This concept was used in many occasions in SFC. Lesellier [10] demonstrated how a difficult separation of structurally-similar polynuclear aromatic hydrocarbons was most easily achieved with the combined selectivities of two different C18 phases. Deschamps and co-workers [11] also employed this concept with silica and propanediol-bonded phases to obtain regularly-spaced class fractions for semi-preparative SFC of wheat glycolipids. More recently, Abrahamsson et al. [12] combined a C18 phase to an ethylpyridine phase to achieve the separation of carotenoid pigments and chlorophylls extracted from microalgae. In these examples, a solvent gradient was used, reinforcing the effect of the column position on the final separation. Delahaye and Lynen [13] demonstrated that a selectivity-optimized separation concept, coupling different stationary phases with varied column lengths with a computer-assisted column selection was feasible in low-density conditions (6% methanol in carbon dioxide, 40°C). However, despite isocratic elution conditions, the selectivity was strongly impacted by the stationary phase order and retention could be strongly affected when changing column length significantly.

Coupling chiral columns is also an interesting option [14–16], especially to achieve the full resolution of mixtures containing several stereoisomers, when more than one chiral center is present and when a single enantioselective column does not resolve them all. In particular, Pirkle and Welch addressed this question [17] and concluded that the systematic use of coupled columns should not be recommended but may be beneficial to "complex mixtures". Finally, the combination of achiral and chiral columns was also explored in a few instances, to achieve the most complete view of both achiral impurities or dia- and enantio-stereoisomers in a single run [18,19], or to purify one target compound from both achiral and chiral impurities [20]. However, resulting from the above-described change in internal pressure in the first column, the order in which the columns are arranged is significant on the final outcome, and reversing the position sometimes yields very different separations, unless the mobile phase and operating conditions are selected to minimize fluid compressibility, or unless backpressure is adjusted to take account of such changes [16].

It is not our intention to provide an extensive review of the literature on this topic but further references can be found elsewhere [1].

One significant difficulty when coupling columns having different stationary phases is that they must provide complementary selectivities but must not provide opposite behaviors. Indeed, when two analytes are separated at the outlet of the first column, the second column should not undo this separation in merging them again into a single peak. Thus orthogonal behavior is desirable, where one column would provide some selectivity where the other column cannot and vice-versa. A good understanding of column selectivity is then highly necessary, to avoid unproductive column coupling [21].

In the present study, we were willing to explore the interest of coupling two columns that were previously demonstrated to offer complementary selectivities in SFC (an octadecyl-bonded silica phase and a sulfobetaine-bonded silica phase) [22] to achieve the most complete view of moderately complex samples, namely synthetic drugs containing impurities. For this purpose and because the method should be applicable as a generic tool and not specifically optimized for a single sample, twenty-five samples of drug candidates containing impurities were used to compare single-column and tandem-column systems. The two combinations of columns were tested and compared to the single-column methods based on selectivity, efficiency and capability for impurity profiling.

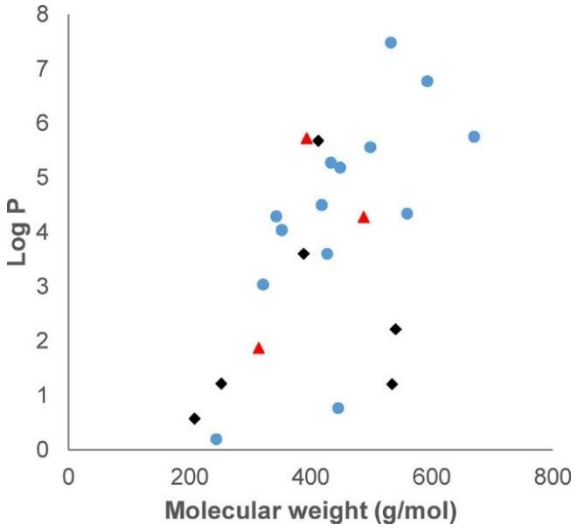


Fig. 2. Physico-chemical properties of the 25 active pharmaceutical ingredients selected in this study, 6 neutral species (black diamonds), 14 basic species (blue circles) and 3 acidic species (red triangles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Material and method

2.1. Chemicals, solvents and reagents

The twenty-five drug candidates in this set were selected so as to reflect the diversity of samples to be processed every day in the target laboratory, *i.e.* (i) the molecular weight and hydrophobicity ranges were rather large (Fig. 2) and covered the space normally encountered in this laboratory, (ii) they included a majority of basic compounds, but also neutral and acidic compounds and (iii) they included some compounds with high purity (above 95%) and others with lower purity (as low as 40%) and small (2) or large (50) numbers of impurities.

Individual solutions of these compounds were prepared in ethanol at concentrations of 1000 ppm.

Solvents used were HPLC-grade methanol (MeOH) and ethanol provided by VWR (Fontenay-sous-Bois, France); carbon dioxide of industrial grade 99.5% was provided by Messer (Puteaux, France). Ammonium hydroxide solution was provided by Fisher Scientific (Illkirch, France); ultra-pure water was provided by an Elga UHQ system from Veolia (Wissous, France).

2.2. Stationary phases

Two columns were used, based on our previous works [22]: an ACQUITY UPC^2 HSS C18 SB column (100 × 3.0 mm, 1.8 µm fully porous silica) from Waters and a Nucleoshell HILIC column (100 × 3.0 mm, 2.7 µm superficially porous silica) from Macherey-Nagel.

2.3. Instrumentation

The UHPSFC system was a Waters Corporation (Millford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography™ (UPC^2 ®). It was equipped with a binary solvent delivery pump compatible with mobile phase flow rates up to 4 mL/min and pressures up to 414 bar, an autosampler that included partial loop vol- ume injection system, a back-pressure regulator (BPR), 2-position column oven compatible with 150 mm length columns and two detectors: a photodiode-array (PDA) detector and an ACQUITY QDa single-quadrupole mass detector with electrospray ionization source. An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was then split by the on-board flow-splitter assembly. With this system, most of the column flow goes to the back-pressure regulator and only an unknown portion goes to the MS. Empower® 3 software was used for system control and data acquisition.

2.4. Chromatographic conditions

The UHPSFC analyses were performed at 15 MPa (outlet pressure), 25°C; the mobile phase composition was CO_2 with 5–50% MeOH comprising 20 mM ammonium hydroxide and 2% water in a gradient elution program, based on optimized conditions developed in our previous works [23]:

(1) For a single column with dimensions of 100 × 3.0 mm: flow rate was 1 mL min^{-1}; co-solvent proportion was increased over 10 min. Inlet pressure at the beginning and end of the gradient program varied from: 21.5–33 MPa on the HSS C18 SB column, and 19–27 MPa on the Nucleoshell HILIC column. Naturally, the 2.7 µm superficially porous particles in the HILIC phase caused less pressure drop than the 1.8 µm fully porous particles in the C18 phase. The measurement of pressure values were repeated with a flow rate of 0.8 mL/min: inlet pressure values then varied from 20.5 to 31.5 MPa on the HSS C18 SB column and from 17.5 to 22 MPa on the Nucleoshell HILIC column.

(2) For the tandem-column method with dimensions of 200 × 3.0 mm: flow rate was 0.8 mL min^{-1}; co-solvent proportion was increased over 25 min. Inlet pressure at the beginning and end of the gradient program varied from 23 to 37 MPa respectively, whatever the order of the columns (C18-HILIC or HILIC-C18). The two columns were linked with a capillary tubing of 60 mm × 70 µm, generating a pressure drop of 0.3–0.6 MPa, which should be negligible.

Thus gradient steepness was consistent between the two systems (one or two columns), but linear velocity could not be kept identical because the upper pressure limit of the pumping system (41.4 MPa) did not allow maintaining a 1 mL/min flow rate when a 20 cm column was used. Variations between the average column pressure values were not compensated with outlet pressure. The differences in average pressure resulting from different flow rate, different column dimensions and particle type and size will be further discussed in the results and discussion section.

2 µL of each sample solution were injected with a 10 µL-loop and MeOH was used to rinse the system.

The extracted wavelength for UV detection was fixed at 210 nm. Frequency was set at 20 pts/s and resolution at 1.2 nm.

The mass detector unit was pre-optimized by the manufacturer. The studied compounds were detected in positive and negative electrospray ionization mode (m/z 150–750), scan rate 10 pts/s, capillary voltage 0.8 kV, cone voltage 15 V, sampling frequency 8 Hz, ion source temperature 150 ° C and probe temperature 600 °C. Nitrogen was used as nebulizing gas. Make-up flow was 0.45 mL/min with 98% methanol – 2% water comprising 1% formic acid. As the pre-selected ionization parameters caused only little fragmentation, only the precursor ions were considered ($[M+H]^+$ in ESI^+ mode, $[M-H]^-$ in ESI^-).

3. Results and discussion

3.1. Optimized UHPSFC methods

The aim of this work was to make an objective comparison of single-column and tandem-column UHPSFC impurity profil-

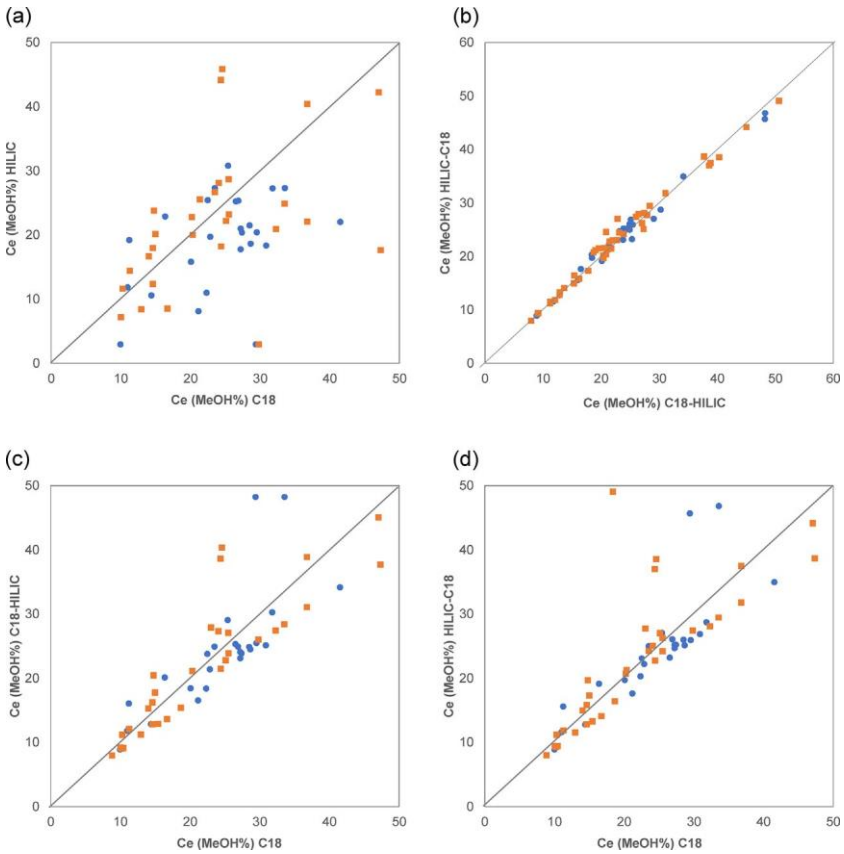


Fig. 3. Comparison of elution compositions (C_e, calculated according to Eq. (1)) between the different systems. (a) single-column systems compared (HILIC vs. C18), (b) tandem-column systems compared (HILIC-C18 vs. C18-HILIC), (c) and (d) best single-column system (C18) compared to tandem-column systems (C18-HILIC and HILIC- C18 respectively). The first bisector shows identical elution composition values. Blue circles are drug candidates, orange squares are major impurities (above 1%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ing methods. In two previous papers [22,23], we have described the development of two complementary UHPSFC methods to be applied in a pharmaceutical research and development (R&D) laboratory. The two methods were based on two different stationary phases (ACQUITY HSS C18 SB with fully porous sub–2 μm particles and Nucleoshell HILIC, a sulfobetaine-bonded silica with superficially porous sub–3 μm particles) but with identical mobile phase composition. For the purpose of simplicity, the two columns will be simply designated as "C18" and "HILIC" in the following. It was shown that these two columns provided a high level of orthogonality, but also that the first one (C18 phase) provided significantly superior performance to the second one (HILIC phase), especially in terms of the proportion of peaks eluted with satisfying peak shapes [24]. Based on these observations, an optimal strategy to achieve impurity profiling would be to use the C18 phase as first-choice method, then use the HILIC phase when an orthogonal method is desired to confirm that all impurities were detected. Our purpose in the following will be to determine whether a quickest and/or more efficient strategy could be proposed with the combination of the two columns in a single analysis.

3.2. Comparison of performance between single-column and tandem-column methods

3.2.1. Selectivity issues

A first interrogation we had when coupling columns was on the benefit of two complementary selectivities. In other words, it was interesting to verify whether a tandem-column system would indeed bring different selectivity from a single column. For this purpose, and because the single- and tandem-column systems were operated in different conditions, the retention times measured for each API and major impurities were converted to the elution composition, that is to say the percentage of methanol in which the analyte is eluted from the column(s). The elution composition C_e (corresponding to the percentage of co-solvent when the analyte of interest is eluted from the column) can be calculated with Eq. (1) [25]:

$$C_e = C_i + \frac{(C_f - C_i)}{t_G} * (t_R - t_D) \quad (1)$$

Where C_i and C_f are the initial and final compositions of the gradient, respectively; t_G is the gradient time; t_R is the retention time of the compound and t_D is the system dwell time. The dwell time of UHPSFC system (t_D) was measured to be 0.46 min at 1 mL/min and 0.58 at 0.8 mL/min.

The comparison of elution compositions is shown in Fig. 3. First, it can be seen on Fig. 3a that the two columns taken individually do indeed provide excellent complementarity, as the points representing all analytes considered (drug candidates and their major impurities) are well scattered in the elution space.

Secondly, on Fig. 3b, the values observed for the two tandem-column systems show that, whatever the column order (C18-HILIC or HILIC-C18), the analytes elute at very close elution compositions. Indeed, most points are closely fitted along the first bisector, with little dispersion.

Thirdly, when comparing the favorite single-column system (the C18 column) to the tandem-column systems, some complementarity appears (Fig. 3c and d). Naturally, because the C18 phase also participates in the process, the orthogonality cannot be as significant as observed in Fig. 3a, but still some selectivity change is

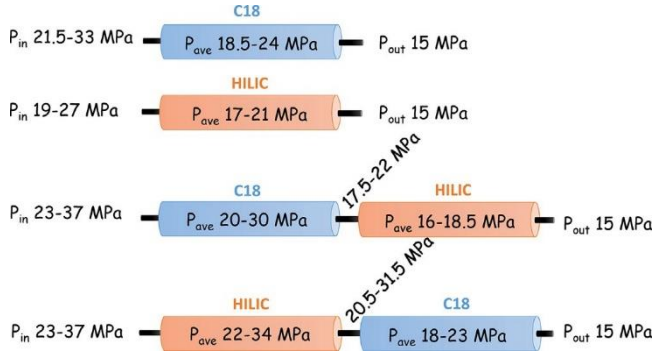


Fig. 4. Variations of inlet and average pressure values from beginning to end of the elution gradient with the different systems. C18 refers to the ACQUITY UPC^2 HSS C18 SB column and HILIC to the Nucleoshell HILIC column. Conditions: 25 °C, gradient elution 5–50% methanol, flow rate 1 mL/min for single columns and 0.8 mL/min for tandem columns.

observed, as the points are not well fitted to a trend line. A large portion of the points appear to be aligned below the first bisector (Fig. 3c and d), indicating that the retention was usually higher when the C18 phase was used alone. This can be explained in part by the fact that average pressure in the single-column system was lower than in the tandem-column system, resulting in lower mobile phase density thus lower elution strength of the mobile phase. To better understand this notion, the average pressure values were determined based on inlet pressure values measured in all cases (Fig. 4). It appears that, although the mobile phase flow rate was lower in the tandem-column system, the average pressure in the column placed in first position is significantly higher than when the same column is used alone, explaining the deviation of points from the first bisector in Fig. 3. Moreover, for a tandem-column system, the compounds enter the second column with a mobile phase containing a higher percentage of methanol than in the single-column system. This results in lower retention in the column placed in second position than when the same column is used alone. However, a significant number of points deviate from the bottom trend line, showing that the second column did bring some change in selectivity. Whether this change of selectivity would be beneficial to impurity profiling or not remains to be proven, and will be discussed below.

3.2.2. Peak capacity

As discussed in the introduction, a longer column (20 cm instead of 10 cm) would be expected to improve column efficiency. As the systems were operated with a gradient elution program, efficiency will be better translated by the measurement of peak capacity. Peak capacity (P_c) is defined as the maximal number of peaks which can be separated during the gradient time, with a resolution of 1 [25] and can be calculated following Eq. (2):

$$P_c = 1 + \frac{t_g}{1.7 * \omega_{50\%}} \quad (2)$$

In this equation, t_g (min) is the gradient time and $w_{50\%}$ (min) represents the measured peak width at 50% height. In our case, the comparison was based on the peak width measured on the 22 compounds that could be detected in UV with all four methods (two single-column and two tandem-column methods). The average peak capacity (calculated as the average of peak capacities measured for the 22 compounds) with the preferred single-column method (C18 phase) was then calculated to be 107, while the average peak capacity on the HILIC phase was a little lower with a value of 82. Coupling columns in the order C18-HILIC resulted in an average peak capacity of 171 while reversing the order of the columns as HILIC-C18 yielded an average peak capacity of 149. Plotting the peak capacity values measured on all peaks for one system vs. another confirmed that the average peak capacity values were representative of the general trend (not shown).

As expected, peak capacity was larger when 20 cm columns were used than with 10 cm column. However, we may note that the increase in peak capacity was not as much as one would have expected. Indeed, in both tandem-column systems, it was inferior to the sum of peak capacities measured on the single columns. In addition, it was not anticipated that changing the order of columns would have so significant impact. Clearly, placing the C18 phase in first position was more favorable as it yielded a peak capacity that was 15% larger than the other column combination.

Several factors may explain the discrepancies: flow rate changes, pressure variations, and mobile phase composition variations.

Firstly, the lower flow rate employed for the tandem-column systems (0.8 vs. 1 mL/min) may be partly responsible for relatively low efficiency, as it is expected that such flow rates would both be below the optimum flow rate. This is clearly a drawback of this strategy but is only dependent on the current inability of our pumping system to deliver higher pressures. If the pump were capable to deliver a higher inlet pressure, the full benefit of coupling columns on efficiency would be restored.

Secondly, looking again at Fig. 4, it appears that when using the C18-HILIC combination, the average pressure inside the C18 column is naturally larger than when using the C18 column alone, especially as no back-pressure adjustment was made (back-pressure was maintained constant in all experiments). However, the pressure increase in this case remained moderate because the superficially porous particles in the HILIC column caused only a moderate pressure drop. On the other hand, when the HILIC column is placed in first position in the tandem-column system, the average pressure in this column is significantly higher than in the

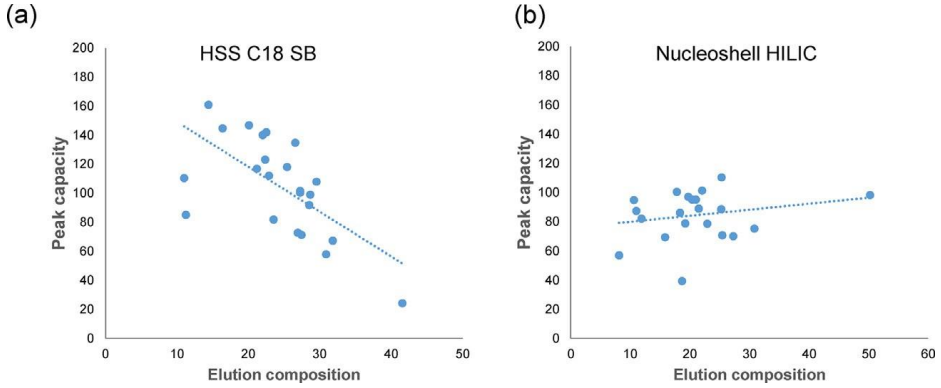


Fig. 5. Comparison of peak capacity based on the measurement of peak width at half height (Eq. (2)) variation with elution composition (Eq. (1)) on all active ingredients. The interrupted line is the linear regression line showing (a) negative tendency on the HSS C18 SB column or (b) absence of tendency on the Nucleoshell HILIC column.

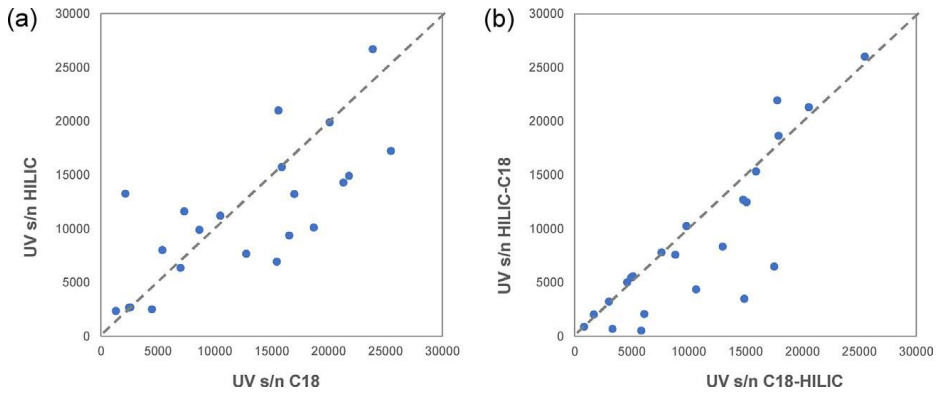


Fig. 6. Comparison of sensitivity (signal-to-noise ratio measured in UV at 210 nm) between. (a) the single-column systems (b) the tandem-column systems. The interrupted line is the bisector, showing theoretical identical sensitivity.

HILIC column alone, because the fully porous 1.8 µm particles in the C18 column caused a high pressure drop. As the mobile phase density in the HILIC column would be higher, the fluid viscosity would also be increased, causing decreased efficiency.

Thirdly, as pointed out above, when an analyte enters the second column in a tandem system, the mobile phase composition contains a larger proportion of methanol than when the column is used alone. However, we observed that the peak capacity on the C18 phase employed alone was following a general trend of decreasing measured peak capacity when the elution composition C_e increased (Fig. 5a). However, on the HILIC phase, no such trend was observed (Fig. 5b). When this trend had been observed on the two columns, we could have concluded that diminution of diffusion coefficients in the mobile phase when methanol percentage is high was responsible for this trend. While this process may occur, it cannot be the sole explanation or both columns would show the same effect. Consequently, we must admit that different solvation of the stationary phases occurs, that is detrimental to mass transfer of the solutes with the C18 phase. Considering this, it seems logical that the HILIC-C18 tandem system should be further disadvantaged compared to the C18-HILIC system, where analytes enter the C18 column with a mobile phase composition and elution strength that are more similar to those in the single C18 column.

3.2.3. Comparison of sensitivity of the methods

The requirements of impurity profiling in the local context of this R&D department (at Servier research laboratories) at this stage of drug development is to estimate the relative concentration of the API and the impurities based on UV detection at 210 nm, unless the API is known to have low UV absorbance and a charged-aerosol detector (CAD) is used. Besides, MS detection is used to confirm the identity of the main compound and may provide additional information on the impurities but is not used to estimate concentration thus MS sensitivity is not an issue.

To compare the sensitivity of the four methods (single-column or tandem-column), baseline noise and signal-to-noise (s/n) values were measured for the API with UV at 210 nm in the four methods. Here again, the average values for all compounds eluted were calculated and it was verified that data distribution was well reflected by the average values.

First, it was observed that UV baseline noise was essentially the same between the four methods, so any variation in UV signal-to-noise should be attributed to signal.

Regarding UV signal-to-noise, the C18 column alone yielded the best results. The HILIC column and tandem C18-HILIC systems were equivalent and the HILIC-C18 system was the worse. Comparison

Table 1
Significant figures of impurity profiling on 24 drug candidates with the four different systems (two single-column systems and two tandem-column systems).

API number	Relative purity of API				Total number of impurities			
	C18	HILIC	C18-HILIC	HILIC-C18	C18	HILIC	C18-HILIC	HILIC-C18
S-01	98.8	98.9	98.7	98.8	4	3	4	4
S-02	95.8	99.5	97.9	97.6	9	2	4	6
S-03	97.4	97.9	98.6	98.7	2	2	2	2
S-04	76.5	80.4	81.2	78.6	17	14	16	14
S-05	98.4	98.9	98.9	99.0	3	1	2	2
S-06	91.0	–	96.1	95.4	11	–	12	11
S-07	94.0	97.5	96.6	97.7	10	6	11	8
S-08	96.8	96.7	97.3	96.1	14	12	11	15
S-09	99.7	98.7	99.6	99.7	4	2	3	3
S-10	93.4	92.4	92.3	93.1	9	7	16	13
S-11	89.8	90.9	91.1	91.7	8	12	12	6
S-12	87.5	70.5	66.0	65.5	22	18	34	27
S-13	92.0	95.9	90.4	90.5	13	10	13	10
S-14	90.7	–	85.2	88.8	4	–	6	5
S-15	97.8	99.0	98.3	98.4	6	4	9	6
S-16	96.8	92.9	97.6	97.7	12	18	14	12
S-17	92.2	97.2	91.0	91.3	11	5	11	10
S-18	84.5	91.8	–	77.2	8	9	–	10
S-19	39.2	38.2	37.4	39.3	25	29	24	29
S-20	78.2	77.2	76.2	75.3	21	15	22	23
S-21	90.1	89.8	88.8	90.7	14	15	21	15
S-22	53.7	46.7	52.9	50.7	18	26	24	16
S-23	88.2	86.8	87.6	83.8	14	10	12	14
S-24	83.1	74.2	79.5	80.7	25	28	47	37

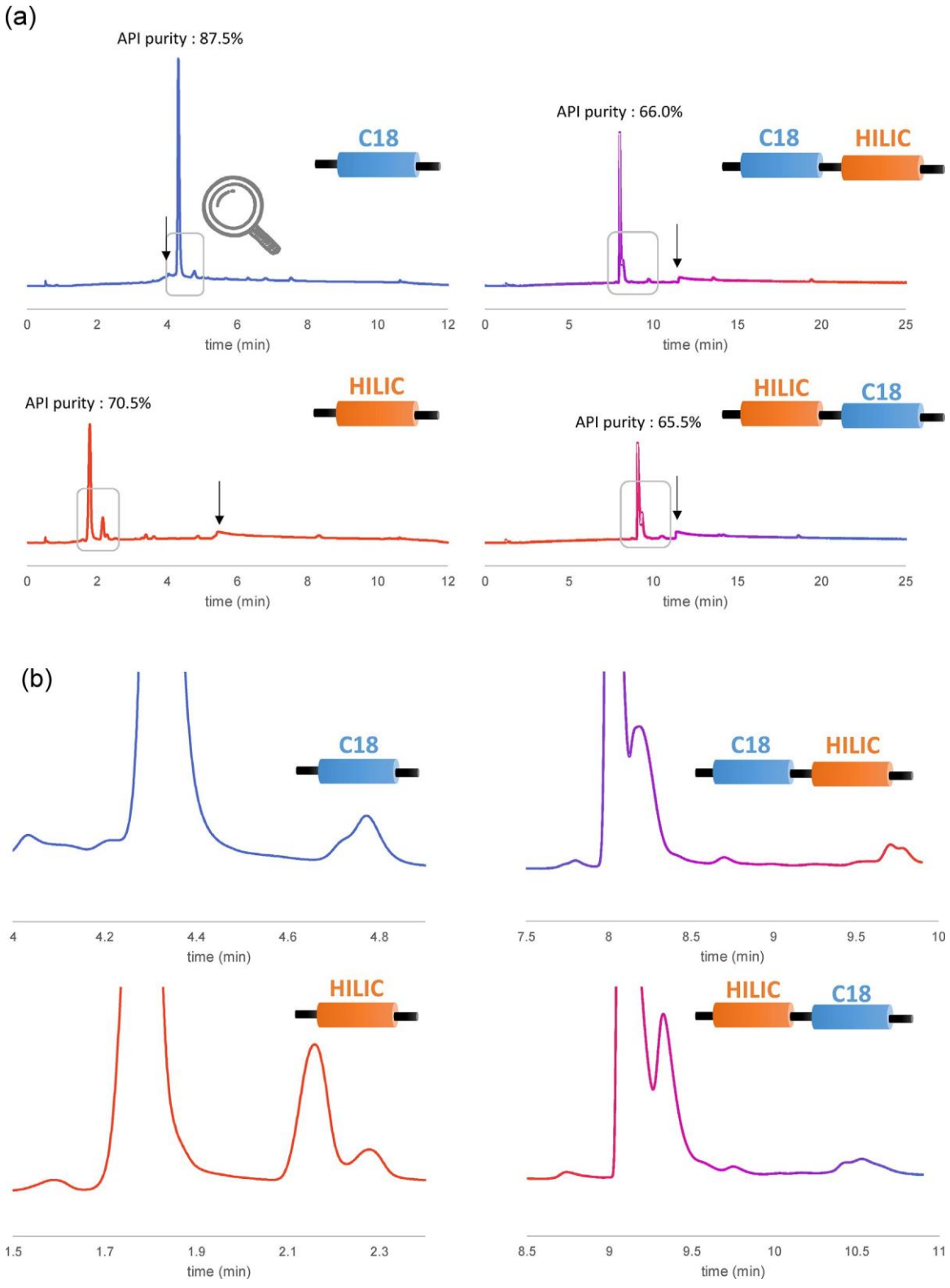


Fig. 7. Sample chromatograms illustrating the improvement brought by tandem columns for the resolution of minor impurities from the major peak. (a) Large view; (b) zoom. The black arrows indicate breakthrough of the mobile phase additive and is not an impurity from the sample.

between the two single-column and the two tandem-column systems can be observed in Fig. 6.

Between the two tandem-column systems, the superiority of the C18-HILIC system seems logical when related to the above-described (Section 3.2.2) larger peak capacity values: thinner peaks should yield higher peaks, hence better sensitivity. It was indeed verified that the points corresponding to larger UV s/n values were related to thinner peaks.

3.2.4. *Comparison of capability for impurity profiling*

In this laboratory and at the stage of API development where this impurity profiling method must apply, identity confirmation

and purity level of the main compound is the major information required, prior to a possible purification step. In addition, some information on impurities is expected: first, estimation of the relative concentration of all impurities above 0.04% of the main peak (based on UV response); second, structure elucidation of impurities with estimated concentration above 1% (UV response).

Firstly, we compared the estimated purity of the API in the 25 samples analyzed with the four methods. As one sample was eluted only with the single C18 column and not with other systems, only 24 cases were compared. The most effective method should be the one providing the lowest value of API purity, indicating that less impurities should remain co-eluted with the major peak. A difference of the API estimated purity of 0.1% was considered insignificant in this case, thus two methods could be considered equivalent. A difference of less than 1% in the estimated purity was still considered as acceptable, although not equivalent.

Secondly, the impurities with estimated concentrations above 0.04% were all counted for each of the four methods. The best method, in this case, should be the one providing the largest number of impurities detected, as less co-elutions should remain. In that case, a method providing no more than one impurity less than the best method was still considered acceptable.

For each of the 24 analytes, we then considered the two criteria at the same time. One method would be the best if it provided the best results both in API purity (smallest value) and in number of impurities (highest value).

All results are indicated in Table 1.

When considering only the single-column systems, the C18 column provided the best results in 12 cases out of 24 (50%). The HILIC column was the best in 5 cases out of 24 (21%). In the remaining 7 cases, one method could be the best for one criterion and the worst for the other criterion, thus none of the two was the best when considering the two criteria. The relative success and complementarity of the two columns was not surprising as the C18 and HILIC column had been previously selected both for their separation capabilities and for their complementarity, with superior performance observed for the C18 column.

Then we compared the single C18 column (best of the two single-column systems) to the C18-HILIC tandem-column system. In this case, the C18 column provided the best results in 7 cases out of 24 (29%) while the C18-HILIC system was the best in 10 cases out of 24 (42%). In the seven remaining cases (29%), none of the two methods combined best results for both criteria. Comparing the single C18 column to the other tandem-system (HILIC-C18), the single C18 column was the best in 11 cases out of 24 (46%) while the HILIC-C18 system was the best also in 10 cases out of 24 (42%). 3 cases (12%) remained undetermined between the two system. Thus the C18-HILIC combination was somewhat superior to the HILIC-C18 combination. They provided somewhat different results, with certain cases being improved by both column combinations and other cases improved more significantly by one or the other combination.

When the tandem-column systems were not superior to a single column, it is probable that resolution scrambling occurred, i.e. the elution order of two peaks on the first column was reversed on the second column, resulting in final co-elution.

Sample chromatograms for one case are provided in Fig. 7 where one major impurity that co-eluted with the API when the C18 column was used alone was better resolved with the tandem-column systems, thanks to the selectivity provided by the HILIC column and the improved efficiency.

One question we wanted to explore was the benefit of a single experiment with the best tandem-column system compared to two experiments with the single-column systems. The total analysis time to carry out two single-column experiments is nearly the same as doing a single tandem-column experiment thus economy of time cannot be expected there. However, the time required for data treatment of a single chromatogram is advantageous. When the C18-HILIC tandem-system was compared to the two single-column systems, it was the best in 9 cases out of 24 (38%) while the combined use of two single-column experiments provided best results in 15 cases out of 24 (62%). The statistical advantage of using two orthogonal experiments is obvious, because there were several cases where one experiment provided the best result for one criterion (the lowest value of API purity) while the second experiment provided the best result for the second criterion (largest number of impurities).

However, a significant conclusion from this comparison of the four systems is that, when only one experiment is desired, the C18-HILIC tandem-column system should be the best, providing the best combined results more often than any other system. It was particularly effective in separating and detecting impurities, as indicated by the total number of impurities counted in the 24 cases (330, vs. 298 for the other tandem-column system, 284 for the C18 column alone, or 248 for the HILIC column alone). To fully assess the contribution of improved efficiency to these improved results, a comparison based on a 20 cm-long C18 column would have been helpful, which was not attempted in the present study.

4. Conclusions

Considering all criteria described above (selectivity, peak capacity, sensitivity, API purity, number of impurities), it should be clear that a tandem-column method provides a significant improvement over the single-column methods, with superior peak capacity resulting in improved capability for impurity profiling. Judging from considerations on peak efficiency, the C18-HILIC combination seemed more promising than the reversed (HILIC-C18). Sensitivity with UV detection was however better with the C18 column employed alone, thus any improvement in the results of impurity profiling should be attributed to efficiency and selectivity. The possibility to increase flow rate in the tandem-column system should be advantageous to reach optimal mobile phase velocity allowing for even higher peak capacities.

Whether a tandem-column method should be preferred over two successive experiments with single columns may be debated, as it was observed that two single-column experiments were more informative than one tandem-column experiments.

Finally, one major benefit of this SFC tandem-column system is the easiness with which different stationary phases can be assembled to take advantage of their complementary selectivities, without the technical constraints and issues of two-dimensional methods (change of mobile phase composition, transfer of samples in compressible fluids).

Acknowledgments

Waters Corporation is warmly acknowledged for continuous support through the Centers of Innovation program. We also thank Régis Guyon (Machery-Nagel) for the kind gift of columns. Caroline West acknowledges the support of the Institut Universitaire de France (IUF) of which she is a Junior member.

References

[1] C. Wang, A.A. Tymiak, Y. Zhang, Chapter 6—application of multiple column supercritical fluid chromatography, in: C.F. Poole (Ed.), Supercritical Fluid Chromatography, Elsevier, 2017, pp. 153–172, http://dx.doi.org/10.1016/ B978-0-12-809207-1.00006-9.

[2] T.A. Berger, W.H. Wilson, Packed column supercritical fluid chromatography with 220, 000 plates, Anal. Chem. 65 (1993) 1451–1455, http://dx.doi.org/10.1021/ac00058a024.

[3] K. Gaudin, E. Lesellier, P. Chaminade, D. Ferrier, A. Baillet, A. Tchapla, Retention behaviour of ceramides in sub-critical fluid chromatography in comparison

with non-aqueous reversed-phase liquid chromatography, J. Chromatogr. A 883 (2000) 211–222, http://dx.doi.org/10.1016/S0021-9673(00)00445-3.
[4] D.A. Roston, S. Ahmed, D. Williams, T. Catalano, Comparison of drug substance impurity profiles generated with extended length columns during packed-column SFC, J. Pharm. Biomed. Anal. 26 (2001) 339–355, http://dx.doi.org/10.1016/S0731-7085(01)00405-8.
[5] C. Brunelli, Y. Zhao, M.-H. Brown, P. Sandra, Development of a supercritical fluid chromatography high-resolution separation method suitable for pharmaceuticals using cyanopropyl silica, J. Chromatogr. A 1185 (2008) 263–272, http://dx.doi.org/10.1016/j.chroma.2008.01.050.
[6] E. Lesellier, A. Latos, A.L. de Oliveira, Ultra high efficiency/low pressure supercritical fluid chromatography with superficially porous particles for triglyceride separation, J. Chromatogr. A 1327 (2014) 141–148, http://dx.doi. org/10.1016/j.chroma.2013.12.046.
[7] J. Duval, C. Colas, V. Pecher, M. Poujol, J.-F. Tranchant, É. Lesellier, Contribution of supercritical fluid chromatography coupled to high resolution mass spectrometry and UV detections for the analysis of a complex vegetable oil—application for characterization of a Kniphofia uvaria extract, C. R. Chim. 19 (2016) 1113–1123, http://dx.doi.org/10.1016/j.crci.2015.11.022.
[8] E. Lesellier, C. West, The many faces of packed column supercritical fluid chromatography—a critical review, J. Chromatogr. A 1382 (2015) 2–46, http:// dx.doi.org/10.1016/j.chroma.2014.12.083.
[9] E. Lesellier, C. West, A. Tchapla, Advantages of the use of monolithic stationary phases for modelling the retention in sub/supercritical chromatography: application to cis/trans-β-carotene separation, J. Chromatogr. A 1018 (2003) 225–232, http://dx.doi.org/10.1016/j.chroma.2003.07.014.
[10] E. Lesellier, Analysis of polycyclic aromatic hydrocarbons by supercritical fluid chromatography (SFC), Analusis 27 (1999) 241–248, http://dx.doi.org/10.1051/analusis:1999113.
[11] F.S. Deschamps, E. Lesellier, J. Bleton, A. Baillet, A. Tchapla, P. Chaminade, Glycolipid class profiling by packed-column subcritical fluid chromatography, J. Chromatogr. A 1040 (2004) 115–121, http://dx.doi.org/10.1016/j.chroma.2004.03.052.
[12] V. Abrahamsson, I. Rodriguez-Meizoso, C. Turner, Determination of carotenoids in microalgae using supercritical fluid extraction and chromatography, J. Chromatogr. A 1250 (2012) 63–68, http://dx.doi.org/10.1016/j.chroma.2012.05.069.
[13] S. Delahaye, F. Lynen, Implementing stationary-phase optimized selectivity in supercritical fluid chromatography, Anal. Chem. 24 (86) (2014) 12220–12228, http://dx.doi.org/10.1021/ac503313j.
[14] W.W. Barnhart, K.H. Gahm, S. Thomas, S. Notari, D. Semin, J. Cheetham, Supercritical fluid chromatography tandem-column method development in pharmaceutical sciences for a mixture of four stereoisomers, J. Sep. Sci. 28 (2005) 619–626, http://dx.doi.org/10.1002/jssc.200500005.
[15] C.J. Welch, M. Biba, J.R. Gouker, G. Kath, P. Augustine, P. Hosek, Solving multicomponent chiral separation challenges using a new SFC tandem column screening tool, Chirality 19 (2007) 184–189, http://dx.doi.org/10.1002/chir.20357.
[16] C. Wang, A.A. Tymiak, Y. Zhang, Optimization and simulation of tandem column supercritical fluid chromatography separations using column back pressure as a unique parameter, Anal. Chem. 86 (2014) 4033–4040, http://dx. doi.org/10.1021/ac500530n.
[17] W.H. Pirkle, C.J. Welch, Some thoughts on the coupling of dissimilar chiral columns or the mixing of chiral stationary phases for the separation of enantiomers, J. Chromatogr. A 731 (1996) 322–326, http://dx.doi.org/10. 1016/0021-9673(95)01240-0.
[18] K.W. Phinney, L.C. Sander, S.A. Wise, Coupled achiral/chiral column techniques in subcritical fluid chromatography for the separation of chiral and nonchiral compounds, Anal. Chem. 70 (1998) 2331–2335, http://dx.doi. org/10.1021/ac971060z.
[19] A.J. Alexander, A. Staab, Use of achiral/chiral SFC/MS for the profiling of isomeric cinnamonitrile/hydrocinnamonitrile products in chiral drug synthesis, Anal. Chem. 78 (2006) 3835–3838, http://dx.doi.org/10.1021/ac060326b.
[20] M. Ventura, Use of achiral columns coupled with chiral columns in SFC separations to simplify isolation of chemically pure enantiomer products, Am. Pharm. Rev. (2013), http://www.americanpharmaceuticalreview.com/Featured-Articles/148859-Use-of-Achiral-Columns-Coupled-with-Chiral-Columns-in-SFC-Separations-to-Simplify-Isolation-of-Chemically-Pure-Enantiomer-Products/ (Accessed October 9 2017).
[21] C. West, E. Lesellier, Orthogonal screening system of columns for supercritical fluid chromatography, J. Chromatogr. A 1203 (2008) 105–113, http://dx.doi. org/10.1016/j.chroma.2008.07.016.
[22] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part II. Selection of an orthogonal set of stationary phases, J. Chromatogr. A 1408 (2015) 227–235, http://dx.doi.org/ 10.1016/j.chroma.2015.07.035.
[23] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part I: optimization of mobile phase composition, J. Chromatogr. A 2015 (1408) 217–226, http://dx.doi.org/10.1016/j.chroma.2015.07.037.
[24] E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West, Comparison of ultra-high performance methods in liquid and supercritical fluid chromatography coupled to electrospray ionization—mass spectrometry for impurity profiling of drug candidates, J. Chromatogr. A 2016 (1472) 117–128, http://dx.doi.org/10.1016/j.chroma.2016.10.045.
[25] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, part I: salt gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 33–44, http://dx.doi.org/10.1016/j.jpba.2014.08.035.

VII. Comparaison des méthodes LC et SFC pour le profilage d'impuretés

Après le développement de méthodes en SFC et les performances de cette méthode mises en avant, on veut maintenant appliquer cette méthode au profilage d'impuretés de candidats médicaments. La comparaison des méthodes UHPSFC et UHPLC pour le profilage d'impuretés de candidats médicaments est présenté dans l'article suivant :

E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West

Comparison of ultra-high performance methods in liquid and supercritical fluid chromatography coupled to electrospray ionization − mass spectrometry for impurity profiling of drug candidates

Journal of Chromatography A, Volume 1472, (2016), 117-128

Journal of Chromatography A, 1472 (2016) 117–128

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Comparison of ultra-high performance methods in liquid and supercritical fluid chromatography coupled to electrospray ionization – mass spectrometry for impurity profiling of drug candidates*

Elise Lemasson[a], Sophie Bertin[b], Philippe Hennig[b], Eric Lesellier[a], Caroline West[a,*]

[a] Univ Orléans, Institut de Chimie Organique et Analytique (ICOA), CNRS UMR 7311, Pôle de Chimie, rue de Chartres, B.P. 6759, 45067 Orléans, Cedex 2, France

[b] Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France

ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form
17 September 2016
Accepted 18 October 2016
Available online 18 October 2016

Keywords:
High-resolution separations
Impurity profiling
Orthogonal methods
Performance comparison
Pharmaceutical ingredients

ABSTRACT

Impurity profiling of organic products synthesized as possible drug candidates represents a major analytical challenge. Complementary analytical methods are required to ensure that all impurities are detected. Both high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) can be used for this purpose.

In this study, we compared ultra-high performance HPLC (UHPLC) and ultra-high performance SFC (UHPSFC) using a large dataset of 140 pharmaceutical compounds. Four previously optimized methods (two on each technique) were selected to ensure fast high-resolution separations. The four methods were evaluated based on response rate, peak capacity, peak shape and capability to detect impurities (UV). The orthogonality between all methods was also assessed. The best UHPLC method and UHPSFC methods provided comparable quality for the 140 compounds included in this study. Moreover, they were found to be highly orthogonal. At last, the potential of the combined use of UHPLC and UHPSFC for impurity profiling is illustrated with practical examples.

1. Introduction

Identification and quantification of active ingredients and their impurities is an important task of chromatographers in pharmaceutical companies, as the identity and proportion of impurities must be strictly controlled to guarantee the efficacy and limit toxicity of the active pharmaceutical ingredient (API) [1]. For this purpose, it is necessary to have complementary high-performance chromatographic tools. For this task, ultra-high performance liquid chromatography (UHPLC) in the reversed-phase mode is the gold standard in most companies. However, ultra-high performance supercritical fluid chromatography (UHPSFC) now progresses rapidly in this application field [2–4].

UHPSFC makes use of mobile phases comprising a significant portion of pressurized carbon dioxide mixed to another liquid solvent (most often an alcohol such as methanol) [5]. CO_2 has major advantages over more conventional chromatographic solvents, as it has a low viscosity allowing for high diffusivities of the analytes (hence high efficiencies) and for limited pressure drop over packed columns. As a result, high flow rates can be used without strongly affecting efficiency, and columns packed with sub–2 μm particles can be employed with relatively low-pressure pumping systems (400 bar) [5,6]. The high-throughput capability and economic benefits of UHPSFC [7], but also the "green" aspect of a non-toxic solvent together render UHPSFC very attractive. The recent introduction of new robust instruments dedicated to UHPSFC and the progress in stationary phase technology have also been a great benefit [8].

Several studies compared impurity profiling between UHPSFC and UHPLC. Xu et al. [9] showed the interest of developing methods in SFC instead of HPLC for the analysis of compounds sensitive to water. The absence of water in SFC eliminates the risk of degradation during the analysis, which was a major concern with HPLC. The SFC method developed was sensitive enough to detect the impurities at 0.5 mg/mL level. Wang *et al.* [10] developed a method for the determination of eight impurities and degradation products of mometasone furoate, at 0.05% of API area. Compared to HPLC, SFC provided higher efficiency and faster analyses. Moreover, the SFC

* Selected paper from 14th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology, 27–29 January 2016, Ghent, Belgium.

* Corresponding author.
E-mail addresses: caroline.west@univ-orleans.fr, west caroline@yahoo.fr (C. West).

http://dx.doi.org/10.1016/j.chroma.2016.10.045
0021-9673/

and HPLC methods provided orthogonal selectivity. SFC would thus be interesting to implement as complementary method for the purpose of identifying all species in a complex mixture. Alexander et al. [11] compared impurity profiling of three antiretroviral drugs with SFC and HPLC. The new generation of SFC instrumentation, as used in the present study, was found to exhibit the required UV sensitivity for successful quantification of potential impurities/degradation products at the 0.05–0.1% area level.

UV detection is usually employed to quantify impurities (unless the API contains no chromophoric group). However, the joint use of mass spectrometric (MS) detection is commonplace and essential to confirm peak identity and support peak purity. In this purpose, it was already proven by others that SFC-MS could compete with LC–MS [12–14]. All these studies, however informative, compared the two methods on a limited number of analytes, while we aim at more general conclusions with a larger set of probe compounds.

In the present study, we employed the same single-quadrupole mass detector with both UHPLC and UHPSFC. Previous studies already showed the interest of the use of UHPSFC rather than UHPLC coupled to ESI–MS [15–17].

We compared the performances of UHPLC and UHPSFC coupled to UV and MS detections for impurity profiling, based on the analyses of a large and diverse set of drug candidates. After the optimization of UHPLC and UHPSFC methods to provide fast high-resolution gradient methods, the performances of the four methods were compared in terms of orthogonality of the techniques, response rate, peak capacity and peak shapes, capability to detect impurities and evaluate purity of the major compound.

2. Materialandmethod

2.1. Chemicals and solvents

140 drug candidates were obtained from Servier Research Laboratories (Suresnes, France). The structures are confidential, but they were previously described [18]. Briefly, molecular weights range from 150 to 750 g/mol, and log P values vary between −1.9 and 7.5, with a large majority of positive values. Moreover, as usual in compounds of pharmaceutical interest, a large portion of them have basic functions (80%). 14 mixtures of 10 compounds each were prepared at 1 mg/mL in acetonitrile for UHPLC, or in ethanol for UHPSFC. The composition of mixtures was designed to avoid isobaric compounds being present in the same mixture.

For the sample applications, a subset of 25 drug candidates was selected from the 140 whole set. They were selected particularly for the large number of impurities seen in their chromatograms, and were injected individually in three replicates to evaluate the relative purity of the main component and provide an estimated quantification of major impurities (>1%).

At Univ Orléans, solvents used were HPLC-grade methanol (MeOH) and ethanol provided by VWR (Fontenay-sous-Bois, France); carbon dioxide of industrial grade 99.5% was provided by Messer (Puteaux, France). Ammonium hydroxide solution was provided by Fisher Scientific (Illkirch, France); ultra-pure water was provided by an Elga UHQ system from Veolia (Wissous, France). At Servier Research laboratories, water was obtained from a Milli-Q Purification System from Millipore (Millipore SAS, France), HPLC-grade acetonitrile was purchased from Merck (VWR international SAS, France), methanesulfonic acid and ammonium bicarbonate were provided by Sigma-Aldrich (Sigma Aldrich Chimie, France).

2.2. UHPLC methods

At Servier Research laboratories, it is common practice to use two complementary reversed-phase UHPLC methods that were optimized several years ago to maximize the chances to identify correctly all impurities. Both methods make use of the same chromatographic column (ACQUITY BEH C18, 50 × 2.1 mm, 1.7 μm fully porous silica, from Waters) but with two different mobile phase compositions: (i) acidic conditions with methanesulfonic acid and (ii) basic conditions with ammonium bicarbonate, thereby providing some complementarity. If the preferred method (acidic conditions) is not satisfactory (when the main compound elutes too close to dead volume, to the gradient end, or is incorrectly or not eluted, if co-elution with impurities is observed or in order to get more structural information), the second method (basic conditions) is used. Two UHPLC systems are used in a parallel fashion. These two methods have been routinely used for day-to-day impurity profiling of synthetic products for several years and constitute reference methods at Servier Research laboratories.

The UHPLC systems were both ACQUITY UPLC® I-Class from Waters Corporation. They were equipped with a binary solvent delivery pump compatible with mobile phase flow rates up to 2 mL/min and pressures up to 827 bar, an autosampler that included partial loop volume injection system, 2-position column oven compatible with 150 mm length columns and a photodiode-array (PDA) detector. For analysis performed in acidic conditions, an ACQUITY QDa® single-quadrupole mass detector (Waters Corporation) with electrospray ionization source was used. An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was then splitted by the on-board flow-splitter assembly. With this system, the split ratio is 1/10: only 1/10 of the column flow goes to the MS. For analysis performed in basic conditions, an ACQUITY SQD® single-quadrupole mass detector (Waters Corporation) with electrospray ionization source was used. MassLynx® software (V4.1) was used for system control and data acquisition. Empower®3 was used for integration of peaks for column efficiency measurements. Waters Data Converter (V2.1) was used to convert data from MassLynx to Empower.

Analyses were performed at 0.4 mL/min, 30 °C, with a gradient elution program in the following conditions:

(1) For analyses performed with methanesulfonic acid (MSA) (constant 0.1% in the mobile phase), the mobile phase composition was water with 2 to 98% acetonitrile in 8 min
(2) For analyses performed with ammonium bicarbonate (BICAR) (constant 20 mM in the mobile phase), the mobile phase composition was water with 2 to 80% acetonitrile in 10 min

2 μL of each 10-compound mixture were injected with a 10 μL-loop and acetonitrile was used to rinse the system.

The extracted wavelength for UV detection was fixed at 210 nm. Frequency was set at 20 pts/s and resolution at 1.2 nm.

For analysis performed with MSA, mass detector conditions were based on pre-optimized conditions recommended by the manufacturer: scan rate 10 pts/s, capillary voltage 0.8 kV, cone voltage 15 V, sampling frequency 8 Hz, ion source temperature 150 °C and probe temperature 600 °C. The analytes were detected in positive electrospray ionization mode (m/z 100–800). Make-up flow was 0.4 mL/min with 70% acetonitrile – 30% water comprising 0.1% formic acid.

For analysis performed with BICAR, the studied compounds were detected in positive and negative electrospray ionization mode (m/z 100–1000), scan time 0.3 s, capillary voltage 4 kV (ESI +) or 3 kV (ESI-), cone voltage 20 V (ESI +) or 30 V (ESI-), ion source temperature 150 ° C, desolvatation temperature 250 ° C, gas flow desolvation 500L/h and gas flow cone 0L/h.

2.3. UHPSFC methods

The UHPSFC system was an ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) from Waters Corporation (Millford, MA, USA). It was equipped with a binary solvent delivery pump compatible with mobile phase flow rates up to 4 mL/min and pressures up to 414 bar, an autosampler that included partial loop volume injection system, a back pressure regulator, 2-position column oven compatible with 150 mm length columns and two detectors: a photodiode-array (PDA) detector and an ACQUITY QDa® single-quadrupole mass detector with electrospray ionization source. An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was then splitted by the on-board flow-splitter assembly. With this system, most of the column flow goes to the back- pressure regulator and only an unknown portion goes to the MS. Empower®3 and MassLynx® (V4.1) softwares were used for system control and data acquisition.

Based on our previous works [19], two different columns were used: an ACQUITY UPC² HSS C18 SB column (100 × 3.0 mm, 1.8 μm fully porous silica) from Waters and a Nucleoshell HILIC column (150 × 3.0 mm, 2.7 μm superficially porous silica) from Macherey- Nagel.

In our previous works [18,19], during method development we found that ammonium acetate and ammonium hydroxide introduced in the methanol co-solvent yielded the best performance in terms of chromatographic features and detection sensitivity. Ammonium acetate was somewhat superior to ammonium hydroxide, but the former has a disadvantage as its high UV absorbance causes a significant baseline drift over a gradient elution, together with baseline disturbances caused by breakthrough of the additive when the stationary phase is covered with salt, thus we finally settled to use ammonium hydroxide instead. To prepare the mobile phase co-solvent, a solution of ammonium hydroxide was first prepared at 1 M in water and then diluted down to 20 mM in MeOH. The final composition of mobile phase co-solvent thus comprises 20 mM ammonium hydroxide and 2% water.

2 μL of each 10-compound mixture were injected on a 10-μL loop and methanol was used to rinse the system.

Analyses were performed at 15 MPa (outlet pressure), 25 °C, and a flow rate of 1 mL/min in a gradient elution program in the following conditions:

(1) For the column with dimensions of 100 × 3.0 mm (ACQUITY UPC² HSS C18 SB, 1.8 μm), the mobile phase composition was CO_2 with 5 to 50% MeOH comprising 20 mM ammonium hydroxide and 2% water increased over 10 min. Inlet pressure at the beginning and end of the gradient program varied from 21.5 to 33 MPa respectively.

(2) For the column with dimensions of 150 × 3.0 mm (Nucleoshell HILIC, 2.7 μm), the mobile phase composition was CO_2 with 5 to 50% MeOH comprising 20 mM ammonium hydroxide and 2% water increased over 15 min. Inlet pressure at the beginning and end of the gradient program varied from 19 to 27 MPa respectively.

Thus identical linear velocity and gradient steepness were used with the two columns. Variations between the average column pressure values were not compensated with outlet pressure, as we aimed at a simple operating procedure. The differences in average pressure resulting from different column dimensions and particle type and size should cause only limited differences in elution strength of the mobile phase, because the outlet pressure (15 MPa) and oven temperature (25 °C) conditions selected result in a fluid of limited compressibility.

The extracted wavelength for UV detection was fixed at 210 nm. Frequency was set at 20 pts/s and resolution at 1.2 nm.

The mass detector unit was pre-optimized by the manufacturer. The analytes were detected in positive and negative electrospray ionization mode (m/z 150–750), scan rate 10 pts/s, capillary voltage 0.8 kV, cone voltage 15 V, sampling frequency 8 Hz, ion source temperature 150 °C and probe temperature 600 °C. Nitrogen was used as nebulizing gas. Make-up flow was 0.45 mL/min with 98% methanol –2% water comprising 1% formic acid. As the pre-selected ionization parameters caused only little fragmentation, only the precursor ions were considered ($[M + H]^+$ in ESI^+ mode, $[M–H]^-$ in ESI^-).

2.4. Orthogonality measures

Different methods have been proposed to evaluate the degree of orthogonality between two chromatographic methods. The simplest are based on linear regression of retention data. For instance, the degree of orthogonality can be expressed as Pearson's correlation coefficient (R) or the determination coefficient (R^2) when plotting a regression line between retention factor values. A low value for R^2 indicates a high degree of orthogonality [20]. Instead of R and R^2, the degree of orthogonality may also be calculated by using the selectivity difference. This criterion, defined by Neue et al. [21], s^2 is equal to $1 - R^2$. Quite logically, with this criterion high s^2 values reflect high degrees of orthogonality. The sole use of R^2 or s^2 is however limited to assess orthogonality. First, they are both strongly affected by lever points, thus depend strongly on the analytes selected to evaluate the orthogonality. Secondly, they provide no indications regarding the retention space covered by the two methods. This lack of information can be problematic because the same R^2 can represent two completely different distribution of retention values (for example compounds concentrated in a restraint zone of retention or data adequately scattered in the retention space).

Other methods for the evaluation of orthogonality are based on a geometric approach, where the two-dimensional space occupied by the peaks on the retention diagram is assessed. We followed the method proposed by D'Attoma et al. [22] to determine the retention areas covered by two UHPLC or two UHPSFC methods, or by the combination of one UHPLC to one UHPSFC method. First, as all methods are based on gradient elution, the analytes elute from one chromatographic system with different mobile phase compositions. The methods are then compared in terms of elution composition. We then have a visual idea of the distribution of our compounds in the retention space. The linear regression line can then be plotted. The retention space covered by two methods is constructed by drawing a polygon (Fig. 1). The two diagonal lines are parallel to the regression line and represent the confidence envelope at 95%.

To compare the methods based on different gradient time and limit values, the retention times obtained with each method must be transformed into elution composition (C_e) [23] using Eq. (1):

$$C_e = C_i + \frac{(C_f - C_i)}{t_G} * (t_R - t_D) \quad (1)$$

Where C_e corresponds to the percentage of ACN (for UHPLC analysis) and MeOH (for UHPSFC analysis) required to elute the analyte of interest; C_i and C_f are the initial and final compositions of the gradient, respectively; t_G is the gradient time; t_R is the retention time of the compound and t_D is the system dwell time. The dwell time of UHPSFC system was measured according to the method described in [24] and the value found was 0.46 min. For UHPLC system, the value found was 0.28 min.

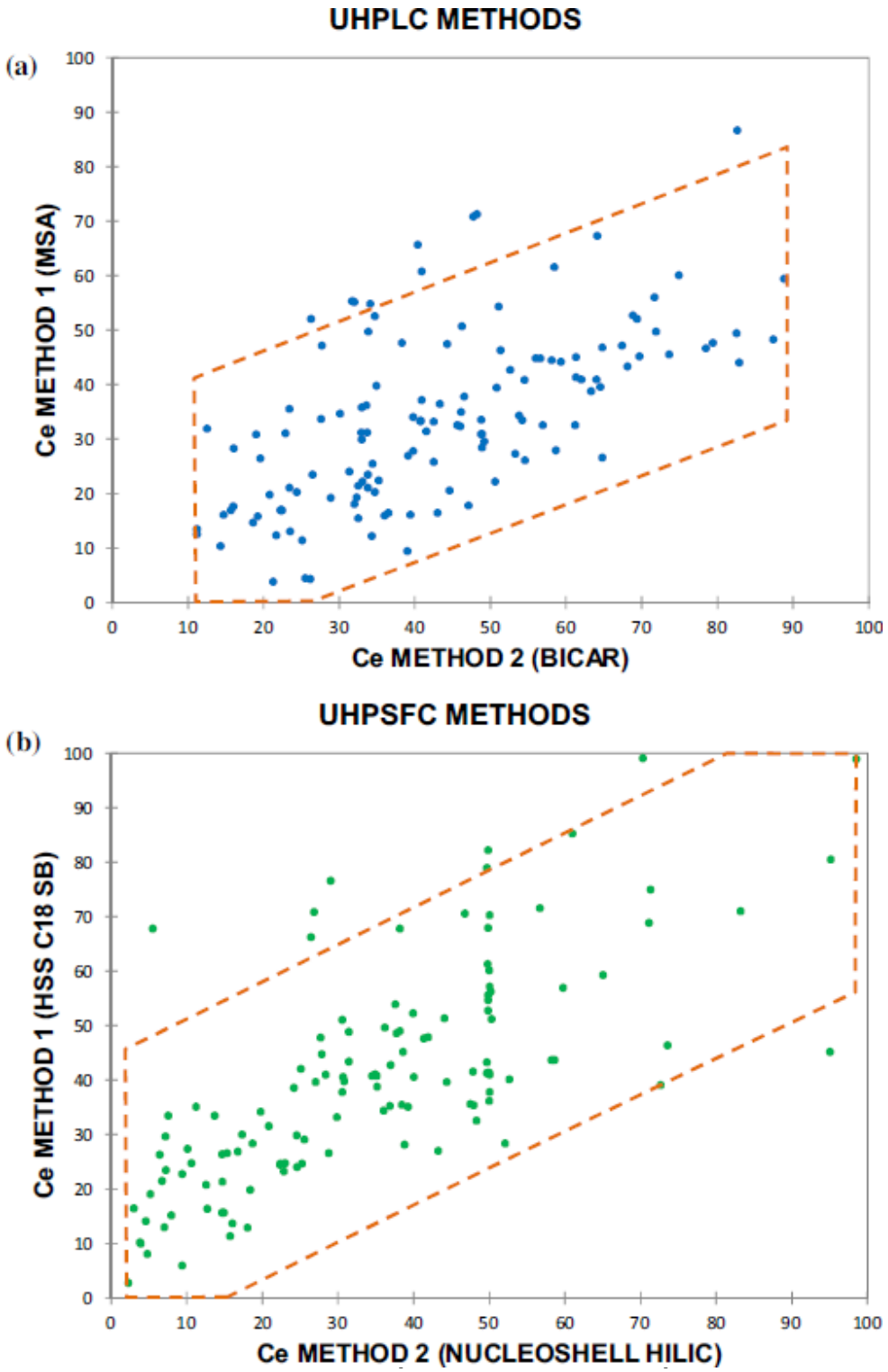


Fig.1. Comparison of elution composition (normalized values) obtained with the pairs of UHPLC methods (a) and UHPSFC methods (b), set of 140 drug candidates. The orange interrupted lines delimit the retention space covered by each technique. Ce is for elution composition, MSA is for methanesulfonic acid, BICAR is for ammonium bicarbonate.

2.4. Peak capacity

Peak capacity (P_c) is defined as the maximal number of peaks which can be separated during the gradient time, with a resolution of 1 [23] and can be calculated following Eq. (2):

$$P_c = 1 + \frac{t_g}{1.7 * W_{50\%}} \quad (2)$$

In this equation, t_g (min) is for gradient time and W_{50} (min) represents the average measured peak width at 50% height. In our case, the comparison was based on the peak width measured on a subset of 25 compounds eluted with all four methods (two UHPLC and two UHPSFC) with symmetrical peaks. The question of peak height at which the measurement of width should be done was raised, judging from the significant portion of non-symmetrical peaks

observed. We considered measuring peak width at a lower height, but partial co-elution with impurities rendered this an unreliable measure. We thus chose to retain the peak width at 50% height, maintaining only symmetrical peaks in the calculation. The peak capacity calculated from this procedure is obviously better than would be if non-symmetrical peaks had been considered. However, as all four methods were evaluated in the same manner, the bias is the same, thus the comparison of methods is valid.

3. Results and discussion

3.1. Orthogonality between UHPLC and UHPSFC methods

First, we can observe orthogonality between the two UHPLC methods, or between the two UHPSFC methods, using both the values of determination coefficients and the retention space covered by our methods (Fig. 1). For UHPLC methods, Fig. 1a shows the elution composition calculated on the ACQUITY UPC^2 BEH C18 column with acidic mobile phase (method 1) or basic mobile phase (method 2). For all methods, the elution compositions were normalized (from 0 to 100%) with regards to the minimum and maximum values of the gradient program (5 to 50% for UHPSFC and 2 to 80 or 98% for UHPLC methods). The vertical orange lines indicate the lowest and the greatest retention of compounds in methods 2 (bicarbonate for UHPLC and Nucleoshell HILIC for UHPSFC). The diagonal orange lines are defined by the 95% confidence limit resulting from the calculation of linear regression. It means that any new compound should have 95% chances to be included in this area.

The correlation between normalized elution compositions as indicated by the determination coefficient is limited (R^2 = 0.39), indicating that the methods are indeed complementary. However, the retention space covered (orange polygon in Fig. 1a) is not very large, restricted to a rather narrow band.

For UHPSFC methods, we compared in Fig. 1b the elution compositions calculated on the ACQUITY UPC^2 HSS C18 SB column (method 1) with those measured on the Nucleoshell HILIC column (method 2). Based on the value of determination coefficient (R^2 = 0.52), the complementarity between the two UHPSFC methods seems not as good as to the one observed between the two UHPLC methods. However, the retention space covered (orange polygon) is larger for the UHPSFC methods than for the UHPLC methods. Based on these observations, the use of retention space seems a better criteria to evaluate chromatographic orthogonality than determination coefficient.

Secondly, orthogonality between each UHPLC method and each UHPSFC method was also assessed (Fig. 2). In the four cases, both determination coefficient and the retention space covered indicate a high level of orthogonality between UHPLC and UHPSFC methods. In conclusion, combining UHPLC and UHPSFC resulted in higher orthogonality compared to employing one single technique. It moreover maximizes the chances of seeing all impurities in only two runs.

3.2. Performance comparison

In this work we aimed at an objective comparison of chromatographic performance of UHPLC and UHPSFC to fully assess their usefulness for impurity profiling. For this purpose, it was necessary to use independently optimized UHPLC and UHPSFC methods. However, high-throughput was not desired thus analysis time was not fully optimized for very high speed, and will not be a criterion for comparison of performance. Different parameters were evaluated: successful elution, peak shapes, peak capacity and sensitivity of the techniques.

Table 1
Comparison of average peak capacity for 25 selected compounds with symmetrical peaks.

	UHPLC		UHPSFC	
	Method 1	Method 2	Method 1	Method 2
t_g	8	10	10	15
Average w_{50} measured	0.06	0.06	0.02	0.03
Average P_c	**108**	**166**	**197**	**191**

3.2.1. Number of analytes successfully eluted and peak shapes

First of all, we measured the response rate of each technique, defined as the number of compounds observed with UV or MS detection (or with both of them). Naturally, the goal is to have a maximum of compounds eluted with the optimized gradient methods. Unseen analytes may be eluted with prolonged gradient times or higher eluting strengths but this was not assessed, as only the routine methods were compared. Indeed, in the usual analysis process followed in this laboratory, when the first chromatographic method is unsuccessful, it is considered a faster and more efficient solution to re-analyze the sample with a different established method rather than trying to understand why the analyte was not successfully eluted with the first method.

Peak symmetry was also assessed based on the measurement of asymmetry (As) at 10% of peak height for each of the 140 compounds. For eluted analytes, we separated symmetrical peaks (0.8 < As <1.4), from non-symmetrical peaks including tailing (As >1.4), fronting (As < 0.8), distortions and shoulders.

The results are summarized in Fig. 3. We obtained very high response rates with all four methods. The response rates obtained with UHPLC methods (95% and 96% respectively for methods 1 and 2) were higher than those obtained with UHPSFC methods (93% and 91% respectively for methods 1 and 2). The highest proportion of symmetrical peaks (measured on UV chromatograms) was obtained with UHPLC method 1 (72% of symmetrical peaks). In that particular case, the methanesulfonic acid additive probably forms ion pairs with basic analytes to favour their elution with good peak shapes. With the other methods, we obtained comparable portions of symmetrical peaks, comprised between 51 and 59% of the analytes eluted. Among the non-symmetrical peaks, a large majority of cases concerned tailing and only a rare cases of fronting were observed.

For the few cases of non-eluted compounds, a majority of them was identical between the four methods. The simple observation of their structure did not provide a clear indication on their particular behavior and no common structural feature could be observed. For these compounds, alternative techniques are currently explored.
Finally, the performance results regarding analytes eluted and peak shapes were close between the four methods, apart from a slight superiority of UHPLC method 1, where a highest number of symmetrical peaks is observed.

3.2.2. Peak capacity evaluation

The results of average peak width (based on 25 analytes) and peak capacity are summarized in Table 1. Maximal peak capacity was obtained with UHPLC method 1, with acidic mobile phase (P_c = 197). With UHPLC method 2, a 3% decrease in peak capacity is observed (P_c = 191). UHPSFC method 2 is somewhat inferior to both UHPLC methods, with P_c = 166. UHPSFC method 1 was the worst, with a significant decrease in peak capacity (Pc = 108).

These figures must be taken with care as only twenty-five analytes with good peak shapes were retained in the calculation. We can conclude that peak capacity was significantly better in UHPLC than UHPSFC.

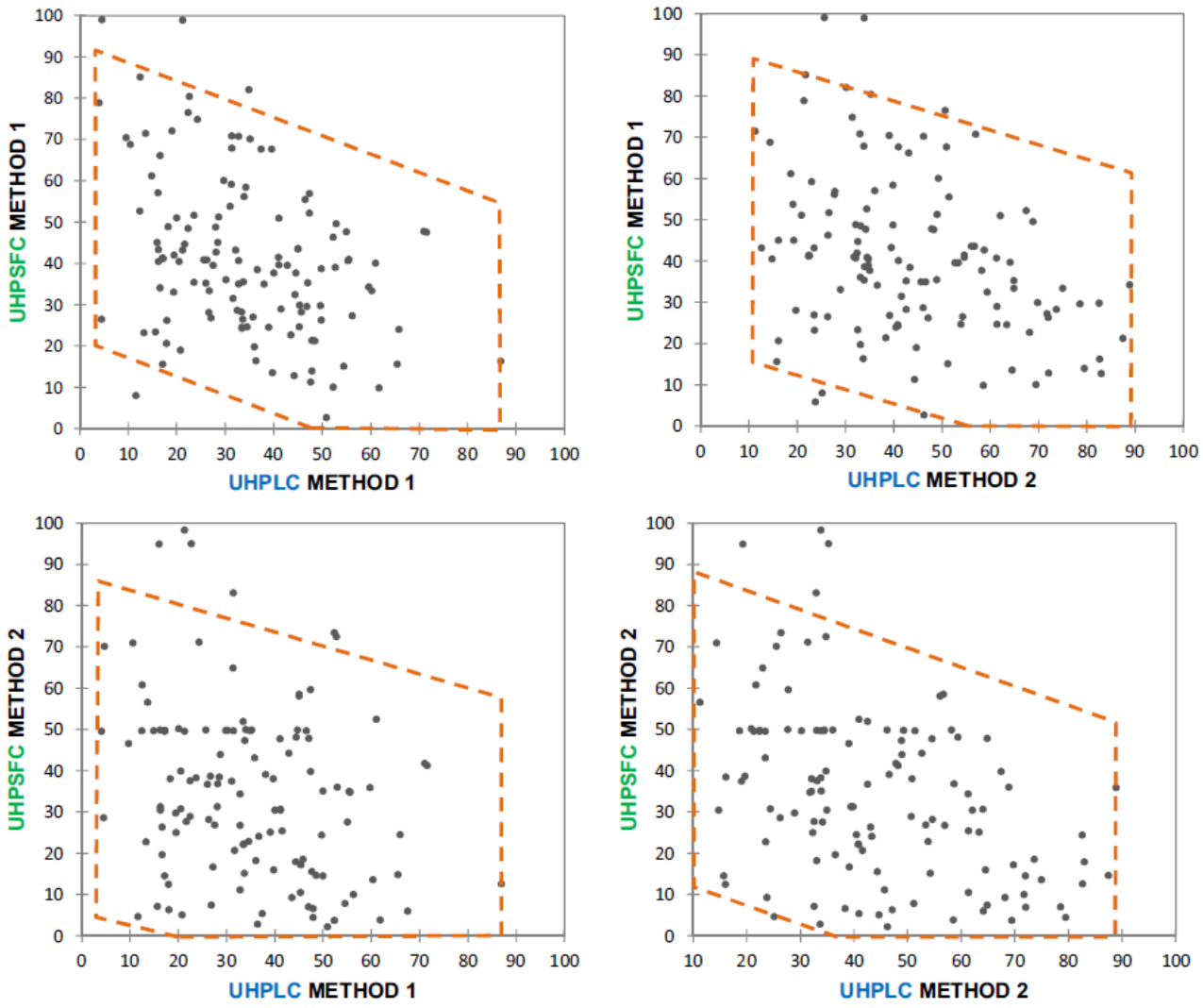


Fig.2. Comparison of elution composition (normalized values) obtained with one UHPLC method and one UHPSFC method, set of 140 drug candidates. The orange interrupted lines delimit the retention space covered.

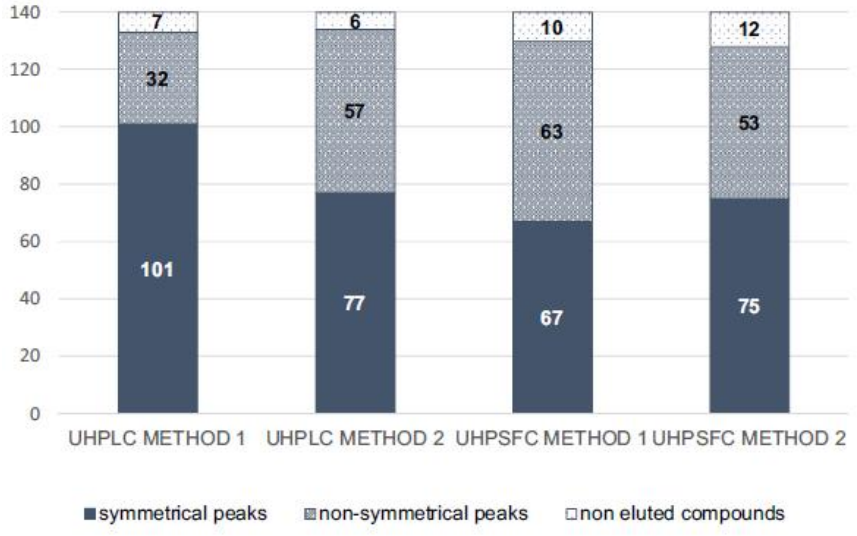


Fig.3. Comparison of response rate, proportion of symmetrical and non-symmetrical peaks (tailing, fronting, shoulders), with an identical set of 140 compounds in all cases.

Table 2
Comparison of impurity profiling methods (Case 1, chromatograms in Fig. S1).

UHPSFC (method 1: NH_4OH)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	5.35	89.70	560.3	
Impurities ≥ 1%				
IMP 1	7.43	6.18	556.3	278.8
IMP 2	6.37	2.22	574.3	
undetected				
undetected				
undetected				
Impurities ≤ 1%				
IMP A	2.34	0.05		
IMP B	4.88	0.07		
IMP C	5.78	0.36		
IMP D	5.89	0.36		
IMP E	6.14	0.08		
IMP F	6.49	0.15		
IMP G	6.56	0.32		
IMP H	6.80	0.41		
IMP I	6.98	0.10		
UV s/n	1.45E + 04			
MS s/n	2.44E + 02			

UHPLC (method 1: MSA)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	2.46	81.03	560.3	
Impurities ≥ 1%				
IMP 1	2.41	8.81	556.2	
IMP 2	3.08	3.28	574.3	
IMP 3	2.36	3.25	542.2	
IMP 4	3.26	1.12	548.4	
undetected				
Impurities ≤ 1%				
IMP A	1.11	0.08		
IMP B	1.18	0.04		
IMP C	1.23	0.19		
IMP D	1.93	0.04		
IMP E	1.96	0.09		
IMP F	2.00	0.08		
IMP G	2.02	0.05		
IMP H	2.07	0.40		
IMP I	2.15	0.04		
IMP J	2.89	0.54		
IMP K	3.06	0.12		
IMP L	3.14	0.22		
IMP M	3.20	0.06		
IMP N	3.22	0.28		
IMP O	3.28	0.28		
UV s/n	4.85E + 03			
MS s/n	9.83E + 01			

UHPSFC (method 2: NH_4OH)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	4.51	87.18	560.3	
Impurities ≥ 1%				
IMP 1	5.74	6.89	556.3	
IMP 2	5.44	2.31	574.3	
undetected				
undetected				
undetected				
Impurities ≤ 1%				
IMP A	1.99	0.05		
IMP B	3.39	0.04		
IMP C	4.44	0.42		
IMP D	4.78	0.11		
IMP E	4.89	0.04		
IMP F	5.13	0.04		
IMP G	5.21	0.04		
IMP H	5.99	0.06		

Table 2 (*Continued*)

UHPSFC (method 2: NH_4OH)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
IMP I	6.04	0.09		
IMP J	6.18	0.08		
IMP K	6.99	0.36		
IMP L	7.30	0.56		
IMP M	7.56	0.50		
IMP N	7.65	0.09		
IMP O	8.14	0.05		
IMP P	8.42	0.11		
IMP Q	8.50	0.15		
IMP R	9.44	0.19		
IMP S	9.53	0.35		
IMP T	10.01	0.08		
IMP U	10.53	0.15		
UV s/n	1.92E + 04			
MS s/n	2.48E + 02			
UHPLC (method 2: BICAR)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	5.59	88.09	560.6	
Impurities ≥ 1%				
IMP 1	4.56	5.55	278.8	
undetected				
undetected				
undetected				
IMP 5	4.59	2.40	279.0	
Impurities ≤ 1%				
IMP A	1.48	0.16		
IMP B	1.55	0.08		
IMP C	3.64	0.09		
IMP D	4.13	0.16		
IMP E	4.23	0.34		
IMP F	4.34	0.52		
IMP G	4.85	0.39		
IMP H	5.01	0.11		
IMP I	5.38	0.50		
IMP J	5.47	0.09		
IMP K	5.76	0.19		
IMP L	5.86	0.39		
IMP M	5.90	0.06		
IMP N	5.97	0.35		
IMP O	6.02	0.10		
IMP P	6.09	0.11		
IMP Q	6.11	0.13		
IMP R	6.15	0.06		
IMP S	6.26	0.12		
UV s/n	1.45E + 04			
MS s/n	9.33E + 02			

3.2.3. Capability for impurity profiling: purity assessment and impurities detected

At Servier Research laboratories where these methods are employed, at the stage of API development where this impurity profiling method must apply, only limited information is required:

(i) identity confirmation (based on MS information) and purity level of the main compound (based on UV integrated chromatogram) is the major information required;
(ii) proposed structure elucidation (based on MS information) of impurities with estimated concentration above 1% (based on UV response);
(iii) indication of all impurities with estimated concentration above 0.04% (based on UV response).

The analyte is also always analyzed with nuclear magnetic resonance and high-resolution mass spectrometry, and all measurements are confronted before to conclude on the API purity.

At this early stage, the impurities are essentially unknown, thus obviously not available in pure powders for accurate quantification. The rough estimation of proportions based on UV detection is of course not accurate, judging that API and impurities have different absorbance of UV light. However, it is considered sufficient at this stage to decide on the necessity of a purification step through preparative chromatography.

To assess the capability of our four methods for the requested task described above, we used a subset of 25 compounds selected from the larger set of 140 compounds. They were selected so as to reflect the diversity of samples to be processed everyday at the laboratory: they included some compounds with high purity (above 95%) and others with lower purity and a large number of impurities. Each of them was analyzed individually (instead of mixtures for the larger set) in the four chromatographic systems.

Different pieces of information were explored at this stage: the estimated purity of API, the number and relative concentrations of detected impurities (identified with their molecular mass when estimated concentration was above 1%), and the comparison of

elution orders and retention patterns. A summary of results can be found in Supplementary information (Table S1).

First of all, the quantification method for API purity was questioned. The usual procedure is to integrate all peaks at 210 nm. We considered employing other wavelengths but it proved inefficient as baseline noise was higher. Peak purity of the API was assessed with the overlay of UV spectra measured at the front, middle and tail of the peak. No deviations could be observed, indicating that peak purity was good. Finally, triplicate analyses (with blank analyses in-between) showed excellent repeatability in the measurement of API purity. An example is provided in Table S2.

Secondly, estimated purity of the API was compared between the four methods. It appeared that the four methods agreed rather well in most instances. Considering that the method with highest performance should be able to separate and detect a maximum of impurities, the best method should also be the one providing the lowest estimated purity for the API. In this respect, we proceeded to rank the four methods. UHPLC method 1 appeared to be often the best according to this criterion, while the other three methods were found to be equivalent, on average.

Secondly, the number of impurities with estimated proportion > 1%, or comprised between 0.04 and 1% were counted. The best method should be the one providing the largest number of separated and detected impurities. Here again, UHPLC method 1 was often superior to other methods, while the other three methods provided comparable results, on average.

It is worth noting that any of the four methods could be the best one in particular cases, but UHPLC method 1 was most frequently the best. This may be related to the superior chromatographic performance described above: a smaller number of non-eluted analytes; best peak symmetry; highest peak capacity. However, UV sensitivity may not be involved.

Indeed, similar values for background noise were observed between the two UHPSFC-UV methods and UHPLC-UV method 1 (about 0.1 mAU). It is worth noting that methanesulfonic acid had been chosen for UHPLC method 1 especially because it is transparent in UV and it was already pointed out above that ammonium hydroxide was preferred in UHPSFC method for the same reason. For UHPSFC-UV, some studies showed the lack of sensitivity specifically for the profiling and quantification of impurities present in very low concentrations [10,25]. However, in the present study, we worked with a reference wavelength compensation mode with the ACQUITY UPC^2 PDA detector, which permitted to increase the sensitivity [26]. This resulted in the possible UV estimation of impurities down to the required 0.04%, in both UHPLC and UHPSFC methods. Considering the results, we can conclude that sensitivities achieved in UV with UHPLC method 1 and both UHPSFC methods were very similar. UHPLC method 2 was inferior in this respect, as sodium bicarbonate is causing higher baseline noise.

Moreover, it appeared here that the mass detector could provide some mass information for impurities with an estimated proportion above 1%, both in UHPLC and UHPSFC. Again, MS information for lower concentrations was not required. This demonstrated the satisfying performance of a rather simple mass detector coupled either to UHPLC or to UHPSFC system, as the response did adequately meet the requirements.

To illustrate this performance evaluation, three case studies are presented, illustrating the three scenarios observed: (1) some cases where a UHPLC method yielded a lower estimated purity for the API than UHPSFC (most frequent case), (2) some cases where the opposite occurred (a lower estimated purity for the API obtained with a UHPSFC method) and (3) close percentages for API and impurities between UHPSFC and UHPLC methods.

The situation (1) often occurred when more impurities were found in UHPLC than in UHPSFC, probably because certain impurities did not elute in UHPSFC, or co-eluted with other impurities

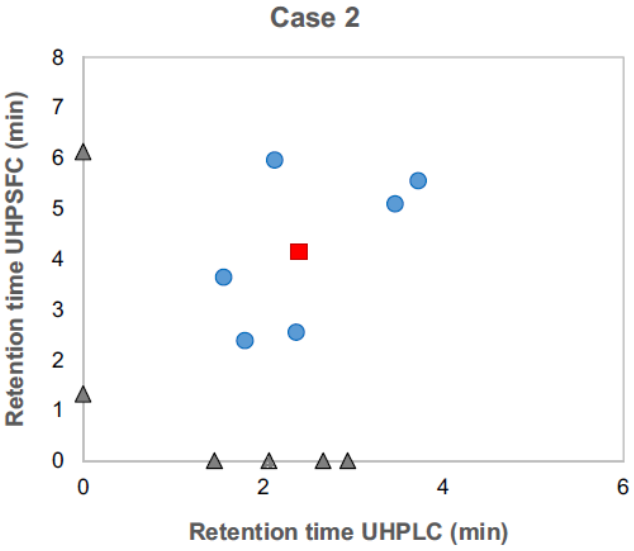


Fig. 4. Comparison of retention times obtained for the API (red square) and impurities (blue circles for impurities observed with both methods, grey triangles for impurities observed with only one method) in UHPLC method 1 (acidic conditions) and UHPSFC method 1 (HSS C18 SB column) in case study number 2 (data in Table 3, chromatograms in Fig. S2).

or with the API. Fig. S1 illustrates this case. In the example proposed, more impurities were found in UHPLC (Fig. S1a) than in UHPSFC (Fig. S1b). In Table 2, the API and all impurities with a concentration above 0.04% are listed for both methods. We can notice in Table 2 that several impurities found in UHPLC method 1 were undetected with the other three methods. In this example, the API was found with a purity of 81.0% with UHPLC method 1, 88.1% with UHPLC method 2, 89.7% with UHPSFC method 1 and 87.2% with UHPSFC method 2. This kind of situation where UHPLC method 1 provided the lowest estimate for API purity occurred 16 times in 25.

In case (2), the differences between UHPLC and UHPSFC profiling were not so strong. In this example, among the 4 impurities detected with concentrations above 1% with UHPSFC method 1, 1 remained undetected with UHPSFC method 2, while 3 impurities were undetected by UHPLC methods (Fig. S2 and Table 3). Judging from the estimated purity of the API between UHPLC and UHPSFC methods 1, respectively 95.0% and 92.1% (Table 3), some impurities probably coeluted with the API in UHPLC method 1. This kind of situations where UHPSFC method 1 provided lower API purity than UHPLC method 1 occurred 9 times in 25.

Finally, in case (3), all UHPLC and UHPSFC methods yielded highly similar results for the API and impurities percentages (Fig. S3 and Table 4). In these cases, a large number of identical impurities was observed with all methods (identical masses and close estimated concentration).

Moreover, in all cases we observed different elution orders and selectivities for the API and impurities between UHPLC and UHPSFC methods. This is better appreciated with Fig. 4, where the API and impurities detected in both methods 1 for the sample case 2 appear to be scattered in a non-linear fashion, as was expected based on the observations in Section 3.1.

4. Conclusions

To compare UHPLC and UHPSFC methods, different parameters were evaluated like orthogonality, peak shapes, peak capacity, and sensitivity. As expected, different selectivities are obtained in UHPLC with two different mobile phases (acidic and basic conditions), whereas they are achieved in UHPSFC with different stationary phases (C18 and sulfobetaine). It was shown that the

Table 3
Comparison of impurity profiling methods (Case 2, chromatograms in Fig. S2).

UHPSFC (method 1: NH_4OH)				
	RT (min)	%	MS ($[M + H]^+$) peak 1	MS ($[M + H]^+$) peak 2
API	4.15	92.09	344.2	
Impurities ≥ 1%				
IMP 1	6.13	0.97	177.2	
IMP 2	2.55	1.01	186.2	
IMP 3	3.65	3.03	267.3	
IMP 4	1.32	2.30	214.2	
undetected				
Impurities ≤ 1%				
IMP A	2.39	0.14		
IMP B	5.09	0.08		
IMP C	5.55	0.28		
IMP D	5.96	0.10		
UV s/n	2.44E + 04			
MS s/n	2.57E + 02			

UHPLC (method 1: MSA)				
	RT (min)	%	MS ($[M + H]^+$) peak 1	MS ($[M + H]^+$) peak 2
API	2.40	95.03	344.2	
Impurities ≥ 1%				
undetected				
undetected				
IMP 3	1.56	3.08	267.3	
undetected				
undetected				
Impurities ≤ 1%				
IMP A	1.46	0.06		
IMP B	1.80	0.13		
IMP C	2.06	0.05		
IMP D	2.12	0.11		
IMP E	2.36	0.40		
IMP F	2.67	0.05		
IMP G	2.94	0.06		
IMP H	3.46	0.37		
IMP I	3.72	0.66		
UV s/n	1.15E + 04			
MS s/n	7.33E + 01			

UHPSFC (method 2: NH_4OH)				
	RT (min)	%	MS ($[M + H]^+$) peak 1	MS ($[M + H]^+$) peak 2
API	1.57	93.21	344.2	
Impurities ≥ 1%				
IMP 1	6.64	1.16	177.2	
IMP 2	3.50	1.09	186.2	
IMP 3	1.84	3.52	267.3	
undetected				
undetected				
Impurities ≤ 1%				
IMP A	1.28	0.05		
IMP B	1.35	0.36		
IMP C	1.91	0.10		
IMP D	2.14	0.47		
IMP E	3.57	0.04		
UV s/n	2.72E + 04			
MS s/n	2.99E + 02			

UHPLC (method 2: BICAR)				
	RT (min)	%	MS ($[M + H]^+$) peak 1	MS ($[M + H]^+$) peak 2
API	6.77	93.68	344.3	
Impurities ≥ 1%				
IMP 1				
IMP 2	5.91	3.20	267.3	
undetected				
undetected				
IMP 5	2.65	1.68	228.0	
Impurities ≤ 1%				
IMP A	2.52	0.31		
IMP B	4.61	0.09		
IMP C	8.14	0.33		
IMP D	8.32	0.40		
IMP E	9.11	0.31		
UV s/n	2.33E + 03			
MS s/n	3.18E + 02			

Table 4
Comparison of impurity profiling methods (Case 3, chromatograms in Fig. S3)

UHPSFC (method 1: NH_4OH)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	4.31	98.51	413.3	
Impurities ≥ 1%				
IMP 1	2.81	1.37	397.3	
Impurities ≤ 1%				
IMP A	4.13	0.07		
IMP B	5.67	0.05		
UV s/n	3.92E + 04			
MS s/n	1.48E + 02			
UHPLC (method 1: MSA)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	3.68	98.70	413.3	
Impurities ≥ 1%				
IMP 1	6.02	1.26	397.2	
Impurities ≤ 1%				
IMP A	1.66	0.04		
UV s/n	1.37E + 04			
MS s/n	6.13E + 01			
UHPSFC (method 2: NH_4OH)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	6.978	98.81	413.3	
Impurities ≥ 1%				
IMP 1	6.637	1.15	397.3	
Impurities ≤ 1%				
IMP A	7.368	0.04		
UV s/n	2.95E + 04			
MS s/n	1.62E + 02			
UHPLC (method 2: BICAR)				
	RT (min)	%	MS ($[M+H]^+$) peak 1	MS ($[M+H]^+$) peak 2
API	3.680	98.77	413.4	
Impurities ≥ 1%				
IMP 1	7.12	1.23	x	
Impurities ≤ 1%				
UV s/n	2.53E + 03			
MS s/n	1.71E + 02			

preferred UHPLC method yielded somewhat superior chromatographic quality to the other three methods, which were rather comparable in these terms. However, UHPLC and UHPSFC were found most orthogonal.

In most pharmaceutical laboratories, two UHPLC methods are preferably used. However, the above study indicates that UHPLC and UHPSFC methods combined should be a better combination, as the strongly different selectivities maximize the chances of seeing all impurities. Furthermore, the use of UHPSFC method might present a significant advantage when a purification step was necessary thereafter, or when the studied compounds were sensitive to water.

Acknowledgment

Waters Corporation is warmly acknowledged for continuous support through the Centers of Innovation program. We also thank Régis Guyon (Machery-Nagel) for the kind gift of columns.

AppendixA. Supplementarydata

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.10. 045.

References

[1] E. Abbott, T.D. Veenstra, H.J. Issaq, Clinical and pharmaceutical applications of packed-column supercritical fluid chromatography, J. Sep. Sci. 31 (2008) 1223–1230, http://dx.doi.org/10.1002/jssc.200700579.
[2] E. Lemasson, S. Bertin, C. West, Use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification: other techniques, J. Sep. Sci. 39 (2016) 212–233, http://dx.doi.org/10.1002/jssc.201501062.
[3] M.B. Hicks, E.L. Regalado, F. Tan, X. Gong, C.J. Welch, Supercritical fluid chromatography for GMP analysis in support of pharmaceutical development and manufacturing activities, J. Pharm. Biomed. Anal. 117 (2016) 316–324, http://dx.doi.org/10.1016/j.jpba.2015.09.014.
[4] C. Galea, D. Mangelings, Y.V. Heyden, Method development for impurity profiling in SFC: The selection of a dissimilar set of stationary phases, J. Pharm. Biomed. Anal. 111 (2015) 333–343, http://dx.doi.org/10.1016/j.jpba.2014.12.043.

[5] E. Lesellier, C. West, The many faces of packed column supercritical fluid chromatography – a critical review, J. Chromatogr. A. 1382 (2015) 2–46, http://dx.doi.org/10.1016/j.chroma.2014.12.083.
[6] L. Nováková, A. Grand-Guillaume Perrenoud, I. Francois, C. West, E. Lesellier, D. Guillarme, Modern analytical supercritical fluid chromatography using columns packed with sub-2 μm particles: a tutorial, Anal. Chim. Acta. 824 (2014) 18–35, http://dx.doi.org/10.1016/j.aca.2014.03.034.
[7] G. Cudiamat, Market profile: supercritical fluid chromatography (SFC), Column. 9 (2013) 8–9.
[8] C. West, E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, An improved classification of stationary phases for ultra-high performance supercritical fluid chromatography, J. Chromatogr. A. 2016 (1440) 212–228, http://dx.doi.org/10.1016/j.chroma.2016.02.052.
[9] J. Xu, R. Thompson, B. Li, Z. Ge, Application of packed column supercritical fluid chromatography for separation of bromosulfone from process related impurities, J. Liq. Chromatogr. Amp Relat. Technol. 25 (2002) 1007–1018, http://dx.doi.org/10.1081/JLC-120003420.
[10] Z. Wang, H. Zhang, O. Liu, B. Donovan, Development of an orthogonal method for mometasone furoate impurity analysis using supercritical fluid chromatography, J. Chromatogr. A. 1218 (2011) 2311–2319, http://dx.doi.org/ 10.1016/j.chroma.2011.02.027.
[11] A.J. Alexander, L. Zhang, T.F. Hooker, F.P. Tomasella, Comparison of supercritical fluid chromatography and reverse phase liquid chromatography for the impurity profiling of the antiretroviral drugs lamivudine/BMS-986001/efavirenz in a combination tablet, J. Pharm. Biomed. Anal. 78-79 (2013) 243–251, http://dx.doi.org/10.1016/j.jpba.2013.02.019.
[12] J.D. Pinkston, D. Wen, K.L. Morand, D.A. Tirey, D.T. Stanton, Comparison of LC/MS and SFC/MS for screening of a large and diverse library of pharmaceutically relevant compounds, Anal. Chem. 78 (2006) 7467–7472, http://dx.doi.org/10.1021/ac061033l.
[13] Y. Hsieh, L. Favreau, J. Schwerdt, K.-C. Cheng, Supercritical fluid chromatography/tandem mass spectrometric method for analysis of pharmaceutical compounds in metabolic stability samples, J. Pharm. Biomed. Anal. 40 (2006) 799–804, http://dx.doi.org/10.1016/j.jpba.2005.06.032.
[14] M.C. Ventura, W.P. Farrell, C.M. Aurigemma, M.J. Greig, Packed column supercritical fluid Chromatography/Mass spectrometry for high-Throughput analysis, Anal. Chem. 71 (1999) 2410–2416, http://dx.doi.org/10.1021/ac981372h.
[15] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Coupling state-of-the-art supercritical fluid chromatography and mass spectrometry: from hyphenation interface optimization to high-sensitivity analysis of pharmaceutical compounds, J. Chromatogr. A. 1339 (2014) 174–184, http:// dx.doi.org/10.1016/j.chroma.2014.03.006.
[16] L. Nováková, M. Rentsch, A. Grand-Guillaume Perrenoud, R. Nicoli, M. Saugy, J. Veuthey, D. Guillarme, Ultra high performance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents. II: Analysis of biological samples, Anal. Chim. Acta. 853 (2015) 647–659, http://dx.doi.org/10.1016/j.aca.2014.10.007.
[17] D. Spaggiari, F. Mehl, V. Desfontaine, A. Grand-Guillaume Perrenoud, S. Fekete, S. Rudaz, D. Guillarme, Comparison of liquid chromatography and supercritical fluid chromatography coupled to compact single quadrupole mass spectrometer for targeted in vitro metabolism assay, J. Chromatogr. A. 1371 (2014) 244–256, http://dx.doi.org/10.1016/j.chroma.2014.10.055.
[18] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part I: optimization of mobile phase composition, J. Chromatogr. A. 2015 (1408) 217–226, http://dx.doi.org/10.1016/j.chroma.2015.07.037.
[19] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part II. Selection of an orthogonal set of stationary phases, J. Chromatogr. A. 1408 (2015) 227–235, http://dx.doi.org/ 10.1016/j.chroma.2015.07.035.
[20] J. Pellett, P. Lukulay, Y. Mao, W. Bowen, R. Reed, M. Ma, R.C. Munger, J.W. Dolan, L. Wrisley, K. Medwid, N.P. Toltl, C.C. Chan, M. Skibic, K. Biswas, K.A. Wells, L.R. Snyder, Orthogonal separations for reversed-phase liquid chromatography, J. Chromatogr. A. 1101 (2006) 122–135, http://dx.doi.org/10.1016/j.chroma.2005.09.080.
[21] U.D. Neue, J.E. O'Gara, A. Méndez, Selectivity in reversed-phase separations, J. Chromatogr. A. 1127 (2006) 161–174, http://dx.doi.org/10.1016/j.chroma.2006.06.006.
[22] A. D'Attoma, C. Grivel, S. Heinisch, On-line comprehensive two-dimensional separations of charged compounds using reversed-phase high performance liquid chromatography and hydrophilic interaction chromatography. Part I: Orthogonality and practical peak capacity considerations, J. Chromatogr. A. 1262 (2012) 148–159, http://dx.doi.org/10.1016/j.chroma.2012.09.028.
[23] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part I: Salt gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 33–44, http://dx.doi.org/10.1016/j.jpba.2014.08.035.
[24] J.W. Dolan, Dwell volume revisited, LCGC N. Am. 24 (2006).
[25] A. Dispas, P. Lebrun, E. Ziemons, R. Marini, E. Rozet, P. Hubert, Evaluation of the quantitative performances of supercritical fluid chromatography: from method development to validation, J. Chromatogr. A. (2014), http://dx.doi.org/10.1016/j.chroma.2014.01.046.
[26] L. Subbarao, J. Cole, R. Chen, Enhancement of UV detection sensitivity in SFC using reference wavelength compensation, LC-GC N Am. 5 (2009)

VIII. Conclusion

Dans ce chapitre, le développement de méthodes SFC pour le profilage d'impuretés de candidats médicaments a été présenté. L'optimisation de la composition de la phase mobile ainsi qu'une sélection adaptée de phases stationnaires ont permis le développement de deux méthodes SFC complémentaires en gradient. Ces méthodes sont obtenues dans des conditions analytiques identiques mais avec deux phases stationnaires différentes : la colonne ACQUITY HSS C18 SB (une phase C18 non-endcapped) et la colonne Nucleoshell HILIC (greffée avec des groupements sulfobétaine).

La robustesse des méthodes gradient a été établie avec un jeu de colonnes différent. Les méthodes développées sont robustes d'une colonne à une autre, mais également d'une dimension de colonnes à une autre.

Des méthodes en gradient focus et isocratique ont également été développées sur ces mêmes phases stationnaires, afin de disposer d'un ensemble complet de méthodes analytiques pour l'analyse de candidats médicaments, et se rapprocher des pratiques utilisées en HPLC à l'Institut de Recherches Servier où une méthode gradient générique et isocratique sont souvent associées pour s'assurer que le profilage d'impuretés est complet. En définitive, l'utilisation de la méthode gradient générique en SFC (criblage de 5 à 50 % de co-solvant) fournit à la fois la pureté du PA la plus basse et le nombre d'impuretés le plus haut dans une majorité de cas. Les méthodes de seconde intention (gradient focus et isocratique) n'apportent que peu d'informations supplémentaires et leur utilisation en complément du gradient n'est donc pas nécessaire en SFC. S'il faut utiliser une seconde méthode, une méthode orthogonale (colonne HILIC) ou un couplage de colonnes semble plus indiqué.

On s'est ensuite intéressé au couplage en série des phases stationnaires C18 et HILIC. Il est apparu que la combinaison des colonnes C18-HILIC permet d'augmenter la capacité de pics par rapport à l'utilisation d'une seule colonne (C18) et est donc bénéfique pour le profilage d'impuretés. Cependant, on observe également que deux gradients successifs, réalisés sur la colonne C18 seule puis HILIC seule, sont plus informatifs (nombre d'impuretés plus haut et pureté relative du PA plus faible) qu'une expérience unique avec les colonnes couplées.

Enfin, on compare les performances des méthodes HPLC utilisées comme méthodes de première intention chez Servier et des méthodes SFC développées, ainsi que leur capacité à séparer des impuretés des PA. Des sélectivités différentes sont

obtenues en HPLC avec l'emploi de conditions acides ou basiques sur une phase stationnaire unique, alors qu'en SFC elles sont obtenues dans des conditions de travail identiques mais avec deux phases stationnaires différentes. La méthode HPLC de première intention, en conditions acides sur phase C18, fournit des performances chromatographiques supérieures aux autres. Cependant, l'utilisation combinée d'une méthode HPLC et SFC est plus performante que deux méthodes HPLC pour maximiser les chances d'identifier l'ensemble des impuretés.

Stratégie d'analyse du candidat médicament : utilisation des méthodes HPLC et SFC Conclusions générales et perspectives

Conclusions

Au commencement de ce travail, plusieurs méthodes en chromatographie en phase liquide étaient déjà en application à l'Institut de Recherche Servier pour réaliser le profilage d'impuretés. La méthode de première intention était une méthode RPLC sur phase C18 en conditions acides (présence d'AMS à 0.1% dans la phase mobile). En effet, les excellentes performances chromatographiques obtenues en font une méthode de choix (article Chapitre 2, V et article Chapitre 3, VII). Elle permet entre autres l'élution de 99% des composés étudiés avec une bonne symétrie de pics dans 74% des cas. Néanmoins, cette méthode échoue parfois, soit car la rétention du PA n'est pas suffisante (C_e<20%, ce qui représente 22% des cas étudiés) ou parce que le résultat chromatographique est insatisfaisant (dégradation du composé, coélution entre le PA et des impuretés). Deux autres méthodes en RPLC étaient donc employées et avaient été développées pour offrir des solutions complémentaires à la première. Une première méthode employait la même phase stationnaire C18 mais avec des conditions d'élution basiques alors qu'une seconde méthode employait une phase stationnaire pentafluorophényle en conditions acides.

L'objectif principal de cette thèse était d'évaluer les performances et la pertinence de ces méthodes, et de proposer d'autres alternatives chromatographiques, notamment en phase supercritique. Pour cela, nous nous sommes appuyés sur un ensemble conséquent (140) et divers de composés issus de la recherche Servier afin d'offrir une évaluation et des comparaisons pertinentes des méthodes.

Dans le Chapitre 2, nous nous sommes intéressés aux trois méthodes HPLC déjà employées chez Servier, et nous avons développé deux nouvelles méthodes en phase liquide en mode mixte (phase stationnaire bimodale RPLC-WCX et phase stationnaire trimodale RPLC-SCX-WAX). Les performances des cinq méthodes disponibles ont été comparées et nous ont permis de conclure notamment que (i) la méthode de référence était statistiquement la plus performante mais (ii) les autres méthodes pouvaient apporter des solutions ponctuelles pour les composés peu retenus.

Dans le Chapitre 3, nous avons développé des méthodes en phase supercritique. Après avoir évalué douze compositions de phase mobile différente sur un large panel de phases stationnaires, une composition optimale de la phase mobile a été déterminée : les meilleures performances ont été obtenues avec un gradient générique de 5 à 50% de co-solvant (MeOH + 20 mM d'ammoniaque et 2% d'eau). Puis un large criblage de colonnes (23) a permis d'identifier 2 colonnes achirales complémentaires adaptées à l'analyse des

candidats médicaments : les colonnes HSS C18 SB et Nucleoshell HILIC. La robustesse des méthodes développées a été démontrée sur un jeu de colonnes différent. Le couplage des deux colonnes en série a permis d'augmenter le nombre d'impuretés détectées, grâce à l'augmentation de la capacité de pics. Cependant, il a également été établi que deux analyses consécutives, réalisées sur la phase C18 seule et HILIC seule, restent plus informatives pour le profilage d'impuretés. Pour terminer, les performances des méthodes SFC et des méthodes RPLC sur phase C18 ont été comparées. Cette comparaison a confirmé la supériorité de la méthode RPLC de référence (C18 en milieu acide) mais a démontré la pertinence des méthodes SFC en termes d'orthogonalité.

La méthode RPLC C18 en conditions acides fournissant des performances chromatographiques supérieures à toutes les autres méthodes évaluées, elle sera donc toujours utilisée en premier lieu. Cependant, si la rétention du PA est insuffisante (C_e<20%), différentes options pourront être envisagées selon la nature du composé étudié pour augmenter sa rétention (Figure 4.11 ci-dessous). Pour les composés acides et les neutres, les méthodes RPLC PFP (en conditions acides, 0.1% de TFA dans la phase mobile ACN et H_2O) et SFC (avec 2% H_2O et 20 mM d'hydroxyde d'ammonium dans le co-solvant méthanol) permettent une augmentation de la rétention par rapport à la méthode de référence (Figure 4.11). Pour la méthode RPLC PFP, l'analyse en gradient sera, comme pour la méthode de référence, suivie d'une analyse isocratique pour détecter des co-élutions. Pour la partie SFC, les méthodes complémentaires développées (gradient focus et isocratique) n'apportent que peu d'informations supplémentaires par rapport au gradient générique (5-50% de co-solvant). La stratégie d'analyse proposée en SFC sera donc plutôt la suivante : (i) réaliser une analyse unique en couplant les colonnes HSS C18 SB et Nucleoshell HILIC en série ou (ii) réaliser deux analyses successives en gradient avec la colonne HSS C18 SB d'abord puis la colonne Nucleoshell HILIC. Ces deux alternatives en SFC apportent davantage d'informations sur le composé analysé (sur le nombre d'impuretés détectées et la pureté relative du PA) qu'une analyse en gradient générique suivie d'un gradient focus ou une élution isocratique.

Enfin, pour les composés basiques, trois méthodes peuvent être envisagées pour augmenter leur rétention (Figure 4.11) : la SFC (dans les mêmes conditions que pour les composés acides et neutres), la RPLC sur C18 en conditions basiques (présence de 20 mM de bicarbonate d'ammonium dans la phase mobile) et l'HPLC mixed-mode sur phase bimodale (en conditions neutres, phase aqueuse contenant 60 mM d'acétate d'ammonium à pH 7). Parmi ces méthodes, on notera la meilleure orthogonalité obtenue par rapport à la méthode de référence avec les méthodes SFC C18 et HPLC MM bimodale.

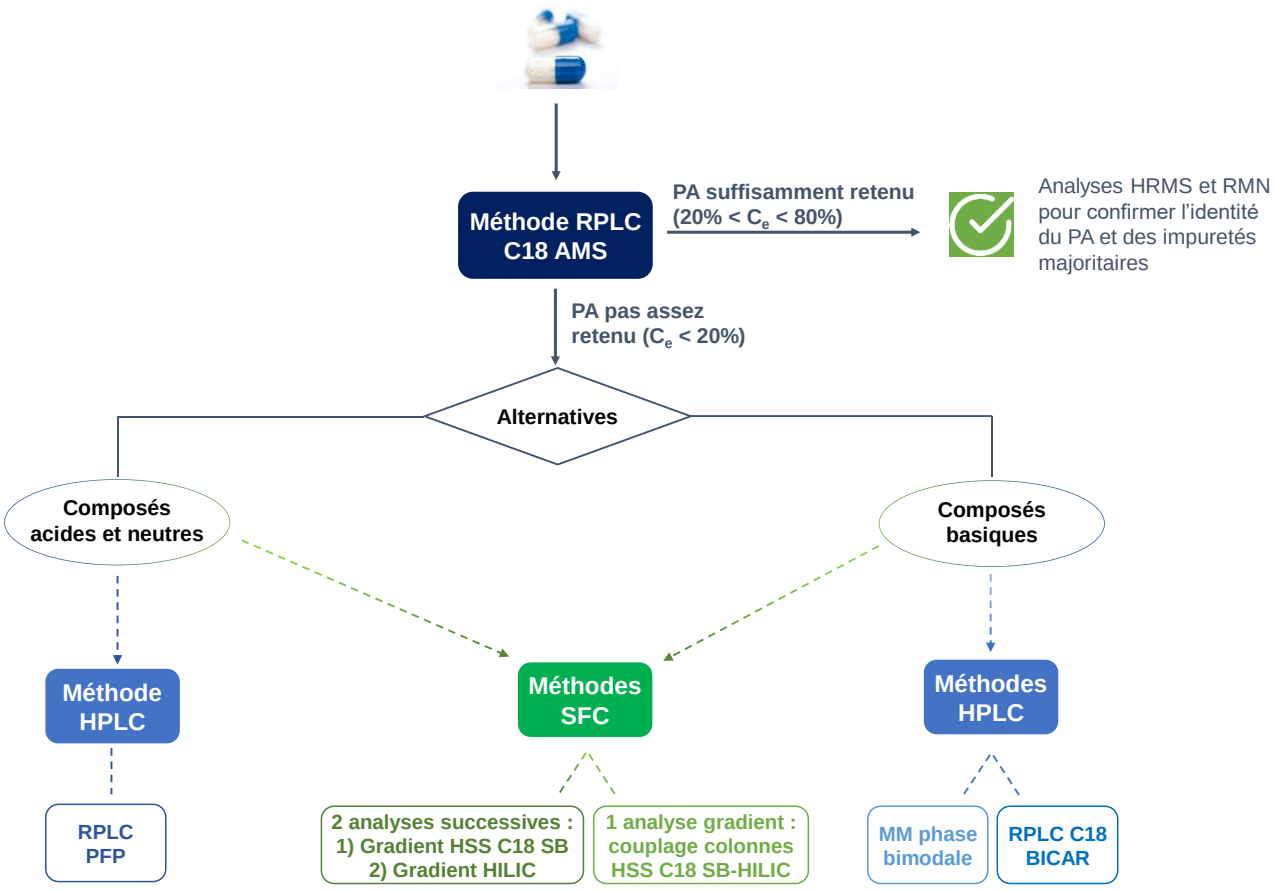


Figure 4.11 - Schéma décisionnel pour l'analyse du PA

De façon générale et pour sortir du cadre strict du laboratoire Servier pour lequel cette étude a été conduite, il apparait à travers nos résultats que la SFC peut être utilisée comme méthode alternative à la RPLC sur phase C18 pour tous types de composés (acides, bases, neutres) n'étant pas suffisamment retenus avec la méthode de référence. Bien que la SFC soit de plus en plus implantée dans les laboratoires pharmaceutiques, l'UHPLC reste néanmoins la méthode dominante. Lorsqu'un laboratoire n'est pas équipé de SFC, une autre méthode HPLC peut être utilisée comme alternative (RPLC PFP ou HPLC mixed-mode selon la nature du composé). Si le laboratoire d'analyse est équipé d'un système SFC, les méthodes SFC devraient être implantées en complément de la méthode RPLC de référence car ce sont elles qui offrent la meilleure complémentarité pour tous types de composés. Cette technique présente de plus un grand intérêt lorsque les composés étudiés sont sensibles à l'eau et instables ou lorsqu'un transfert à l'échelle préparative est envisagé par la suite.

Perspectives

Les travaux présentés dans cette thèse portent sur l'évaluation de la pureté chimique des candidats médicaments, donc concernent tous l'analyse achirale à l'échelle analytique. Cependant, la détermination de la pureté énantiomérique à l'échelle analytique et la purification des principes actifs (chirale ou achirale) grâce à la chromatographie préparative représentent également une part importante du travail des chromatographistes des laboratoires pharmaceutiques. Le temps consacré à l'étude de chaque échantillon augmente lorsque plusieurs analyses sont nécessaires pour déterminer la pureté chimique et énantiomérique. A l'échelle analytique, l'analyse simultanée de la pureté chimique (profilage d'impuretés) et énantiomérique sur une seule colonne et une seule analyse permet de diminuer le temps consacré à chaque composé. A l'échelle préparative, la purification en un seul run serait également intéressante en termes de productivité.

La séparation simultanée de composés chiraux et achiraux a été effectuée en chromatographique bidimensionnelle (2D) : Venkatramani *et al.* [156] ont développé une méthode 2D RPLC/SFC pour l'analyse d'un PA possédant 3 centres chiraux et 4 paires de diastéréoisomères. La première dimension achirale en RPLC-UV permettait de séparer les diastéréoisomères et autres impuretés du PA (pureté chimique). Chaque paire de diastéréoisomères était ensuite transférée dans la seconde dimension en SFC-MS sur une colonne chirale afin de déterminer la pureté énantiomérique. Zeng *et al.* [157] ont également développé une méthode 2D SFC/SFC/MS pour l'analyse de composés pharmaceutiques. La première dimension sur colonne achirale permettait d'isoler le racémique des autres composés (mesure de la pureté chimique). La fraction contenant le racémique était alors envoyée sur la seconde dimension sur colonne chirale afin de réaliser la mesure de pureté énantiomérique.

Dans notre cas, l'objectif recherché serait de n'utiliser qu'une seule colonne chirale pour réaliser une analyse de la pureté chimique et énantiomérique en une seule expérience. Cette méthode serait aussi plus facile à transférer à l'échelle préparative qu'une analyse bidimensionnelle. Nous avons réalisé des analyses préliminaires en SFC sur le jeu de composés achiraux afin de caractériser la sélectivité achirale des colonnes chirales par rapport aux colonnes achirales précédemment sélectionnées (HSS C18 SB et Nucleoshell HILIC). Les 140 composés ont été criblés sur 10 colonnes chirales (colonnes Chiralpak® IA, IB, IC, ID, IE, IF, IG, Whelk O®-1, Chiralpak® AD et Chirobiotic® TAG), en utilisant le même gradient générique que précédemment (de 5 à 50% de co-solvant, MeOH + 20 mM d'ammoniaque + 2% H_2O). Les fonctions de Derringer ont été utilisées

pour classer les colonnes selon 4 critères : (i) le nombre de composés élués, (ii) le nombre de pics symétriques, (iii) la valeur de la C_e moyenne (idéalement située entre 10 et 25% MeOH afin d'augmenter la solubilité de l'analyte et la productivité à l'échelle préparative), (iv) la distribution des composés (idéalement homogène entre 5 et 50%). A l'issue de ce criblage, les 6 colonnes les mieux classées avec les fonctions de Derringer ont été conservées pour la suite de l'étude (Chiralpak® IA, IB, IC, ID, Whelk O®-1, Chiralpak® AD). Un ensemble de 42 composés chiraux issus de la Recherche Servier ont par la suite été analysés sur les 6 colonnes, le traitement des résultats est en cours et va se poursuivre avec une ingénieure d'études à l'ICOA, recrutée pour une période d'un an afin de poursuivre la collaboration avec l'Institut de Recherches Servier. La transposition à l'échelle préparative sera également un point clé pour déterminer la portée de cette expérience.

Différents points d'intérêt n'ont pas pu être traités au cours de la thèse. Parmi eux, l'analyse des diastéréoisomères en SFC intéresse particulièrement le laboratoire Servier, car il arrive souvent que ces composés ne soient pas correctement séparés à l'aide des méthodes RPLC sur phase C18. Or, la SFC est généralement une technique performante dans la séparation des isomères. Un autre axe de travail concerne l'analyse en SFC des composés sensibles à l'eau ou aux alcools. Nous nous étions également intéressés au développement de méthode selon les principes du Quality by Design. Le travail amorcé pendant la thèse a permis de répondre à différentes problématiques, d'autres restent encore à explorer, peut être au travers d'un autre sujet de thèse.

Références bibliographiques

[1] International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. Impurities in new drug substances Q3A(R2), 2006, (n.d.). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3A_R2/Step4/Q3A_R2__Guideline.pdf (accessed October 13, 2017).

[2] R. Holm, D.P. Elder, Analytical advances in pharmaceutical impurity profiling, Eur. J. Pharm. Sci. 87 (2016) 118–135. doi:10.1016/j.ejps.2015.12.007.

[3] S. Fekete, I. Molnár, D. Guillarme, Separation of antibody drug conjugate species by RPLC: A generic method development approach, J. Pharm. Biomed. Anal. 137 (2017) 60–69. doi:10.1016/j.jpba.2017.01.013.

[4] R. Nageswara Rao, V. Nagaraju, An overview of the recent trends in development of HPLC methods for determination of impurities in drugs, J. Pharm. Biomed. Anal. 33 (2003) 335–377. doi:10.1016/S0731-7085(03)00293-0.

[5] D. Guillarme, J.-L. Veuthey, Alternative Strategies to Reversed-Phase Liquid Chromatography for the Analysis of Pharmaceutical Compounds, Am. Pharm. Rev. (2017).

[6] M.R. Euerby, A.P. McKeown, P. Petersson, Chromatographic classification and comparison of commercially available perfluorinated stationary phases for reversed-phase liquid chromatography using Principal Component Analysis, J. Sep. Sci. 26 (2003) 295–306. doi:10.1002/jssc.200390035.

[7] P. Cummins, K. Rochfort, B. O'Connor, Ion-Exchange Chromatography: Basic Principles and Application, in: D. Walls, S.T. Loughran (Eds.), Protein Chromatogr., Springer New York, 2017: pp. 209–223. doi:10.1007/978-1-4939-6412-3_11.

[8] A.J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds, J. Chromatogr. A. 499 (1990) 177–196. doi:10.1016/S0021-9673(00)96972-3.

[9] A. Periat, A. Grand-Guillaume Perrenoud, D. Guillarme, Evaluation of various chromatographic approaches for the retention of hydrophilic compounds and MS compatibility, J. Sep. Sci. 36 (2013) 3141–3151. doi:10.1002/jssc.201300567.

[10] M.A. Strege, S. Stevenson, S.M. Lawrence, Mixed-mode anion-cation exchange/hydrophilic interaction liquid chromatography-electrospray mass spectrometry as an alternative to reversed phase for small molecule drug discovery, Anal. Chem. 72 (2000) 4629–4633.

[11] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part I: Optimization of mobile phase composition, J. Chromatogr. A. 1408 (2015) 217–226. doi:10.1016/j.chroma.2015.07.037.

[12] E. Lemasson, S. Bertin, P. Hennig, H. Boiteux, E. Lesellier, C. West, Development of an achiral supercritical fluid chromatography method with ultraviolet absorbance and mass spectrometric detection for impurity profiling of drug candidates. Part II. Selection of an orthogonal set of stationary phases, J. Chromatogr. A. 1408 (2015) 227–235. doi:10.1016/j.chroma.2015.07.035.

[13] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Comparison of ultra-high performance supercritical fluid chromatography and ultra-high performance liquid chromatography for the analysis of pharmaceutical compounds, J. Chromatogr. A. 1266 (2012) 158–167. doi:10.1016/j.chroma.2012.10.005.

[14] E. Lesellier, C. West, The many faces of packed column supercritical fluid chromatography - A critical review, J. Chromatogr. A. 1382 (2015) 2–46. doi:10.1016/j.chroma.2014.12.083.

[15] A. Periat, J. Boccard, J.-L. Veuthey, S. Rudaz, D. Guillarme, Systematic comparison of sensitivity between hydrophilic interaction liquid chromatography and reversed phase liquid chromatography coupled with mass spectrometry, J. Chromatogr. A. 1312 (2013) 49–57. doi:10.1016/j.chroma.2013.08.097.
[16] B. Buszewski, S. Noga, Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique, Anal. Bioanal. Chem. 402 (2012) 231–247. doi:10.1007/s00216-011-5308-5.
[17] B. Bolaños, M. Greig, M. Ventura, W. Farrell, C.M. Aurigemma, H. Li, T.L. Quenzer, K. Tivel, J.M.R. Bylund, P. Tran, C. Pham, D. Phillipson, SFC/MS in drug discovery at Pfizer, La Jolla, Int. J. Mass Spectrom. 238 (2004) 85–97. doi:10.1016/j.ijms.2003.11.021.
[18] C. Brunelli, Y. Zhao, M.-H. Brown, P. Sandra, Pharmaceutical analysis by supercritical fluid chromatography: Optimization of the mobile phase composition on a 2-ethylpyridine column, J. Sep. Sci. 31 (2008) 1299–1306. doi:10.1002/jssc.200700555.
[19] E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, C. West, Comparison of ultra-high performance methods in liquid and supercritical fluid chromatography coupled to electrospray ionization – mass spectrometry for impurity profiling of drug candidates, J. Chromatogr. A. 1472 (2016) 117–128. doi:10.1016/j.chroma.2016.10.045.
[20] A. Grand-Guillaume Perrenoud, J. Boccard, J.-L. Veuthey, D. Guillarme, Analysis of basic compounds by supercritical fluid chromatography: Attempts to improve peak shape and maintain mass spectrometry compatibility, J. Chromatogr. A. 1262 (2012) 205–213. doi:10.1016/j.chroma.2012.08.091.
[21] K. Zhang, X. Liu, Reprint of “Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications,” J. Pharm. Biomed. Anal. 130 (2016) 19–34. doi:10.1016/j.jpba.2016.09.013.
[22] H. Qiu, A.K. Mallik, M. Takafuji, S. Jiang, H. Ihara, New poly(ionic liquid)-grafted silica multi-mode stationary phase for anion-exchange/reversed-phase/hydrophilic interaction liquid chromatography, Analyst. 137 (2012) 2553–2555. doi:10.1039/C2AN35348B.
[23] J.R. Mazzeo, U. D.Neue, M. Kele, R.S. Plumb, Advancing LC Performance with Smaller Particles and Higher Pressure, Anal. Chem. 77 (2005) 460 A-467 A. doi:10.1021/ac053516f.
[24] J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Ultrahigh-Pressure Reversed-Phase Liquid Chromatography in Packed Capillary Columns, Anal. Chem. 69 (1997) 983–989. doi:10.1021/ac961094r.
[25] Ultra Performance LC™ Separation Science Redefined - Supplement to LCGC North America, (2005). http://www.waters.com/webassets/cms/library/docs/720001136en.pdf (accessed October 17, 2017).
[26] S. Fekete, I. Kohler, S. Rudaz, D. Guillarme, Importance of instrumentation for fast liquid chromatography in pharmaceutical analysis, J. Pharm. Biomed. Anal. 87 (2014) 105–119. doi:10.1016/j.jpba.2013.03.012.
[27] M.E. LaCourse, W.R. LaCourse, Chapter 17 - General instrumentation in HPLC*, in: S. Fanali, P.R. Haddad, C.F. Poole, M.-L. Riekkola (Eds.), Liq. Chromatogr. Second Ed., Elsevier, 2017: pp. 417–429. doi:10.1016/B978-0-12-805393-5.00017-8.
[28] S. Fekete, J. Schappler, J.-L. Veuthey, D. Guillarme, Current and future trends in UHPLC, TrAC Trends Anal. Chem. 63 (2014) 2–13. doi:10.1016/j.trac.2014.08.007.
[29] D.T.-T. Nguyen, D. Guillarme, S. Rudaz, J.-L. Veuthey, Fast analysis in liquid chromatography using small particle size and high pressure, J. Sep. Sci. 29 (2006) 1836–1848. doi:10.1002/jssc.200600189.
[30] J.W. Dolan, Dwell Volume Revisited, LCGC N. Am. 24 (2006) 458–466.
[31] J.W. Dolan, Gradient Elution, Part IV: Dwell-Volume Problems, LC-GC N. Am. 31 (2013) 456–463.

[32] S. Fekete, J. Fekete, The impact of extra-column band broadening on the chromatographic efficiency of 5cm long narrow-bore very efficient columns, J. Chromatogr. A. 1218 (2011) 5286–5291. doi:10.1016/j.chroma.2011.06.045.
[33] F. Gritti, G. Guiochon, On the extra-column band-broadening contributions of modern, very high pressure liquid chromatographs using 2.1mm I.D. columns packed with sub-2µm particles, J. Chromatogr. A. 1217 (2010) 7677–7689. doi:10.1016/j.chroma.2010.10.016.
[34] J.H. Knox, M. Saleem, Kinetic Conditions for Optimum Speed and Resolution in Column Chromatography, J. Chromatogr. Sci. 7 (1969) 614–622. doi:10.1093/chromsci/7.10.614.
[35] C.J. Hudalla, B. Alden, T. Walter, D. Walsh, E. Bouvier, P.C. Iraneta, N. Lawrence, K. Wyndham, Synthesis and Applications of BEH Particles in Liquid Chromatography, LC-GC Eur. 30 (2012) 20–29.
[36] J.J. Destefano, T.J. Langlois, J.J. Kirkland, Characteristics of superficially-porous silica particles for fast HPLC: some performance comparisons with sub-2-microm particles, J. Chromatogr. Sci. 46 (2008) 254–260.
[37] S. Heinisch, A. D'Attoma, C. Grivel, Effect of pH additive and column temperature on kinetic performance of two different sub-2 µm stationary phases for ultrafast separation of charged analytes, J. Chromatogr. A. 1228 (2012) 135–147. doi:10.1016/j.chroma.2011.08.041.
[38] S. Fekete, D. Guillarme, Kinetic evaluation of new generation of column packed with 1.3µm core–shell particles, J. Chromatogr. A. 1308 (2013) 104–113. doi:10.1016/j.chroma.2013.08.008.
[39] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Octadecylsilylated porous silica rods as separation media for reversed-phase liquid chromatography, Anal. Chem. 68 (1996) 3498–3501. doi:10.1021/ac960281m.
[40] K. Cabrera, D. Lubda, H.-M. Eggenweiler, H. Minakuchi, K. Nakanishi, A New Monolithic-Type HPLC Column For Fast Separations, J. High Resolut. Chromatogr. 23 (2000) 93–99. doi:10.1002/(SICI)1521-4168(20000101)23:1<93::AID-JHRC93>3.0.CO;2-2.
[41] K. Cabrera, Applications of silica-based monolithic HPLC columns, J. Sep. Sci. 27 (2004) 843–852.
[42] N. Ishizuka, H. Kobayashi, H. Minakuchi, K. Nakanishi, K. Hirao, K. Hosoya, T. Ikegami, N. Tanaka, Monolithic silica columns for high-efficiency separations by high-performance liquid chromatography, J. Chromatogr. A. 960 (2002) 85–96.
[43] T. Bamba, E. Fukusaki, Y. Nakazawa, A. Kobayashi, Rapid and high-resolution analysis of geometric polyprenol homologues by connected octadecylsilylated monolithic silica columns in high-performance liquid chromatography, J. Sep. Sci. 27 (2004) 293–296. doi:10.1002/jssc.200301664.
[44] D. Guillarme, S. Rudaz, J.-L. Veuthey, Nouveaux développements en chromatographie rapide dans le domaine pharmaceutique, Spectra Anal. (2009) 12–17.
[45] A. Periat, B. Debrus, S. Rudaz, D. Guillarme, Screening of the most relevant parameters for method development in ultra-high performance hydrophilic interaction chromatography, J. Chromatogr. A. 1282 (2013) 72–83. doi:10.1016/j.chroma.2013.01.037.
[46] G. Guiochon, F. Gritti, Shell particles, trials, tribulations and triumphs, J. Chromatogr. A. 1218 (2011) 1915–1938. doi:10.1016/j.chroma.2011.01.080.
[47] S. Fekete, J. Fekete, Fast gradient screening of pharmaceuticals with 5 cm long, narrow bore reversed-phase columns packed with sub-3 µm core–shell and sub-2 µm totally porous particles, Talanta. 84 (2011) 416–423. doi:10.1016/j.talanta.2011.01.053.
[48] S. Fekete, D. Guillarme, M.W. Dong, Superficially Porous Particles: Perspectives, Practices, and Trends, LC-GC N. Am. 32 (2014) 420–433.

[49] Y. Zhang, X. Wang, P. Mukherjee, P. Petersson, Critical comparison of performances of superficially porous particles and sub-2μm particles under optimized ultra-high pressure conditions, J. Chromatogr. A. 1216 (2009) 4597–4605. doi:10.1016/j.chroma.2009.03.071.
[50] F. Gritti, G. Guiochon, Mass transfer mechanism in liquid chromatography columns packed with shell particles: Would there be an optimum shell structure?, J. Chromatogr. A. 1217 (2010) 8167–8180. doi:10.1016/j.chroma.2010.10.075.
[51] F. Gritti, G. Guiochon, Rapid development of core–shell column technology: Accurate measurements of the intrinsic column efficiency of narrow-bore columns packed with 4.6 down to 1.3 μm superficially porous particles, J. Chromatogr. A. 1333 (2014) 60–69. doi:10.1016/j.chroma.2014.01.061.
[52] S. Fekete, E. Olàh, J. Fekete, Fast liquid chromatography: The domination of core–shell and very fine particles, J. Chromatogr. A. 1228 (2012) 57–71. doi:10.1016/j.chroma.2011.09.050.
[53] A.C. Sanchez, G. Friedlander, S. Fekete, J. Anspach, D. Guillarme, Pushing the performance limits of reversed-phase ultra high performance liquid chromatography with 1.3 μm core–shell particles, J. Chromatogr. A. 1311 (2013) 90–97. doi:10.1016/j.chroma.2013.08.065.
[54] G. Vanhoenacker, P. Sandra, High temperature and temperature programmed HPLC: possibilities and limitations, Anal. Bioanal. Chem. 390 (2008) 245–248. doi:10.1007/s00216-007-1671-7.
[55] S. Heinisch, J.-L. Rocca, Sense and nonsense of high-temperature liquid chromatography, J. Chromatogr. A. 1216 (2009) 642–658. doi:10.1016/j.chroma.2008.11.079.
[56] Y.V. Kazakevich, R. LoBrutto, HPLC for Pharmaceutical Scientists, John Wiley & Sons, 2007.
[57] F. Gritti, G. Guiochon, Critical contribution of nonlinear chromatography to the understanding of retention mechanism in reversed-phase liquid chromatography, J. Chromatogr. A. 1099 (2005) 1–42. doi:10.1016/j.chroma.2005.09.082.
[58] E. Lesellier, A. Tchapla, A simple subcritical chromatographic test for an extended ODS high performance liquid chromatography column classification, J. Chromatogr. A. 1100 (2005) 45–59. doi:10.1016/j.chroma.2005.09.016.
[59] J. Nawrocki, The silanol group and its role in liquid chromatography, J. Chromatogr. A. 779 (1997) 29–71. doi:10.1016/S0021-9673(97)00479-2.
[60] F. Gritti, G. Guiochon, Effect of the endcapping of reversed-phase high-performance liquid chromatography adsorbents on the adsorption isotherm, J. Chromatogr. A. 1098 (2005) 82–94. doi:10.1016/j.chroma.2005.08.045.
[61] G.B. Cox, The influence of silica structure on reversed-phase retention, J. Chromatogr. A. 656 (1993) 353–367. doi:10.1016/0021-9673(93)80809-M.
[62] U.D. Neue, Y.-F. Cheng, Z. Lu, B.A. Alden, P.C. Iraneta, C.H. Khoebe, K.V. Tran, Properties of reversed phase packings with an embedded polar group, Chromatographia. 54 (2001) 169–177. doi:10.1007/BF02492239.
[63] J.E. O'Gara, B.A. Alden, T.H. Walter, J.S. Petersen, C.L. Niederlaender, U.D. Neue, Simple preparation of a C8 HPLC stationary phase with an internal polar functional group, Anal. Chem. 67 (1995) 3809–3813. doi:10.1021/ac00116a032.
[64] D.V. McCalley, Comparison of the performance of conventional C18 phases with others of alternative functionality for the analysis of basic compounds by reversed-phase high-performance liquid chromatography, J. Chromatogr. A. 1–2 (1999) 23–38. doi:10.1016/S0021-9673(99)00250-2.
[65] E. Lesellier, C. West, A. Tchapla, Classification of special octadecyl-bonded phases by the carotenoid test, J. Chromatogr. A. 1111 (2006) 62–70. doi:10.1016/j.chroma.2006.01.107.

[66] C.R. Silva, S. Bachmann, R.R. Schefer, K. Albert, I.C.S.F. Jardim, C. Airoldi, Preparation of a new C18 stationary phase containing embedded urea groups for use in high-performance liquid chromatography, J. Chromatogr. A. 948 (2002) 85–95.
[67] A. Abbood, C. Smadja, C. Herrenknecht, Y. Alahmad, A. Tchapla, M. Taverna, Retention mechanism of peptides on a stationary phase embedded with a quaternary ammonium group: A liquid chromatography study, J. Chromatogr. A. 1216 (2009) 3244–3251. doi:10.1016/j.chroma.2009.02.031.
[68] T. Taylor, Getting the Most from Phenyl Stationary Phases for HPLC, (n.d.). http://www.chromatographyonline.com/getting-most-phenyl-stationary-phases-hplc (accessed October 25, 2017).
[69] S. Bocian, B. Buszewski, Phenyl-bonded stationary phases—The influence of polar functional groups on retention and selectivity in reversed-phase liquid chromatography, J. Sep. Sci. 37 (2014) 3435–3442. doi:10.1002/jssc.201400764.
[70] M. Przybyciel, Fluorinated HPLC Phases — Looking Beyond C18 for Reversed-Phase HPLC, LC-GC Eur. 19 (2006) 19–27.
[71] C. West, E. Lemasson, S. Khater, E. Lesellier, An attempt to estimate ionic interactions with phenyl and pentafluorophenyl stationary phases in supercritical fluid chromatography, J. Chromatogr. A. 1412 (2015) 126–138. doi:10.1016/j.chroma.2015.08.009.
[72] M.R. Euerby, P. Petersson, W. Campbell, W. Roe, Chromatographic classification and comparison of commercially available reversed-phase liquid chromatographic columns containing phenyl moieties using principal component analysis, J. Chromatogr. A. 1154 (2007) 138–151. doi:10.1016/j.chroma.2007.03.119.
[73] D.J. Carrier, C. Eckers, J.-C. Wolff, “In-source” fragmentation of an isobaric impurity of lamotrigine for its measurement by liquid chromatography tandem mass spectrometry after pre-concentration using solid phase extraction, J. Pharm. Biomed. Anal. 47 (2008) 731–737. doi:10.1016/j.jpba.2008.03.002.
[74] K.M. Alsante, P. Boutros, M.A. Couturier, R.C. Friedmann, J.W. Harwood, G.J. Horan, A.J. Jensen, Q. Liu, L.L. Lohr, R. Morris, J.W. Raggon, G.L. Reid, D.P. Santafianos, T.R. Sharp, J.L. Tucker, G.E. Wilcox, Pharmaceutical impurity identification: a case study using a multidisciplinary approach, J. Pharm. Sci. 93 (2004) 2296–2309. doi:10.1002/jps.20120.
[75] E.F. Hewitt, P. Lukulay, S. Galushko, Implementation of a rapid and automated high performance liquid chromatography method development strategy for pharmaceutical drug candidates, J. Chromatogr. A. 1107 (2006) 79–87. doi:10.1016/j.chroma.2005.12.042.
[76] E.L. Regalado, W. Schafer, R. McClain, C.J. Welch, Chromatographic resolution of closely related species: Separation of warfarin and hydroxylated isomers, J. Chromatogr. A. 1314 (2013) 266–275. doi:10.1016/j.chroma.2013.07.092.
[77] Guidance for Industry Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research, (2008). file:///C:/Users/lemasson/AppData/Roaming/Zotero/Zotero/Profiles/3nhk6629.default/zotero/storage/C356QA6S/fda-2008-d-0629-gdl.pdf (accessed November 8, 2017).
[78] J. Wang, S. Yang, K. Zhang, A simple and sensitive method to analyze genotoxic impurity hydrazine in pharmaceutical materials, J. Pharm. Biomed. Anal. 126 (2016) 141–147. doi:10.1016/j.jpba.2016.04.038.
[79] K. Grigori, Y.L. Loukas, A. Malenović, V. Samara, A. Kalaskani, E. Dimovasili, M. Kalovidouri, Y. Dotsikas, Chemometrically assisted development and validation of LC–MS/MS method for the analysis of potential genotoxic impurities in meropenem active pharmaceutical ingredient, J. Pharm. Biomed. Anal. 145 (2017) 307–314. doi:10.1016/j.jpba.2017.06.061.

[80] C. Pan, F. Liu, M. Motto, Identification of Pharmaceutical Impurities in Formulated Dosage Forms, J. Pharm. Sci. 100 (2011) 1228–1259. doi:10.1002/jps.22376.
[81] R.P. Shah, S. Singh, Identification and characterization of a photolytic degradation product of telmisartan using LC-MS/TOF, LC-MSn, LC-NMR and on-line H/D exchange mass studies, J. Pharm. Biomed. Anal. 53 (2010) 755–761. doi:10.1016/j.jpba.2010.05.005.
[82] Qureshi, Smith, Delost, Njarðarson, 2016 Top 200 Pharmaceutical Prescription Sales Poster, (n.d.). http://njardarson.lab.arizona.edu/sites/njardarson.lab.arizona.edu/files/2016Top200PharmaceuticalPrescriptionSalesPosterLowResV2.pdf (accessed October 26, 2017).
[83] G. Walsh, Biopharmaceutical benchmarks 2014, Nat. Biotechnol. 32 (2014) nbt.3040. doi:10.1038/nbt.3040.
[84] A.S. Rathore, H. Winkle, Quality by design for biopharmaceuticals, Nat. Biotechnol. 27 (2009) nbt0109-26–26. doi:10.1038/nbt0109-26.
[85] S. Fekete, J.-L. Veuthey, D. Guillarme, New trends in reversed-phase liquid chromatographic separations of therapeutic peptides and proteins: Theory and applications, J. Pharm. Biomed. Anal. 69 (2012) 9–27. doi:10.1016/j.jpba.2012.03.024.
[86] K. Sandra, I. Vandenheede, P. Sandra, Modern chromatographic and mass spectrometric techniques for protein biopharmaceutical characterization, J. Chromatogr. A. 1335 (2014) 81–103. doi:10.1016/j.chroma.2013.11.057.
[87] T.M. Dillon, P.V. Bondarenko, M. Speed Ricci, Development of an analytical reversed-phase high-performance liquid chromatography–electrospray ionization mass spectrometry method for characterization of recombinant antibodies, J. Chromatogr. A. 1053 (2004) 299–305. doi:10.1016/j.chroma.2004.08.058.
[88] S. Fekete, S. Rudaz, J. Fekete, D. Guillarme, Analysis of recombinant monoclonal antibodies by RPLC: Toward a generic method development approach, J. Pharm. Biomed. Anal. 70 (2012) 158–168. doi:10.1016/j.jpba.2012.06.021.
[89] A. Goyon, A. Beck, O. Colas, K. Sandra, D. Guillarme, S. Fekete, Evaluation of size exclusion chromatography columns packed with sub-3µm particles for the analysis of biopharmaceutical proteins, J. Chromatogr. A. 1498 (2017) 80–89. doi:10.1016/j.chroma.2016.11.056.
[90] A. Goyon, V. D'Atri, O. Colas, S. Fekete, A. Beck, D. Guillarme, Characterization of 30 therapeutic antibodies and related products by size exclusion chromatography: Feasibility assessment for future mass spectrometry hyphenation, J. Chromatogr. B. 1065–1066 (2017) 35–43. doi:10.1016/j.jchromb.2017.09.027.
[91] S. Fekete, A. Beck, J.-L. Veuthey, D. Guillarme, Ion-exchange chromatography for the characterization of biopharmaceuticals, J. Pharm. Biomed. Anal. 113 (2015) 43–55. doi:10.1016/j.jpba.2015.02.037.
[92] J. Kittelmann, K.M.H. Lang, M. Ottens, J. Hubbuch, Orientation of monoclonal antibodies in ion-exchange chromatography: A predictive quantitative structure–activity relationship modeling approach, J. Chromatogr. A. 1510 (2017) 33–39. doi:10.1016/j.chroma.2017.06.047.
[93] R. Gahoual, A. Beck, E. Leize-Wagner, Y.-N. François, Cutting-edge capillary electrophoresis characterization of monoclonal antibodies and related products, J. Chromatogr. B. 1032 (2016) 61–78. doi:10.1016/j.jchromb.2016.05.028.
[94] M. Han, B.M. Rock, J.T. Pearson, D.A. Rock, Intact mass analysis of monoclonal antibodies by capillary electrophoresis—Mass spectrometry, J. Chromatogr. B. 1011 (2016) 24–32. doi:10.1016/j.jchromb.2015.12.045.
[95] V. Dotz, R. Haselberg, A. Shubhakar, R.P. Kozak, D. Falck, Y. Rombouts, D. Reusch, G.W. Somsen, D.L. Fernandes, M. Wuhrer, Mass spectrometry for glycosylation analysis of biopharmaceuticals, TrAC Trends Anal. Chem. 73 (2015) 1–9. doi:10.1016/j.trac.2015.04.024.

[96] E. Higgins, Carbohydrate analysis throughout the development of a protein therapeutic, Glycoconj. J. 27 (2010) 211–225. doi:10.1007/s10719-009-9261-x.
[97] E. Klesper, A.H. Corwin, D.A. Turner, High-pressure gas chromatography above critical temperature, J. Org. Chem. 27 (1962) 700–706. doi:10.1021/jo01049a069.
[98] J.C. Giddings, M.N. Myers, L. McLaren, R.A. Keller, High Pressure Gas Chromatography of Nonvolatile Species, Science. 162 (1968) 67–73. doi:10.1126/science.162.3849.67.
[99] S.T. Sie, W.V. Beersum, G.W.A. Rijnders, High-Pressure Gas Chromatography and Chromatography with Supercritical Fluids. I. The Effect of Pressure on Partition Coefficients in Gas-Liquid Chromatography with Carbon Dioxide as a Carrier Gas, Sep. Sci. (1966). doi:10.1080/01496396608049460.
[100] R.E. Jentoft, T.H. Gouw, Pressure-Programmed Supercritical Fluid Chromatography of Wide Molecular Weight Range Mixtures, J. Chromatogr. Sci. 8 (1970) 138–142. doi:10.1093/chromsci/8.3.138.
[101] S.R. Springston, M. Novotny, Kinetic optimization of capillary supercritical fluid chromatography using carbon dioxide as the mobile phase, Chromatographia. 14 (1981) 679–684. doi:10.1007/BF02259454.
[102] M. Novotny, S.R. Springston, P.A. Peaden, J.C. Fjeldsted, M.L. Lee, Capillary supercritical fluid chromatography, Anal. Chem. 53 (1981) 407–414. doi:10.1021/ac00226a002.
[103] D.R. Gere, R. Board, D. McManigill, Supercritical fluid chromatography with small particle diameter packed columns, Anal. Chem. 54 (1982) 736–740. doi:10.1021/ac00241a032.
[104] T.A. Berger, Separation of polar solutes by packed column supercritical fluid chromatography, J. Chromatogr. A. 785 (1997) 3–33. doi:10.1016/S0021-9673(97)00849-2.
[105] T.A. Berger, J.F. Deye, Role of additives in packed column supercritical fluid chromatography: suppression of solute ionization, J. Chromatogr. A. 547 (1991) 377–392. doi:10.1016/S0021-9673(01)88661-1.
[106] T.A. Berger, J.F. Deye, Effect of Basic Additives on Peak Shapes of Strong Bases Separated by Packed-Column Supercritical Fluid Chromatography, J. Chromatogr. Sci. 29 (1991) 310–317. doi:10.1093/chromsci/29.7.310.
[107] D. Berger, Supercritical Fluid Chromatography (SFC): A Review of Technical Developments, LC-GC Eur. 20 (2007) 164–166.
[108] K. Anton, J. Eppinger, L. Frederiksen, E. Francotte, T.A. Berger, W.H. Wilson, Chiral separations by packed-column super- and subcritical fluid chromatography, J. Chromatogr. A. 666 (1994) 395–401. doi:10.1016/0021-9673(94)80399-4.
[109] C. White, Integration of supercritical fluid chromatography into drug discovery as a routine support tool: Part I. Fast chiral screening and purification, J. Chromatogr. A. 1074 (2005) 163–173. doi:10.1016/j.chroma.2005.03.073.
[110] C.J. Welch, M. Biba, J.R. Gouker, G. Kath, P. Augustine, P. Hosek, Solving multicomponent chiral separation challenges using a new SFC tandem column screening tool, Chirality. 19 (2007) 184–189. doi:10.1002/chir.20357.
[111] E.R. Francotte, Enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers, J. Chromatogr. A. 906 (2001) 379–397. doi:10.1016/S0021-9673(00)00951-1.
[112] M.L. de la Puente, P. López Soto-Yarritu, J. Burnett, Supercritical fluid chromatography in research laboratories: Design, development and implementation of an efficient generic screening for exploiting this technique in the achiral environment, J. Chromatogr. A. 1218 (2011) 8551–8560. doi:10.1016/j.chroma.2011.09.029.
[113] W. Ren-Qi, O. Teng-Teng, N. Siu-Choon, T. Weihua, Recent advances in pharmaceutical separations with supercritical fluid chromatography using chiral stationary phases, TrAC Trends Anal. Chem. 37 (2012) 83–100. doi:10.1016/j.trac.2012.02.012.

[114] C. West, Enantioselective Separations with Supercritical Fluids - Review, Curr. Anal. Chem. 10 (2014) 99–120. doi:10.2174/1573411011410010009.
[115] D. Speybrouck, E. Lipka, Preparative supercritical fluid chromatography: A powerful tool for chiral separations, J. Chromatogr. A. 1467 (2016) 33–55. doi:10.1016/j.chroma.2016.07.050.
[116] S. Khater, C. West, E. Lesellier, Characterization of five chemistries and three particle sizes of stationary phases used in supercritical fluid chromatography, J. Chromatogr. A. 1319 (2013) 148–159. doi:10.1016/j.chroma.2013.10.037.
[117] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, The use of columns packed with sub-2 µm particles in supercritical fluid chromatography, TrAC Trends Anal. Chem. 63 (2014) 44–54. doi:10.1016/j.trac.2014.06.023.
[118] L. Nováková, A. Grand-Guillaume Perrenoud, I. Francois, C. West, E. Lesellier, D. Guillarme, Modern analytical supercritical fluid chromatography using columns packed with sub-2 µm particles: A tutorial, Anal. Chim. Acta. 824 (2014) 18–35. doi:10.1016/j.aca.2014.03.034.
[119] T.A. Berger, Demonstration of High Speeds with Low Pressure Drops Using 1.8 µm Particles in SFC, Chromatographia. 72 (2010) 597–602. doi:10.1365/s10337-010-1699-2.
[120] A.G.-G. Grand-Guillaume Perrenoud, W.P. Farrell, C.M. Aurigemma, N.C. Aurigemma, S. Fekete, D. Guillarme, Evaluation of stationary phases packed with superficially porous particles for the analysis of pharmaceutical compounds using supercritical fluid chromatography, J. Chromatogr. A. 1360 (2014) 275–287. doi:10.1016/j.chroma.2014.07.078.
[121] E. Lesellier, Extension of the carotenoid test to superficially porous C18 bonded phases, aromatic ligand types and new classical C18 bonded phases, J. Chromatogr. A. 1266 (2012) 34–42. doi:10.1016/j.chroma.2012.09.068.
[122] M. Ashraf-Khorassani, G. Isaac, P. Rainville, K. Fountain, L.T. Taylor, Study of UltraHigh Performance Supercritical Fluid Chromatography to measure free fatty acids with out fatty acid ester preparation, J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 997 (2015) 45–55. doi:10.1016/j.jchromb.2015.05.031.
[123] J. Duval, C. Colas, V. Pecher, M. Poujol, J.-F. Tranchant, E. Lesellier, Hyphenation of ultra high performance supercritical fluid chromatography with atmospheric pressure chemical ionisation high resolution mass spectrometry: Part 1. Study of the coupling parameters for the analysis of natural non-polar compounds, J. Chromatogr. A. 1509 (2017) 132–140. doi:10.1016/j.chroma.2017.06.016.
[124] B. Andri, A. Dispas, R. Klinkenberg, B. Streel, R.D. Marini, E. Ziémons, P. Hubert, Is supercritical fluid chromatography hyphenated to mass spectrometry suitable for the quality control of vitamin D3 oily formulations?, J. Chromatogr. A. 1515 (2017) 209–217. doi:10.1016/j.chroma.2017.07.057.
[125] C. Desmortreux, M. Rothaupt, C. West, E. Lesellier, Improved separation of furocoumarins of essential oils by supercritical fluid chromatography, J. Chromatogr. A. 1216 (2009) 7088–7095. doi:10.1016/j.chroma.2009.08.080.
[126] S. Khater, C. West, Development and validation of a supercritical fluid chromatography method for the direct determination of enantiomeric purity of provitamin B5 in cosmetic formulations with mass spectrometric detection, J. Pharm. Biomed. Anal. 102 (2015) 321–325. doi:10.1016/j.jpba.2014.09.036.
[127] E. Lesellier, D. Mith, I. Dubrulle, Method developments approaches in supercritical fluid chromatography applied to the analysis of cosmetics, J. Chromatogr. A. 1423 (2015) 158–168. doi:10.1016/j.chroma.2015.10.053.
[128] J. Crepier, A. Le Masle, N. Charon, F. Albrieux, S. Heinisch, Development of a supercritical fluid chromatography method with ultraviolet and mass spectrometry detection for the characterization of biomass fast pyrolysis bio oils, J. Chromatogr. A. 1510 (2017) 73–81. doi:10.1016/j.chroma.2017.06.003.

[129] D. Thiébaut, Separations of petroleum products involving supercritical fluid chromatography, J. Chromatogr. A. 1252 (2012) 177–188. doi:10.1016/j.chroma.2012.06.074.
[130] J.L. Bernal, M.T. Martín, L. Toribio, Supercritical fluid chromatography in food analysis, J. Chromatogr. A. 1313 (2013) 24–36. doi:10.1016/j.chroma.2013.07.022.
[131] Y. Huang, G. Tang, T. Zhang, M. Fillet, J. Crommen, Z. Jiang, Supercritical fluid chromatography in traditional Chinese medicine analysis, J. Pharm. Biomed. Anal. 147 (2018) 65–80. doi:10.1016/j.jpba.2017.08.021.
[132] R. Rosset, A. Jardy, M. Caude, Chromatographies en phases liquide et supercritique, Paris, France, Italie, Espagne, 1991.
[133] G. Guiochon, A. Tarafder, Fundamental challenges and opportunities for preparative supercritical fluid chromatography, J. Chromatogr. A. 1218 (2011) 1037–1114. doi:10.1016/j.chroma.2010.12.047.
[134] T. Caudell, Practical Supercritical Fluid Chromatography and Extraction, CRC Press, 1999. https://books.google.fr/books?id=FgN-6I_vAYUC&pg=PA56&lpg=PA56&dq#v=onepage&q&f=false.
[135] G. Madras, C. Kolluru, R. Kumar, Synthesis of biodiesel in supercritical fluids, Fuel. 83 (2004) 2029–2033. doi:10.1016/j.fuel.2004.03.014.
[136] M. Herrero, A. Cifuentes, E. Ibañez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review, Food Chem. 98 (2006) 136–148. doi:10.1016/j.foodchem.2005.05.058.
[137] M.Z. Ozel, F. Gogus, A.C. Lewis, Subcritical water extraction of essential oils from Thymbra spicata, Food Chem. 82 (2003) 381–386. doi:10.1016/S0308-8146(02)00558-7.
[138] R.L. Holliday, J.W. King, G.R. List, Hydrolysis of Vegetable Oils in Sub- and Supercritical Water, Ind. Eng. Chem. Res. 36 (1997) 932–935. doi:10.1021/ie960668f.
[139] S.S. Toor, H. Reddy, S. Deng, J. Hoffmann, D. Spangsmark, L.B. Madsen, J.B. Holm-Nielsen, L.A. Rosendahl, Hydrothermal liquefaction of Spirulina and Nannochloropsis salina under subcritical and supercritical water conditions, Bioresour. Technol. 131 (2013) 413–419. doi:10.1016/j.biortech.2012.12.144.
[140] I.M. Svishchev, D.A. Guzonas, Supercritical water and particle nucleation: Implications for water chemistry control in a GEN IV supercritical water cooled nuclear reactor, J. Supercrit. Fluids. 60 (2011) 121–126. doi:10.1016/j.supflu.2011.06.013.
[141] D.E. Raynie, Warning concerning the use of nitrous oxide in supercritical fluid extractions, Anal. Chem. 65 (1993) 3127–3128. doi:10.1021/ac00069a028.
[142] S.F.Y. Li, C.P. Ong, M.L. Lee, H.K. Lee, Supercritical fluid extraction and chromatography of steroids with Freon-22, J. Chromatogr. A. 515 (1990) 515–520. doi:10.1016/S0021-9673(01)89348-1.
[143] S.K. Yeo, C.P. Ong, H.K. Lee, S.F.Y. Li, Some applications of Freon-22 in supercritical fluid chromatographic analysis of environmental pollutants, Environ. Monit. Assess. 19 (1991) 47–53. doi:10.1007/BF00401296.
[144] International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. Pharmaceutical development Q8(R2), 2009, (n.d.). https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf (accessed October 12, 2017).
[145] A. Dispas, V. Desfontaine, B. Andri, P. Lebrun, D. Kotoni, A. Clarke, D. Guillarme, P. Hubert, Quantitative determination of salbutamol sulfate impurities using achiral supercritical fluid chromatography, J. Pharm. Biomed. Anal. 134 (2017) 170–180. doi:10.1016/j.jpba.2016.11.039.
[146] B. Andri, P. Lebrun, A. Dispas, R. Klinkenberg, B. Streel, E. Ziemons, R.D. Marini, P. Hubert, Optimization and validation of a fast supercritical fluid chromatography method for the quantitative determination of vitamin D3 and its related impurities, J. Chromatogr. A. 1491 (2017) 171–181. doi:10.1016/j.chroma.2017.01.090.

[147] V. Desfontaine, A. Tarafder, J. Hill, J. Fairchild, A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, A systematic investigation of sample diluents in modern supercritical fluid chromatography, J. Chromatogr. A. 1511 (2017) 122–131. doi:10.1016/j.chroma.2017.06.075.
[148] S.R. Needham, P.R. Brown, K. Duff, D. Bell, Optimized stationary phases for the high-performance liquid chromatography–electrospray ionization mass spectrometric analysis of basic pharmaceuticals, J. Chromatogr. A. 869 (2000) 159–170. doi:10.1016/S0021-9673(99)00986-3.
[149] E.L. Regalado, A.A. Makarov, R. McClain, M. Przybyciel, C.J. Welch, Search for improved fluorinated stationary phases for separation of fluorine-containing pharmaceuticals from their desfluoro analogs, J. Chromatogr. A. 1380 (2015) 45–54. doi:10.1016/j.chroma.2014.12.025.
[150] E.L. Regalado, P. Zhuang, Y. Chen, A.A. Makarov, W.A. Schafer, N. McGachy, C.J. Welch, Chromatographic Resolution of Closely Related Species in Pharmaceutical Chemistry: Dehalogenation Impurities and Mixtures of Halogen Isomers, Anal. Chem. 86 (2014) 805–813. doi:10.1021/ac403376h.
[151] T.A. Berger, W.H. Wilson, Separation of basic drugs by packed-column supercritical fluid chromatography 3. Stimulants, J. Pharm. Sci. 84 (1995) 489–492. doi:10.1002/jps.2600840419.
[152] Transfer of a UPLC Screening Method to Preparative HPLC Purification using CSH Column Technology and Focused Gradients, (n.d.). http://www.waters.com/webassets/cms/library/docs/720004228en.pdf (accessed September 13, 2017).
[153] K.F. Blom, B. Glass, R. Sparks, A.P. Combs, Preparative LC-MS purification: improved compound-specific method optimization, J. Comb. Chem. 6 (2004) 874–883. doi:10.1021/cc049890v.
[154] P. Koza, V. Gonnot, J. Pelleter, Right-first-time isocratic preparative liquid chromatography-mass spectrometry purification, ACS Comb. Sci. 14 (2012) 273–279. doi:10.1021/co200197b.
[155] UPC2 Strategy for Scaling SFC Methods: Applications for Preparative Chromatography, (n.d.). http://www.waters.com/webassets/cms/library/docs/720005064en.pdf (accessed September 13, 2017).
[156] C.J. Venkatramani, M. Al-Sayah, G. Li, M. Goel, J. Girotti, L. Zang, L. Wigman, P. Yehl, N. Chetwyn, Simultaneous achiral-chiral analysis of pharmaceutical compounds using two-dimensional reversed phase liquid chromatography-supercritical fluid chromatography, Talanta. 148 (2016) 548–555. doi:10.1016/j.talanta.2015.10.054.
[157] L. Zeng, R. Xu, Y. Zhang, D.B. Kassel, Two-dimensional supercritical fluid chromatography/mass spectrometry for the enantiomeric analysis and purification of pharmaceutical samples, J. Chromatogr. A. 1218 (2011) 3080–3088. doi:10.1016/j.chroma.2011.03.041.

Annexes

Annexe 1 : Chapitre 3 - IV : résumé des cas étudiés pour la comparaison des méthodes SFC gradient, gradient focus et isocratique

	Pureté relative du PA			Nb impuretés >1%			Nb impuretés >0.04%		
Composé	Gradient C18	Focus C18	Isocra-tique C18	Gradient C18	Focus C18	Isocra-tique C18	Gradient C18	Focus C18	Isocra-tique C18
S10375	93.4	94.0	93.7	2	2	2	7	6	6
S10419	91.0	93.6	93.9	3	2	2	8	8	8
S10986	94.0	97.7	96.2	2	1	1	8	5	5
S12068	89.8	91.4	96.4	3	2	1	5	6	10
S15124	96.8	96.5	96.3	0	0	0	14	14	17
S18543	87.5	88.8	91.4	4	4	3	18	15	20
CMN314	98.8	99.1	98.3	0	0	1	4	2	3
S3685	95.8	95.9	97.0	2	2	1	7	8	9
S37857	83.1	79.5	82.3	3	6	5	22	23	16
S10385	92.0	91.9	91.4	2	2	2	11	9	11
S14367	90.7	88.9	72.4	2	3	2	2	0	1
S14512	97.4	98.0	97.6	2	1	1	0	1	1
S15855	97.8	97.6	98.0	0	0	0	6	8	6
S18949	96.8	95.3	96.7	0	0	0	12	9	11
S21599	92.2	92.4	91.6	2	2	2	9	9	11
S40363	39.2	45.5	40.4	10	7	11	15	11	7
S76937	78.2	78.2	83.4	6	6	4	15	15	2
S9106	40.2	40.2	40.6	3	4	3	11	12	12
Sdia9106	49.9	49.4	48.4	-	-	-	-	-	-
S16051	53.7	59.1	49.2	8	7	8	10	6	12
S18272	88.2	88.9	91.9	2	3	3	12	8	7
S39656	76.5	77.8	77.6	5	5	7	12	11	9
S38098	32.7	32.3	34.5	0	0	0	4	4	3
Sdia38098	67.0	67.3	65.1	-	-	-	-	-	-
S33434	98.4	98.8	99.0	1	1	1	2	2	0

Annexe 1 (suite)

Composé	Pureté relative du PA			Nb impuretés >1%			Nb impuretés >0.04%		
	Gradient HILIC	Focus HILIC	Isocra-tique HILIC	Gradient HILIC	Focus HILIC	Isocra-tique HILIC	Gradient HILIC	Focus HILIC	Isocra-tique HILIC
S10375	92.4	92.7	93.1	3	3	2	4	3	5
S10419	x	x	x	x	x	x	x	x	x
S10986	97.5	97.9	97.2	1	1	1	5	3	5
S12068	90.9	91.9	91.4	2	2	2	10	4	11
S15124	96.7	95.9	94.4	0	0	1	12	14	20
S18543	70.5	72.0	73.1	8	6	6	10	8	14
CMN314	98.9	99.8	98.9	0	0	0	3	3	4
S3685	99.5	99.1	98.7	0	0	0	2	2	4
S37857	74.2	78.2	75.1	8	8	10	20	14	18
S10385	95.9	96.5	96.5	2	1	1	8	7	7
S14367	x	x	x	x	x	x	x	x	x
S14512	97.9	97.9	98.9	1	1	1	1	0	x
S15855	99.0	99.5	99.3	0	0	0	4	3	4
S18949	92.9	95.7	97.7	1	0	0	17	11	12
S21599	97.2	89.0	90.1	1	1	2	4	5	5
S40363	38.2	40.0	38.2	11	11	11	18	14	13
S76937	77.2	79.2	77.6	5	5	6	10	11	13
S9106	39.5	39.3	40.2	4	3	3	11	13	6
Sdia9106	50.3	50.1	50.3	-	-	-	-	-	-
S16051	46.7	48.4	51.1	13	9	7	13	17	7
S18272	86.8	86.0	88.1	4	5	4	6	8	4
S39656	80.4	80.4	75.9	4	4	7	10	9	8
S38098	98.7	99.3	99.7	1	0	0	1	1	1
Sdia38098	x	x	x	-	-	-	-	-	-
S33434	98.9	99.0	99.0	1	1	1	0	0	0

Elise LEMASSON

Stratégies chromatographiques en phase liquide et supercritique pour l'analyse de candidats médicaments

Résumé :

Le profilage d'impuretés de candidats médicaments est une préoccupation majeure des industries pharmaceutiques. L'identification et la quantification des impuretés doivent être strictement contrôlées pour assurer l'efficacité et la toxicité limitée du principe actif. Il est donc nécessaire de disposer de méthodes analytiques performantes afin de s'assurer que l'ensemble des impuretés est identifié. L'HPLC phase inverse sur phase C18 reste aujourd'hui la méthode de choix pour cette tâche. Cependant, il arrive que cette méthode échoue, notamment lorsque le principe actif n'est pas suffisamment retenu sur la colonne ou que les impuretés ne sont pas parfaitement séparées du composé principal. Il est alors essentiel de pouvoir se tourner vers des méthodes analytiques alternatives et complémentaires.

Ce travail de recherche traite du développement et de l'évaluation de méthodes analytiques alternatives à l'HPLC phase inverse sur phase C18 pour le profilage d'impuretés de principes actifs pharmaceutiques. L'HPLC phase inverse sur d'autres phases stationnaires, l'HPLC mixed-mode ainsi que la SFC ont été explorées et leurs performances chromatographiques comparées. La comparaison et l'étude des différentes méthodes ont permis de proposer une stratégie d'analyse du candidat médicament.

Mots clés : développement de méthodes, HPLC, mixed-mode, SFC, candidats médicaments

Liquid and supercritical chromatographic strategies for analysis of drug candidates

Summary:

Impurity profiling of drug candidates is a significant concern of pharmaceutical industries. The identification and quantification of impurities must be strictly controlled to ensure the efficacy and limited toxicity of the active ingredient. It is therefore necessary to have efficient analytical methods to ensure that all impurities are identified. Today, reversed-phase HPLC with C18 column remains the method of choice for this task. However, this method sometimes fails, particularly when the active pharmaceutical ingredient is not sufficiently retained on the column or when the impurities are not resolved from the main compound. It is therefore essential to turn to alternative and complementary analytical methods.

This work deals with the development and evaluation of alternative analytical methods to reversed-phase HPLC on C18 phase for impurity profiling of pharmaceuticals. Reversed-phase HPLC on other stationary phases, mixed-mode HPLC as well as SFC were explored and their chromatographic performances compared. The comparison and the study of the different methods allowed proposing a strategy of analysis of the drug candidate.

Keywords: method development, HPLC, mixed-mode, SFC, drug candidates

Institut de Chimie Organique et Analytique
Université d'Orléans, CNRS UMR 7311, BP 6759
45067 Orléans Cedex 2, France