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Adrien Raimbault

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ÉCOLE DOCTORALE
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Institut de Chimie Organique et Analytique

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Adrien RAIMBAULT

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Discipline : **Chimie Analytique**

**Analyse de molécules d'intérêt biologique en
chromatographie supercritique et chromatographie
unifiée – Etudes fondamentales et applications**

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1) A chiral unified chromatography–mass spectrometry method to analyze free amino acids

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2) Effects of high concentrations of mobile phase additives on retention and separation mechanisms on a teicoplanin aglycone stationary phase in supercritical fluid chromatography

A. Raimbault, C. West

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3) Analysis of free amino acids with unified chromatography–mass spectrometry – Application to food supplements

A. Rimbault, A. Noireau, C. West

Journal of Chromatography A, In press, Article 460772

4) Cinchona-based zwitterionic stationary phases: Exploring retention and enantioseparation mechanisms in supercritical fluid chromatography with a fragmentation approach

A. Rimbault, C.M.A. Ma, M. Ferri, S. Bäurer, P. Bonnet, S. Bourg, M.

Lämmerhofer, C. West

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5) Optimised pressurized liquid extraction of native amino acids from *Capsella bursa-pastoris* and their analysis with unified chromatography

A. Rimbault, M. Dorebska, E. Destandau, C. West

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6) First inter-laboratory study of a supercritical fluid chromatography method for the determination of pharmaceutical impurities

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Communications orales

- 1) Ions and SFC: an improbable match?

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

- 2) Improving our understanding of enantioseparation in supercritical fluid chromatography

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

- 3) Effects of acidic and basic additives on retention mechanisms on a teicoplanin aglycon (TAG) stationary phase

A. Raimbault, C. West
SFC 2017, Rockville, MD (USA)

- 4) Effets d'additifs sur le comportement d'acides aminés en chromatographie supercritique

A. Raimbault, C. West.
Journées de la section Régionale Centre-Ouest de la Société Chimique de France (SCF) 2017, Tours (France). **Prix de la meilleure communication**

- 5) Expanding the possibilities of SFC with the enantioresolution of native amino acids

A. Rimbault, C. West.

SFC 2018, Strasbourg (France)

- 6) Extension des possibilités en SFC avec l'énantiorésolution d'acides aminés natifs

A. Rimbault, C. West.

SEP 2019, Congrès international de l'AFSEP (Association francophone des sciences séparatives), Paris (France)

- 7) SFC-ESI-MS methods to analyse polar compounds in traditional phytotherapy

A. Rimbault, C. West.

HPLC 2019, Milan (Italie)

- 8) Interest of chiral stationary phases for achiral separations in supercritical fluid chromatography

C. West., A. Noireau, A. Rimbault

12th Balaton Symposium on high-performance separation methods 2019, Siófok (Hongrie)

Communications par affiche

- 1) Comparaison de phases stationnaires énantiosélectives en chromatographie supercritique

A. Rimbault, S. Khater, C. West.

SEP 2017, Congrès international de l'AFSEP (Association francophone des sciences séparatives), Paris (France) **Nominé pour le prix du meilleur poster**

- 2) Study of achiral selectivity of chiral columns in SFC: application for impurity profiling of drug candidates

E. Lemasson, A. Rimbault, S. Bertin, F. Mauge, P. Hennig, E. Lesellier, C. West

HPLC 2017, Prague (République Tchèque)

- 3) Quinine-Based Zwitterionic Stationary Phases: Exploring Retention and Enantioseparation Mechanisms in Supercritical Fluid Chromatography

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Liste des abréviations

ACN : acétonitrile
AMS : acide méthanesulfonique
ATFA : ammonium trifluoroacétate
BuOH : butanol
DEDL : détecteur évaporatif à diffusion de lumière
DSS : dioctyl sodium sulfosuccinate
EFLC : chromatographie liquide à fluidité améliorée
ESI : ionisation par électrospray
EtOH : éthanol
FID : détecteur par ionisation de flamme
GC : chromatographie gazeuse
HPGC : chromatographie gazeuse à haute pression
HPLC : chromatographie liquide haute performance
IPA : isopropylamine
iPrOH : isopropanol
log D : mesure de log P à un pH donné
log P / log $K_{o/w}$: coefficient de partage octanol/eau
LSER : relation linéaire de l'énergie de solvatation
MeOH : méthanol
MS : spectromètre de masse
MWE : extraction assistée par micro-ondes
Pc : pression critique
PFE : extraction par fluide pressurisé
pKa : logarithme de la constante de dissociation
SAX : échangeur fort d'anion
SFC : chromatographie en phase supercritique
SFE : extraction par fluide supercritique
Tc : température critique

TEA : triéthylamine

TFA : acide trifluoroacétique

UC : chromatographie unifiée entre l'HPLC et la SFC

UHPLC : HPLC à ultra haute performance

UHPSFC : SFC à ultra haute performance

UV : ultra-violet

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Introduction générale

La chromatographie en phase supercritique (ou SFC) fut longtemps le vilain petit canard de la chromatographie. Cependant durant ces dernières années, tel le phénix, cette technique renaquit de ses cendres. En effet, cette technique chromatographique est restée de nombreuses années dans l'ombre de techniques plus performantes comme la chromatographie liquide haute performance (HPLC) ou encore de la chromatographie en phase gazeuse (GC), en raison d'appareillages de plus en plus innovants et plus performants que les systèmes SFC. Seules les applications chirales, et en particulier les utilisations en chromatographie préparative (purification énantiomérique par exemple) ont permis de maintenir en vie la SFC. Les avantages de la SFC pour la purification sont la consommation moindre de solvant et également la réduction du temps d'évaporation du solvant, étant donné que la majorité du solvant est fait de dioxyde de carbone qui s'évapore directement en sortie du système.

La renaissance de la SFC dans les années 2010 a été mise en œuvre par la commercialisation d'appareils performants et beaucoup plus robustes, ce qui permet d'avoir une meilleure répétabilité des injections, une meilleure robustesse des méthodes. Depuis cette période, de nombreuses applications se développent sur cette technique, surtout sur des petites molécules peu polaires. Ceci peut s'expliquer par la faible polarité du CO₂ supercritique, souvent comparé à de l'hexane en termes de force éluante.

Pendant longtemps, le CO₂ supercritique a donc été utilisé pour l'analyse de composés apolaires. Mais en ajoutant un co-solvant dans la phase mobile, les propriétés de polarité vont changer. La majorité des solvants sont miscibles au CO₂ pressurisé et peuvent être utilisés comme l'hexane, le méthanol (MeOH), l'isopropanol (iPrOH), l'acétonitrile (ACN). Ces solvants vont donc augmenter la polarité de la phase mobile et permettre d'élué des molécules de plus en plus polaires. Des additifs et de l'eau peuvent aussi être ajoutés pour moduler la polarité, en étant prudent de ne pas entrer dans la zone de démixtion puisque l'eau est très peu miscible au CO₂ supercritique.

L'objectif de cette thèse est donc de voir les possibilités de la SFC dans l'analyse de molécules de plus en plus polaires, quelles méthodes peuvent être mises en place pour parvenir à l'analyse de molécules de forte polarité.

Dans le premier chapitre de ce manuscrit, une synthèse bibliographique est réalisée : après un bref historique des fluides supercritiques et de leurs utilisations en chromatographie, l'utilisation de la chromatographie en phase supercritique pour des applications « non conventionnelles », c'est-à-dire des molécules d'une polarité élevée, sera détaillée. Dans un premier temps, nous nous sommes intéressés aux applications concernant les molécules polaires non ionisables, puis aux molécules ionisables comme des molécules acides, basiques ou encore zwitterioniques. Ce chapitre va permettre de comprendre les conditions nécessaires à l'analyse de molécules polaires en SFC.

Le second chapitre est consacré à l'étude de deux phases stationnaires. La première est la colonne Astec Chirobiotic™ TAG. L'étude de cette phase stationnaire va nous apporter des informations sur le comportement des molécules polaires ionisables en milieu supercritique. En effet, la molécule greffée à cette phase stationnaire est un peptide macrocyclique, la teicoplanine aglycone. Un set de molécules achirales et chirales a été injecté dans des conditions de phases mobiles différentes. Les mécanismes de rétention seront donc déterminés grâce au modèle LSER (Linear Solvation Energy Relationship) qui modélise toutes les interactions que vont pouvoir faire les molécules analysées avec la phase stationnaire. La deuxième colonne étudiée a été la colonne Chiralpak® ZWIX. Cette phase stationnaire est déclinée en deux modèles : Chiralpak® ZWIX (+) et Chiralpak® ZWIX (-), ces deux greffons sont des diastéréoisomères l'un de l'autre. Ce sont des phases stationnaires qui ont été conçues pour répondre à la problématique d'énantioséparation des acides aminés natifs. Le greffon étant un zwitterion, cela favorise l'énantioséparation de molécules comme des acides aminés qui sont aussi des zwitterions. Plusieurs phases stationnaires similaires aux colonnes Chiralpak® ZWIX ont été fabriquées à façon par l'équipe du professeur Michael Lämmerhofer à l'Université de Tübingen en Allemagne. Ces phases stationnaires diffèrent des colonnes de référence Chiralpak® ZWIX par le retrait d'un groupement fonctionnel situé sur le greffon des phases Chiralpak® ZWIX. L'impact de ces faibles variations au niveau des greffons va être étudié grâce à un set de molécules achirales et chirales, comme précédemment sur la Chirobiotic™ TAG. L'objectif était ici de mieux comprendre les interactions des analytes ioniques avec la phase stationnaire employée en SFC.

La dernière partie de ce manuscrit de thèse est consacrée au développement de méthodes analytiques pour l'analyse de composés polaires, et plus particulièrement les acides aminés. Une méthode générique a été développée dans le but d'atteindre l'énantioséparation de tous les acides aminés natifs protéinogéniques (sauf la glycine qui ne présente pas de centre asymétrique). Pour ce faire, la méthode employée n'est plus précisément de la chromatographie supercritique mais peut être qualifiée de « chromatographie unifiée » (UC) car le gradient d'élution commence en conditions SFC et se termine en conditions HPLC. Cette méthode générique a ensuite été améliorée en modifiant la phase mobile et en jouant sur les paramètres tels que la contrepression et le débit. Cette nouvelle méthode a été utilisée pour l'analyse des acides aminés dans diverses applications. La première application a été la validation d'une méthode de quantification des acides aminés dans des compléments alimentaires. La deuxième application a été l'optimisation de l'extraction des acides aminés natifs dans une plante médicinale, la Bourse à Pasteur (*Capsella bursa-pastoris*).

Après une conclusion générale qui fait le bilan de l'ensemble des travaux, une ouverture est effectuée sur d'autres manipulations faites dans le cadre de la thèse. Ces expériences ont été faites sur d'autres familles moléculaires telles que des antibiotiques, des anthocyanes, ou des iridoïdes.

Chapitre 1

Synthèse bibliographique de l'utilisation de la chromatographie supercritique pour des applications non conventionnelles

I. Les fluides supercritiques et leur utilisation dans le domaine de la chromatographie

1. Historique des fluides supercritiques

Lors de ses expériences le Baron Cagniard de Latour, un ingénieur et physicien français, a mis en évidence un état supercritique lors de ses expériences en 1822. Il tenta de volatiliser, en vase clos, dans un volume beaucoup plus grand que le volume du liquide, de l'éther, de l'alcool et de l'eau, chacun individuellement. Il y arriva à une température relativement élevée. Cette découverte est une des bases expérimentales de la théorie des gaz. La notion de point critique se dégage grâce à cette expérience et il définit l'état de fluide supercritique.

Il faut attendre les années 1970 pour avoir une réelle exploitation de la technologie des fluides supercritiques dans le monde industriel. La première application utilisant l'extraction supercritique relate l'extraction des graines de moka pour la décaféination ainsi que des travaux sur du houblon, des feuilles de tabac et des épices [1]. Plusieurs autres domaines ont aussi utilisé ces fluides supercritiques, comme la pétrochimie ou l'industrie du charbon [1].

Les premiers travaux utilisant un fluide supercritique comme phase mobile de chromatographie ont été l'œuvre de Klesper *et al.* [2] en 1962 avec un système HPGC « High-Pressure Gas Chromatography » pour la séparation de porphyrines sur une colonne polyéthylène glycol en utilisant du dichlorodifluorométhane ($T_c = 112^\circ\text{C}$) et du monochlorodifluorométhane ($T_c = 96^\circ\text{C}$) comme fluide supercritique. Sie et Rijnders ont aussi publié une série d'articles dans les années 1966-1967 sur l'utilisation du dioxyde de carbone supercritique comme phase mobile [3–6]. Mais la chromatographie en phase supercritique ou SFC (selon l'acronyme anglo-saxon pour Supercritical Fluid Chromatography) ne s'est pas bien établie par la suite. Il y a plusieurs raisons qui pourraient expliquer ce retard d'implantation. La première, et sûrement plus importante, est l'avènement, à la même période, de 'HPLC. A l'époque des premiers articles en SFC, la GC était déjà une technique bien établie avec des instruments disponibles de plusieurs constructeurs. Quand les besoins des

scientifiques se sont tournés vers l'analyse de composés thermolabiles, ou encore des composés polaires, non-volatiles qui ne pouvaient pas être séparés en GC, l'HPLC avait le potentiel pour répondre à toutes ces demandes. Le développement de la SFC a donc été fortement ralenti à cause de celui de l'HPLC qui a pris une place très importante dans la fin des années 1960 et 1970.

En revanche, depuis plusieurs années, la SFC connaît un regain d'intérêt, en particulier dû aux nouveaux instruments disponibles sur le marché, qui sont beaucoup plus robustes que les instruments précédemment disponibles, les analyses sont plus reproductibles avec un bruit de fond en UV beaucoup plus faible. Également le couplage à la spectrométrie de masse maintenant mieux maîtrisé a renforcé les applications de cette technique. Ceci est illustré dans la figure 1.1 ci-dessous qui représente le nombre d'articles écrits sur le domaine de la SFC depuis la fin des années 1960. Cet histogramme peut être nuancé par les nombreux articles traitant de l'extraction par fluide supercritique (SFE), et non pas de SFC, surtout dans les années difficiles pour la SFC (avant les années 2010).

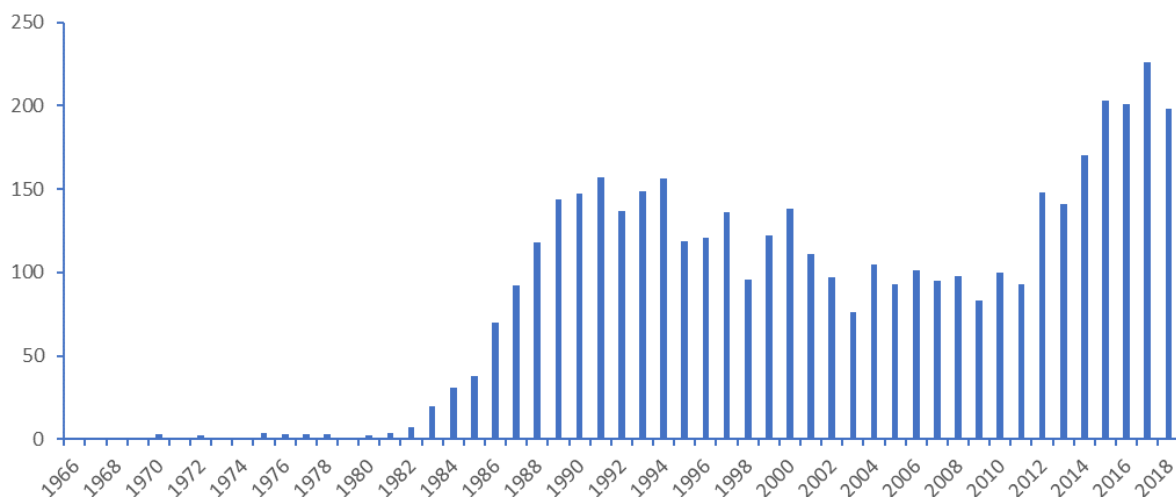


Figure 1.1 : Histogramme des publications répertoriées sur Scopus avec les mots clés "supercritical fluid chromatography" et "subcritical fluid chromatography"

Ce regain d'intérêt corrèle avec l'apparition des nouveaux appareils plus performants sur le marché, et également des colonnes plus performantes comme celles trouvées en HPLC. Ce sont des colonnes avec des diamètres de particules plus

faibles (sub-2 μm) ou à porosité superficielle, afin d'accroître les performances chromatographiques. Ces colonnes vont permettre d'atteindre de très hautes performances, des pics très fins avec de très hautes valeurs d'efficacité. Le terme d'UHPSFC (chromatographie en phase supercritique à très haute performance) est donc utilisé pour désigner la chromatographie utilisant les colonnes de faibles particules et des instruments modernes offrant des volumes morts réduits. Ce terme peut être comparé avec l'UHPLC (chromatographie liquide à très haute performance), qui est la variante à plus haute performance de l'HPLC avec des colonnes à petites particules (sub-2 μm) et des appareils plus performants avec, par exemple, des pompes pouvant aller à plus de 1000 bar.

Dans une mise au point [7], Caroline West a montré la répartition de l'utilisation de colonnes en fonction de la taille des particules dans les publications récentes (2016-2018). On voit sur la figure 1.2, que les colonnes de faibles particules, avec un diamètre inférieur à 2 μm , dominent les applications achirales en SFC. En revanche, ces colonnes ne sont pas encore bien implantées dans les applications chirales en raison de la faible disponibilité des phases stationnaires chirales à des faibles diamètres de particules. L'UHPSFC est donc bien implantée dans les mœurs concernant les applications achirales.

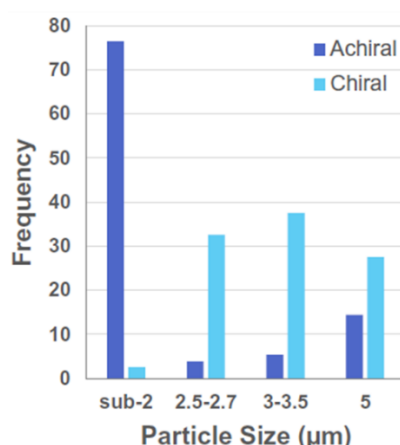


Figure 1.2 : Fréquence d'utilisation des phases stationnaires à diamètres de particules différents lors d'applications achirales et chirales en SFC entre 2016 et 2018. [7]

On peut voir sur les diagrammes de Van Deemter ci-dessous (figure 1.3) que la vitesse optimale est plus haute en SFC que celle retrouvée en HPLC et que le terme

C de la courbe (remontée après l'optimum) est plus faible. Ceci se traduit par des efficacités similaires retrouvées en HPLC mais avec des temps d'analyse beaucoup plus courts pour la SFC puisque l'on travaillera à des plus hauts débits. La même comparaison peut être faite entre l'UHPLC et l'UHPSFC où l'on peut voir, pour cette dernière, un plateau allant de 5 mm/s à plus de 12 mm/s. Les limites sont ici les performances de l'appareil chromatographique puisque les pompes sont limitées à 40 MPa, et plus récemment à 66 MPa. Il sera donc souvent impossible de travailler à de telles vitesses linéaires.

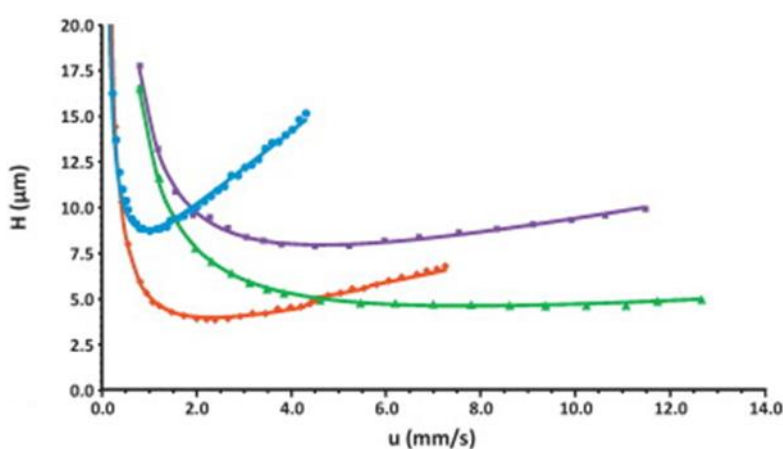


Figure 1.3 : Courbes de Van Deemter du butylparabène en HPLC (bleu), SFC (violet), UHPLC (rouge), UHPSFC (vert). Ces études ont été réalisées sur des colonnes de différentes granulométrie : 1,7 μm pour les tests en UHPLC et UHPSFC ; 3,5 μm pour les tests en HPLC et SFC. [8]

Un des avantages des fluides supercritiques se trouve dans leurs propriétés physico-chimiques. Comme il est montré dans le tableau 1.1, les fluides supercritiques présentent une masse volumique importante combinée à une faible viscosité. Le CO_2 aura une grande diffusivité, ce qui augmentera les performances chromatographiques de la SFC vis-à-vis de l'HPLC.

La proportion de co-solvant observée dans la plupart des applications SFC est inférieure à 50%. Il existe une technique intermédiaire entre la SFC et l'HPLC. C'est une technique avec des pourcentages importants de co-solvants et des proportions plus faibles de CO_2 . Lorsque la proportion de co-solvant est supérieure à 50%, la technique est généralement appelée EFLC (Enhanced-Fluidity Liquid Chromatography, ou chromatographie liquide à fluidité améliorée) [9]. L'utilisation

d'une faible proportion de CO₂ sous pression introduite dans un solvant hydro-organique a pour principal objectif de réduire la viscosité de la phase mobile et d'augmenter les coefficients de diffusion des analytes. Il est à noter que la plupart des instruments actuellement disponibles pour le SFC permettent d'utiliser toute la gamme de composition de phase mobile, allant de 100% de CO₂ à 100% de co-solvant. Les deux techniques chromatographiques SFC et HPLC sont alors combinées dans une forme de chromatographie unifiée (UC). Bien que cette idée ait été exprimée il y a 20 ans par Chester [10], elle a été démontrée beaucoup plus tard, notamment par le groupe de T. Bamba pour l'analyse des analytes lipophiles et hydrophiles au cours d'une même analyse [11]. Dans cette étude, Taguchi *et al.* ont utilisé un gradient d'élution allant de 2 à 100% de co-solvant à la fin du gradient. Ce large gradient était nécessaire pour l'élution des vitamines liposolubles et hydrosolubles dans la même analyse. Une autre méthodologie similaire a été proposée par Desfontaine *et al.* [12] où les métabolites non polaires et polaires (y compris certains acides aminés natifs) ont été analysés avec une élution à gradient unique, également comprise entre 2 et 100% de co-solvant.

2. Diagramme d'état et fluide supercritique

Tous les composés, lorsqu'ils sont purs, peuvent se retrouver sous trois états de la matière différents : gaz, liquide et solide. Cet état dépend de la pression et de la température dans lesquelles se trouve ce composé. Des diagrammes existent et permettent de représenter ces différents états de la matière en fonction de la pression et de la température, comme le montre la figure 1.4.

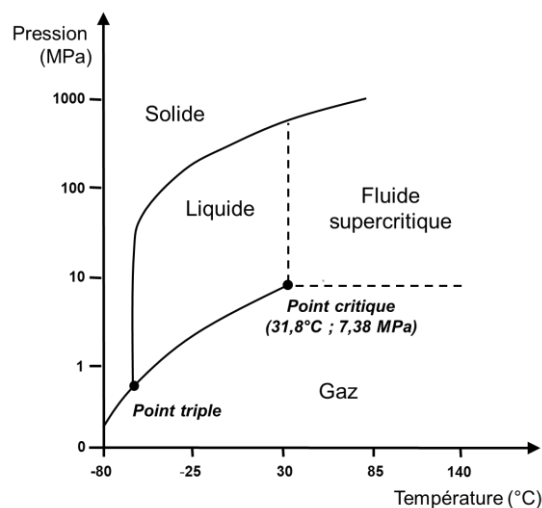


Figure 1.4 : Diagramme de phases d'un corps pur (exemple : le dioxyde de carbone)

Sur cette figure, les courbes représentent les changements d'état pouvant intervenir entre ces trois états de la matière : la sublimation entre le solide et le gaz, la fusion entre le solide et le liquide et enfin la vaporisation entre le liquide et le gaz . Ces diagrammes d'état mettent en évidence deux points particuliers : le point triple et le point critique. Le point triple est défini par la coexistence des trois états de la matière, seul endroit du diagramme d'état où l'on retrouve cet équilibre. Sur les courbes de changement d'état, le composé se retrouve en équilibre entre deux états de la matière. Et partout ailleurs ce composé existe sous un seul état de la matière. Le deuxième point particulier est le point critique, il se trouve à l'extrémité de la courbe de vaporisation. Ce point se caractérise par une pression critique, P_c , et une température critique, T_c . Au-delà de ces deux paramètres, le composé se retrouvera dans un état appelé supercritique. Les transitions entre la phase liquide et supercritique, et de même entre la phase gazeuse et supercritique, sont définies par des changements continus des propriétés physiques à la différence de tout autre changement d'état, marqué par un trait continu sur le diagramme d'état. Ce phénomène de continuité est illustré sur la figure 1.5, où l'on remarque la disparition de l'interface liquide/vapeur, présente sur la représentation la plus à gauche, lorsque l'on élève la température et la pression. Cette figure traduit l'homogénéité d'un fluide à l'état supercritique.

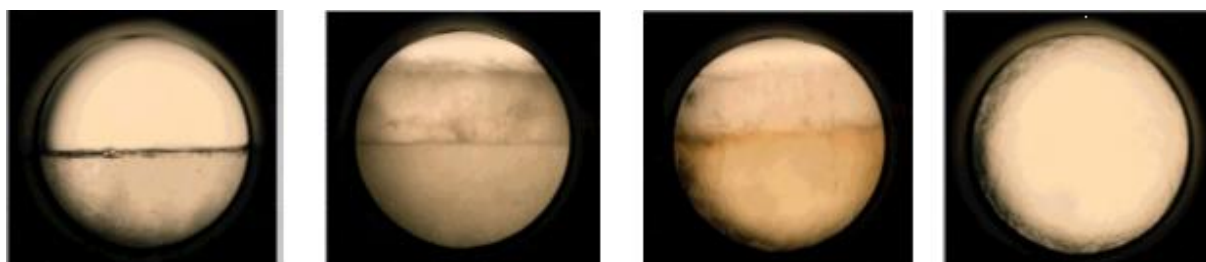


Figure 1.5 : Evolution de l'interface liquide/vapeur en fonction de l'augmentation de la température et de la pression, passage d'un mélange biphasique à un mélange homogène [13]

3. Propriétés physico-chimiques d'un fluide supercritique

Cet état supercritique confère des propriétés physico-chimiques particulières au fluide concerné. L'état supercritique étant un état de la matière intermédiaire entre le liquide et le gaz, les fluides supercritiques vont partager des propriétés intermédiaires de ces deux états. La viscosité d'un fluide supercritique est proche de celle d'un gaz, alors que sa masse volumique est proche d'un liquide. Un autre paramètre physico-chimique, le coefficient de diffusion, est quant à lui intermédiaire entre celui d'un liquide et d'un gaz. Ces résultats sont visibles dans le tableau 1.1.

Tableau 1.1 : Propriétés physiques des liquides, fluides supercritiques et gaz [14]

	Viscosité (cPoise)	Coefficient diffusion D_m (cm ² /s)	Masse volumique ρ (g/cm ³)
Liquides (30°C, 1atm)	0,2 – 3	$(0,2 – 3,5) \times 10^{-5}$	0,6 – 1,6
Fluides supercritiques (conditions variables)	0,01 – 0,1	$(0,2 – 7) \times 10^{-4}$	0,2 – 1,1
Gaz (30°C, 1atm)	0,01 – 0,03	0,1 – 0,4	$(0,6 – 2) \times 10^{-3}$

Ces propriétés intermédiaires entre un liquide et un gaz confèrent aux fluides supercritiques la possibilité de travailler à un haut débit, grâce à la faible viscosité. En ajoutant la forte masse volumique et un coefficient de diffusion intermédiaire, ce fluide aura une grande diffusivité et permettra à la SFC d'être une technique à haute efficacité.

4. Utilisation du dioxyde de carbone (CO₂) comme fluide supercritique

Plusieurs fluides peuvent être utilisés à l'état supercritique en chromatographie. On les retrouve dans le tableau 1.2.

Tableau 1.2 : P_c et T_c de fluides pouvant être utilisés en chromatographie [15]

	T_c (°C)	P_c (bar)
<i>Trifluorométhane</i>	26	48
<i>Dioxyde de carbone</i>	31	74
<i>Oxyde nitreux</i>	37	73
<i>Ammoniaque</i>	132	113
<i>Méthanol</i>	240	80
<i>Eau</i>	374	221

Comme le présente le tableau, certains points critiques ne sont pas aussi accessibles que le CO₂. Par exemple l'eau nécessite une température de 374°C et une pression de 221 bar pour passer à l'état supercritique. Certains articles utilisent cependant l'eau subcritique (eau chauffée sous haute pression) pour faire de l'extraction de molécules d'intérêt provenant de plantes, comme des anti-oxydants provenant du romarin [16]. Une étude tente aussi de comparer les méthodes utilisant l'eau subcritique avec des méthodes traditionnelles, comme l'extraction au Soxhlet, sur des clous de girofle [17]. L'eau subcritique a également permis d'extraire des huiles essentielles de la plante *Thymbra spicata* [18] ou bien l'extraction de lipides de microalgues (*Spirulina platensis* et *Nannochloropsis salina*) [19]. Le méthanol (MeOH) a aussi été utilisé comme fluide supercritique pour la synthèse de biocarburants et aussi de nanoparticules d'oxyde de zinc [20]. Un fluide, aux valeurs critiques proche de celles du dioxyde de carbone, a été utilisé fin des années 90 : il s'agit du trifluorométhane [21,22]. Mais, parmi tous ces fluides, celui qui s'est très vite imposé comme étant accessible et facile d'utilisation a été le CO₂.

Le CO₂ présente de nombreux avantages vis-à-vis de tous les autres fluides pouvant être utilisés en SFC. Le premier a déjà été cité dans ce manuscrit, c'est la facilité d'atteindre les conditions supercritiques du CO₂ avec une température et une

pression critique assez basses à la différence de tous les autres exemples du tableau 1.2. Il est moins contraignant, d'un point de vue instrumental, de devoir chauffer le CO₂ au-delà de 31°C que l'eau à 374°C. L'oxyde nitreux pourrait être une bonne alternative mais une étude a montré que c'est un oxydant relativement fort et qu'il peut provoquer des explosions [23].

Le deuxième avantage du CO₂ est économique et écologique. Il est non inflammable, non toxique, chimiquement inerte et non corrosif. Il est produit en grande quantité au niveau industriel, car il s'agit d'un sous-produit de réaction dans quelques processus industriels à grande échelle. C'est donc un solvant relativement peu coûteux et disponible en haute pureté.

II. Applications « naturelles » de la SFC

Depuis les années 1960 où elle a vu le jour, en grande partie grâce aux travaux de Klesper et al [2], la SFC (nommée HPGC à l'époque) a mis du temps à s'imposer dans le domaine de la chromatographie en grande partie à cause de l'avènement de l'HPLC, comme dit précédemment dans la première partie.

Durant les années 1980, la SFC est vue comme une technique intermédiaire entre la GC et à l'HPLC mais en raison de la faible fiabilité des appareillages et de la mauvaise reproductibilité des analyses, les applications sont encore restreintes. Seul le domaine pétrochimique l'utilise et « beaucoup de pionniers de l'industrie pharmaceutique qui ont testé l'instrumentation disponible dans les années 1980 ont constaté que la technologie était très limitée, voire inutile en raison de sa faible reproductibilité » et de considérer la SFC comme « une technique de séparation considérée révolutionnaire à son introduction mais dont la réputation s'est détériorée au fil des années » [24]. Malgré tout, un système dédié à la SFC a été développé et commercialisé par Hewlett-Packard. Il reprend les parties d'une chaîne d'HPLC avec un régulateur de pression en plus pour maintenir le fluide à l'état supercritique [25]. Les auteurs ont démontré, dans cet article, que la SFC donnait des efficacités supérieures pour des particules allant de 3 µm à 10 µm, essentiellement pour des vitesses linéaires plus grandes lorsque l'on compare à l'HPLC. Ce constat est le même

que l'on peut faire sur les courbes de Van Deemter présentées plus tôt dans ce manuscrit sur la figure 4. De plus, durant cette décennie, Novotny et al ont introduit en 1981 la SFC sur colonnes capillaires, colonnes semblables à celles retrouvées en GC [26]. Malgré ces avancées, la SFC est restée dans l'ombre de la GC et de l'HPLC durant cette décennie.

Dans les années 90, la SFC connaît un regain d'intérêt grâce aux travaux de celui que beaucoup de chromatographistes considèrent comme le père de la SFC moderne, Terry Berger. Il a étudié les composés polaires en SFC utilisant cette fois des colonnes remplies (ou « packed columns » en anglais) (et non plus des colonnes capillaires qui dominaient la décennie précédente) ainsi que l'impact de l'ajout d'un additif dans la phase mobile sur la forme des pics [27–29]. Deux personnes ont publié des livres sur l'utilisation de la SFE et SFC avec colonnes remplies dans les années 1994-1995 [30,31]. A cette époque, la SFC est retrouvée dans plusieurs domaines, comme par exemple en pharmaceutique avec l'analyse de barbituriques en CO₂ pur [32], de sulfonamides [33,34] ou encore d'alkaloïdes opiacés [35]. Des pesticides ont également été analysés durant cette décennie [36,37]. Plusieurs applications chirales ont vu le jour [38–42] avec des études des phases stationnaires différentes, comme une phase chirale de type Pirkle [39], une phase polysaccharide [40], ou d'autres sélecteurs chiraux plus exotiques [41,42]. Plusieurs de ces études comparent les performances obtenues en HPLC vis-à-vis de la SFC, et les efficacités sont généralement meilleures et les temps de rétention plus courts [38,40,42]. Ces applications chirales ont permis à la SFC de se développer pendant les années 90. Et jusqu'au début des années 2010, seules ces séparations chirales, et surtout l'intérêt de la SFC chirale préparative, en raison de l'économie de solvant réalisée comparé à l'HPLC préparative, ont maintenu la SFC en vie.

Ces dernières années, plusieurs développements relatifs à la SFC ont participé au nouvel essor de cette technique chromatographique. Les premiers développements se sont faits sur les appareils, avec l'apparition de systèmes chromatographiques beaucoup plus performants (système Aurora d'Agilent et UPC² de Waters), qui ont résolu en grande partie les problèmes que l'on pouvait avoir avec les anciens systèmes (bruit de fond en UV trop important, mauvaise répétabilité, grands volumes extra-colonne). Les améliorations du couplage à la spectrométrie de masse ont également permis d'ouvrir la SFC à plus d'applications. En effet, il s'agit d'un mode de détection

spécifique et c'est le plus utilisé désormais. Dernièrement, un nouvel appareil a été commercialisé par Shimadzu (Nexera UC) qui permet de coupler en ligne un système d'extraction par fluide supercritique (SFE) avec un système de SFC. Le système complet est donc SFE-SFC-UV-MS.

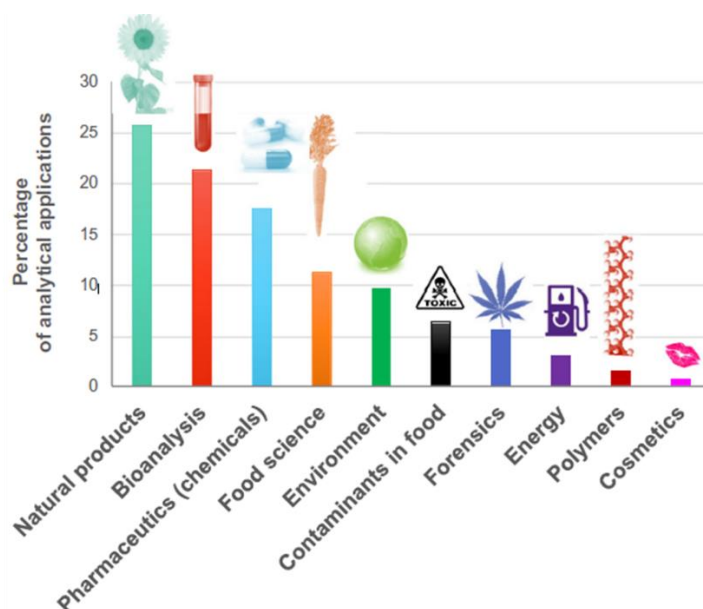


Figure 1.6 : Domaines d'application de la SFC entre 2014 et 2018 [7].

Concernant les applications de la SFC ces dernières années, elles sont en grande partie dirigées vers les produits naturels, les bio-analyses et également le domaine pharmaceutique comme on peut le voir sur la figure 1.6.

Le CO₂ supercritique est un solvant peu polaire ou non polaire. La valeur de sa constante diélectrique, qui est liée à la polarité d'un solvant, est proche de celle des alcanes. Etant donné sa faible polarité, le CO₂ supercritique pur est utilisé pour l'analyse de composés apolaires. Des espèces modérément polaires peuvent être éluées en augmentant la densité du fluide grâce à un gradient de pression. En revanche, par ajout d'un co-solvant dans la phase mobile, les propriétés de polarité vont changer. Des solvants miscibles au CO₂ pressurisé, comme le MeOH ou encore l'ACN vont pouvoir être ajoutés à ce dernier afin d'augmenter la polarité de la phase mobile et permettre d'analyser des molécules de plus en plus polaires. La polarité de la phase mobile peut encore être améliorée par ajout d'un additif et/ou d'eau en son

sein. Le seul problème à cet ajout d'eau est que celle-ci n'est pas miscible au CO₂ pressurisé en toutes proportions et donc la présence d'un co-solvant assure la miscibilité du mélange ternaire, en étant prudent de ne pas entrer dans la zone de démixtion. Il reste donc des choses à faire dans le domaine de l'analyse des composés polaires en SFC en essayant d'aller au plus près de cette zone de démixtion.

III. Analyse de composés polaires non ionisables en SFC

1. Qu'est-ce que la polarité ?

La polarité d'une molécule est une caractéristique qui décrit la répartition des charges partielles positives et négatives au sein de celle-ci. La polarité est due à la différence d'électronégativité de deux atomes, un atome va attirer à lui les deux électrons engagés dans une liaison. Plus la répartition de ses charges est symétrique, et plus la molécule sera apolaire. Un exemple avec le CO₂ qui est une molécule plane, deux doubles liaisons lient l'atome de carbone avec chacun des atomes d'oxygène. Il y a une différence d'électronégativité entre le carbone et l'oxygène, ce dernier est plus électronégatif, il attire plus à lui les électrons. Cette différence d'électronégativité va induire des charges partielles δ^+ , portées par l'atome ayant l'électronégativité la plus faible, celui qui « cède » une partie de ses électrons, et δ^- , portées par l'atome ayant l'électronégativité la plus forte, celui qui « récupère » une partie des électrons de la liaison. Ces deux charges partielles forment un moment dipolaire partiel entre ses deux atomes. Mais étant donné que cette molécule est linéaire et symétrique, les deux moments dipolaires partiels vont s'annuler en s'additionnant. A l'inverse, la molécule d'eau (H₂O) n'est pas linéaire, les deux hydrogènes sont liés à l'oxygène et forment un angle de 104,45°, les deux moments dipolaires ne s'annulent donc plus à l'addition et créent ainsi un moment dipolaire permanent non nul. L'eau est donc une molécule polaire à la différence du CO₂ qui est apolaire. La figure 1.7 illustre bien ces propos.

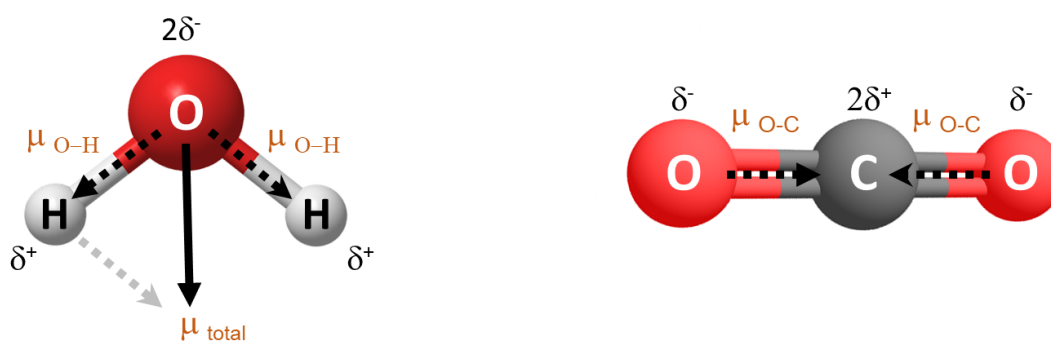


Figure 1.7 : Structure des molécules d'eau et de dioxyde de carbone illustrant la différence entre une molécule apolaire (le dioxyde de carbone) et une molécule polaire (l'eau).

Cependant, le moment dipolaire d'une molécule ne suffit pas à caractériser ses capacités d'interaction dans un système chromatographique. Nous pouvons prendre comme autre définition le coefficient de partition octanol-eau, $\log P$ ou $\log K_{o/w}$ (ou $\log D$ puisque nous nous intéressons à des espèces ionisables). Cela revient donc à s'intéresser aux capacités d'interaction avec l'eau, considérée comme le solvant le plus polaire dans notre cas. Il faut cependant faire une distinction entre les composés polaires neutres et les composés polaires ionisables.

La polarité est une notion assez vague, elle varie d'un chimiste à un autre. Les composés polaires pourraient être considérés comme étant des composés ayant une valeur de $\log P$ inférieur à 0, c'est-à-dire un composé qui sera plus soluble dans l'eau que dans l'octanol.

2. Composés polaires non ionisables en SFC

Dans une mise au point, Caroline West [43] présente les possibilités de la SFC pour analyser les composés polaires. Tous les articles cités emploient une phase stationnaire polaire pour l'analyse de composés polaires. Il peut s'agir de phase stationnaire silice, ou encore des phases greffées avec un ligand aminopropyl, un cyanopropyl ou un propanediol. La phase polaire ne suffit pas pour éluer les composés polaires, il faut également une phase mobile capable de les « décrocher » de la phase

stationnaire, donc avec une certaine polarité. Elle se demande aussi si l'eau, considérée comme le meilleur additif (ou co-solvant ?) n'est pas la solution. Cette eau permet d'augmenter la polarité de la phase mobile, et améliorerait la forme des pics [44].

La SFC est une technique qui est bien définie pour toutes les applications de composés apolaires. Cependant, comme dit précédemment en fin de partie II, il resterait à définir les limites en termes de gamme de polarité des molécules analysables en SFC.

En SFC, il est dit que l'on peut analyser facilement des composés qui sont solubles à hauteur de 1 mg/mL dans le MeOH [45]. En revanche, la SFC est généralement reconnue comme étant incapable d'analyser des composés solubles dans l'eau [29]. Cependant, plusieurs études ont montré que l'analyse de composés solubles dans l'eau était possible, avec par exemple l'analyse d'une molécule dissoute dans l'eau [46], les pics n'étaient pas bien définis mais l'ajout d'1 mM d'acide citrique comme additif a permis d'avoir un pic bien défini mais un peu dissymétrique. Des travaux de Mukherjee et Cook [47,48] sur des molécules pharmaceutiques ont également montré des analyses de composés polaires solubles dans l'eau. La liste des molécules analysées est récapitulée dans le tableau 1.3 à la fin de cette sous-partie.

Deux choses sont donc nécessaires pour analyser des composés polaires en SFC :

- Une phase stationnaire assez polaire pour pouvoir retenir les composés.
- Une phase mobile plutôt polaire pour permettre une bonne solubilité des composés et pour également éluer les composés assez rapidement.

Dès 1990, des études ont été effectuées dans notre institut, l'ICOA, sur des composés polaires non ionisables avec des glucopyranosides, tel que le glucose, sur une colonne cyanopropyle [49] avec une phase mobile composée d'un mélange CO₂/MeOH (93,75/6,25, v/v). Dans ces conditions, le glucose est élué assez rapidement mais le pic est large. D'autres études du même groupe ont été faites sur d'autres sucres (mannose, fructose, xylose, ...) sur des phases polaires de type propanediol, nitropropyle ou cyanopropyle [50]. Une autre équipe française a également réussi à éluer des sucres sur une colonne plutôt apolaire de type C18, avec

seulement 7,5% de MeOH dans le dioxyde de carbone [51]. Plus récemment, Pauk *et al.* [52] ont analysé des monosaccharides en gradient d'élution allant de 5% à 30% de co-solvant, un mélange de MeOH, d'eau et d'acide formique (91:5:4 ; v/v/v) . Des glycosphingolipides, qui sont des chaînes glucidiques liées à une molécule de sphingosine, ont également été analysés avec une détection à ionisation de flamme (FID) avec des colonnes capillaires [53]. Ces molécules sont amphiphiles du fait de la partie lipidique, ce qui les rend plus apolaires que des sucres simples. Un autre article de Deschamps *et al.* traite de ces molécules en SFC sur colonne remplie [54], où ils sont analysés en gradient avec jusqu'à 40% de MeOH. Le détecteur le plus utilisé pour les glucides est le détecteur évaporatif à diffusion de lumière (DEDL) [55–57]. L'utilisation du FID n'est maintenant plus possible à cause de l'ajout d'un co-solvant polaire afin d'obtenir une meilleure solubilité des glucides. A l'ICOA, Salvador *et al.* [57] ont donc utilisé la SFC-DEDL pour la séparation de glucides. Une séparation de huit monosaccharides a été obtenue en moins de dix minutes avec une phase mobile composée de 20% de co-solvant dans le CO₂ sur une colonne aminopropyle. Le co-solvant étant constitué d'un mélange MeOH/H₂O/TEA dans les proportions suivantes : 91,5/8,0/0,5. Toujours dans notre laboratoire, Morin-Allory et Herbreteau [55] utilisent la SFC-DEDL pour l'analyse de douze sucres et des polyols, et Herbreteau *et al.* [58], pour l'analyse de sucres méthylés et cyclodextrines, ont comparé la SFC-DEDL avec la SFC-MS, cette dernière pouvant donner des informations structurales sur le nombre de groupes méthyles et leurs positions. Une dernière étude, en EFLC, a été décrite par Bennett et Olesik [59] sur l'inuline, un fructane, qui est un polysaccharide trouvé dans de nombreuses plantes. Cette molécule est analysée en mélange ternaire avec de très faibles proportions de CO₂, et un mélange de MeOH et d'eau. La proportion de CO₂ varie de 20% à 5% au cours de l'analyse. Le co-solvant utilisé est un mélange hydroalcoolique à différentes proportions. De la triéthylamine est ajoutée, à hauteur de 2%, dans le co-solvant afin d'améliorer la forme des pics. Toutes ces molécules sont analysées sur des phases polaires (cyanopropyle, aminopropyle ou propanediol) avec de faibles pourcentages en CO₂ aux alentours de 10%. Ces molécules ne nécessitent pas d'additifs ou de forts pourcentages de co-solvant pour être éluées.

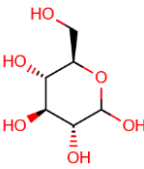
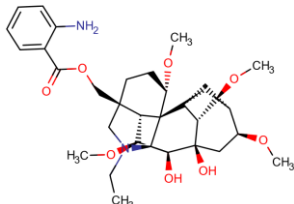
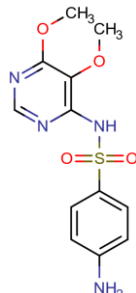
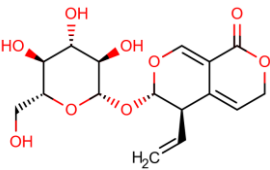
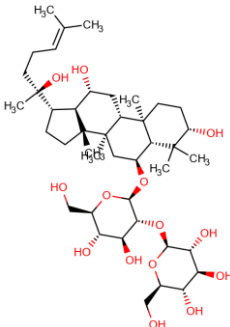
Une autre famille de composés polaires non ionisables a été bien étudiée, il s'agit de la famille des benzamides ou sulfonamides. Ce sont des composés possédant une fonction amide, composée d'un groupement carbonyle lié à un atome d'azote. Selon

les calculs délivrés par le site Chemicalize (consulté le 03/05/2019), ces composés ont un log P aux alentours de 0, ils sont donc autant solubles dans une phase apolaire que dans une phase polaire. Perkins *et al.* [34] s'est penché sur la séparation de dix sulfonamides avec huit d'entre eux entièrement séparés en dix minutes sur des phases polaires de type silice ou aminopropyle. Un gradient d'élution de 15% de co-solvant à 25% dans le CO₂ a permis d'atteindre cette séparation. Des conditions similaires ont été utilisées par Dost *et al.* [60] pour la séparation de six sulfonamides en quatorze minutes avec cette fois ci une phase de type cyanopropyle en utilisant une double détection UV-MS. Terry Berger s'est également intéressé à l'analyse de composés amidés [61,62]. Dans la plus ancienne publication (1993) [61], un additif basique, l'isopropylamine, a été ajouté à la phase mobile afin de symétriser les pics puisqu'il s'intéressait à un mélange de dérivés d'aniline, de benzylamine et de benzamide. Ces deux premières molécules étant basiques, un additif est recommandé pour améliorer la forme des pics. Concernant uniquement les benzamides, une séparation de cinq molécules a été atteinte en vingt minutes sur une colonne de type silice en mode isocratique avec 10% de MeOH dans le CO₂. Dans la publication la plus récente (2013) [62], il utilise une colonne remplie de particules sub-2 µm et arrive à séparer 9 sulfonamides en 4 min. La colonne utilisée est encore une fois une silice, avec une granulométrie de 1,8 µm, il s'agit donc d'UHPSFC. Un couplage de colonnes est aussi possible comme le montre Combs *et al.* [63] avec le couplage d'une colonne de type silice avec une colonne aminopropyle pour l'analyse de huit sulfonamides. Différents couplages ont été testés entre une colonne cyanopropyle suivie d'une aminopropyle ou alors une colonne silice suivie d'une aminopropyle afin d'éliminer des coélutions, et des différences de sélectivité ont été constatées. Berry *et al.* [33] ont également analysé des sulfonamides avec une phase stationnaire faite de silice. Mais ils ont aussi étudié d'autres composés polaires comme des xanthines (caféine, théophylline et théobromine) et des pesticides avec les mêmes conditions, c'est-à-dire des analyses sur des phases polaires (silice ou aminopropyle) en isocratique avec des proportions de MeOH entre 10% et 15% dans le CO₂. A travers ces travaux, on remarque que des phases polaires (silice, cyanopropyle, aminopropyle, propanediol) sont recommandées, et très fréquemment utilisées, pour analyser les molécules amidées et sulfamidées.

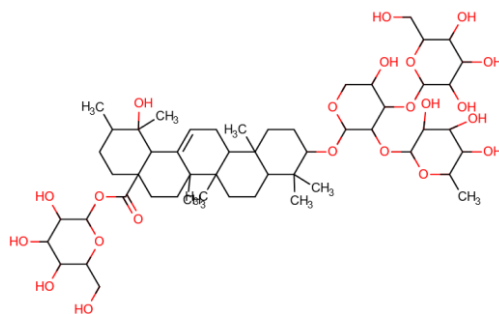
Des molécules naturelles ont été extraites pour ensuite être analysées en SFC. C'est le cas des feuilles de maté et du saule blanc. Alexandre Grand-Guillaume Perrenoud *et al.* [64] décrit l'analyse d'extraits de ces plantes contenant plusieurs familles de molécules comme des alcaloïdes, des terpènes, des flavonoïdes et aussi des iridoïdes. Cette dernière famille est intéressante car il s'agit de petits terpènes polaires, portant généralement un résidu glucose. Les iridoïdes possèdent plusieurs propriétés pharmaceutiques, telles que des propriétés cardiovasculaires, anti-inflammatoires ou encore antitumorales. Ces analyses sont réalisées en UHPSFC avec une colonne sub-2 μm , la Torus Diol. La phase mobile utilisée est un mélange de MeOH contenant 10 mM de formiate d'ammonium ainsi que 2% d'eau. Ce co-solvant étant bien évidemment mélangé dans le CO_2 . Les analyses sont réalisées en gradient commençant à 2% de co-solvant et se finissant à 40%. Grâce à la double détection UV et MS, un iridoïde a pu être identifié et il s'agit de la molécule de gentiopicroside ($\log P = -3,03$), c'est donc une molécule très polaire, 1000 fois plus soluble dans l'eau que dans l'octanol. Dans ces conditions, cette molécule est éluée en cinq minutes, vers la fin du gradient où le pourcentage de co-solvant est le plus fort et donc la polarité de la phase mobile plus importante. Un autre exemple de molécule polaire non ionisable naturelle a été décrit par Huang *et al.* [65]. Dans cet article, des nucléosides, des nucléobases et des ginsenosides sont analysés dans des extraits de Ginseng. Ces composés sont porteurs de nombreuses fonctions hydroxyles, ce qui les rend polaires. Ils ont étudié les effets du co-solvant utilisé ainsi que de l'additif présent dans la phase mobile et ont conclu que le mélange idéal est du MeOH avec 5 mM d'acétate d'ammonium. Les analyses se déroulent une nouvelle fois sur une phase stationnaire polaire de type silice, avec un gradient d'élution allant de 15% à 35% de phase mobile. Ce même groupe a également étudié des saponines triterpénoïdes, comme les kudinosides, des triterpènes glycosylés (de xylose, rhamnose ou glucose) [66]. Ce sont des molécules amphiphiles avec des $\log P$ négatifs. Une phase silice a été utilisée pour la séparation de six kudinosides. Un gradient d'élution commençant à 30% de co-solvant et finissant à 50% a été employé ce qui a permis à ces six kudinosides d'être séparées en dix minutes. L'impact de l'eau, en tant qu'additif, a été discuté et il s'avère qu'elle est nécessaire. En effet, lors des essais sans eau, les résultats sont décevants, signal sur bruit très faible et mauvaise séparation. Dès lors que de l'eau est ajoutée, la séparation s'améliore et les pics s'affinent. 10% d'eau dans le MeOH a été retenu comme étant les meilleures conditions d'analyse pour la suite. Ils ont ensuite évalué

l'impact d'ajout d'un additif et encore une fois on observe une meilleure réponse avec des pics plus fins. Le co-solvant final est donc un mélange de MeOH avec 10% d'eau et 0,05% d'acide formique. Un dernier exemple de molécules naturelles concerne les ginkgolides [67], des diterpènes riches en oxygène (lactones et hydroxyles, $\log P \approx 0$) provenant du *Ginkgo biloba*, un arbre qui aurait survécu à l'explosion de la bombe atomique à Hiroshima. Ils analysent ces molécules en utilisant un gradient allant de 10% à 35% de co-solvant, un mélange de MeOH, de 5% d'eau et 10 mM d'acétate d'ammonium.

Tableau 1.3 : Molécules polaires non ionisables (noms, structures, log P*, références)
 *Les log P sont déterminés sur Chemicalize (ChemAxon)

Nom	Structure	log P	Référence
Glucose (et dérivés)		-2,93	[49–52, 55–58]
Inuline		0,88	[59]
Sulphadoxine (Sulfonamides)		0,58	[34,60–63]
Gentiopicroside (Iridoïdes)		-1,38	[64]
Ginsenosides		0,68	[65]

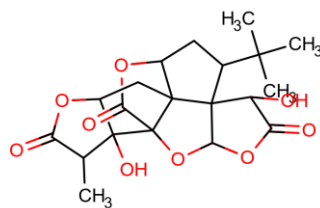
Kudinosides



-0,68

[66]

Ginkgolides



0,34

[67]

IV. Analyse de composés polaires ionisables en SFC

Des composés polaires non ionisables sont donc analysés en SFC. Qu'en est-il alors des composés ionisables ? Un composé ionisable est un composé qui peut exister sous différentes formes, moléculaire et ionique, en fonction du pH de la phase mobile. En SFC, lorsque l'on travaille avec une phase mobile classique contenant un pourcentage modéré de MeOH dans le CO₂, le pH apparent de cette phase mobile sera aux alentours de 5 [68]. A ce pH, des composés peuvent se trouver sous leurs formes ioniques. C'est le cas pour des acides forts ou bases fortes par exemple. Le pH sera supérieur au pKa des acides, ils seront donc déprotonés et inversement pour les bases qui se trouveront protonées à un pH inférieur à leurs pKa.

1. Molécules polaires acides

Des molécules polaires avec des fonctions acides ionisables ont été analysées en SFC. Il peut s'agir de groupements carboxylates, qui se trouveront déprotonés au pH apparent de la phase mobile ou encore de groupements phosphates, phosphonates ou sulfoniques. La liste des molécules analysées est récapitulée dans le tableau 1.4 à la fin de cette sous-partie.

Dans leur article, Berger et Deye [27] ont étudié l'effet des additifs sur la séparation de trois acides benzoïques et ce sur cinq phases stationnaires différentes. Un des acides étudiés est l'acide trimellitique, un cycle aromatique portant trois fonctions acides carboxyliques, son log P est aux alentours de 1. 0,2% d'additifs ont été ajoutés dans du MeOH et ils ont remarqué que lorsque les acides utilisés en tant qu'additifs ne sont pas plus forts que les acides étudiés, il n'y a pas d'amélioration de la forme des pics. Il faut donc utiliser en additif un acide plus fort que les acides que l'on veut analyser. Berger et Deye ont également étudié les acides hydroxybenzoïques [69]. Ils ont tous un log P aux alentours de 1 et ils parviennent à séparer dix acides hydroxybenzoïques en deux minutes sur une phase cyanopropyle, avec une phase mobile constituée de 7,4% de MeOH dans le CO₂. Un additif a été ajouté, afin d'améliorer la forme des pics des acides étudiés, il s'agit de l'acide citrique à 0,6%.

Des essais ont été faits sans additifs et il a un impact significatif sur la rétention et surtout sur la forme des pics. Jiang *et al.* [70] ont étudié des acides phénoliques en SFC. Ils ont testé plusieurs phases stationnaires différentes pour ces analyses : une phase BEH de silice hybride ; une phase BEH 2-éthylpyridine ; une phase CSH pentafluorophényl ; et une phase HSS C18 SB. Avec la phase HSS C18 SB, la moins polaire, les composés sont élués le plus rapidement sans une bonne séparation. Concernant les trois autres phases, les meilleurs résultats en termes de forme de pics ont été obtenus sur la BEH. Ils ont aussi évalué la meilleure phase mobile à utiliser entre du MeOH pur, de l'ACN pur ou alors un mélange des deux. Les meilleures conditions furent une phase mobile contenant du CO₂ avec un co-solvant fait d'un mélange de MeOH et d'ACN avec 1% de l'acide trifluoroacétique (TFA) en gradient d'élution allant de 12% à 40% de co-solvant. L'additif, un acide fort, est ajouté en raison de l'analyse de composés ioniques. Il a été démontré que les additifs améliorent la forme des pics [27,71].

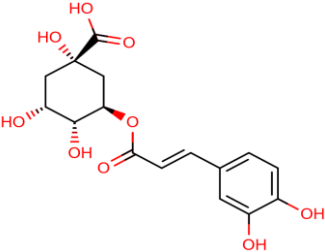
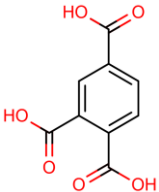
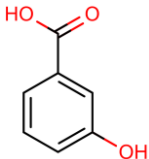
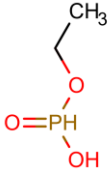
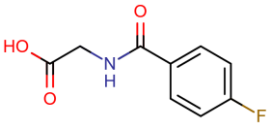
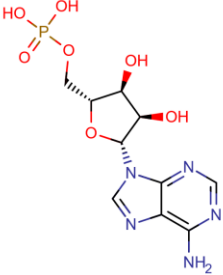
Des études se portent à la fois sur des acides et des bases. Comme Ishibashi *et al.* [72] qui se penchent sur l'analyse de pesticides par SFC-MS. Ils comparent trois phases stationnaires différentes, une phase C18 hydrophobe, une autre phase C18 avec un groupement polaire intercalé et une phase cyanopropyle. L'analyse se fait en gradient d'élution allant de 5% à 40% de co-solvant, un mélange de CO₂ et de MeOH avec 0,1% de formiate d'ammonium. Un additif basique a été ajouté ici puisque cet article traite à la fois de pesticides acides et basiques. Un pesticide polaire acide analysé est le Fosetyl, il s'agit d'un fongicide pour la viticulture ou encore utilisé contre le mildiou. Ce pesticide porte un groupement phosphonate et son log P est de -2,70 (log D = -5,3 à pH 5,5) [72], ce qui en fait un composé très polaire. Sur la phase C18 hydrophobe, ce pesticide est élué rapidement en raison de sa forte affinité pour la phase mobile. En revanche avec les deux phases plutôt polaires, ce pesticide est plus retenu et les dix-sept pesticides sont quasiment complètement séparés en onze minutes lors de l'analyse avec la phase C18 avec un groupement polaire intercalé, l'Inertsil ODS-EP. L'hydrazide maléique, un pesticide basique, utilisé comme régulateur de croissance, est analysé de la même façon. C'est une molécule très polaire puisque son log P est inférieur à -2. Une autre étude porte sur des métabolites acides et basiques de la flupirtine, un analgésique, dans les urines [73]. Les métabolites polaires acides sont les acides 4-fluoro hippurique et 3,4-difluoro

hippurique. Ces molécules sont plutôt polaires, elles ont un log P de 0,67 et 0,81. Elles sont analysées sur la Lux Amylose-2, une phase polysaccharide avec un gradient d'éluion allant de 5% à 25% de co-solvant, un mélange de MeOH avec 0,225% d'eau et 0,075% d'ammoniaque.

Un dernier exemple est l'étude de nucléotides, comme l'adénosine monophosphate. Philibert et Olesik [74] étudient ces nucléotides avec l'EFLC. Les nucléotides sont analysés en EFLC sur une phase amide. Les conditions finales d'analyse ont été 90% de MeOH et 10% d'un mélange d'une solution de 75 mM de phosphate d'ammonium avec 5 mM de DBN (1,5-diazabicyclo[4,3,0]non-5-ène, une base forte) et 0,2 M de chlorure de sodium. Ils ont étudié l'impact qu'avait l'ajout de concentration croissante de CO₂ au sein de ce mélange complexe, et ont conclu que cela retardait l'éluion des composés polaires mais améliorait la séparation, en raison de son apolarité. L'ajout d'un additif impacte significativement la rétention des composés également.

Globalement, sur tous ces exemples, l'ajout d'un additif est quasiment indispensable pour favoriser la forme des pics ainsi qu'un certain pourcentage de co-solvant pour permettre l'éluion des composés polaires. Concernant les phases stationnaires, il faut aussi qu'elles possèdent des fonctions polaires pour permettre un minimum de rétention.

Tableau 1.4 : Molécules polaires acides (noms, structures, log P*, références)
 *Les log P sont déterminés sur Chemicalize (ChemAxon)

Nom	Structure	log P	Référence
Acide chlorogénique		-0,27	[70]
Acide trimellitique		0,95	[27]
Acide 3-hydroxybenzoïque		1,33	[69]
Fosetyl		-0,41	[72]
Acide 4-fluoro hippurique		0,67	[73]
Adénosine monophosphate		-4,75	[74]

2. Molécules polaires basiques

Des molécules polaires avec des fonctions basiques ont également été analysées. Ce sont généralement des fonctions amines qui vont être protonées à un pH de 5. La liste des molécules analysées est récapitulée dans le tableau 1.5 à la fin de cette sous-partie.

De nombreux articles traitent de l'analyse de composés pharmaceutiques basiques en SFC [75–78]. Parmi ces quatre exemples, trois antibiotiques sont traités. Le premier est l'isoniazide par Prajapati et Agrawal [75]. Cet antibiotique, agissant sur les bacilles de Koch, ainsi que deux de ses impuretés sont analysés sur une colonne C18. Cet antibiotique porte une fonction amine primaire et sera donc sous forme ammonium à un pH de 5. La phase mobile utilisée est un mélange de 85% de CO₂ avec 15% de co-solvant. Le co-solvant est un mélange de dichlorométhane avec du MeOH, de l'acétate d'éthyle et de l'acide formique dans les proportions 70/30/0,5/0,1. Cette même équipe de recherche s'est également penchée sur un autre antibiotique, l'éthambutol [76]. Ils l'analysent encore une fois sur une colonne C18, l'Inertsil ODS-C18. Du fait que son log P soit plus élevé (-0,06 pour l'éthambutol contre -0,69 pour l'isoniazide), il faut un pourcentage moins élevé de co-solvant polaire pour l'éluer. Ceci se traduit par le fait qu'une élution isocratique à 90/10 de CO₂/MeOH suffit pour éluer l'éthambutol en seulement trois minutes. Le troisième antibiotique étudié est la rifampicine, ainsi que ses impuretés, par Li *et al.* [77]. Ils font un criblage de plusieurs phases stationnaires afin de choisir la meilleure entre la phase UPC² BEH, Torus Diol, Torus 1-AA et Torus 2-Pic. Sur les deux dernières colonnes, la rifampicine est trop retenue dans toutes les conditions testées. Il reste donc la BEH et la Diol sur lesquelles ils vont tester l'impact de l'ajout d'un additif : formiate d'ammonium, acide formique, triéthylamine (TEA) et eau. Sur la BEH, le meilleur additif est la TEA pour l'amélioration de la forme des pics. En revanche, pour la Torus Diol, la meilleure combinaison d'additifs est un mélange de 0,1% de formiate d'ammonium et 2% d'eau. Les analyses ont été effectuées en gradient d'élution, partant de 15% de co-solvant et se finissant à 40%. Dans ces conditions, la rifampicine et ses 6 impuretés sont séparées en moins de quatre minutes. Après ces 3 antibiotiques, un autre exemple est celui d'un bêta-bloquant, le métoprolol [78]. Celui-ci et ses treize impuretés ont été analysés sur une phase aminopropyle en mode isocratique avec un mélange de 92% de CO₂ et de 8% de

MeOH sans additifs dans la phase mobile. Dans ces conditions, la séparation est effective en vingt minutes et les pics sont assez larges. Une amélioration pourrait être envisageable avec les résultats précédents, en ajoutant un additif puisque l'on a vu que cela favorisait la forme des pics, et aussi diminuait légèrement la rétention. Ramsey *et al.* [79] analyse entre autres le triméthoprim (log P = 0,79), un anti-infectieux utilisé dans le traitement des cystites ou de plusieurs infections (antibiotique aminopyrimidine). Cette molécule est éluée en dix minutes sur une phase stationnaire aminopropyle avec un gradient d'éluion allant d'une phase contenant 100% de CO₂ à un mélange 80/20 CO₂/MeOH. Une autre molécule pharmaceutique polaire basique a été étudiée dernièrement, il s'agit du salbutamol (log P = 0,34). Dispas *et al.* [80,81] ont développé une méthode d'analyse en SFC du salbutamol pour la comparer, et améliorer celle de référence. La méthode de référence d'analyse dans la Pharmacopée Européenne est une méthode de cinquante minutes utilisant du sodium heptanesulfonate comme agent d'appariement d'ions, c'est une méthode longue et difficile à mettre en place. Plusieurs études ont montré que la SFC pouvait être une alternative pour le contrôle qualité de médicaments [82–85]. Une méthode SFC a donc été créée utilisant la colonne Torus Diéthylamine (DEA) avec un gradient allant de 5% de co-solvant à 35% en fin de gradient. Le co-solvant utilisé a été du MeOH avec 0,1% d'hydroxyde d'ammonium. Avec cette méthode, le temps d'analyse est réduit de cinquante minutes à dix minutes. Une étude inter-laboratoires à laquelle nous avons participé a été menée suite à ce développement de méthode réunissant 19 laboratoires possédant une chaîne UPC² commercialisée par Waters afin d'étudier la reproductibilité inter-laboratoires de la SFC [81]. Il s'agit de la première étude inter-laboratoires en SFC, et les répétabilités et reproductibilités sont trouvées similaires ou meilleures que celles obtenues pour des méthodes HPLC, ce qui montre le travail des fournisseurs d'instruments analytiques pour que les appareils de SFC soient robustes et produisent des analyses répétables.

Des molécules polaires présentes dans les fluides et tissus biologiques ont également été analysées en SFC. Parmi ces molécules, on peut citer une cytokine, l'histamine, une substance de signalisation cellulaire [86]. Cette molécule a été étudiée par Dispas *et al.* au cours d'un plan d'expériences. L'utilisation de ce genre de méthodologie dans le développement de méthodes analytiques est assez innovante. Des exemples de développement de méthodes existent pour une séparation de 19

molécules contre la malaria [87], ou encore une séparation de 16 molécules pharmaceutiques candidates pour une administration par voie gingivo-jugale [88]. Elle est de plus en plus demandée dans les industries pharmaceutiques pour apporter de la robustesse aux méthodes développées en un temps assez court. Les plans d'expériences se caractérisent par l'obtention d'un « Design Space » ou espace de conception, zone où la méthode répond aux spécifications imposées au départ. Avec cet espace de conception, une nouvelle validation de méthode n'est pas nécessaire si l'on se trouve encore dans cet espace, ce qui permet de gagner du temps si des légères modifications de méthode sont nécessaires. Les guidelines ICH commencent à encourager l'utilisation de cette méthodologie [89]. Dans l'étude de Dispas *et al.*, deux plans ont été utilisés : un plan de criblage et un plan d'optimisation. Le plan de criblage est utilisé pour définir quels sont les facteurs qui sont significativement impactant sur la réponse regardée. Le plan d'optimisation est utilisé pour obtenir une zone de robustesse de la méthode et un point optimum où tous les critères fixés sont atteints. Le plan de criblage a permis de choisir la phase stationnaire idéale, ainsi qu'une phase mobile et un additif pour permettre l'élution de tous les composés en moins de quinze minutes. Le résultat fut une analyse sur la phase éthylpyridine avec du MeOH comme co-solvant et du TFA en additif. Le plan d'optimisation a permis de fixer tous les autres paramètres tels que la pente du gradient, la température ainsi que la concentration en additif. Les résultats finaux sont un gradient de 5% à 40% de co-solvant et un ajout de 25 mM de TFA.

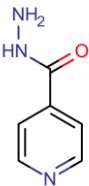
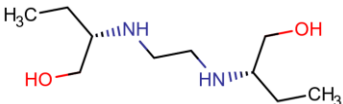
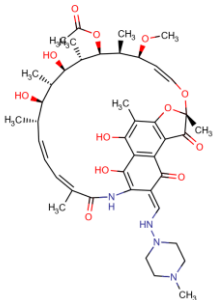
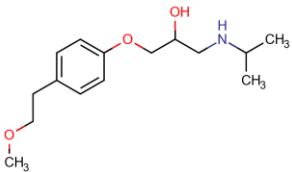
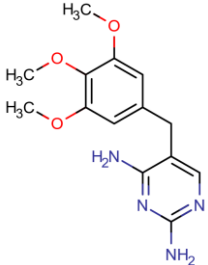
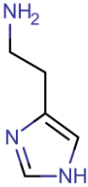
D'autres molécules biologiques ont été analysées, des nucléobases porteuses de fonctions basiques comme la cytosine [90]. Beaucoup d'expériences ont été réalisées pour trouver la bonne combinaison de phase stationnaire, phase mobile et additif afin d'avoir une séparation des quatre nucléobases. Les colonnes testées ont été des colonnes polaires de type cyanopropyle, propanediol ou éthylpyridine. Une très faible qualité de séparation a été observée avec la colonne Diol quel que soit l'alcool utilisé en co-solvant (MeOH, EtOH, PrOH, BuOH). Deux nucléobases, la thymine et l'uracile ont donné de très bonnes formes de pics lors de tests sans additifs, alors que les deux autres étaient larges et nécessitaient la présence d'un additif pour avoir une belle forme. Des tests avec l'eau comme additif ont été faits et cela a donné une bonne séparation des nucléobases avec des pics plutôt fins. Les conditions finales d'analyse ont été la phase éthylpyridine avec du MeOH en co-solvant et 5 mM d'acétate


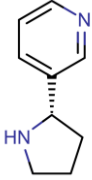
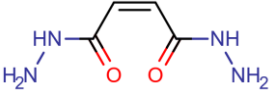
d'ammonium avec 5% d'eau. Les analyses sont réalisées en gradient partant de 20% de co-solvant et terminant à 50%, c'est une phase mobile relativement polaire qui est nécessaire pour l'élution de ces nucléobases. Un autre article traite des nucléobases en SFC [91] avec l'élution de cinq nucléobases en moins de 10 min à l'aide d'un mélange ternaire contenant du CO₂, de l'éthanol (EtOH) et un tampon formiate d'ammonium / acide formique.

La nornicotine est un alcaloïde présent dans diverses plantes, et notamment la plante de tabac. C'est un analogue de la nicotine puisque seulement un groupement méthyle les sépare. C'est une molécule chirale, polaire et qui contient une amine secondaire. Une étude de cette molécule a été effectuée sur quatre colonnes polysaccharides [92]. Les auteurs ont remarqué que l'élution de cette nornicotine n'est possible sur aucune des phases stationnaires testées lorsque l'on n'utilise pas d'additifs dans la phase mobile, même avec 40% de co-solvant. Cependant, dès l'ajout de 0,05% d'ammoniaque, on retrouve l'élution et l'énantioséparation des deux formes de la nornicotine. En revanche, si l'on remplace l'ammoniaque par de l'acide formique, l'énantioséparation est perdue. Un gradient est utilisé allant de 4% de co-solvant à 40%, le co-solvant étant du MeOH avec 0,05% d'ammoniaque. Parmi les quatre colonnes polysaccharides étudiées, la colonne immobilisée Chiralpak® IC-3 s'est montrée la meilleure avec la séparation complète des deux énantiomères de la nornicotine en quatre minutes. Sur les trois autres colonnes (Waters Trefoil AMY1, CEL1 et CEL2) la séparation est incomplète mais pourrait facilement s'améliorer en faisant quelques changements dans les conditions analytiques.

La même observation que pour les composés polaires acides est faite ici avec l'amélioration des paramètres chromatographiques, tel que l'asymétrie, la rétention, le rapport signal sur bruit, qui s'améliore grandement lorsqu'un additif est ajouté [77,90,92]. L'utilisation conjointe d'eau et d'un additif basique, acide ou d'un sel semble être une bonne solution dans la majorité des cas.

Tableau 1.5 : Molécules polaires basiques (noms, structures, log P*, références)
 *Les log P sont déterminés sur Chemicalize (ChemAxon).

Nom	Structure	log P	Référence
Isoniazide		-0,69	[75]
Ethambutol		-0,06	[76]
Rifampicine		0,77	[77]
Métoprolol		1,76	[78]
Triméthoprime		1,28	[79]
Histamine		-0,70	[86]

Cytosine		-1,24	[90]
Nornicotine		0,78	[92]
Hydrazide maléique		-2,25	[72]

3. Molécules polaires ioniques

Certaines molécules sont chargées sans influence du pH, par exemple un ammonium quaternaire ou encore un oxygène impliqué dans une double et une simple liaison comme dans les anthocyanes ou encore dans les phospholipides (porteurs d'une fonction phosphorique chargée). Ce sont des molécules amphiphiles, composées d'une « tête » polaire porteuse du groupement phosphorique et de longues chaînes carbonées hydrophobes. La liste des molécules analysées est récapitulée dans le tableau 1.6 à la fin de cette sous-partie.

Suto *et al.* ont analysé deux molécules alcaloïdes en 1997, la berbérine et la palmatine [93]. Ce sont des molécules qui contiennent un azote chargé positivement puisqu'il est impliqué dans quatre liaisons. Ce sont des molécules polaires, leur log P est de -1,28 et -1,22 respectivement. Ils ont extrait ces deux molécules d'une des cinquante plantes fondamentales de la médecine traditionnelle chinoise, Huáng bǎi (ou *Cortex phellodendri*) avec une technique d'extraction par SFE en utilisant la même composition de phase mobile que pour l'analyse en SFC après l'extraction, un mélange de CO₂ avec du MeOH et 100 mM de Dioctyl sodium sulfosuccinate (DSS), un surfactant chargé négativement. Une paire d'ions est alors formée entre la charge positive portée par l'azote de la berbérine et de la palmatine avec la charge négative du sulfonate. Cette paire d'ions « neutralise » la molécule et diminue sa polarité en apportant des chaînes hydrophobes comme on peut le voir sur la figure 1.8, ce qui augmente sa solubilité dans le CO₂.

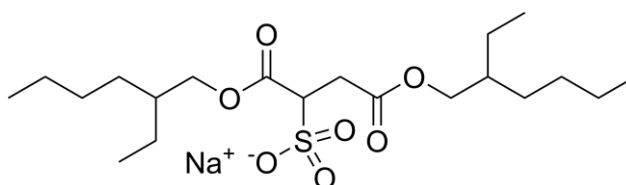


Figure 1.8 : Structure du dioctyl sodium sulfosuccinate

L'élution des deux molécules se fait sur une phase stationnaire polaire faite de silice avec une phase mobile constituée de 85% de CO₂ et de 15% du co-solvant utilisé pour l'extraction.

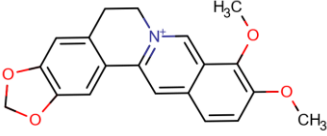
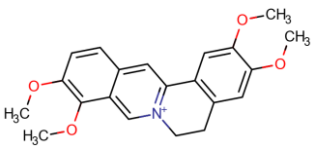
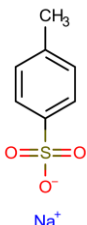
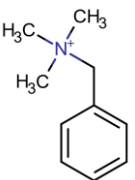
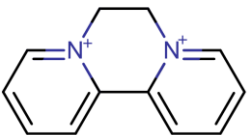
Des agents de paires d'ions ont également été utilisés dans d'autres travaux de Zheng *et al.* [94,95] Dans le premier article de 2005 [94], ils analysent trois arylsulfonates de sodium, dont deux sont très apolaires en raison de longues chaînes d'hydrocarbure, mais le dernier, le p-toluènesulfonate de sodium, est quant à lui polaire car son log P est aux alentours de 1. Plusieurs additifs ont été testés : acétate de lithium, acétate d'ammonium, acétate de tétraméthylammonium, acétate de tétrabutylammonium, chlorure d'ammonium. Trois phases stationnaires ont également été criblées : deux phases cyanopropyle et une phase silice. Les premiers tests effectués ont montré qu'un additif était nécessaire pour l'élution de ces composés puisqu'avec du MeOH pur, aucun des composés n'a été élué. Ensuite tous les additifs, à 2,5 mM, ont été testés sur toutes les phases stationnaires. Concernant la molécule qui nous intéresse, le p-toluènesulfonate de sodium, les meilleures conditions utilisent la phase silice avec de l'acétate d'ammonium ou de l'acétate de tétrabutylammonium. Avec les autres additifs, ou même sans additifs, le pic correspondant à cette molécule était très large et peu intense. L'additif est donc indispensable pour avoir des performances chromatographiques convenables. L'autre article de Zheng *et al.* [95] porte sur d'autres molécules chargées, dont le chlorure de benzyltriméthylammonium qui est une molécule fortement polaire avec un log P de -2,25. Le même constat que précédemment est effectué avec la non-élution des composés avec du MeOH seul en tant que co-solvant alors qu'elle redevient effective dès l'ajout d'un additif. Le chlorure de benzyltriméthylammonium a été élué convenablement sur une phase éthylpyridine avec 20% de co-solvant contenant du MeOH et 2,5 mM d'acétate d'ammonium. Cependant le pic reste assez large et dissymétrique. Une autre nouvelle méthode a été abordée dans cet article, celle de l'échange d'ions. Une colonne SAX (strong anion exchange) a été utilisée et l'on remarque une nette amélioration de la forme des pics lors de l'utilisation de cette colonne avec un additif dans la phase mobile ; lors des analyses avec du MeOH pur, les pics restent larges et peu intenses. Le pourcentage de co-solvant a également son importance sur la forme des pics. En effet à 30%, le pic du chlorure de benzyltriméthylammonium est assez large et trainant alors qu'à 40% il est plutôt fin, intense et élué en seulement deux minutes.

Le dernier exemple pris est à nouveau celui des pesticides décrits par Ishibashi *et al.* [72]. Dans les pesticides étudiés se trouve le Diquat dibromure, une molécule contenant deux charges positives portées par deux azotes. Les analyses sont

effectuées sur une colonne C18 avec un groupement polaire, l'Inertsil ODS-EP en gradient d'élution allant de 5% à 40% de co-solvant, un mélange de CO₂ et de MeOH avec 0,1% de formiate d'ammonium. Ce pesticide est élué tardivement, pendant le plateau à 40% en fin de gradient, ce qui prouve bien qu'il nécessite une phase mobile d'une certaine polarité pour être décroché de la phase stationnaire.

En conclusion de cette partie sur l'analyse de molécules polaires ionisables et ioniques (acides, basiques ou porteuses d'une charge permanente), le constat est sans appel. Il est quasiment impératif d'utiliser une phase stationnaire polaire ou possédant une fonction polaire (comme les C18 à groupement polaire inséré) pour offrir une rétention nécessaire aux composés que l'on veut analyser. En termes de phase mobile, une très grande majorité des cas utilisent le MeOH comme co-solvant avec l'ajout d'un additif afin d'obtenir des pics plus fins et plus intenses. Pour l'analyse de molécules ioniques, l'additif n'agit pas toujours de la même façon. En effet, il peut très bien entraîner des suppressions d'ions, modifier le pH apparent de la phase mobile, être compétiteur dans un mécanisme d'échange d'ions ou être agent d'appariement d'ions.

Tableau 1.6 : Molécules polaires ioniques (noms, structures, log P*, références)
 *Les log P sont déterminés sur Chemicalize (ChemAxon).

Nom	Structure	log P	Référence
Berbérine		-1,28	[93]
Palmatine		-1,22	[93]
p-Toluènesulfonate de sodium		1,67	[94]
Chlorure de benzyltriméthylammonium		-2,25	[95]
Diquat dibromure		-7,03	[72]

4. Analyse de molécules zwitterioniques en SFC (acides aminés)

Certaines molécules polaires peuvent contenir à la fois un groupement acide et un groupement basique. C'est-à-dire qu'à un pH tel que l'on retrouve en SFC, ces molécules seront sous leurs formes zwitterioniques, protonées au niveau de la fonction basique et déprotonées au niveau de la fonction acide, c'est le cas des acides aminés.

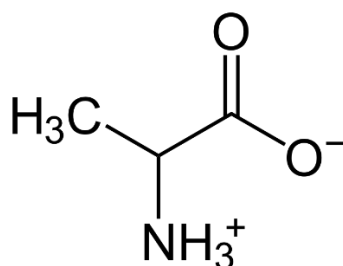


Figure 1.9 : Représentation de l'alanine, un acide aminé, sous sa forme zwitterionique avec le groupement carboxylate COO^- et le groupement ammonium NH_3^+ .

Les acides aminés sous leurs formes natives, c'est-à-dire sans aucune dérivation au niveau de leurs groupements acides et amines, se retrouvent sous forme zwitterionique à un pH de 5, pH apparent d'une phase mobile CO_2 -MeOH en SFC [68]. Les pKa des fonctions acides et amines sont respectivement de 2-3 et de 9-10. Ce sont de petites molécules polaires (le log P de l'alanine en figure 1.9 est de -2,84) et souvent chirales. Ils peuvent s'associer entre eux pour donner des peptides, puis des protéines. Ces molécules ont été analysées pour la première fois en 1992 en SFC, sous leurs formes natives [96] car plusieurs articles traitaient déjà des acides aminés dérivés [97–99]. Camel *et al.* [96] ont alors développé une méthode d'analyse des acides aminés non dérivés. Ils ont fait un criblage de huit phases stationnaires différentes, parmi elles une phase C18 a été testée mais n'a pas été conservée car tous les acides aminés injectés n'étaient pas retenus. La colonne retenue a été la Lichrosorb Diol et différentes phases mobiles sont utilisées en fonction des acides aminés analysés. Le co-solvant utilisé, un mélange MeOH / H_2O / TEA / Pyridine dans les proportions 87,95/7/0,05/5, reste le même mais les proportions de ce co-solvant dans le CO_2 changent. La phase mobile peut être un mélange de 85% de CO_2 avec 15% de co-solvant lors de l'analyse des acides aminés avec une chaîne secondaire apolaire. Mais cela peut être aussi un gradient de 30% à 40% de co-solvant lors de l'analyse des acides aminés avec une chaîne basique. Dans cet article, la détection

utilisée est l'UV avec le DEDL. La spectrométrie de masse n'est pas utilisée en raison de l'incompatibilité de la phase mobile avec ce détecteur. Et l'énantioséparation des acides aminés n'est pas non plus abordée dans ce travail.

Chronologiquement, le deuxième article traitant des acides aminés natifs en SFC date de 2016 par Sanchez-Hernandez *et al.* [100]. Dans cette étude, seuls les trois acides aminés dotés d'un groupement aromatique sont étudiés, il s'agit de la phénylalanine, de la tyrosine et du tryptophane. La détection UV est donc utilisée puisque tous les composés possèdent un groupement chromophore. L'énantioséparation des trois acides aminés est atteinte en moins de sept minutes sur la colonne Chirobiotic™ T2. Cette colonne est intéressante car la silice est liée à un antibiotique polaire, la teicoplanine. Cette teicoplanine possède quatre cavités dans sa structure qui servent de sélecteurs chiraux. Les analyses sont effectuées en isocratique avec un pourcentage de co-solvant variant de 35% à 60% avec de 2% à 10% d'eau ajoutée. Les phases mobiles sont donc plutôt polaires et permettent d'éluer rapidement les acides aminés natifs. Cependant, les pics sont relativement larges, l'ajout d'un additif à cette phase mobile permettrait sans nul doute d'améliorer les performances chromatographiques.

Le troisième article est l'œuvre de Wolrab *et al.* en Autriche. Ils se sont intéressés à l'analyse d'acides aminés en utilisant un couplage avec la spectrométrie de masse [101]. Les séparations ont été effectuées sur la colonne Phenomenex Luna HILIC avec un gradient d'élution allant de 15% à 45% de co-solvant. En ce qui concerne le co-solvant, ils en ont comparé trois différents : MeOH, EtOH et un mélange 1:1 MeOH/ACN. Dans ce co-solvant ils ont testé deux additifs basiques, le formiate d'ammonium et l'acétate d'ammonium. Ils ont également essayé un mélange d'additif en associant le formiate d'ammonium soit avec l'acide formique soit avec de l'eau. Les meilleures conditions ont été celles avec du MeOH seul, moins rétentif que l'EtOH ou le mélange MeOH/ACN (certains acides aminés ne sont pas élués avec l'EtOH ou avec MeOH/ACN). En termes de rapport signal sur bruit la différence est flagrante également, nettement meilleure avec le MeOH seul comparé avec l'ajout d'ACN dans le MeOH. L'additif choisi a été un mélange de formiate d'ammonium avec de l'eau. Aucune énantioséparation n'était recherchée dans cet article. Avec cette méthode, ils parviennent à éluer 20 acides aminés en moins de sept minutes et obtenir une détection satisfaisante en spectrométrie de masse.

L'article le plus récent traitant des acides aminés natifs vient de France, de Lipka *et al.* [102]. Ils ont étudié l'énantioséparation de quatorze acides aminés natifs sur huit phases stationnaires différentes de type polysaccharide. Ils ont évalué l'impact de la phase mobile sur la séparation, et surtout l'impact que peut avoir l'ajout d'un additif sur les performances chromatographiques. Une phase mobile différente a été utilisée en fonction de l'acide aminé étudié. Le pourcentage de co-solvant peut varier de 20% à 40%. Le co-solvant utilisé a été l'EtOH car il a été le meilleur solvant dans les études préliminaires, des séparations de certains couples d'énantiomères était constatées avec l'EtOH et pas avec le MeOH. La phase polysaccharide retenue comme étant optimale fut la Lux iCellulose-5, commercialisée par Phenomenex. Un additif a été testé afin d'améliorer la séparation et affiner les pics et il s'est avéré que la triéthylamine a apporté un bon compromis entre la forme des pics, le temps d'analyse et la résolution des paires d'énantiomères. Ils ont remarqué que lors de leurs expériences avec de l'eau comme additif, elle n'apportait aucune amélioration en termes de performances chromatographiques. Cependant, cet article ne montre que très peu de résultats sur les acides aminés les plus basiques (histidine, glutamine et lysine).

L'étude d'acides aminés natifs est donc possible en SFC avec tous ces exemples, mais qu'en est-il des peptides ? Zheng *et al.* [103] ont analysé en 2005 des peptides allant jusqu'à quarante unités d'acides aminés. Ils ont développé ces méthodes sur trois phases stationnaires différentes : une colonne échangeuse d'anions, une éthylpyridine et une aminopropyle. Ils ont étudié l'impact de la présence d'un additif dans la phase mobile sur plusieurs polypeptides. Les premiers étaient des peptides simples allant de trois à neuf acides aminés. Les essais sur ces peptides montrent que la séparation est meilleure lorsque l'on utilise du MeOH pur, puisque des coélutions sont constatées dès qu'un additif est ajouté. Cependant, des petites modifications au niveau du gradient, comme une diminution de la pente et du pourcentage final, ont permis de retrouver la séparation avec des pics plus fins. Les deuxièmes tests sont faits sur des peptides comportant des acides aminés basiques. Les analyses sont faites sur la colonne éthylpyridine et un comparatif des additifs a, une nouvelle fois, été effectué. Les meilleurs résultats ont été vus avec 5 mM ou 13 mM de TFA où les pics sont les plus fins. Des peptides plus longs ont ensuite été analysés allant pour les plus longs à quarante acides aminés. Encore une fois les meilleures conditions sont

celles avec 13 mM de TFA, la sauvagine n'est pas éluée avec du MeOH pur ou même avec 5 mM de TFA. En revanche, elle l'est avec 13 mM d'ATFA (Ammonium Trifluoroacétate) comme la plupart des peptides analysés mais les résultats sont quand même meilleurs avec le TFA. Les paires d'ions ne sont pas le seul mécanisme mis en jeu ici, l'acidité du TFA est aussi importante pour l'éluion de ces peptides.

A travers ces exemples, nous pouvons voir que l'analyse de biomolécules est possible en SFC, même des polypeptides qui étaient considérés comme trop polaires pour être analysés. Il faut, en revanche, adapter les conditions d'analyses avec une phase stationnaire polaire et une phase mobile avec de forts pourcentages de co-solvants et la présence d'additifs, et parfois d'eau.

V. Conclusion de la synthèse bibliographique

Bien que la SFC ait vu le jour dans les années 1960, il a fallu attendre les années 90 pour qu'elle soit vraiment adoptée parmi les techniques chromatographiques. Et ces dernières années, par l'apparition sur le marché de nouveaux appareils innovants et performants, la SFC s'est bien ancrée dans les pensées des chromatographistes.

Du fait de la faible polarité du CO₂, cette technique a longtemps été imaginée incapable d'analyser correctement les composés polaires. Avec l'apparition de phases stationnaires polaires permettant d'assurer la rétention de ces composés, et l'accroissement de la polarité des phases mobiles avec l'ajout d'un co-solvant polaire ainsi que des additifs, et même des faibles pourcentages d'eau, cette technique se révèle capable d'analyser des composés de plus en plus polaires. Comme montré par ces différents exemples, la SFC est capable d'analyser des composés polaires non ionisables, tels que des sucres, des sulfamides ou des xanthines. Mais elle est également capable d'analyser des composés polaires ionisables (acides ou basiques) ou ioniques avec des molécules ayant des log P inférieurs à -2. Pour l'analyse de ces composés, les additifs sont quasiment indispensables pour avoir des pics fins et intenses. Sans additifs, les pics sont généralement larges et asymétriques.

Chapitre 2

Caractérisation de phases stationnaires

I. Introduction

Comme nous l'avons vu dans le chapitre précédent, pour développer une méthode permettant d'analyser des molécules polaires, qu'elles soient ionisables ou non, la nécessité d'une phase stationnaire polaire est requise dans la majorité des cas. L'objectif de ma thèse étant de trouver des moyens d'analyser des molécules de plus en plus polaires, et de comprendre le comportement de celles-ci dans un mélange de CO₂ supercritique et co-solvant, il faut commencer par comprendre comment se comportent les phases stationnaires dans différents milieux, et également y expliquer les mécanismes de rétention. La méthodologie LSER (Linear Solvation Energy Relationship ou Relation Linéaire de l'Energie de Solvatation) va être utilisée, comme décrit par de nombreux articles du groupe de C. West [104–108]. Cette méthodologie permet de mettre en évidence toutes les interactions que peuvent faire les molécules analysées avec la phase stationnaire et la phase mobile.

Dans un premier temps l'étude de la phase stationnaire Astec Chirobiotic™ TAG sera effectuée, avec l'analyse de molécules achirales et chirales dans des conditions différentes de phase mobile, comprenant des concentrations élevées d'additifs acide ou basique. La colonne Chirobiotic™ TAG est greffée d'un peptide macrocyclique, la teicoplanine aglycone (TAG). Le log P de cette molécule est inférieur à 0, ce qui en fait une molécule polaire et ionisable. Un set de composés achiraux et chiraux a été analysé sur cette colonne. Les différences d'interaction seront donc discutées en fonction de la phase mobile utilisée et permettront de comprendre le comportement de ce peptide macrocyclique en conditions variables de pH apparent.

La deuxième partie de ce chapitre sera consacrée à l'étude d'une phase stationnaire destinée à la rétention et l'énantioséparation des acides aminés natifs, sans aucun groupement protecteur. Cette phase stationnaire est la colonne Chiralpak® ZWIX, qui existe sous 2 formes, la ZWIX(+) et ZWIX(-) formées de ligands zwitterioniques dérivés de la quinine et la quinidine, et qui sont des diastéréoisomères. Ces phases stationnaires vont être utilisées dans les applications du chapitre 3, mais avant tout cela, une étude est effectuée pour comprendre les mécanismes de rétention et d'énantioséparation. Pour réaliser ces études, 9 autres phases stationnaires sont étudiées (un total de 11 phases stationnaires en rajoutant les deux colonnes

Chiralpak® ZWIX). Parmi ces 9 colonnes, 2 sont commerciales, il s'agit des Chiralpak® QN-AX et Chiralpak® QD-AX qui sont des phases échangeuses d'anions. Les 7 autres, comprenant des phases zwitterioniques et échangeuses de cations, ont été fabriquées à façon par l'équipe du Professeur Michael Lämmerhofer de l'université de Tübingen, en Allemagne. Ces colonnes comportent toutes des parties communes au niveau du greffon par rapport aux phases Chiralpak® ZWIX de référence. En revanche, sur chaque phase stationnaire, il manque une ou plusieurs fonctions chimiques présentes sur le greffon principal afin de voir l'impact de cette fonction manquante sur la rétention et l'énantioséparation. Des molécules achirales et chirales, ainsi que des acides aminés ont été analysées. Le modèle LSER est, encore une fois, utilisé afin de modéliser la rétention des composés achiraux sur ces différentes colonnes et de corrélérer ces modifications de rétention avec la présence ou l'absence d'un ou plusieurs groupements fonctionnels. Des analyses discriminantes permettent de déchiffrer le mécanisme d'énantioséparation.

Ces différentes études ont permis de mieux comprendre le comportement de deux phases stationnaires : l'une utilisée pour l'analyse des acides aminés, des composés que nous souhaitons analyser dans le cadre de ma thèse, et l'autre colonne greffée d'un peptide macrocyclique, s'apparentant donc aux analytes qui nous intéressent.

II. Etude de la phase stationnaire Chirobiotic™ TAG – Effet de la composition de phase mobile

Cette étude a été effectuée sur la colonne Astec Chirobiotic™ TAG, commercialisée par Supelco (Merck). Des essais ont été effectués avec 11 phases mobiles différentes, utilisant soit un additif acide, l'acide trifluoroacétique (TFA), ou un additif basique, l'isopropylamine (IPA) sur 54 molécules achirales et 24 molécules chirales afin d'observer les modifications de rétention et d'énantioséparation sur cette phase stationnaire. L'objectif de cette étude est donc de comprendre le comportement d'un peptide macrocyclique dans différents milieux (acides ou basiques).

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Effects of high concentrations of mobile phase additives on retention and separation mechanisms on a teicoplanin aglycone stationary phase in supercritical fluid chromatography

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ABSTRACT

The objective of this study is to understand the behavior of a peptide in a medium containing supercritical carbon dioxide mixed with an alcohol (methanol) and acidic or basic additives in uncommonly high concentrations. Chirobiotic TAG is a chromatographic column made of silica bonded with a macrocyclic peptide, teicoplanin aglycone. With this stationary phase, two additives (trifluoroacetic acid and isopropylamine) were tested under extreme concentration conditions to observe the behavior of this peptide. Indeed, concentrations exceeding 1 M in the methanol co-solvent (>0.1 M overall concentration in the CO₂-methanol mixture) were used whereas usual additive concentrations employed in supercritical fluid chromatography (SFC) rarely exceed 50 mM in the co-solvent. One purpose was to modify the apparent pH of the fluid, which is normally slightly acidic (around 5) and consequently possibly changing the ionization state of the peptide. Firstly, the effect of acidic and basic additives on the polarity and the apparent pH were evaluated with the help of color indicators. This served to assess the ionization state of the peptide under the selected operating conditions. Secondly, 54 achiral and 24 chiral molecules were injected in the chromatographic column at different levels of additives. The achiral species served at establishing retention models based on linear solvation energy relationships (LSER), while the chiral species were examined for their enantioresolution. From the LSER equations and observation of chromatograms, it appeared that specific interactions between the peptide-based stationary phase and the analytes evolved when increasing the concentration of additives, particularly hydrogen bonds and ionic interactions. A bare silica stationary phase (Acquity BEH) served as reference to deconvolute the contributions of silica support and bonded peptide. This study, with these extreme conditions of mobile phase, could be useful to understand the behavior of such peptides in SFC mobile phases and also improve the knowledge of the effects of additives in SFC, which should be helpful in the future prospect of analyzing large biomolecules in SFC.

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1. Introduction

Supercritical Fluid Chromatography (SFC) has faced a renewed interest in recent years [1,2], thanks to the introduction of modern analytical systems [3] and because it is considered as a green analytical technique with a low consumption of solvents, thereby producing little waste. Indeed, the main solvent employed as mobile phase is pressurized carbon dioxide (CO₂) which is easily renewable and has a low toxicity. SFC was previously mainly used for chiral applications [4–7], especially at the preparative scale in the pharmaceutical industry. Currently, more and more achiral and analytical-scale applications are being developed with this tech-

nique [1]. In particular the analysis of biomolecules is raising expectations. While several recent papers have demonstrated the feasibility of eluting small polar biomolecules like nucleotides or nucleosides [8–12], the elution of large biomolecules was only rarely shown. Lipophilic peptides and proteins were analyzed with SFC in a few occasions [13–15]. Hydrophilic peptides and proteins were explored recently with SFC [16,17] or enhanced-fluidity liquid chromatography (EFLC) in the hydrophilic interaction liquid chromatographic mode (HILIC) [18]. EFLC typically employs a larger portion of liquid solvent than is usually done in SFC, and this liquid solvent often contains a significant percentage of water. The question of peptides and proteins solubility and stability in a typical SFC mobile phase, where the portion of liquid solvent remains moderate (typically inferior to 50%), is thus still vacant [17].

This work aims at understanding the behavior of free (non-derivatized) peptides when they are placed in typical SFC mobile

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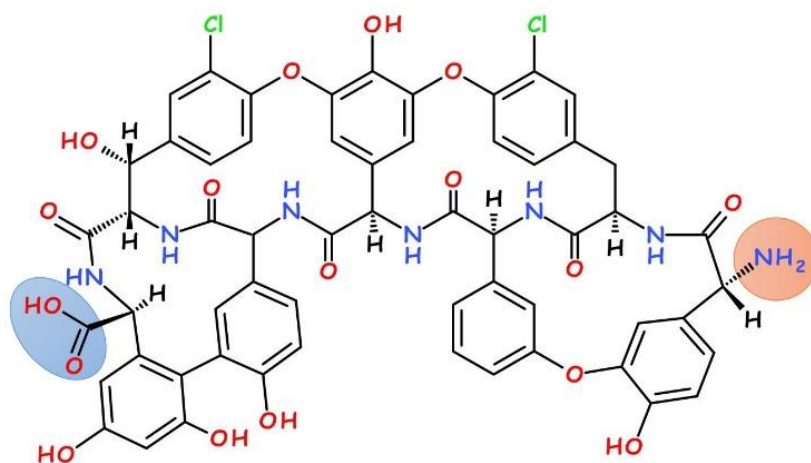


Fig. 1. Structure of Teicoplanin Aglycone (TAG), the chiral selector in the stationary phase examined.

phases, i.e. a mobile phase made of a pressurized CO₂-alcohol mixture. In addition, the effect of acidic and basic additives introduced in the methanol co-solvent is of interest. While the effects of additives in SFC mobile phases have long been explored [19–23], they are still not fully understood. It is generally accepted that they adsorb on the stationary phase (like any other mobile phase component), thereby changing the surface in contact with analytes [24], may suppress ionization [20] and/or form ion pairs with ionic or ionizable analytes [16,25]. Typical concentrations used in current SFC practice range in the 1–50 mM or 0.1–1% in the alcohol co-solvent. The behavior of a CO₂-alcohol mixture with higher concentrations of additive has only rarely been explored in SFC. A recent study from Speybroeck and co-workers explored the effects of large concentrations of additive (10% isopropylamine) on enantioresolution of drug-like molecules with polysaccharide stationary phases [26]. Unusual effects were observed like reversals of elution order of the enantiomers, suggesting that the retention mechanism had been deeply modified.

There may be two different ways to study the behavior of a molecule in a fluid with chromatography. The first method is to directly inject this molecule on any column with different mobile phases to control the evolution of its retention under several conditions. The second approach is to study this molecule as a stationary phase (coated or bonded on a support) and to inject known molecules in varied conditions, to observe how the interactions that the target molecule (as stationary phase) is able to do vary with the operating conditions. The latter option was selected in the present study, with the use of a commercial chromatographic column packed with silica particles bonded with a macrocyclic peptide. The chosen column was Chirobiotic TAG based on teicoplanin aglycone, whose structure is presented in Fig. 1. It is a complex macrocyclic peptide possessing four cavities within its structure. This stationary phase is normally employed as a chiral selector, where each of these four cavities may participate in the enantio-recognition process. Note that the structure presented in Fig. 1 is not the exact structure of the stationary phase, as the TAG molecule is bonded to silica gel, probably through the amine function [27], and perhaps also through the phenolic groups [28]. Teicoplanin, which has 3 additional sugar units relative to the aglycone molecule, is an antibiotic produced by the fermentation of *Actinoplanes teicomyceticus*. While teicoplanin-bonded silica was first introduced as a chiral selector [29], it was later modified to remove the glycosyl groups and obtain different enantio-recognition capabilities [30]. This column was already cited in many studies in HPLC, notably for pharmaceutical applications [31–33], and some-

times in SFC [34–37]. In addition, although it is an enantioselective stationary phase, it may also be useful to the analysis of achiral molecules [38].

The TAG structure comprises two free ionizable functions: a carboxylic acid function and a primary amine function. The latter may however serve as an attachment to the silica support and may then be converted to a urea function. However, the three-step bonding protocol employed to prepare the stationary phase [30] also involves modifying the silica surface with amino-propyl groups prior to bonding of the peptide. It is therefore likely that amine groups also remain on the silica surface, along with possible residual silanol groups. All these ionizable functions will have different ionization states depending on the mobile phase pH and would therefore contribute differently to the interaction capabilities. This in turn would affect retention and possibly enantio-recognition of the analytes injected in this column. Similarly, the acid and amine ionizable functions would be present in all linear and non-derivatized peptides as well as in free amino acids, and their ionization state would have a significant influence on their solubility in the mobile phase fluid and on their retention properties in any stationary phase.

It is likely that large concentrations of acidic or basic additives in SFC could affect the ionization state of biomolecules like peptides, thereby impacting their solubility in the CO₂-alcohol mobile phase and their interaction properties, whether they are immobilized on a stationary phase or injected as analytes. One objective of the present paper was thus to assess whether the ionization state of a peptide could be manipulated through the concentration of acidic or basic additives.

Two additives were tested: trifluoroacetic acid (TFA) and isopropylamine (IPA) at different concentrations in methanol mixed to 90% pressurized CO₂. These additive concentrations were pushed to the extreme to go above 1 M in methanol, representing an overall concentration greater than 100 mM in the mobile phase. Mathematical models based on Linear Solvation Energy Relationships (LSER) [39,40] were used to model the possible interactions between probe molecules and the peptide stationary phase to better understand the evolution of these interactions depending on the nature and concentration of additives in the mobile phase. A modified version of the solvation parameter model, comprising five Abraham descriptors [41], two additional descriptors to account for ionic interactions [42], and two more descriptors that were previously developed to characterize enantioselective stationary phases [43], was employed. These models were made following the injection of 54 achiral molecules under 11 different mobile phase con-

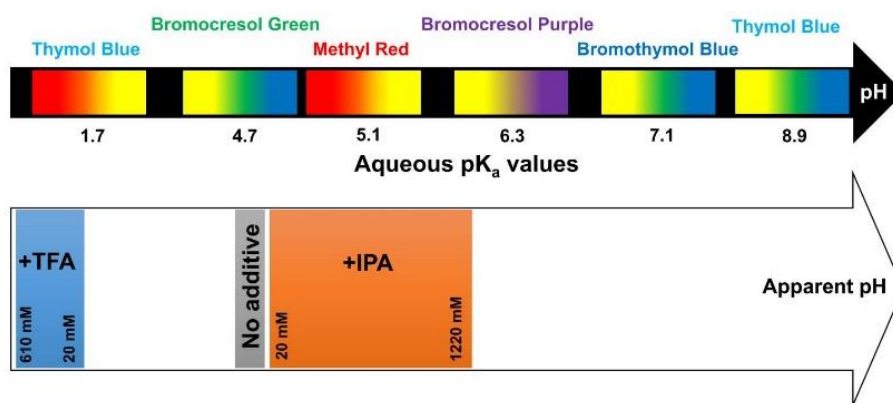


Fig. 2. Evaluation of apparent pH when large concentrations of acidic and basic additives are employed. TFA is trifluoroacetic acid, IPA is isopropylamine.

ditions. 24 racemates were also analyzed in order to see the evolution of enantioseparation with the presence of additives. A silica stationary phase (Acquity BEH) served as reference material to deconvolute the effects related to the silica support or the bonded peptide.

2. Material and methods

2.1. Chemicals and solvents

Nile Red, Thymol Blue, Bromocresol Green, Methyl Red, Bromocresol Purple and Bromothymol Blue were provided by Sigma-Aldrich (Sigma Aldrich Chimie, France). Their structures and UV-visible spectra can be observed in supplementary material (Fig. S1 and S2). Solutions were prepared in methanol. The colors and aqueous pK_a values associated to each acid-base pair are presented in Fig. 2.

54 achiral and 24 chiral molecules were purchased from Sigma Aldrich. The complete list of analytes together with molecular descriptors can be found in electronic supplementary information (Tables S1 for achiral probes and S2 for chiral probes). Although the set may be considered rather small, the achiral molecules were selected so as to provide a good distribution of properties to achieve meaningful LSER description, according to the recommendations of Vitha and Carr [44]. As a rule of thumb dictates that a minimum of three analytes per variable should be employed, 54 adequately chosen analytes should be largely sufficient to account for the effects of 9 variables. Descriptors for the chiral molecules are also indicated for information although they did not serve to establish models but only to assess the structural variance among this set. Solutions of all analytes were prepared in methanol. Ultrapure water was supplied by an Elga UHQ system from Veolia (Wis-sous, France). The HPLC-grade methanol used as the mobile phase was supplied by VWR (Fontenay-sous-Bois, France). CO_2 , with a purity of 99.995%, was delivered by Air Liquide (Paris, France). Isopropylamine (IPA) and trifluoroacetic acid (TFA) were provided by Sigma Aldrich (Sigma Aldrich Chimie, France).

2.2. Instruments and stationary phases

All LSER experiments were carried out on two columns: Chirobiotic TAG column (150×4.6 mm; $5 \mu m$), provided by Merck and Acquity UPC² BEH (100×3.0 mm; $1.7 \mu m$) provided by Waters, that served for comparison purposes. The charge state of teicoplanin aglycon depending on mobile phase pH was calculated based on aqueous pK_a values determined with Chemicalize program (<http://www.chemicalize.org/>).

The supercritical fluid chromatography system used 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, a column oven compatible with 150 mm length columns and a photodiode-array (PDA) detector. Empower® 3 (Waters Corporation) was used to process the chromatograms.

2.3. Analytical methods

The mobile phase was a mixture of 90% CO_2 and 10% MeOH, comprising additive or not. Two additives commonly employed in chiral SFC, an acidic one (trifluoroacetic acid) and a basic one (isopropylamine), were selected. 11 mobile phase compositions were tested: no additive; 20 mM, 122 mM, 366 mM and 610 mM trifluoroacetic acid in methanol; and 20 mM, 61 mM, 122 mM, 366 mM, 610 mM and 1220 mM isopropylamine in methanol. As a note of caution, it is worth saying that a very thorough rinsing of the system is absolutely necessary after operation, as such high concentrations of additives tend to leave traces in the system for a long while (especially isopropylamine). Obviously, the adsorption on the stationary phase is also quite extensive thus the columns must be rinsed for a long time to restore the initial state. The 54 achiral and 24 chiral molecules (Tables S1 and S2) were injected with each of these mobile phase conditions. The flow rate was 3.0 mL/min for the Chirobiotic TAG column, and 1 mL/min for the BEH column, to allow for comparable internal pressure in the two columns and avoid reaching the pump upper pressure limit. The backpressure was maintained at 15 MPa and the column oven was heated to 25 °C. The injection volume was set at 2 μL . The dead time and retention times of all compounds were recorded to calculate the log k value, required to achieve the LSER models.

2.4. Evaluation of polarity and apparent pH

Measurements of UV-visible spectra in the carbon dioxide – methanol – additive compositions were done as previously described [45]. Briefly, solutions of the color indicators (Nile Red polarity indicator and pH indicators) were injected in the Acquity BEH column to ensure that the analyte would have time to be desolvated from the dilution solvent plug (methanol), and would thus reflect the mobile phase polarity and acidity, with operating conditions identical to the LSER experiments described above. UV-visible spectra were recorded with the diode-array detector between 300 and 700 nm with 1.2 nm intervals. The spectra for pH indicators were first recalculated for 1 nm intervals, then they were

normalized and compared to UV–visible spectra measured in aqueous phases with controlled pH values through principal component analyses (PCA). PCA were computed with XLStat 2015.2.02 software (Addinsoft, New York, NY). The reader is referred to previous works for full details on this procedure [45].

2.5. LSER model

The LSER equation usually employed in chromatography, based on Abraham descriptors, is:

$$\log k = c + eE + sS + aA + bB + vV \quad (1)$$

In this equation, capital letters represent the solute descriptors, related to particular interaction properties, each molecule has its own molecular descriptors. While lower case letters represent the system constants, related to the complementary effect of the two phases (stationary and mobile). 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; V is the McGowan characteristic volume in units of $\text{cm}^3 \text{mol}^{-1}/100$. The system constants (e, s, a, b, v), 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.

However, in previous works we have had many occasions to point out that the lack of terms to account for ionic interactions in Eq. (1) is a problem when dealing with ionizable analytes. To take account of electrostatic interactions with anionic and cationic species, we have previously proposed to extend Eq. (1) with two additional terms [42], as follows:

$$\log k = c + eE + sS + aA + bB + vV + d^- D^- + d^+ D^+ \quad (2)$$

where D^- represents the negative charge carried by anionic and zwitterionic species, and D^+ represents the positive charge carried by cationic and zwitterionic species. They are calculated with the apparent pH (that may be different from effective pH^*) of the mobile phase and the pK_a values of the analyte. For neutral species, D^- and D^+ are zero so that Eq. (2) reverts to Eq. (1). We had initially designed Eq. (2) for hydrophilic interaction liquid chromatography (HILIC) [42,46,47], but they were also applied to other HPLC systems [48,49]. We have recently showed that it is also well suited to the description of SFC systems [24,50,51], provided we admit to some approximations (aqueous pK_a values and apparent pH equal to 5).

Finally, two more descriptors (F and G), previously introduced to characterize enantioselective stationary phases [43,52], are needed to provide information related to the 3D behavior of the probe molecules interacting with chiral selectors. As described in previous works, the F descriptor reflects the flexibility of the molecule (fraction of rotatable bonds), which is important for chiral recognition because flexible molecules have more conformers, thus more ways for intermolecular interactions, which is generally detrimental to enantioresolution. The G descriptor accounts for globularity (or sphericity) and can be related to steric impedance to insertion into the stationary phase but also to the possibility for a small molecule to better fit on the walls of a spherical cavity thus establish several interactions at a time. While F and G are often found to contribute very little to retention mechanisms, they are both highly significant to enantiorecognition. The full LSER equation employed in the present work is then:

$$\log k = c + eE + sS + aA + bB + vV + fF + gG + d^- D^- + d^+ D^+ \quad (3)$$

This 9-term equation was never employed before and should allow for a most complete assessment of interactions with the complex, enantioselective and ionizable stationary phase.

Multiple linear regressions (MLR) were performed using XLStat 2015.2.02 software. The quality of the MLR fits was estimated using the adjusted determination coefficient (R^2_{adj}), standard error in the estimate (RMSE) and Fisher F statistic. The statistical significance of individual coefficients was evaluated with the 95% confidence intervals.

Because the selection of probe molecules is rather small (54 achiral molecules for 9 independent variables), the statistical quality of the models is moderate (R^2_{adj} values ranging from 0.77 to 0.93). However, no prediction capability was desired, only some understanding of the interactions contributing to retention and their variation with mobile phase composition. For this reason, only the terms that vary significantly will be discussed in the following.

3. Results and discussion

3.1. Evaluation of changes in polarity with a solvatochromic dye

Nile Red was selected as a solvatochromic probe to assess possible changes in the polarity of the mobile phase when large concentrations of additives were present. Its structure and UV–visible spectra are visible in electronic supplementary information (Fig. S1). UV–visible spectra of Nile Red were recorded in SFC conditions with all levels of acidic and basic additives as described in methods section. Based on the first derivative of smoothed spectra, the wavelength at maximum absorbance was measured, from which the transition energy E_{NR} was calculated using the Planck–Einstein equation. As appears in the spectra (Fig. S1), large concentrations of additives yielded very small shifts in the absorbance maximum. Namely, large concentrations of trifluoroacetic acid (TFA) caused a small bathochromic shift (ENR decreases from 55.9 to 55.8 kcal.mol^{-1}), which is comparable to the polarity increase that would result from increasing the percentage of methanol co-solvent from 10 to 11% [45]. Conversely, large concentrations of isopropylamine (IPA) caused a small hypsochromic shift (ENR increases from 55.9 to 56.0 kcal.mol^{-1}), which is comparable to the polarity decrease that would result from decreasing the percentage of methanol co-solvent from 10 to 9%. Thus the two additives have opposite effects on polarity: TFA slightly increases the polarity in the cybotactic region (solvation sphere around the analyte) while IPA slightly decreases it. This conclusion is not so surprising considering the structures of the two molecules, compared to CO_2 and methanol, as TFA possesses a high proportion of heteroatoms with significant electronegativity (O and F) while IPA possesses a larger portion of hydrocarbon moieties. In the following, we shall consider these changes as negligible.

3.2. Evaluation of the ionization state of teicoplanin aglycone

In previous works, we had demonstrated how a selection of color indicators of pH could be used to obtain some information on the apparent pH of the SFC mobile phases [45]. In particular, we had shown that additives in usual concentrations could modify the apparent pH of the fluid. Indeed, mixtures of carbon dioxide and alcohols comprising no additives are slightly acidic, with an apparent pH close to 4.5–5, due to the formation of alkyloxycarbonic acid. In usual concentrations, acidic additives can reduce the apparent pH down to 1–2 while basic additives would cause only a moderate increase (5–6). As mentioned in the introduction, typical concentrations used in current SFC practice range in the 1–50 mM or 0.1–1% in the alcohol co-solvent. At such low concentrations, a

basic additive is not susceptible to compensate for the large concentration of alkylloxycarbonic acid, possibly explaining why the increase in apparent pH is rather small. However, higher concentrations of basic additive may create a really basic environment for the analytes and stationary phase. With these further experiments, we wished to verify this point and the extent of apparent pH increase.

In Fig. 2, the selection of five color indicators of pH (one of which has two pK_a values) with their aqueous pK_a values can be seen. As detailed in Section 2.4 above, UV-visible spectra were measured for each of the color indicators of pH with the diode-array detector of the SFC system. The spectra were then compared to spectra measured in aqueous phases of controlled pH. All spectra can be observed in electronic supplementary information (Fig. S2). A simple comparison of spectra is not sufficient. As was demonstrated in our previous study, the use of principal component analysis (PCA) to compare all spectral data is most informative to obtain a better estimate of apparent pH. In the present case, PCA were computed for each color indicator individually, and also with combinations of color indicators that proved the most informative. One interesting combination can be observed in electronic supplementary information (Fig. S3) with a PCA score plot computed with the spectral data measured for Thymol Blue, Bromocresol Green, Methyl Red and Bromocresol Purple. On this plot, the first principal component (PC1) is essentially a pH axis between 2 and 8, while the second principal component (PC2) is a pH axis in the ranges 1–2 and 8–10. Based on the examination of all spectra and PCA plots, the estimated apparent pH values can be observed in Fig. 2.

We had demonstrated before that the mobile phase comprising no additive has an apparent pH close to 5, or somewhat smaller depending on the proportion of methanol (around 4.5–5). When large concentrations of TFA are introduced, the apparent pH is strongly decreased, around 1–2. Indeed, all spectra in these conditions show the acidic form for each of the five color indicators. When large concentrations of IPA are introduced, the apparent pH is slowly increasing and reaches a value of about 6. This is demonstrated by the spectra of Bromocresol Purple, where the absorption of the basic form is observed at the largest concentrations of IPA, but still in equilibrium with a significant portion of the acidic form. The apparent pH is therefore never above the pK_a value of Bromocresol Purple (6.3). Furthermore, the basic forms of Bromothymol Blue and Thymol Blue are never observed (pK_a values 7.1 and 8.9).

It must be mentioned again here that all these values are estimates of apparent pH, based on aqueous pK_a values, which are obviously not identical in pressurized CO_2 -methanol environment.

The structure of teicoplanin aglycon was processed with the Chemicalize program to compute aqueous pK_a values for each of the ionizable function (carboxylic acid, amine, phenolic and amide groups). Based on these values, a curve of charge state vs. mobile phase pH was computed and can be seen in Fig. 3. On this figure, the pK_a values of the principal ionizable functions is pointed with arrows, and the estimated pH domains are presented. Not all of these ionizable functions may be available, as the amine [27] or phenol groups [28] may both have served as attachment points to the supporting silica gel. The availability of the amine function is clearly a matter of debate as some authors report bonding via the amine through a urea attachment [27], while other bonding protocols have maintained a quaternary ammonium function as the binding site [53]. Besides, several papers have demonstrated that interaction with the protonated amine function was significant to enantioselectivity [54], suggesting that the amine was still free in the bonded phase. Our own works [55] demonstrate that interactions with anionic compounds are significant to successful enantioseparation. This may result from positive interactions with an

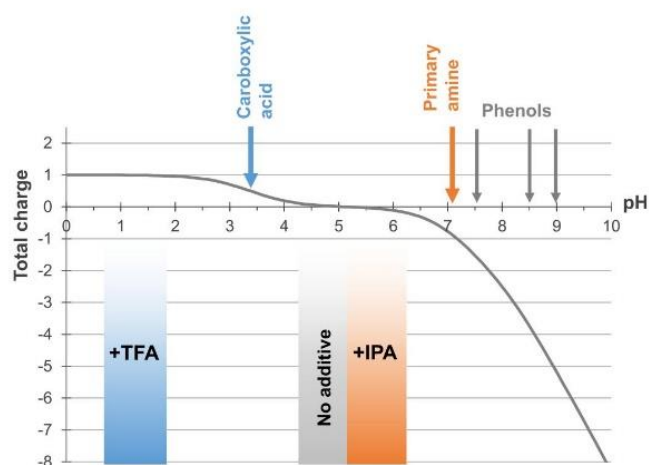


Fig. 3. Ionization states of teicoplanin aglycon depending on mobile phase pH, according to aqueous pK_a values.

amine group, but whether this group is present in the peptide ligand and on in the aminopropyl ligands cannot be told. Both options shall be considered in the following. According to these estimations, we can however suppose that:

- In the CO_2 -methanol mobile phase comprising no additive, both the acid and amine functions should be ionized, thus the net charge for the ligand would be zero. If the amine function is unavailable, the net charge is -1 .
- When IPA is introduced, deprotonation of a free amine function would not occur, thus the net charge would still be the same;
- When TFA is introduced, the acid function should protonate, thus the net charge would be $+1$, or zero in case the amine function is not free.

These observations should be kept in mind when interpreting retention and separation behavior, as will be done below.

3.3. LSER characterization of the effects of additives

In a previous paper, we had characterized three macrocyclic peptide stationary phases (Chriobiotic V, T and TAG based respectively on vancomycin, teicoplanin and teicoplanin aglycon) [55]. LSER methodology was employed, based on Eq. (2), to describe the retention mechanisms in a carbon dioxide-methanol environment, without additives. In the present paper, a most complete LSER model, based on Eq. (3), is employed, with the intention to describe the effects of additives. The full LSER models obtained from Eq. (3) can be observed in supporting information, in histograms (Figure S1a for the TAG column and Figure S1b for the silica column) or in the tables reporting the statistical features of each model (Tables S3 and S4).

To some extent, the mobile phase employed here (carbon dioxide – methanol, with or without an additive) should be comparable to polar organic and polar ionic modes where only organic solvents (methanol and/or acetonitrile) are used as eluents, together with additives to adjust the acidity in the polar ionic mode.

On both columns, the most significant variations were observed for the a , b , d^- and d^+ terms, related to hydrogen bonding and ionic interactions. Thus only these four selected system constants will be discussed and are represented in Fig. 4a (Chriobiotic TAG) and 4b (Acquity BEH). On the figures, the evolution of the system constants with increasing apparent mobile phase pH is represented from left to right, corresponding on the left to the highest concentration of TFA (610 mM in methanol co-solvent) and on the right to the highest concentration of IPA (1220 mM in methanol).

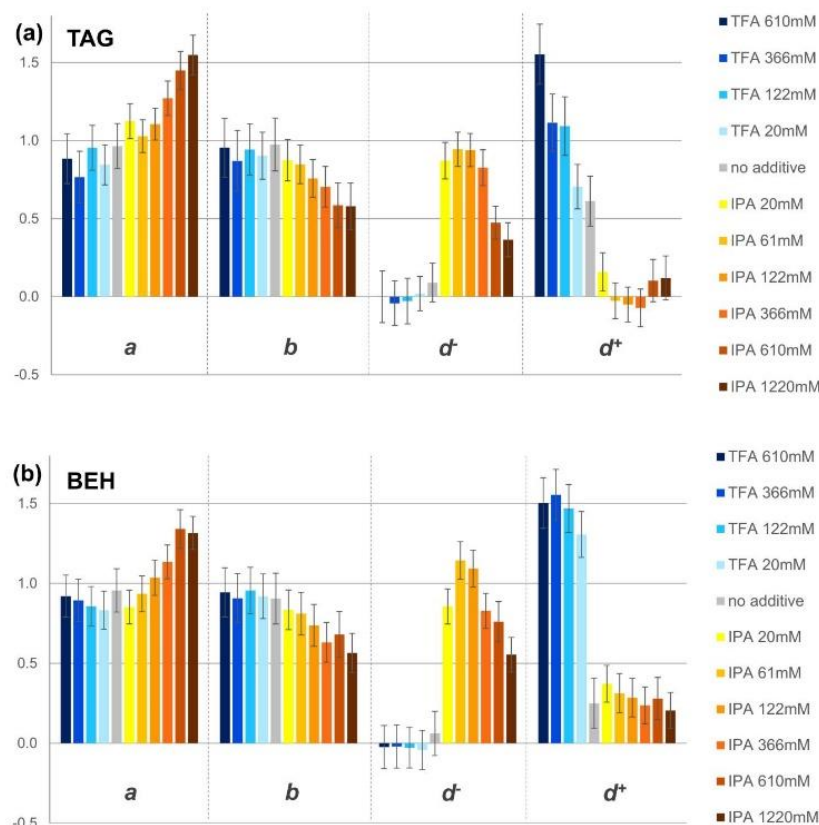


Fig. 4. Selected system constants from the LSER models of (a) the teicoplanin aglycone stationary phase (Chirobiotic TAG) and (b) a bare silica stationary phase (Acquity BEH), retaining only the 4 system constants that vary significantly with a change in the nature or concentration of the additive (TFA or IPA). Mobile phase: carbon dioxide – methanol (with or without additive according to legend, from left to right) 90:10 (v/v), 25 °C, 15 MPa, 3 mL/min on TAG or 1 mL/min on BEH.

3.3.1. Evolution of retention with IPA concentration

3.3.1.1. Hydrogen bonds. With both stationary phases, when IPA concentration increases, a significant variation in the a and b system constants related to hydrogen bonding is observed, with opposite trends for a and b . Indeed, an increase in the a term (related to retention of proton donors) and a decrease in the b term (related to retention of proton acceptors) are observed when the concentration of IPA increases from no additive to 1220 mM IPA in the methanol co-solvent. Moreover, the trends of a and b variations are nearly collinear (they vary with the same slopes). This suggests that the reason for the variation is identical on both columns. In the LSER equation, each system constant is related to the difference in the interactions between analyte and stationary phase on the one hand, analyte and mobile phase on the other hand. As a result, the increase in one system constant may be due to (i) increase of analyte – stationary phase interactions or (ii) decrease of analyte – mobile phase interactions. Conversely, the decrease in one system constant may be due to (i) decrease of analyte – stationary phase interactions or (ii) increase of analyte – mobile phase interactions.

Considering previous observations on a large variety of stationary phases [24], the increase in a term on both stationary phases may be explained by an adsorption phenomenon of the additive on the surface of the stationary phase. Indeed, as should be the case for any other component of the mobile phase, the additive gets adsorbed at the surface of the stationary phase, which modifies the possible interactions between the analytes and stationary phase. The higher the concentration of additive in the mobile phase, the greater the quantity of additive adsorbed on the stationary phase. In this case, because isopropylamine is ba-

sic, hydrogen-bonding interactions with proton donors (a term) increase.

Basic analytes do not interact favorably with isopropylamine thus the adsorbed additive should not cause particularly favorable interactions with electron donors (b term). On the opposite, the adsorption of isopropylamine is increasingly shielding proton-donor interaction sites of the stationary phases. Additionally, isopropylamine likely acts as a competitor for adsorption of basic analytes on the stationary phases. As a result of both effects (adsorption and increasing elution strength towards basic compounds), the retention of basic compounds reduces when IPA concentration increases (b term decreases). This is illustrated in Fig. 5 where it appears that the retention time of the two enantiomers of 5-methyl-5-phenylhydantoin, an electron-donor chiral compound, significantly decreases with increasing IPA concentration. However, the peak shapes are improved and good resolution is maintained in that case.

3.3.1.2. Ionic interactions. The d^- and d^+ terms are related to interactions with anionic and cationic species respectively. Again their variation on both columns is most similar, indicating that the effects observed probably have the same causes.

The d^- term varies significantly in a non-monotonous manner. First, it is near zero on both stationary phases when no additive is present. Anionic compounds may be partly repulsed by the stationary phases. Indeed, at the apparent pH obtained with such a mobile phase (around 5), some accessible residual silanol groups and the carboxylic acid function of the teicoplanin aglycone may be partly deprotonated. Therefore, there would be some repulsion between the negative charges of the analytes and the negative

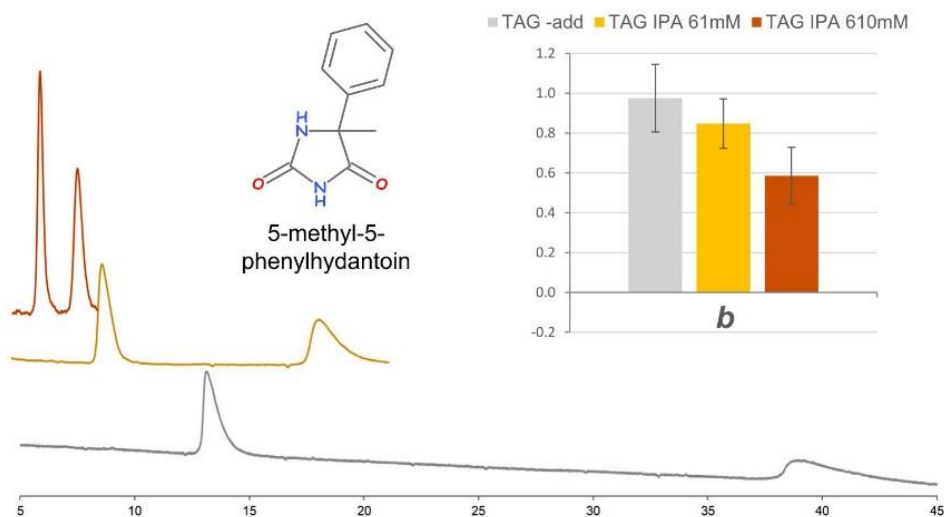


Fig. 5. Chromatograms of *rac*-5-methyl-5-phenylhydantoin on the Chirobiotic TAG column with increasing concentrations of IPA from bottom to top: without additive (gray), IPA 61 mM (orange) and IPA 610 mM (brown). The inserted histograms show the related variation of LSER b term when IPA concentration increases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

charges on the stationary phases. Thus negatively charged species experience rather low retention in these conditions.

The d^- term increases strongly when IPA is first introduced in the mobile phase (20 mM in methanol co-solvent). This is again consistent with adsorption of isopropylamine on the stationary phase as anionic compounds are expected to interact favorably with adsorbed isopropylamine molecules (which may be in the neutral or protonated form).

Further increases in IPA however see the d^- term reach a maximum then a subsequent decrease. Considering the above observations on ionization state of teicoplanin aglycone, deprotonation of free amine functions (whether from the peptide or the aminopropyl groups) should not contribute to this effect. However, the decrease in retention when adding high concentrations of IPA can be explained by two phenomena. First, at high IPA concentrations, the stationary phase is saturated with additive and then more and more free IPA is found in the mobile phase, creating ion pairs with the anionic analytes, which increases favorable interactions with the mobile phase, resulting in a decrease in the overall value of the d^- constant. Second, we may infer that, at such high base concentration, the methoxycarbonic acid present in the mobile phase due to the reaction of carbon dioxide with methanol may be titrated by isopropylamine, and the mobile phase apparent pH is now somewhat increasing towards more neutral values (up to 6).

We had previously demonstrated a similar reversal of dominant behavior – with a dominant effect of adsorption at low concentrations and dominant effect of mobile phase properties at high concentrations – when examining the effects of the nature of the co-solvent with porous graphitic carbon stationary phases [56].

The trend in d^- term is illustrated by the chromatograms in Fig. 6 of an acidic compound, aspirin (aqueous $pK_a = 3.41$), which, at an apparent pH greater of about 6, should be predominantly in its deprotonated form. It can be observed that the low concentrations of IPA cause a sharp increase in retention compared to the “no additive” mobile phase composition, while further increases of IPA concentration cause retention decrease.

Concerning the retention of cationic species (d^+ term), comparing the “no additive” composition and 20 mM IPA in methanol, the first introduction of small concentrations of IPA in mobile phase causes a significant decrease in the d^+ term from positive values to near zero on the TAG column, indicating that cationic species are much less retained when IPA is present in mobile phase.

Further increases in IPA concentration cause no significant changes. On the silica column, the introduction of IPA in the mobile phase is mostly insignificant. Therefore, this is the only case where the introduction of IPA causes different variations between the two columns. We may attribute these differences to the different interaction capability of the “bare” stationary phases (uncovered with additive): on the silica surface, only silanol groups (neutral or ionized) should interact with cationic species while on the teicoplanin aglycone stationary phase, the deprotonated carboxyl group should also contribute to interaction with cationic species.

3.3.2. Effect of IPA on enantioseparations

From a chirality point of view, overall, a loss of enantioseparation is observed when adding high concentrations of IPA for most of the compounds that were resolved when no additive was used. This is in contradiction with previous observations from Phinney and Sander, where the introduction of isopropylamine in the SFC mobile phase was seen to slightly diminish separation factors on a vancomycin stationary phase, without loss of separation [23]. However, the enantioselectivity site may be significantly different between vancomycin and teicoplanin aglycone.

An example of chromatograms is presented in Fig. 7 where the ketoprofen enantiomers show some debut separation when the mobile phase does not contain any additive, but even the smallest concentrations of IPA cause a co-elution of the enantiomers. This molecule should also be partly anionic at the apparent pH of the mobile phase (aqueous $pK_a = 4.45$), that is to say that this molecule has a non-zero D^- descriptor value, and behaves according to the variations of d^- term, with a sharp increase of retention at low IPA concentrations, followed by a decrease in retention when IPA concentration increases.

A single exception was observed with dichlorprop, for which the separation factor improved with IPA compared to no additive (for instance, $\alpha = 1.65$ with 120 mM IPA compared to 1.43 without additive). In all other cases examined, separation factors decreased when IPA was used. The loss of enantioresolution can have two possible origins. Firstly, the chiral cavities may be rendered inaccessible to the enantiomers by the thick layer of adsorbed additive. Secondly, ionizable analytes for which the pK_a values are close to the apparent pH may change protonation state, thereby affecting the way they interact with the stationary phase. The ionizable functions are known to contribute significantly to enantioselectivity.

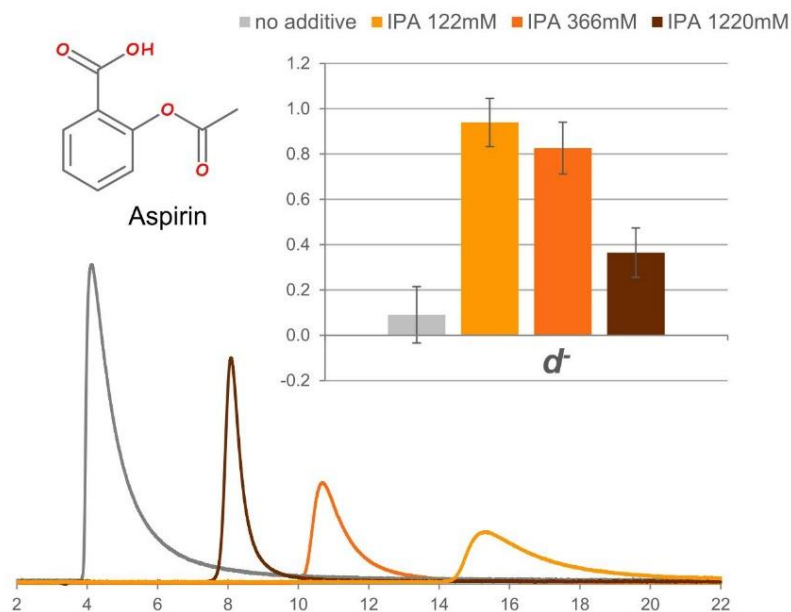


Fig. 6. Chromatograms of aspirin on the Chirobiotic TAG column with increasing concentrations of IPA: no additive (gray), IPA 122 mM (light orange), IPA 366 mM (dark orange) and IPA 1220 mM (brown). The inserted histograms show the variation of related LSER d^+ term when IPA concentration increases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

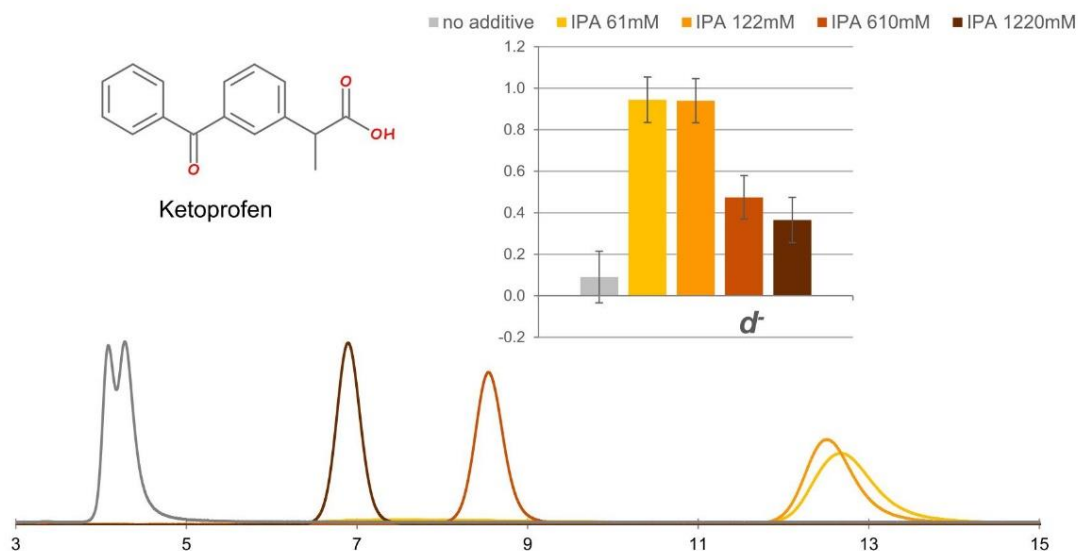


Fig. 7. Chromatograms of *rac*-ketoprofen on the Chirobiotic TAG column with increasing concentrations of IPA: no additive (gray), IPA 61 mM (yellow), IPA 122 mM (orange), IPA 610 mM (light brown), IPA 1220 mM (dark brown). The inserted histograms show the variation of related LSER d^+ term when IPA concentration increases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nitium in this stationary phase. This can however not contribute to changes for non-ionizable species like methylphenylsulfoxide or chlormezanone.

Interestingly, a recent paper from Pokrovskiy et al. [57] showed that the manipulation of ionic interactions through the selection and concentration of additives in the SFC mobile phase was significant to enantioresolution on another antibiotic stationary phase, erythromycin.

3.3.3. Evolution of retention with TFA concentration

On both columns, the only significant effect of TFA introduction is observed with the d^+ term, namely, increasing the concentration of TFA causes a sharp increase of d^+ values, indicating that cationic species are more retained when TFA is present in the mobile phase. Without an additive, the d^+ term was already pos-

itive on both columns due to interactions between cationic analytes and the silanol groups or carboxyl function of the teicoplanin aglycone. At the largest concentration of TFA tested (610 mM in methanol), the d^+ term is the most significant contributor to retention.

This is perhaps counter-intuitive as one might expect TFA to act as an ion-pairing agent to favor the elution of protonated basic compounds. Besides, as the carboxylic acid function of the teicoplanin aglycone should protonate, strong coulombic interactions between the stationary phase and cationic analytes were expected to decrease. The opposite behavior observed is most likely related to the same phenomenon as described for IPA: TFA adsorbs on the stationary phase and creates a layer on top of the surface and ligands. Because TFA interacts strongly with cationic species, the interactions between analyte and stationary phase strongly increase.

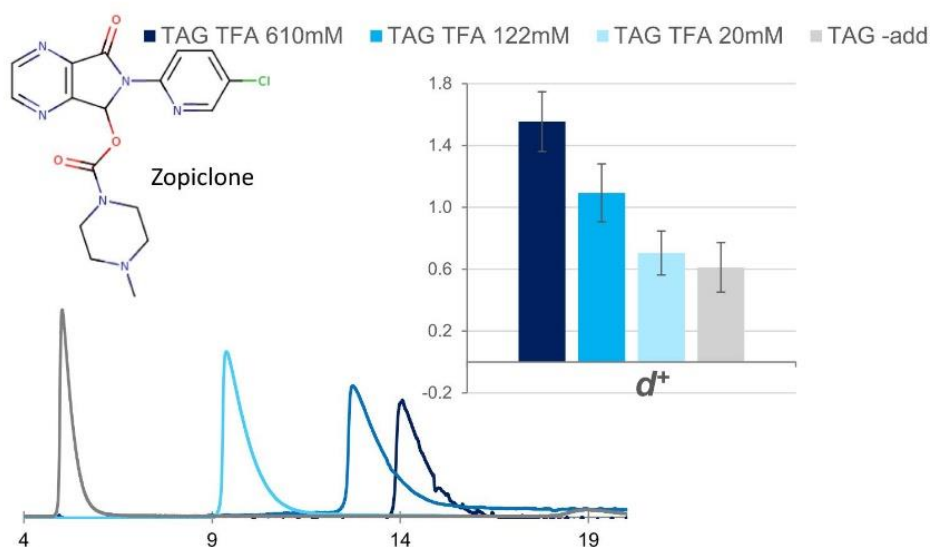


Fig. 8. Chromatograms of zopiclone on the Chirobiotic TAG column with increasing concentrations of TFA: no additive (gray), TFA 20 mM (light blue), TFA 122 mM (blue), TFA 610 mM (dark blue). The inserted histograms show the variation of related LSER d^+ term when TFA concentration varies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Contrary to the observations with IPA, no reversal of trend was observed at the largest concentrations on the TAG column (although we may suspect some of it on the BEH column). But then it was not possible to increase the concentration as high as in the IPA experiments due to solubility issues, thus if a reversal should occur at higher TFA concentrations, it was impossible to verify.

An example is given in Fig. 8 with the chromatograms of zopiclone, where the retention is seen to increase continuously with the concentration of TFA.

3.3.4. Effect of TFA on enantioseparations

Similarly to the effect of IPA, TFA had a negative influence on separation factors. Apart from one exception, separation factors decreased when TFA was introduced, compared to no additive. In one case, 5-methyl-5-phenylhydantoin, for which the separation factor was already very large without an additive ($\alpha = 3.07$), it increased further with the introduction of TFA ($\alpha = 3.30$ with 120 mM TFA).

The loss of enantioselectivity when an acidic additive is introduced may again have different origins, including obstruction of the chiral cavities by the adsorption of a large amount of TFA, varying charge state of ionizable analytes, or the protonation of the carboxylic group of the stationary phase, which may be deleterious to enantioselectivity.

4. Conclusion

In this paper, we have evaluated how large concentrations of acidic and basic additives influenced the retention and enantioselectivity behavior of a complex macrocyclic peptide-bonded stationary phase. It was first demonstrated that these highly concentrated additives had negligible influence on the mobile phase polarity. The influence on mobile phase acidity was however more significant, with TFA inducing strong decrease in the apparent pH (from 4.5–5 to 1–2), and IPA inducing some moderate increase (up to 6). This could cause changes in the ionization state of the carboxylic acid function of teicoplanin aglycone, but should have limited effect on the ionization state of free amine functions. Therefore, the probable unavailability of the amine function in teicoplanin aglycone could not be assessed through these means.

Considering these elements, the retention and enantioselectivity behavior were examined with the help of LSER methodology,

with a most complete description achieved with nine molecular descriptors. Based on the comparison to the effects observed on a bare silica phase, many effects could be explained by the adsorption of additives on the stationary phase. With the high concentrations that could be achieved for isopropylamine, it was observed that the primary effect of additives on retention mechanisms is through adsorption on the stationary phase, then when even higher concentrations are used, the effects in the mobile phase dominate the retention mechanisms. With trifluoroacetic acid, only adsorption effects were observed, but then it was not possible to increase its concentration as high as that of isopropylamine, due to solubility issues.

These results should be helpful in further understanding the effects of mobile phase additives in SFC, but also to understand the behavior of large biomolecules analyzed in SFC.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2019.460494.

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Effects of High Concentrations of Mobile Phase Additives on Retention and Separation Mechanisms on a Teicoplanin Aglycon Stationary Phase in Supercritical Fluid Chromatography

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Supporting Information

Figure S1: UV visible spectra of Nile Red showing polarity shift with additives

Figure S2: UV-visible spectra of the color indicators of pH

Figure S3: Principal component analysis score plot based on UV-visible spectra of color indicators in aqueous phase and SFC mobile phases

Figure S4: Full LSER model for (a) Chirobiotic TAG and (b) Acquity BEH

Table S1 List of achiral probe compounds with LSER descriptors

Table S2: List of chiral probe compounds with LSER descriptors

Table S3: Full LSER models for Chirobiotic TAG with statistical features

Table S4: Full LSER models for Acquity BEH with statistical features

Figure S1: UV visible spectra of Nile Red showing polarity shift with additives

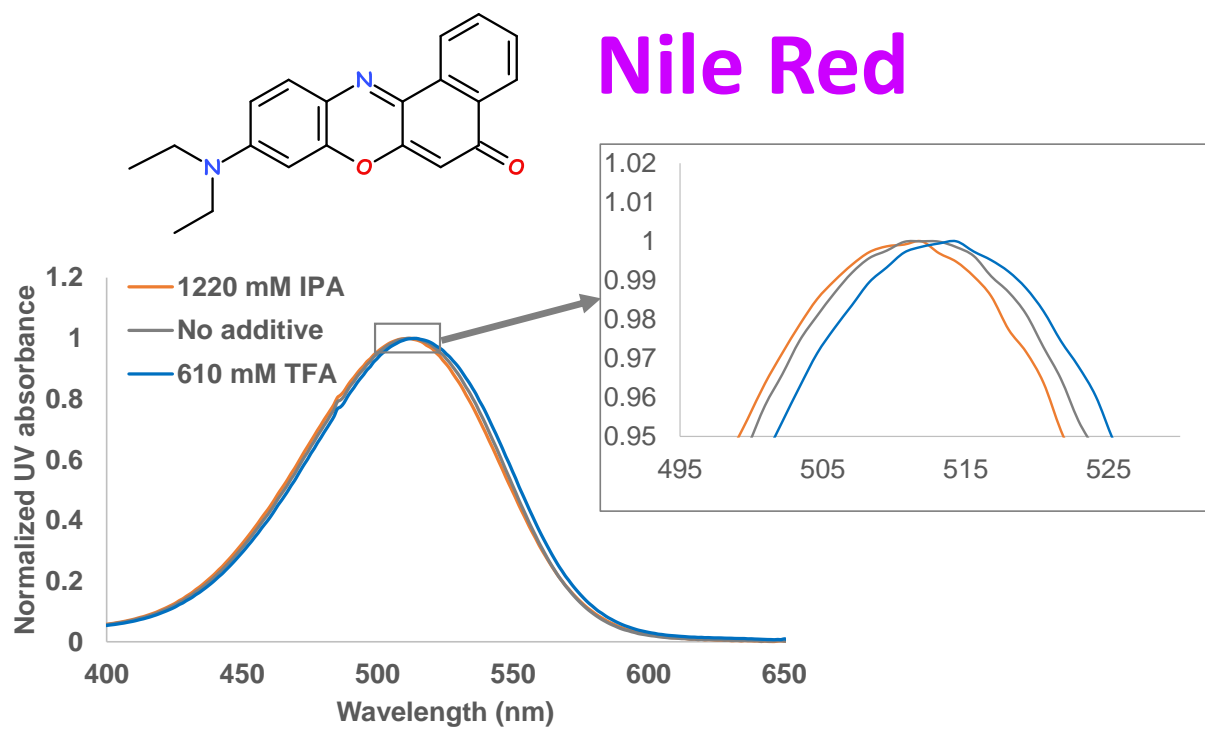
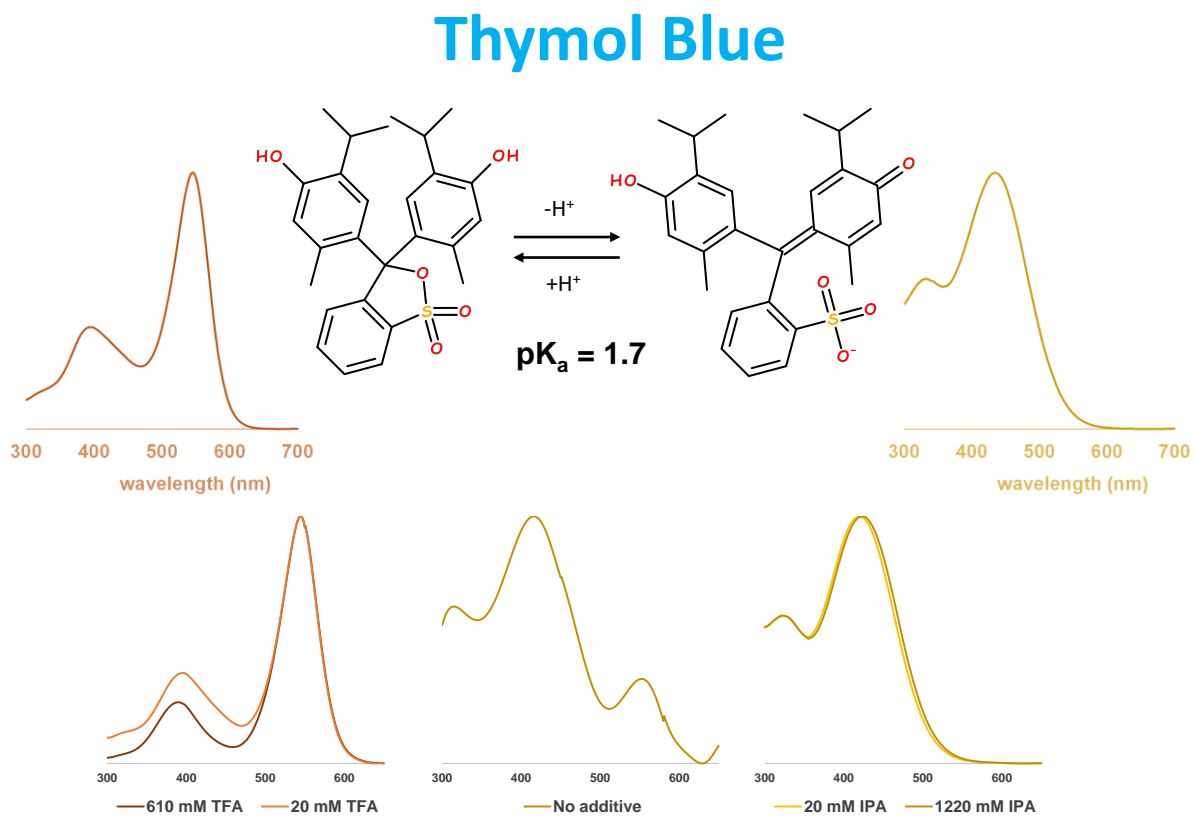
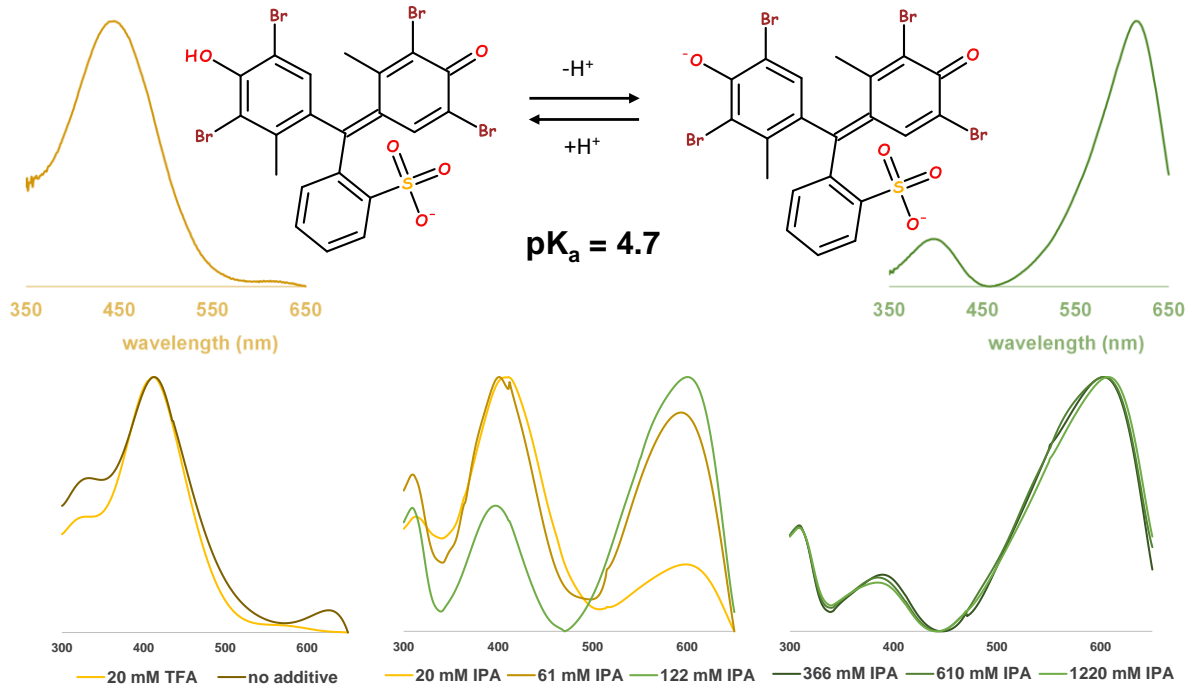


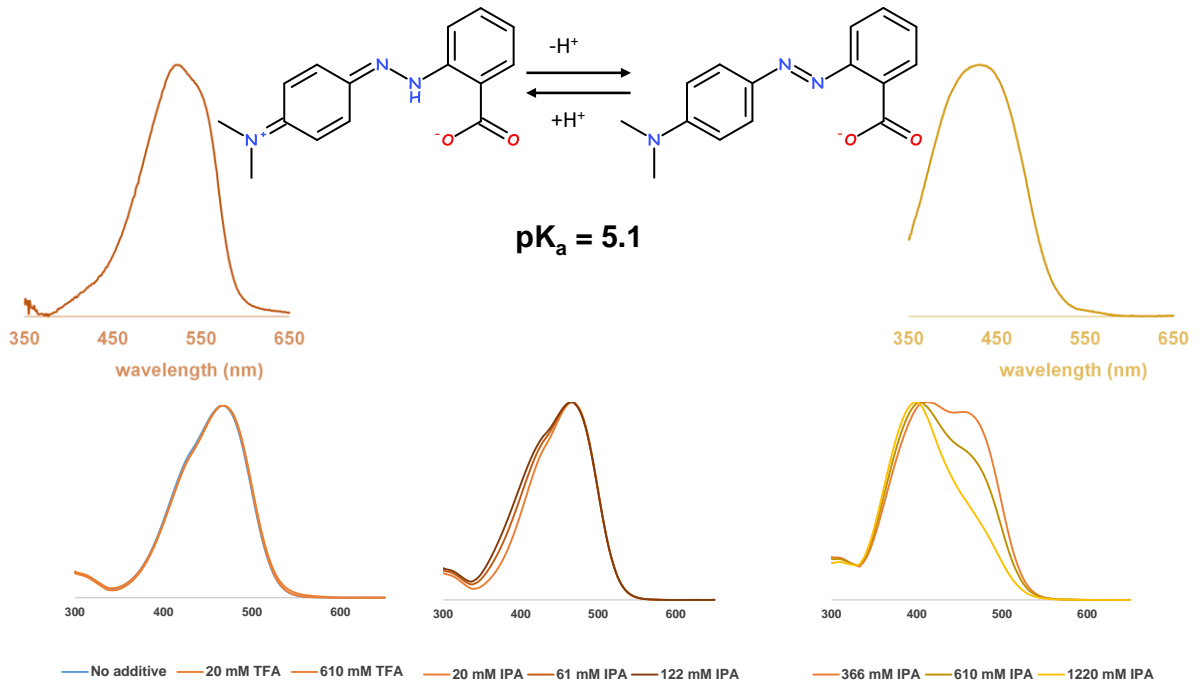
Figure S2: UV-visible spectra of the color indicators of pH. For each color indicator, the top spectra were acquired in aqueous liquid phase (see ref. [25] for details), the bottom spectra were acquired in SFC mobile phases (this work).



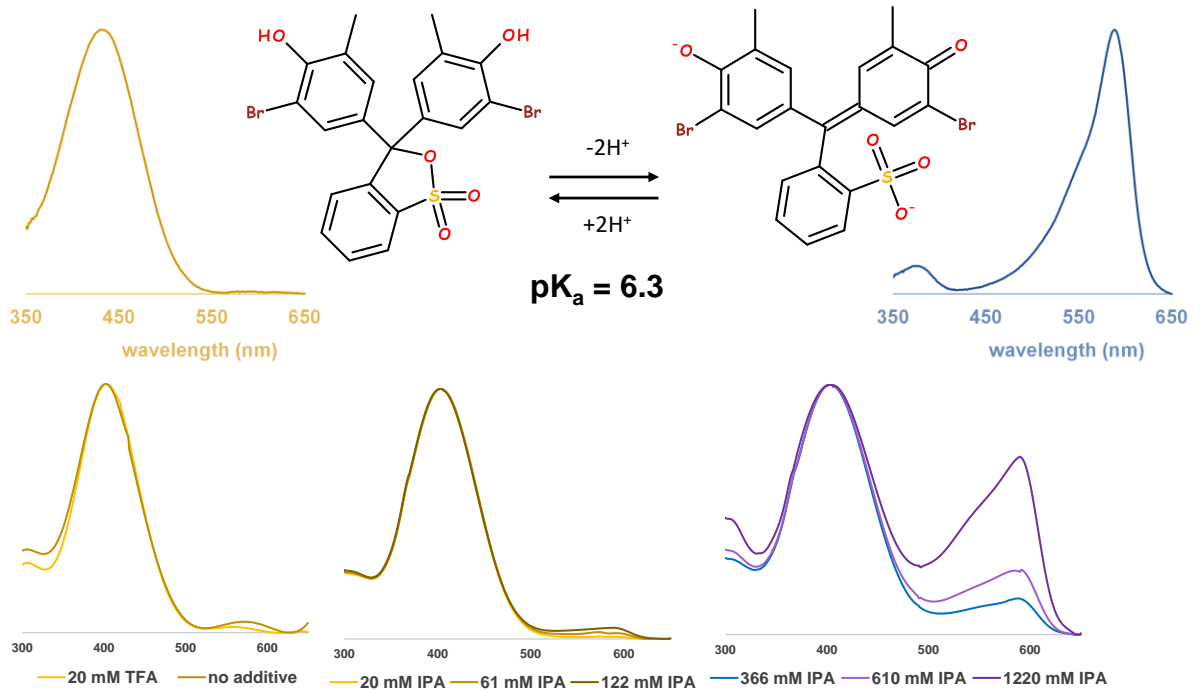
Bromocresol Green



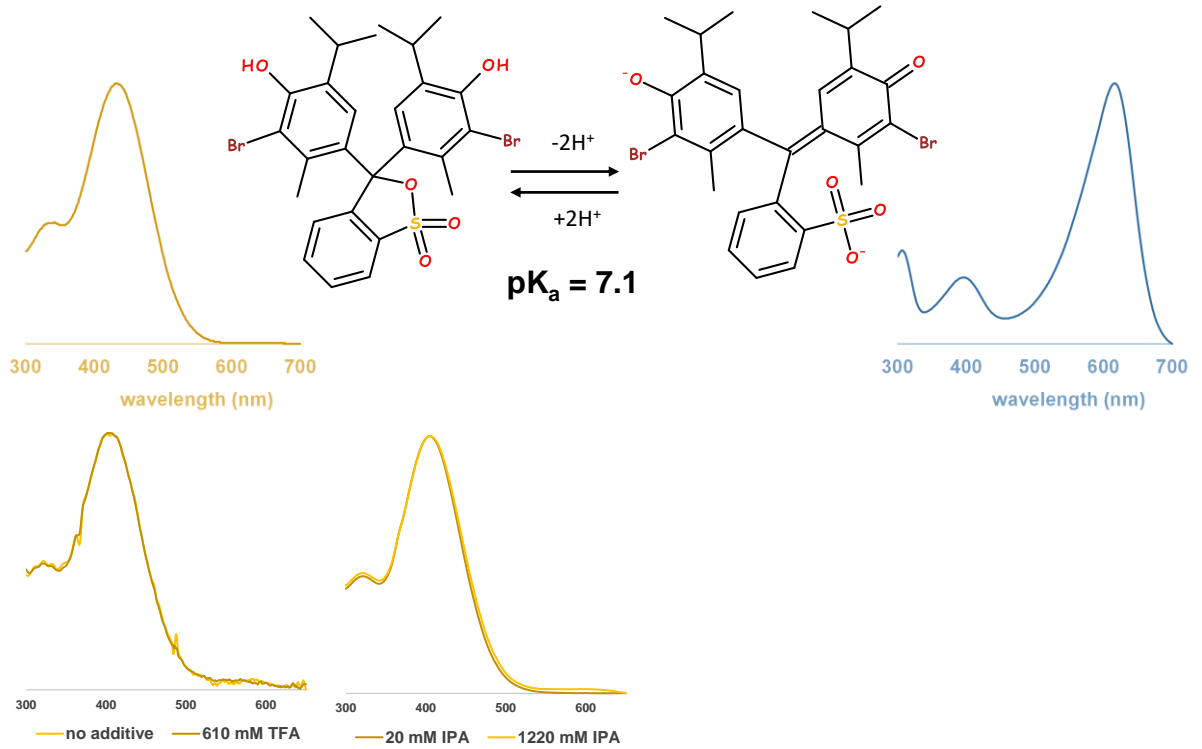
Methyl Red



Bromocresol Purple



Bromothymol Blue



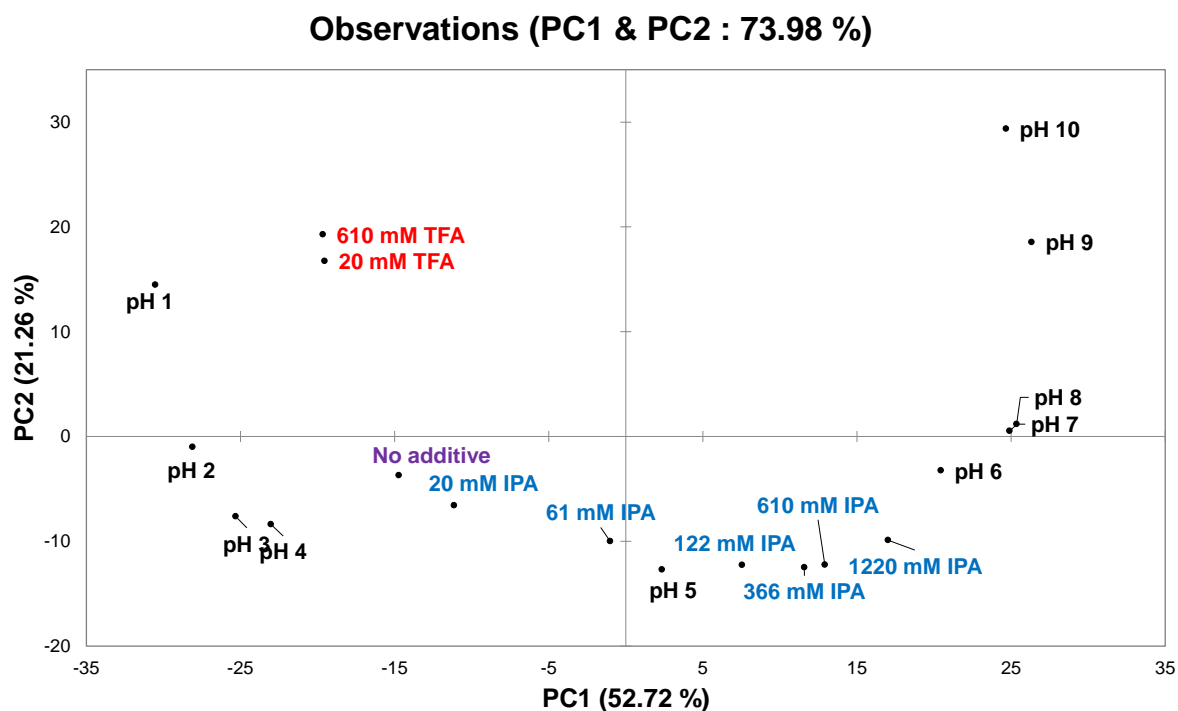


Figure S3: Principal component analysis score plot based on UV-visible spectra of color indicators in aqueous phase (ref. [25]) and SFC mobile phases (this work). The spectral data from Thymol Blue, Bromocresol Green, Methyl Red and Bromocresol Purple served as variables.

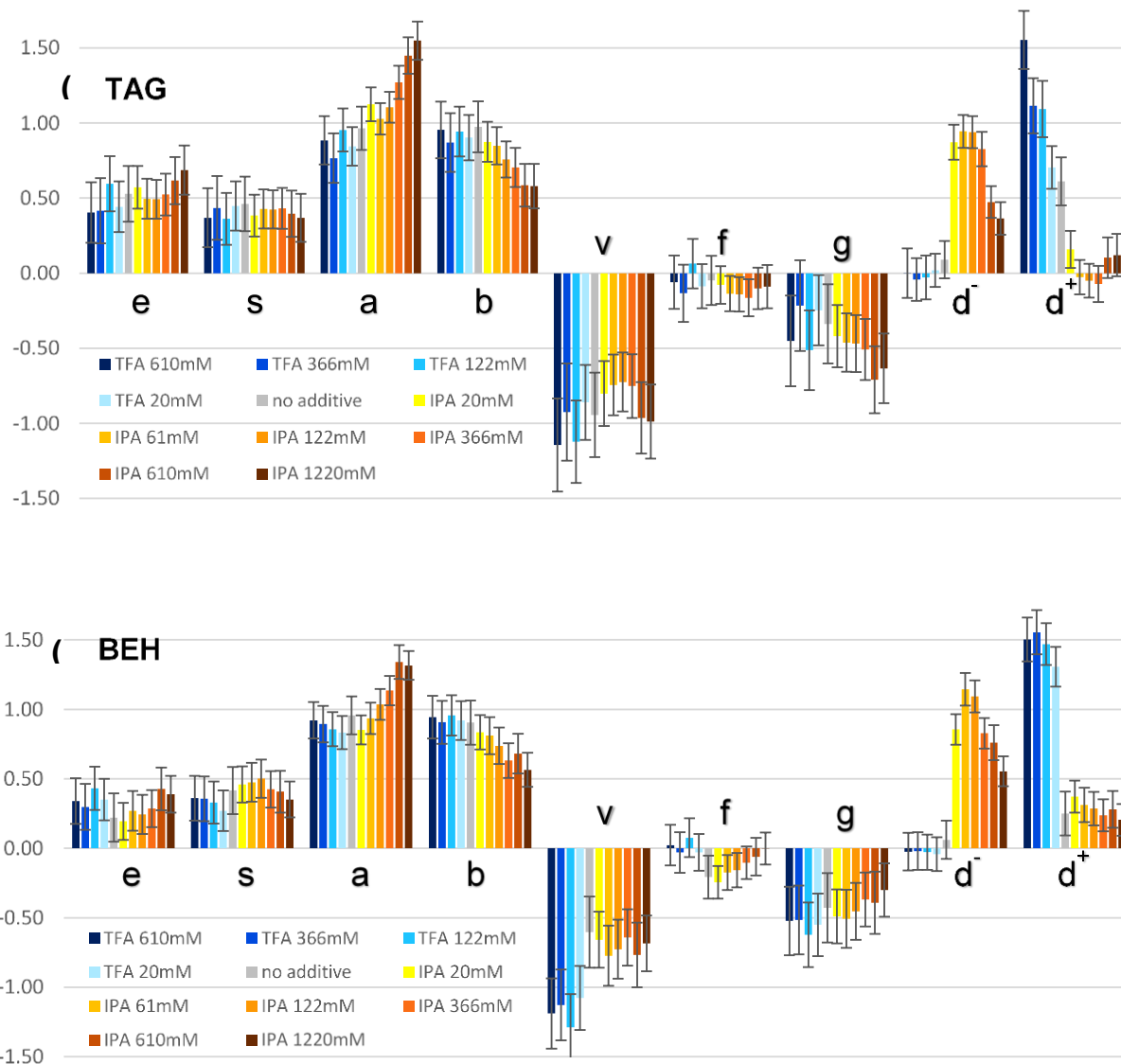


Figure S4: Full LSER models (according to Eq. (3)) for (a) the Chirobiotic TAG and (b) the Acquity BEH columns. Mobile phase: carbon dioxide – methanol (with or without additive according to legend, from left to right) 90:10 (v/v), 25°C, 15 MPa, 3 mL/min on TAG or 1 mL/min on BEH. For each descriptor, from left to right: TFA 610 mM, TFA 366 mM, TFA 122 mM, TFA 20 mM, no additives, IPA 20 mM, IPA 61 mM, IPA 122 mM, IPA 366 mM, IPA 610 mM and IPA 1220 mM.

Table S1: List of achiral probe compounds with LSER descriptors

Compound name	E	S	A	B	V	F	G	D ⁻	D ⁺
1-Methylnaphthalene	1.344	0.92	0.00	0.20	1.2263	0.000	1.720	0.00	0.00
1-Ethyl-naphthalene	1.371	0.88	0.00	0.20	1.3672	0.385	1.615	0.00	0.00
Benzyl alcohol	0.803	0.87	0.39	0.56	0.9160	0.625	1.833	0.00	0.00
Naphthalene methanol	1.530	1.27	0.39	0.62	1.2850	0.385	1.676	0.00	0.00
Methylbenzilate	1.350	1.40	0.17	1.03	1.8800	0.790	1.231	0.00	0.00
Anisole	0.708	0.75	0.00	0.29	0.9160	0.625	1.960	0.00	0.00
Acetophenone	0.818	1.01	0.00	0.48	1.0139	0.556	1.807	0.00	0.00
Valerophenone	0.795	0.95	0.00	0.50	1.4366	1.667	1.292	0.00	0.00
Nitrobenzene	0.871	1.11	0.00	0.28	0.8906	0.556	1.869	0.00	0.00
Iodobenzene	1.188	0.82	0.00	0.12	0.9750	0.000	1.851	0.00	0.00
Fluoronaphthalene	1.144	0.97	0.00	0.13	1.1030	0.000	1.785	0.00	0.00
Iodonaphthalene	1.928	1.22	0.00	0.16	1.3436	0.000	1.676	0.00	0.00
Phenol	0.805	0.89	0.60	0.30	0.7751	0.000	1.984	0.00	0.00
Eugenol	0.946	0.99	0.22	0.51	1.3544	1.250	1.554	0.00	0.00
Vanillin	1.040	1.33	0.29	0.69	1.1313	0.909	1.644	0.00	0.00
α -Naphthol	1.520	1.05	0.60	0.37	1.1441	0.000	1.777	0.00	0.00
Thioanisole	1.063	0.68	0.00	0.32	1.0208	0.625	1.751	0.00	0.00
<i>o</i> -Cresol	0.840	0.86	0.52	0.30	0.9160	0.000	1.884	0.00	0.00
<i>m</i> -Nitrotoluene	0.874	1.10	0.00	0.25	1.0315	0.500	1.691	0.00	0.00
<i>p</i> -Methylacetophenone	0.842	1.00	0.00	0.52	1.1550	0.500	1.631	0.00	0.00
Ethylbenzoate	0.689	0.85	0.00	0.46	1.2135	0.909	1.399	0.00	0.00
Diethylphthalate	0.729	1.40	0.00	0.88	1.7106	1.250	1.397	0.00	0.00
Dibutylphthalate	0.700	1.40	0.00	0.86	2.2742	2.000	1.185	0.00	0.00
Ethylparabene	0.860	1.35	0.69	0.45	1.2722	0.834	1.372	0.00	0.00
Butylparabene	0.860	1.33	0.71	0.46	1.5540	1.429	1.077	0.00	0.00
Anthracene	2.290	1.34	0.00	0.28	1.4544	0.000	1.541	0.00	0.00
Fluoranthene	2.600	1.52	0.00	0.25	1.5846	0.000	1.505	0.00	0.00
<i>p</i> -Terphenyl	2.040	1.48	0.00	0.30	1.9320	0.500	0.997	0.00	0.00
Acetanilide	0.900	1.39	0.48	0.67	1.1137	0.500	1.557	0.00	0.00
Antipyrine	1.320	1.50	0.00	1.48	1.4846	0.334	1.460	0.00	0.00
Paracetamol	1.060	1.63	1.04	0.86	1.1724	0.455	1.499	0.00	0.00
Phenobarbital	1.630	1.80	0.73	1.15	1.6999	0.556	1.966	0.00	0.00
Primidone	1.510	2.08	0.51	1.45	1.6842	0.588	1.576	0.00	0.00
Carbazole	1.787	2.01	0.18	0.08	1.3154	0.000	1.590	0.00	0.00
Hydroquinone	1.063	1.27	1.06	0.57	0.8337	0.000	1.916	0.00	0.00
Indazole	1.180	1.22	0.53	0.35	0.9050	0.000	1.956	0.00	0.00
Resorcinol	0.980	1.00	1.09	0.52	0.8338	0.000	1.914	0.00	0.00
Benzamide	0.990	1.50	0.49	0.67	0.9728	0.556	1.848	0.00	0.00
Phenylurea	1.110	1.33	0.79	0.79	1.0726	0.500	1.690	0.00	0.00
Benzenesulfonamide	1.130	1.55	0.55	0.80	1.0971	0.500	1.745	0.00	0.00

Table S1 (continued): Selection of achiral compounds for LSER characterization

Compound name	E	S	A	B	V	F	G	D ⁻	D ⁺
o-Toluidine	0.966	0.92	0.23	0.45	0.9570	0.000	1.874	0.00	0.23
N-Methylaniline	0.948	0.90	0.17	0.43	0.9570	0.625	1.780	0.00	0.32
Pyridine	0.631	0.84	0.00	0.52	0.6753	0.000	2.075	0.00	0.57
N,N-Diethylaniline	0.953	0.80	0.00	0.41	1.3798	1.364	1.570	0.00	0.88
Papaverine	2.190	2.76	0.00	1.47	2.5914	1.111	1.062	0.00	0.91
Amitriptyline	2.246	1.78	0.00	1.00	2.3996	0.652	1.128	0.00	1.00
Procaine	1.135	1.68	0.44	1.23	1.9767	1.765	0.955	0.00	1.00
2-Naphtylacetic acid	1.470	1.40	0.57	0.50	1.4416	0.667	1.438	0.65	0.00
3,3-Diphenylpropionic acid	1.330	1.53	0.57	0.57	1.8213	1.111	1.584	0.67	0.00
Benzoic acid	0.730	0.90	0.59	0.40	0.9317	0.556	1.876	0.89	0.00
4-Biphenylcarboxylic acid	1.480	1.34	0.61	0.51	1.5395	0.625	1.334	0.89	0.00
1-Naphtic acid	1.460	1.20	0.65	0.46	1.3007	0.357	1.684	0.96	0.00
Aspirin	0.781	0.80	0.49	1.00	1.2879	0.769	1.719	0.97	0.00
Cyclobarbital	1.440	1.35	0.49	1.45	1.7859	0.556	2.001	1.99	0.00

Table S2: List of chiral probe compounds with LSER descriptors

Compound name	E	S	A	B	V	F	G	D ⁻	D ⁺
Mandelic acid, methyl ester	0.800	1.01	0.17	0.89	1.2720	1.667	1.441	0.00	0.00
<i>trans</i> -Chlorostilbene oxide	1.570	1.39	0.00	0.45	1.6790	1.111	1.332	0.00	0.00
(2,3-epoxypropyl)-benzene	0.840	0.87	0.00	0.34	1.0890	1.818	2.162	0.00	0.00
Methylphenylsulfoxide	1.104	1.73	0.00	0.88	1.0795	1.111	1.764	0.00	0.00
Phenylvinylsulfoxide	1.220	1.89	0.00	0.85	1.1774	2.000	1.592	0.00	0.00
Isofenphos	1.390	1.38	0.13	1.56	2.6442	3.636	1.150	0.00	0.00
Fenamifos	1.020	2.05	0.13	1.29	2.3467	3.684	0.720	0.00	0.00
Tebupirimfos	1.270	1.10	0.00	1.42	2.4465	3.000	1.032	0.00	0.00
Flavanone	1.650	1.76	0.00	0.73	1.7127	0.526	1.235	0.00	0.00
6-Methoxyflavanone	1.720	1.89	0.00	0.93	1.9123	0.952	1.155	0.00	0.00
Oxazepam	2.350	1.10	0.45	1.60	1.9917	0.455	1.047	0.00	0.00
Chlormezanone	1.440	2.40	0.00	1.39	1.8311	0.556	1.374	0.00	0.00
5-Methyl-5-phenylhydantoin	1.370	1.58	0.44	1.06	1.4024	0.667	2.090	0.00	0.00
Diazepam	2.078	1.57	0.00	1.25	2.0739	0.455	1.137	0.00	0.01
Zopiclone	2.660	3.20	0.00	2.43	2.6228	1.000	0.773	0.00	0.99
Warfarine	2.300	2.18	0.35	1.49	2.3077	1.600	1.101	0.22	0.00
2-phenylpropionic acid	0.730	0.97	0.57	0.48	1.2140	1.818	1.658	0.72	0.00
Flurbiprofen	1.500	1.51	0.57	0.58	1.8389	1.579	1.190	0.79	0.00
(4-Hydroxyphenylacetic)-2-propionic acid	0.908	1.46	0.59	0.68	1.2720	1.667	1.590	0.89	0.00
Suprofen	1.510	1.89	0.57	0.81	1.9026	2.105	1.131	0.91	0.00
Ketoprofen	1.560	1.97	0.57	0.87	1.9779	2.000	1.285	0.93	0.00
Fmoc-Proline	2.310	2.36	0.57	1.15	2.4828	1.429	1.008	0.95	0.00
Fmoc-Threonine(tBu)	2.090	2.37	0.80	1.40	3.0728	1.935	0.908	0.96	0.00
Dichlorprop	1.050	1.40	0.57	0.61	1.5050	2.143	1.560	0.99	0.00

Table S3: Full LSER models (system constants) for Chirobiotic TAG with statistical features (R^2_{adj} is the adjusted correlation coefficient, F is Fisher's statistic, RMSE is the standard error in the estimate).

	TFA				No	IPA					
	610mM	366mM	122mM	20mM	additive	20mM	61mM	122mM	366mM	610mM	1220mM
c	0.90	0.26	0.65	0.06	0.61	0.09	0.31	0.31	0.28	0.79	0.65
e	0.40	0.42	0.59	0.44	0.51	0.57	0.50	0.49	0.52	0.62	0.69
s	0.37	0.43	0.36	0.45	0.49	0.38	0.43	0.42	0.43	0.40	0.37
a	0.88	0.77	0.95	0.84	1.00	1.12	1.03	1.11	1.27	1.45	1.55
b	0.95	0.87	0.94	0.90	1.06	0.87	0.85	0.76	0.70	0.59	0.58
v	-1.14	-0.93	-1.12	-0.86	-1.06	-0.80	-0.74	-0.73	-0.75	-0.96	-0.99
f	-0.06	-0.13	0.06	-0.09	-0.08	-0.08	-0.14	-0.14	-0.16	-0.10	-0.09
g	-0.45	-0.22	-0.51	-0.25	-0.60	-0.42	-0.46	-0.47	-0.51	-0.71	-0.63
d⁻	0.00	-0.04	-0.03	0.02	0.11	0.87	0.94	0.94	0.83	0.47	0.36
d⁺	1.55	1.11	1.09	0.70	0.68	0.16	-0.03	-0.05	-0.07	0.10	0.12
R^2_{adj}	0.81	0.77	0.85	0.85	0.89	0.92	0.92	0.93	0.92	0.90	0.90
F	26	21	31	34	47	65	71	76	71	57	55
RMSE	0.28	0.30	0.25	0.23	0.22	0.20	0.19	0.18	0.20	0.22	0.23

Table S4: Full LSER models (system constants) for Acquity BEH with statistical features (R^2_{adj} is the adjusted correlation coefficient, F is Fisher's statistic, RMSE is the standard error in the estimate).

	TFA				No	IPA					
	610mM	366mM	122mM	20mM	additive	20mM	61mM	122mM	366mM	610mM	1220mM
c	0.19	0.20	0.36	0.26	-1.62	-0.28	-0.17	-0.31	-0.45	-0.53	-0.38
e	0.34	0.30	0.43	0.35	0.34	0.19	0.27	0.24	0.29	0.43	0.39
s	0.36	0.36	0.33	0.27	0.49	0.46	0.48	0.50	0.42	0.41	0.35
a	0.92	0.89	0.86	0.83	1.01	0.85	0.94	1.04	1.14	1.34	1.32
b	0.94	0.91	0.96	0.92	0.92	0.83	0.81	0.74	0.63	0.68	0.56
v	-1.19	-1.13	-1.29	-1.08	-0.62	-0.66	-0.77	-0.73	-0.64	-0.77	-0.68
f	0.02	-0.03	0.08	-0.03	0.04	-0.24	-0.17	-0.16	-0.10	-0.06	0.00
g	-0.52	-0.52	-0.62	-0.55	0.04	-0.49	-0.51	-0.45	-0.37	-0.39	-0.30
d⁻	-0.02	-0.02	-0.03	-0.04	-0.02	0.86	1.14	1.09	0.83	0.76	0.55
d⁺	1.50	1.55	1.47	1.31	-0.10	0.37	0.31	0.29	0.24	0.28	0.20
R^2_{adj}	0.86	0.86	0.87	0.86	0.92	0.91	0.91	0.91	0.91	0.91	0.91
F	37	36	39	37	62	57	59	63	61	56	62
RMSE	0.23	0.23	0.22	0.21	0.16	0.19	0.20	0.20	0.19	0.22	0.19

III. Etude des phases stationnaires Chiralpak® ZWIX – Contribution des éléments structurels du ligand

Cette étude a été effectuée sur les colonnes Chiralpak® ZWIX, commercialisées par Chiral Technologies. Ces colonnes vont être nos références, puisqu'elles sont intéressantes en raison de leur utilisation pour l'énantioséparation des acides aminés, une des problématiques de ma thèse. 9 phases stationnaires structurellement proches des Chiralpak® ZWIX sont étudiées afin de comprendre leurs mécanismes de rétention et d'énantioséparation. Ces phases présentent des dissimilitudes en termes de greffon par rapport aux phases de référence, c'est-à-dire qu'il manque des groupements fonctionnels dans la structure de leur greffon. L'impact de l'absence de certains greffons sur la rétention et l'énantioséparation est discuté. Deux sets de 161 molécules achirales et de 66 molécules chirales sont analysés sur les 11 colonnes en SFC en mode isocratique, avec une seule condition de phase mobile. Des modèles LSER sont donc utilisés, comme précédemment, pour visualiser rapidement les différences entre toutes ces phases stationnaires. D'autres analyses multivariées, les analyses discriminantes, permettent de mettre en évidence les facteurs contribuant à l'énantioséparation.

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Cinchona-based zwitterionic stationary phases: Exploring retention and enantioseparation mechanisms in supercritical fluid chromatography with a fragmentation approach

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Cinchona-based zwitterionic stationary phases: Exploring retention and enantioseparation mechanisms in supercritical fluid chromatography with a fragmentation approach

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ABSTRACT

Chiralpak ZWIX(+) and ZWIX(-), are brush-type bonded-silica chiral stationary phases (CSPs), based on complex diastereomeric *Cinchona* alkaloids derivatives bearing both a positive and a negative charge. In the present study, we aimed to improve the understanding of retention and enantioseparation mechanisms of these CSPs employed in supercritical fluid chromatography (SFC). For this purpose, 9 other stationary phases were used as comparison systems: two of them are commercially available and bear only a positive charge (Chiralpak QN-AX and QD-AX) and the 7 others were designed purposely to be structurally similar to the parent ZWIX phases, but miss some portion of the complex ligand. First, cluster analysis was employed to identify similar and dissimilar behavior among the 11 stationary phases, where ionic interactions appeared to dominate the observed differences. Secondly, the stationary phases were characterized with linear solvation energy relationships (LSER) based on the SFC analysis of 161 achiral analytes and a modified version of the solvation parameter model to include ionic interactions. This served to compare the interaction capabilities for the 11 stationary phases and showed in particular the contribution of attractive and repulsive ionic interactions. Then the ZWIX phases were characterized for their enantioseparation capabilities with a set of 58 racemic probes. Discriminant analysis was applied to explore the molecular structural features that are useful to successful enantioseparation on the ZWIX phases. In particular, it appeared that the presence of positive charges in the analyte is causing increased retention but is not necessarily a favorable feature to enantioselectivity. On the opposite, the presence of negative charges in the analyte favors early elution and enantioselectivity. Finally, a smaller set of 30 pairs of enantiomers, selected by their structural diversity and different enantioselectivity values on the ZWIX phases, were analyzed on all chiral phases to observe the contribution of each structural fragment of the chiral ligand on enantioselectivity. Molecular modelling of the ligands also helped in understanding the three-dimensional arrangement of each ligand, notably the intra-molecular hydrogen bonding or the possible contribution of ionic interactions. In the end, each structural element in the ZWIX phases appeared to be a significant contributor to successful enantioselectivity, whether they contribute as direct interaction groups (ion-exchange functions) or as steric constraints to orientate the interacting groups towards the analytes.

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1. Introduction

Cinchona-based chiral stationary phases were introduced in the late 1980s in form of free quinine bonded to silica [1] and as carbamate derivatives in 1996 by Lämmerhofer and Lindner [2]. They are

brush-type enantioselective stationary phases where the ligand is derived from quinine and quinidine diastereomers bonded on silica gel. Recently, similar phases bonded so superficially porous silica were proposed by Armstrong and co-workers [3]. Because quinine and quinidine both possess a tertiary amine function (quinuclidine ring), which should be protonated in most liquid and supercritical fluid mobile phases, a quinine- or quinidine-based stationary phase should act as an anion-exchanger. One such phase is presented in Fig. 1(QN). Two commercial stationary phases are

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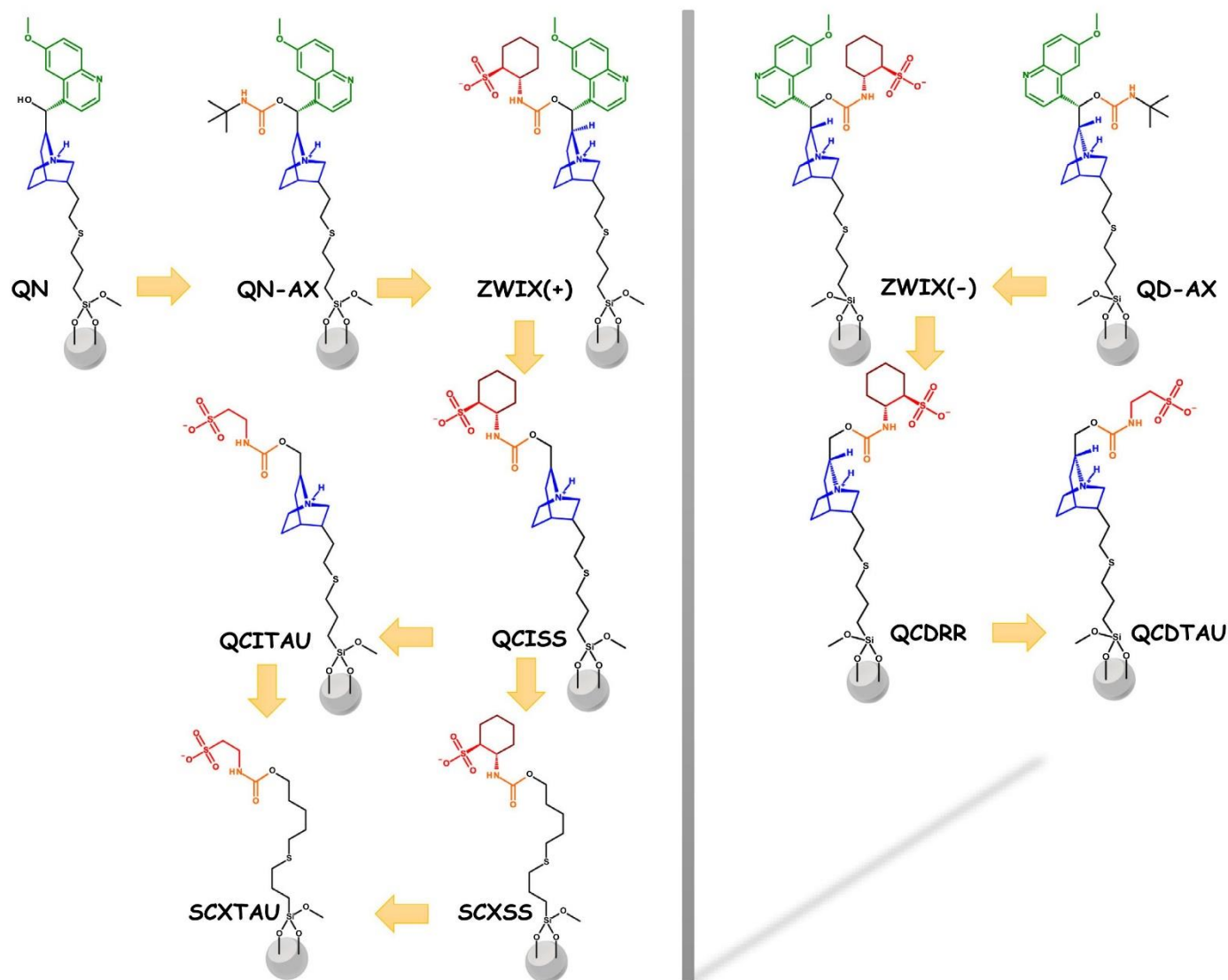


Fig. 1. Structures of the chiral selectors in the 11 stationary phases compared.

available, which were derived from quinine- and quinidine-bonded silica, with a carbamate function modifying the hydroxyl group and retaining the anion-exchange capabilities: Chiralpak **QN-AX** and **QD-AX** (Fig. 1). These stationary phases are mostly employed in HPLC, where they have demonstrated excellent enantioselective properties for a broad range of chiral acids [2,4]. Further modification of the carbamate function to include a chiral sulfonic acid group yielded two zwitterionic stationary phases, commercially available as Chiralpak **ZWIX(+)** and **ZWIX(-)** [5,6]. These phases were notably demonstrated to be particularly useful for the enantioseparation of free or derivatized amino acids and oligopeptides by HPLC [6–13]. Cinchona alkaloids and their derivatives were also employed as chiral selectors in capillary electrophoresis [14,15] and capillary electrochromatography [16,17], where the high efficiency values usually yield excellent resolution.

Supercritical fluid chromatography (SFC) is known to be most effective for enantioselective separations [18,19], as the resolution per unit time is often advantageous compared to HPLC separations, making it suitable for high-throughput enantioselective analysis. In addition, the economic and ecological aspects related to the use of pressurized carbon dioxide as principal component of the mobile phase render it an attractive method for the industry, both

at the analytical and preparative scales. The *Cinchona*-based chiral phases were only rarely employed in SFC. One possible reason is the wrong perception that ionic species cannot exist in the apparently non-polar SFC mobile phase. However, it was proven in several studies that ionic species exist in carbon dioxide – organic solvent mixtures and can even be resolved [20,21] and that ionic interactions exist, which are measurable [4,22,23] and can be modified when acidic and basic additives are introduced in the co-solvent [24]. Thus a few papers relate the use of **ZWIX** phases to resolve N-protected amino acids [25,26] or indole-type analytes [27]. Recently, we have demonstrated that elution and enantioresolution of a wide range of free natural amino acids were possible with a wide elution gradient running from SFC to HPLC conditions [28].

Retention and enantioselective mechanisms on the **ZWIX** phases were explored mostly in liquid phase by the group of Lindner on the one hand [9,29], Ilisz and Péter on the other hand [30], but only little was done in SFC conditions. Pell and Lindner showed that the ion-exchange mechanism existed on **QN-AX** and **QD-AX** phases in SFC conditions, thanks to the presence of methoxycarbonic acid formed by the reaction of CO_2 and acting as counterion in the anion-exchange process [4]. This ion-exchange mechanism

could be tuned with the introduction of acidic or basic additives in the mobile phase. For these stationary phases, the same chiral recognition mechanism was observed between HPLC and SFC experiments. More recently, Wolrab et al. observed major differences between HPLC and SFC separations on the **ZWIX** phases and proceeded to explain the differences [31]. A major conclusion from this work is that mechanistic investigations done on the **ZWIX** phases in HPLC conditions may not all be transferable to SFC conditions. Naturally, the components of the mobile phase can have a significant contribution to the enantioselectivity as they define the environment in which the process occurs [6,30,32], may have different solvation properties towards the chiral analytes and the chiral stationary phase, which is sometimes resulting in different conformations of the stationary phase.

To better understand the behavior of Chiralpak **ZWIX(+)** and **ZWIX(-)** in SFC, a fragment-based approach to deciphering the complex enantioselective ligands was selected: nine other (commercial or in-house) stationary phases were used as comparison. All nine phases had some similarity to the **ZWIX** phases, each one having one less fragment than the parent ligand. The purpose was to understand the contribution of each functional group in the retention mechanisms and appreciate how each functional group was determinant in the enantioselective process. For this purpose, the eleven columns were first characterized by means of quantitative structure-retention relationships (QSRR) with a modified version of the solvation parameter model, to include ionic interactions, based on the analysis of 161 achiral analytes. Cluster analysis and the QSRR models served to compare the columns. Secondly, based on the analysis of 58 racemic probes, discriminant analysis served to identify the molecular features that are most favorable to successful enantioselective separation on the **ZWIX** phases. Finally, 30 racemates with structural diversity were analyzed to explore the differences in enantioselectivity. Molecular modelling assisted in understanding the observed differences.

2. Material and methods

2.1. Stationary phases

All experiments were carried out on the eleven columns. Four columns are commercially available, named Chiralpak **ZWIX(+)**, Chiralpak **ZWIX(-)**, Chiralpak **Chiralpak QN-AX** and Chiralpak **QD-AX**, and were kindly provided by Chiral Technologies Europe (Illkirch, France) with ligands bonded on 3 μm particles. The seven other ligands were prepared in-house, bonded on 3 μm Daisogel silica, 120 Å (Osaka Soda) and packed. Their synthesis and liquid-phase characterization was described in a previous study [33]. All columns had the same dimensions of 150 × 3 mm.

2.2. Chemicals and solvents

161 achiral (Table S1) and 66 chiral molecules (Table S2) in their racemic form were purchased from Sigma-Aldrich (Sigma Aldrich Chimie, France). The achiral analytes were selected to provide a diverse representation of chemical functions to obtain meaningful retention models with the solvation parameter model. The chiral probes in Table S2 were selected to obtain meaningful information from the discriminant analyses with different structures and shapes. These sets were fully described in previous papers [34,35]. The reduced set of chiral analytes (Table S2, in bold) was selected as a subset of the wider set maintaining structural diversity but also included an addition of 8 free amino acids. As the individual enantiomers were not available in most cases, the elution order was not assessed. Solutions of all analytes were prepared at about 1 mg/mL in methanol (MeOH). The HPLC-grade

methanol used for analyte dissolution and as mobile phase co-solvent was supplied by VWR (Fontenay-sous-Bois, France). Ultrapure water was supplied by an Elga UHQ system from Veolia (Wis-sous, France). CO₂, with a purity of 99.995%, was delivered by Air Liquide (Paris, France). Ammonium formate was provided by Sigma Aldrich (Sigma Aldrich Chimie, France).

2.3. Instruments and operating conditions

The supercritical fluid chromatography 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 a photodiode-array (PDA) detector. Empower® 3 was used for integration of peaks for column efficiency measurements. An ACQUITY QDa® single-quadrupole mass detector with electrospray ionization source was also used for amino acids detection. 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.

All analyses (chiral and achiral sets) were performed in isocratic conditions (CO₂-MeOH 90:10 (v/v), 25 °C, 150 bar, 3 mL/min), apart from the analyses of amino acids for which a wide gradient elution (10 to 100% co-solvent) was done with methanol containing 5% H₂O and 50 mM ammonium formate, according to previously developed method [28]. In that case, the flow rate was reduced to 0.5 mL/min to avoid reaching the upper pressure limit of the pumping system. The UV detection wavelength was 210 nm while single-ion recording was used for MS detection of amino acids according to the mass of the protonated molecular ion.

2.4. Data analysis

Abraham descriptors (Tables S1 and S2) were extracted from an in-house database based on all existing literature on the solvation parameter model. Additional structural descriptors were computed as previously described [22,35].

Multivariate data analyses (hierarchical cluster analysis (HCA), multiple linear regression and discriminant analysis (DA)) were done with XLSTAT 19.03 software (Addinsoft, New York, NY, USA). The retention data (log *k* values) for achiral analytes were normalized (centred and reduced to adjust mean value on 0 and standard deviation on 1) prior to HCA calculation, to ensure that equal significance would be associated to each analyte. HCA was based on Ward aggregation method with Euclidean distance.

The quality of QSRR fits was estimated using the adjusted determination coefficient (R^2_{adj}), standard error in the estimate (*SE*) and Fisher *F* statistic. The statistical significance of individual coefficients was evaluated with the 95% confidence intervals.

The linear solvation energy relationship (LSER) equation used in this work is based on the five Abraham descriptors and two additional descriptors to take ionic interactions into account:

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

In this equation, capital letters represent the solute descriptors, related to particular interaction properties, each molecule has its own molecular descriptors. While lower case letters represent the system constants, related to the complementary effect of the two phases (stationary and mobile). *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* quantifies the presence of dipoles and polarizability; *A* and *B* are the

solute overall hydrogen-bond acidity and basicity; V is the McGowan characteristic volume in units of $\text{cm}^3 \text{mol}^{-1}/100$; D^- and D^+ are the solute negative and positive charges respectively, calculated with the pK_a and apparent pH of the mobile phase. The five first descriptors are known as Abraham descriptors and were used in many occasions to characterize chromatographic systems, while the latter two were introduced more recently to characterize hydrophilic interaction liquid chromatography (HILIC) systems [36], and were later demonstrated to be suitable to describe SFC systems, provided we admit to some approximations [22,23]. 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 very strongly with either the mobile phase (if the coefficient is negative) or the stationary phase (if the coefficient is positive). The system constants facilitate the comparison of the separation characteristics of different stationary phases and enable the identification of stationary phases of similar or dissimilar selectivity.

In previous works, we have demonstrated that two other structural features that contributed little to retention were however significant to describe enantioseparation mechanisms on polysaccharide or macrocyclic glycopeptide stationary phases: flexibility (F) and globularity or sphericity (G) [35,37–39]. In the present work, the F and G descriptors were first introduced in Eq. (1) but appeared to be insignificant to explain retention. They were however retained for the evaluation of chiral separations through discriminant analysis. The quality of discriminant analysis was estimated based on ROC (receiver operating characteristics) curves (visible in Fig. S1) and confusion matrices.

2.5. Molecular modelling

The 3D structures of each enantioselective ligand presented in Fig. 1 were prepared from 2D coordinates. The 3D conformations were generated using the Structure Preparation function and hydrogen atoms were added using Protonate3D function in MOE2014.09 (Chemical Computing Group Inc., Montreal, Canada). Then the structures were submitted to conformational search using LowModeMD with default parameters. The MMFF94x force field with Born solvation was used and the lowest energy conformer was retained for comparison of the ligand conformations. The structures were observed and copied from Discovery Studio 4.0 Visualizer (Accelry, San Diego, California, USA).

3. Results and discussion

3.1. Description of the stationary phases

The chiral selectors in the eleven stationary phases compared in this study are presented in Fig. 1.

As can be seen in Fig. 1, apart from the above-cited **ZWIX(+)**, **ZWIX(-)**, **QN**, **QN-AX** and **QD-AX**, six other fragmented ligands were designed. First, removal of the quinoline group from the **ZWIX(+)** and **ZWIX(-)** chiral selectors yielded **QCISS** and **QC-DRR** ligands, respectively. The steric constriction of the sulfonic group was further removed to yield **QCITAU** and **QCDAU**, based on quincorine (QCI) / quincoridine (QCD) and taurine (TAU). Finally, the cationic quincoridine group was removed from **QC-DRR** and **QCDAU** to obtain the strong cation-exchangers 2-aminocyclohexanesulfonic acid-derived **SCXSS** and taurine-derived **SCXTAU**. The ligand coverage on the stationary phase surface was adjusted to ca. $200 \mu\text{mol/g}$ (within $\pm 30 \mu\text{mol/g}$) for all stationary phases [33].

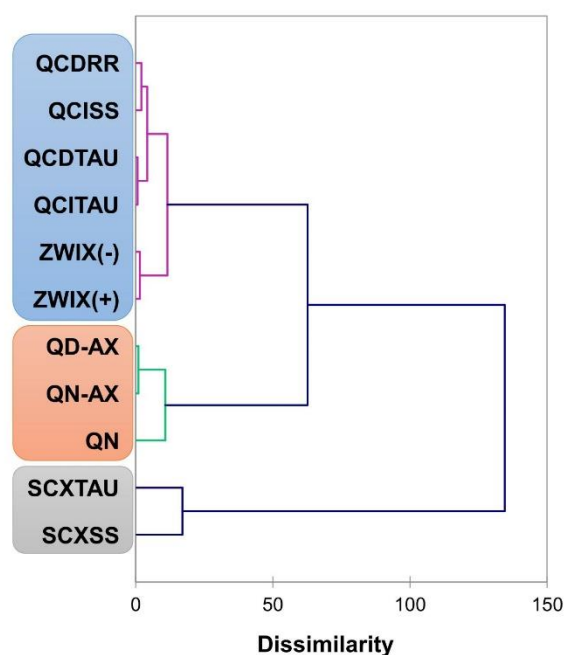


Fig. 2. Hierarchical cluster analysis on the normalized retention data (normalized log k values) measured for the 161 analytes in Table S1 on the 11 stationary phases in Fig. 1. Retention dissimilarity in the abscissa reflects Euclidean distance between the stationary phases. Chromatographic conditions: CO_2 -methanol 90:10 (v/v), 25°C , 15 MPa, 3 mL/min.

All these phases (the seven in-house and the Chiralpak **QN-AX** and **QD-AX**) were highly similar to the **ZWIX** phases, each one having only some portion of the structure less than the original ligand. The purpose was to understand the involvement of each functional group in the retention and enantioseparation mechanisms and measure how each functional group was determinant in the enantioseparation process. Due to the large number of stationary phases and the number of analytes included to obtain most accurate and representative models, only one mobile phase composition was employed, although acidic and basic additives should clearly have an effect on enantioselective capabilities of these phases [31].

3.2. Multivariate data analysis: hierarchical cluster analysis

Hierarchical cluster analysis (HCA) is a useful method to group chromatographic columns according to the similarity in their retention behaviour. Because the retention data were normalized, the similarity will reflect elution order of the analytes, rather than overall retention. The result are presented in a dendrogram (Fig. 2), where the columns that are linked with a short horizontal line are most similar and have close chromatographic selectivity, and the columns that are linked with a long horizontal line are most dissimilar and exhibit different chromatographic selectivity. In Fig. 2, three principal clusters appear:

- In the upper part of the figure, the **ZWIX(+)** and **ZWIX(-)** phases are grouped with the four other zwitterionic phases **QC-ITAU**, **QCISS**, **QCRRR** and **QCDAU**.
- In the middle of the figure, the three phases possessing only a cationic charge are grouped (**QN**, **QN-AX** and **QD-AX**).
- In the lower part of the figure, the two phases possessing only an anionic charge are grouped (**SCXSS** and **SCXTAU**).

Clearly, the clustering is mostly based on ionic interactions, while the presence of quinoline and the hexyl ring on the sulfonic group are much less significant in terms of retention

Table 1
System constants and statistics for the 11 columns tested obtained with Eq. (2).

Column	<i>c</i>	<i>e</i>	<i>s</i>	<i>a</i>	<i>b</i>	<i>v</i>	<i>d</i> ⁻	<i>d</i> ⁺	<i>n</i>	R ² _{adj}	SE
ZWIX(+)	-1.004	0.569	0.291	1.283	0.167	-0.241	0.046	0.190	126	0.876	0.190
ZWIX(-)	0.081	0.056	0.080	0.067	0.084	0.077	0.058	0.086			
	-0.916	0.537	0.283	1.333	0.217	-0.240	0.049	0.176	127	0.899	0.163
	0.068	0.048	0.069	0.059	0.072	0.066	0.053	0.068			
QN-AX	-0.916	0.493	0.179	1.477	-0.055	-0.093	1.154	0.036	145	0.944	0.153
	0.059	0.039	0.060	0.054	0.067	0.036	0.067	0.063			
QD-AX	-0.873	0.482	0.187	1.396	-0.022	-0.089	1.073	0.024	143	0.941	0.155
	0.061	0.040	0.061	0.055	0.069	0.037	0.065	0.064			
QN	-1.132	0.484	0.119	1.229	0.081	0.009	1.205	0.108	150	0.930	0.159
	0.058	0.040	0.059	0.055	0.068	0.037	0.070	0.063			
QCISS	-1.154	0.527	0.141	1.508	0.218	-0.082	-0.094	0.038	150	0.930	0.151
	0.058	0.039	0.060	0.051	0.067	0.036	0.048	0.065			
QCRRR	-0.842	0.481	0.034	1.359	0.297	-0.074	0.260	-0.039	147	0.949	0.122
	0.047	0.032	0.049	0.042	0.056	0.029	0.044	0.053			
QCITAU	-1.079	0.518	0.236	1.438	0.174	-0.142	0.309	-0.044	148	0.935	0.158
	0.058	0.041	0.061	0.053	0.071	0.037	0.050	0.066			
QCDTAU	-1.014	0.490	0.184	1.339	0.185	-0.137	0.212	0.018	147	0.939	0.139
	0.052	0.036	0.054	0.047	0.062	0.033	0.044	0.058			
SCXRR	-1.482	0.554	0.144	0.928	0.658	-0.191	-0.260	1.799	145	0.851	0.215
	0.093	0.058	0.081	0.072	0.086	0.057	0.063	0.163			
SCXTAU	-1.374	0.566	0.242	0.997	0.657	-0.446	-0.202	1.531	134	0.874	0.217
	0.103	0.059	0.084	0.076	0.094	0.063	0.065	0.126			

n is the number of solutes finally retained in the multiple linear regression.
R² is the determination coefficient; SE in the standard error in the estimate.

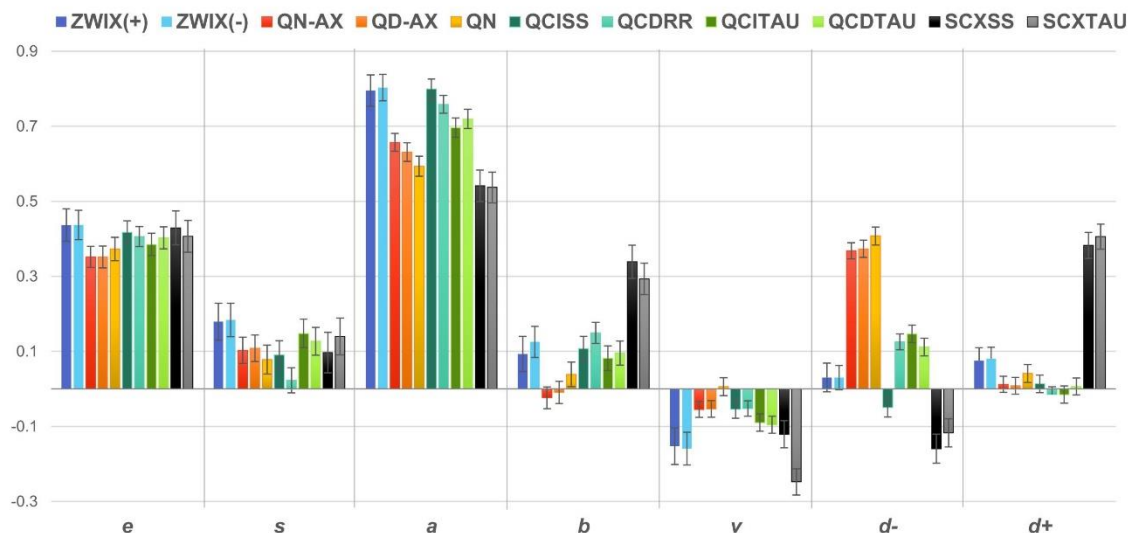


Fig. 3. Coefficients of the normalized LSER models calculated with Eq. (1) and the retention data measured for the 161 achiral analytes in Table S1 for the eleven stationary phases described in Fig. 1. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25 °C, 15 MPa, 3 mL/min.

behaviour. Quite logically, diastereomeric or pseudoenantiomeric phases are most similar (linked with the smallest lines): **ZWIX(+)** and **ZWIX(-)**; **QN-AX** and **QD-AX**; **QCISS** and **QCRRR**; **QCITAU** and **QCDTAU**.

While the dendrogram is a simple way to compare columns, it is not highly informative. Therefore, linear solvation energy relationships were calculated for each column to explore interaction differences more precisely.

3.3. Retention mechanisms examined with achiral compounds

161 achiral molecules (Table S1) were analyzed on the eleven columns studied in order to model their retentive properties. A modified version of the LSER model (Eq. (1)) was used with the five Abraham descriptors and the addition of two descriptors taking ionic interactions into account. The model therefore contains a total of seven different descriptors. The results are presented in Table 1 and Fig. 3. Considering that no retention prediction was de-

sired (only interpretation of interaction terms), the statistics were considered as reasonably good.

Retention mechanisms on all eleven phases are dominated by hydrogen bonding with proton donors (*a* terms) and by interactions with π and *n* electrons (*e*) that must result principally from π -stacking with aromatic analytes. Hydrogen-bonding with proton acceptors (*b* term), the contribution of molecular volume (*v*) and ionic interactions (*d*⁻ and *d*⁺ terms) are significant on certain columns and are clearly discriminant. Dipole-dipole interactions (*s*) contribute moderately and offer little discrimination between the stationary phases examined.

Examining the models obtained on the **ZWIX** phases, a first, quite surprising observation is that the ionic interaction terms (*d*⁻ and *d*⁺) are rather small although significant ionic interactions were expected. The *d*⁺ term is slightly larger than the *d*⁻ term, in accordance with previous reports that the surface of **ZWIX** stationary phases is slightly acidic [40]. Besides, the four other zwitterionic phases (**QCISS**, **QCRRR**, **QCITAU** and **QCDTAU**) also have rather

small ionic interaction terms. This would tend to indicate that the cationic and anionic groups in the ligands must be associated to each other, thereby reducing their availability for interaction with ionic analytes. Indeed, the d^- term, reflecting retention of anionic compounds, is large and positive on the anion-exchange stationary phases (**QN-AX**, **QD-AX** and **QN**) while the d^+ term, reflecting retention of cationic compounds, is large and positive on the cation-exchange stationary phases (**SCXSS** and **SCXTAU**). In addition, the d^+ term, reflecting retention of cationic compounds, is reduced on the anion-exchange stationary phases while the d^- term, reflecting retention of anionic compounds, is large and negative on the cation-exchange stationary phases, probably due to electrostatic repulsion interactions. In the case of simple ion-exchangers (not zwitterionic), the charges on the ligands are thus more freely available to interact with ionic analytes than on the **ZWIX** phases. On the contrary, the phases possessing a single ionic function, whether it is cationic or anionic, display strong ionic interactions. This is consistent with previous observations in HPLC that the sulfonic group “mostly act as intramolecular counterions leading to reduced run times compared to parent anion exchangers” [5]. Another interesting explanation for the moderate values of d^- and d^+ terms may be found in the electrostatic attraction-repulsion (push-and-pull) mechanism recently proposed by Mimini et al. [41]. Basically any anionic analyte should be both attracted by the cationic function of the stationary phase and repulsed by the anionic function, and vice-versa for an anionic analyte. This combined effect of attraction and repulsion could explain the overall moderate retention of ionic species on the **ZWIX** phases. In other words, the two ionic functions in the chiral ligand partly cancel each other.

Another interesting feature is that the v term, reflecting the effect of molecular volume on retention, is negative on all stationary phases but most significant on the **ZWIX** phases. The negative terms indicate that an increase in hydrocarbon volume is causing decreased retention on all columns, as usually observed on polar stationary phases in SFC [42], related to a normal-phase retention mode. The most significant negative value of v term on **ZWIX** phases can be related to the difficulty for large molecules to insert in the stationary phase, due to the bulky ligand leaving little space for insertion, or due to the cohesiveness of the stationary phase resulting from ionic and hydrogen-bonding interactions between them. Only the strong cation exchanger **SCXTAU** had comparably large negative v term, which can be explained by the absence of hydrophobic ligand portion in this stationary phase that could contribute to positive dispersive interactions to compensate for the phase cohesiveness.

Compared to **ZWIX** phases, the four other zwitterionic columns **QCIS**, **QCRR**, **QCITAU** and **QCDAU** are lacking a quinoline group, and two of them are lacking a butyl linker that is constraining the sulfonic group through a cyclohexyl function.

A small increase is observed for the interactions with anionic species (d^-), and a small decrease for the interactions with cationic species (d^+) for the phases that do not possess a quinoline group, which may be attributed to easier access of the ionic analytes to the cationic charge borne by the protonated nitrogen atom, when the bulky quinolic group is absent, causing increased attraction of anions and increased repulsion of cations. Other terms were mostly similar on all zwitterionic phases.

The three phases **QN-AX**, **QD-AX** and **QN** are similar to the **ZWIX** phases apart from the sulfonic group. As mentioned above, the most significant differences observed are in the ionic interactions and v term. On a practical note, this will reflect on the retention of acidic species that should comparatively be more retained on the anion-exchange stationary phases than on the zwitterionic phases, as appears in Fig. 4a. In this figure, it appears that the retention of Chiralpak **ZWIX(+)** and Chiralpak **QN-AX** are most similar, apart from the species that should bear a negative charge in

the present operating conditions. Other small differences are noticeable in Fig. 3: a small decrease of hydrogen bonding with proton donors and acceptors (a and b terms respectively) and interactions with dipoles and polarizable species (s and e terms respectively) are observed for the 3 phases possessing no sulfonic group when compared to the **ZWIX** phases.

The simple cation-exchangers **SCXSS** and **SCXTAU** are the most different from the **ZWIX** phases, not only due to a different pattern of ionic interactions, but also with significantly weaker hydrogen bonding interactions with proton donors (a term) and significantly stronger hydrogen bonding with proton acceptors (b term). Overall, this reflects in generally weaker retention on the cation-exchange phases compared to the zwitterionic phases, as appears in Fig. 4b, where most points fall below the first bisector. However, when a trend line is plotted across hydrophobic analytes (black diamonds), although some dispersion appears for polar non-ionic analytes (open circles), the lower retention of acidic anionic compounds (red triangles) and the stronger retention of basic cationic analytes (blue squares) are evident.

Finally, based on the LSER models, the eleven phases were plotted in the spider diagram reflecting the overall selectivity of the phases (Fig. 5). This figure was previously developed to allow easy comparison of a large number of stationary phases based on the system constants from LSER characterization [23,43]. Quite simply, the figure is a projection of the seven-dimension selectivity space defined by the seven system constants in Eq. (1) on a plane. Most hydrophobic stationary phases are situated on the left of the figure and most polar stationary phases are located on the right. From top to bottom, hydrogen bonding and ionic interactions contribute significantly to scattering the stationary phases. Bubble size is related to the overall strength of interactions (length of the solvation vector). To better appreciate the diversity of selectivities provided by the eleven phases, 36 other stationary phases previously characterized with the same protocol were also plotted. The full list of the columns relating to their number in the figure is presented in Table S3.

A first observation is that all eleven phases are grouped on the right of the figure, with polar stationary phases, but they are clearly scattered along the vertical axis, as a result of their different ionic interactions: the anion-exchange stationary phases are grouped at the bottom of the figure, the cation-exchange phases are situated in the upper part of the figure, and the zwitterionic phases are in-between. Secondly, all eleven phases are singular (not superimposed with any other in the figure) but have some similarity to other phases. The **ZWIX** phases are not too far from the achiral zwitterionic phases based on sulfobetaine ligand (# 22 and 23). They are also close to a stationary phase bonded with amino-anthracene ligand, probably due to the similarity to the quinoline group. The cation-exchange phases are not too far from the bare silica phases (# 31–36) which can also interact with cationic analytes through their silanol groups. Finally, the phases possessing amine or pyridine groups in their bonded ligands (# 21, 24 and 25) are all at the bottom of the figure, close to the zwitterionic and anion-exchange phases characterized in this work.

3.4. Enantioseparation mechanism examined with discriminant analysis

In previous studies on polysaccharide or macrocyclic glycopeptide enantioselective stationary phases, we have demonstrated that discriminant analysis was a useful tool to understand the molecular features that contribute to favorable enantiorecognition [37–39]. Basically, discriminant analysis is applied to a diverse selection of racemates (58 in the present case) analyzed in a single chromatographic system (one stationary phase and one mobile phase composition). The probe molecules are characterized by molecu-

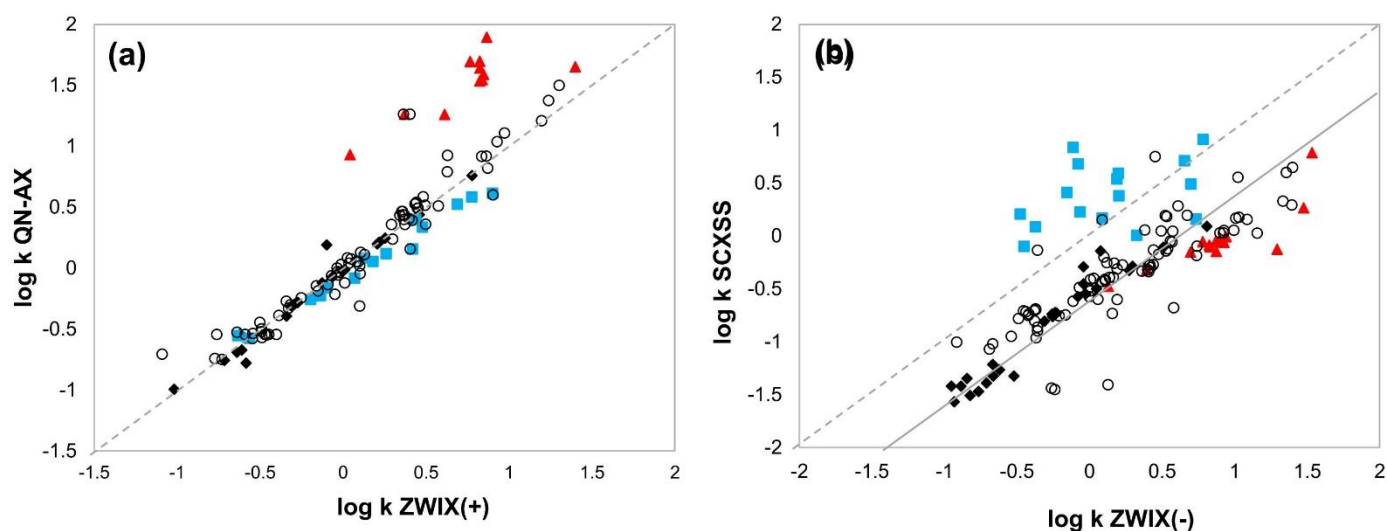


Fig. 4. Plots of logarithms of retention factors for the analytes in Table S1 on the Chiralpak **ZWIX** columns compared to (a) an anion-exchange stationary phase (Chiralpak **QN-AX**) and (b) a cation-exchange stationary phase (the in-house prepared **SCXSS**). Black diamonds are hydrophobic analytes, open circles are polar non-ionizable analytes, red triangles are acidic compounds bearing a negative charge, blue squares are basic compounds bearing a positive charge. The interrupted line is the first bisector indicating identical retention values. The continuous gray line in (b) is showing the trend line between hydrophobic analytes. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25 °C, 15 MPa, 3 mL/min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

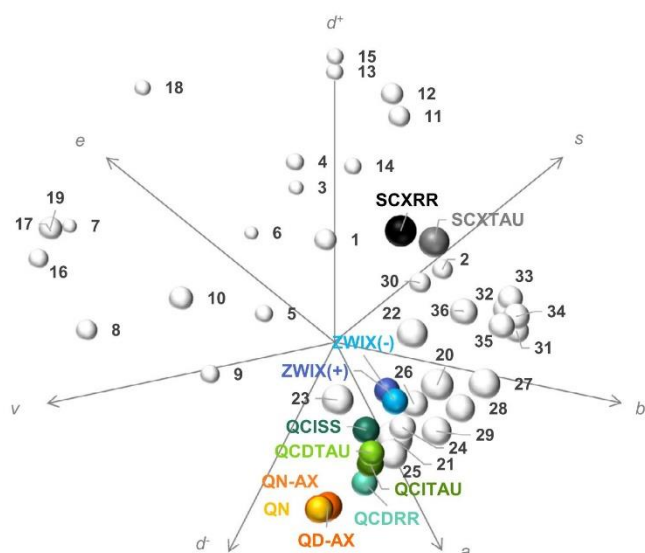


Fig. 5. Spider diagram based on the LSER models in Table 1 to observe the overall selectivity of the eleven columns in Fig. 1. The 36 numbered columns are identified in Table S3. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25 °C, 15 MPa, 3 mL/min.

lar descriptors and by the chromatographic result that can be simplified in two categories: separated (separation factor > 1) or not separated (separation factor = 1). However, we had also observed that applying this strategy to the whole set of racemates was often unsuccessful because not all racemates that were resolved had been discriminated for the same reasons. Thus a discriminant analysis including the whole set of analytes usually results in poor statistics, indicating that the models obtained are not relevant. To improve the significance of the results, a more efficient strategy was defined. First, the experimental retention of the two enantiomers is compared to the theoretical retention predicted with the solvation parameter model (Eq. (1)). In most cases, the two enantiomers both elute earlier than the prediction or they both elute later than the prediction. A first discriminant analysis is then

carried out to observe the features that contribute to early elution or late elution of the enantiomers. At this stage, it is useful to introduce two additional descriptors that are helpful to describe enantio-recognition processes: flexibility (F) and globularity (G). The other descriptors are the same seven used in the LSER models above.

The result of this discriminant analysis can be observed for Chiralpak **ZWIX(-)** in Fig. 6. It appears that late-eluting analytes are mostly flexible molecules (positive F term) and cationic molecules (positive D⁺ term). Flexible molecules have more ways to adapt to a complex, rather rigid ligand, which should be favorable to increased possibilities of interactions between a multi-functional analyte and the multi-functional ligand. The favorable contribution of positive charges may be related to better accessibility of the anionic sulfonic group than the cationic quinuclidine causing stronger retention for cations. Conversely, anionic compounds seem to be repulsed as the presence of negative charges is favorable to early elution (negative D⁻ term). This may be observed on a molecular model of the Chiralpak **ZWIX(-)** ligand in Fig. 7, where the quinuclidine group, being closer to the silica surface, should be most difficult to interact with than the pending sulfonic group.

Secondly, discriminant analysis based on the separated / non separated classes is applied independently on the 32 early-eluting (Fig. 6b) and 26 late-eluting racemates (Fig. 6c). Comparing the two figures, it appears that the structural features that contribute favorably to enantio-recognition are not all identical between early-eluting and late-eluting racemates. More precisely, the same three features (acidic character A, globularity G and negative charges D⁻) appear to be favorable but not in the same proportions. For early-eluting analytes, which should be rather rigid and not cationic, the presence of a negative charge is the most favorable feature (large positive D⁻ term), followed by proton-donor capability (positive A term). We may suppose that the ability to interact favorably with the cationic group (quinuclidine function) and with the quinoline and/or carbamate functions must contribute favorably to enantio-recognition. Late-eluting molecules are mostly cationic and flexible, but these features are not favorable to enantio-recognition for this group (negative F and D⁺ terms).

In both cases (early and late-eluting), globularity is highly significant (positive G term) while small molecular volume is also a

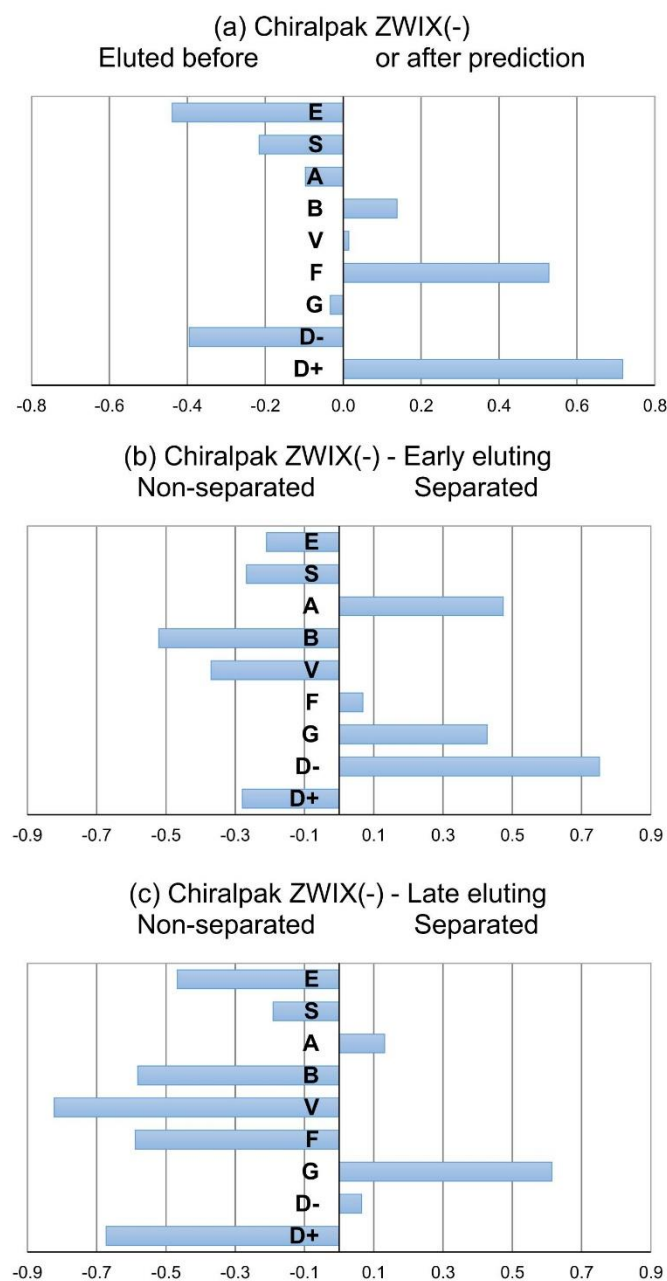


Fig. 6. Selected discriminant analyses between (a) early-eluting and late-eluting racemates, (b) early eluting separated and non-separated racemates and (c) late-eluting separated and non-separated racemates. Conditions: Chiralpak ZWIX(-), CO₂-methanol 90:10 (v/v), 25 °C, 15 MPa, 3 mL/min, 58 racemates in Table S2. Negative features are common to the analytes in the left group; positive features are common to the analytes in the right group; zero features are non-discriminant.

favorable feature (negative V term). This indicates that small spherical molecules are generally better resolved with this chiral selector. The chiral selector is rather constrained with three rather rigid portions: the quinoline ring, the quinuclidine group and the cyclohexanesulfonic function. The three of them define a small space for chiral molecules to fit in. A small spherical molecule should better adapt to this defined space than a large and/or non-spherical (planar or linear) molecule.

Very similar results were obtained in all cases on Chiralpak ZWIX(+).

A subset of the 58 racemates was selected for structural diversity: 22 molecules (written in bold in Table S2) representing different analyte classes and with different separation features on the

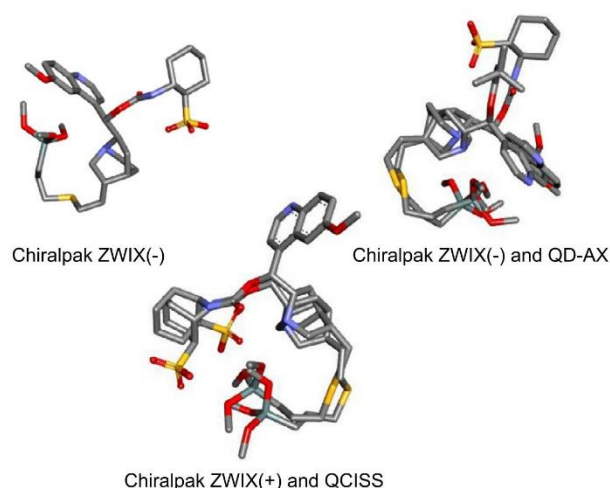


Fig. 7. Molecular modelling of the ligands and superimposition of them for useful comparison.

ZWIX phases (resolved or nor resolved) were chosen. Additionally, 8 free amino acids that were resolved on the ZWIX phases were included in this final test set. The 30 pairs of enantiomers were analysed on the ten chiral phases (thus excluding the non-chiral SCXTAU). Some representative examples are shown in Table 2. Only racemates that could be resolved on one stationary phase at least are presented in this table because the racemates that cannot be (at least partially) resolved on any stationary phase provide little information on enantioselective mechanisms.

One example of the contribution of shape is that of benzodiazepines. For instance, Oxazepam is a large and rather flat molecule. Because of its rigidity, it was eluted rather earlier than predicted but its flatness is probably responsible for the lack of enantioselectivity on the ZWIX phases. Some enantioselectivity was however observed on the QN-AX and QD-AX phases. Molecular modelling allows observing the different three-dimensional orientation of each CSP ligand: the folding of each ligand is highly dependent on intra-molecular interactions and steric constraints. Comparing the structures of the ligands (see superimposed ligands of ZWIX(-) and QD-AX in Fig. 7), it appears that the structure of QN-AX and QD-AX should be more open, less sterically constrained than the structure of ZWIX ligands. The replacement of the large cyclohexylsulfonic group by a smaller t-butyl group should make it easier for a large molecule to adapt to the chiral selector.

On the contrary, 5-methyl-5-phenylhydantoin is a small and rather compact (spherical) molecule that was well resolved on the ZWIX phases, as can be seen in the chromatograms in Fig. 8a. Resolution was also possible on the anion-exchange phases (QN-AX, QD-AX and QN), and it was also possible on the two phases lacking the quinoline group but retaining the cyclohexyl group (QCISS and QCRRR). However, enantioselectivity was lost when the steric constraint on the sulfonic group was not present (QCITAU and QCDTAU). Clearly, the orientation of the sulfonic group, forced by the presence of the cyclohexyl group, is favorable to enantioselectivity. This observation can be contrasted to previous observations in the liquid phase that “the *trans*-2-aminocyclohexanesulfonic acid moiety... is truly beneficial but not essential for the observed enantioselective properties of the zwitterionic chiral stationary phases” [5].

Acidic compounds and proton-rich compounds were generally well resolved on ZWIX phases and anion exchangers, while no resolution could be obtained on the other columns. Only the anion-exchange phases were also favorable to resolution of acids.

Table 2
Examples of enantioseparations for selected racemates on the ten chiral columns studied.

	ZWIX+	ZWIX-	QN-AX	QD-AX	QN	QCISS	QCRR	QCITAU	QCDAU	SCXSS
Oxazepam	×	×	✓	✓	✓	×	×	×	×	×
Mandelic acid, ethyl ester	✓	✓	✓	✓	×	✓	✓	✓	✓	×
5-methyl-5-phenylhydantoin	✓	✓	✓	✓	✓	✓	✓	×	×	×
Suprofen	✓	✓	✓	✓	×	×	×	×	×	×
Warfarin	✓	✓	✓	✓	×	×	×	×	×	×
Alanine	✓	✓	×	×	×	×	×	×	×	×
Glutamine	✓	✓	×	×	×	×	×	×	×	×

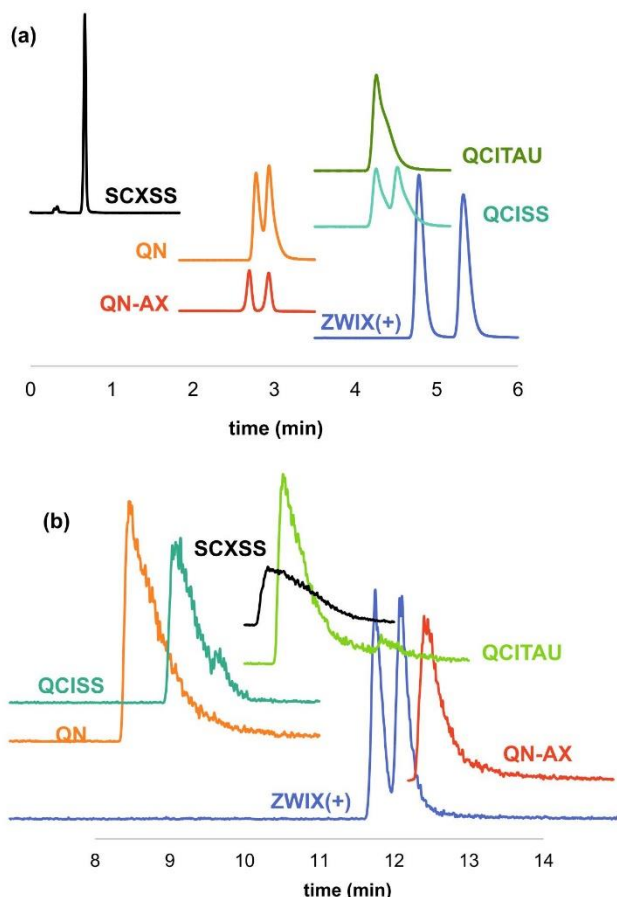


Fig. 8. Chromatograms of (a) 5-Methyl-5-phenylhydantoin and (b) Alanine. Analytical conditions: Chiralpak ZWIX(+) (blue), Chiralpak QN-AX (red), QN (orange), QCISS (light green), QCITAU (dark green), SCXSS (black). Conditions (a) CO₂-MeOH 90:10 (v/v), 25 °C, 150 bar, 3 mL/min, UV detection (210 nm); (b) gradient elution from 10% to 100% co-solvent (methanol containing 50 mM of ammonium formate and 5% water), 25 °C, 150 bar, 0.5 mL/min, ESI(+)-MS detection $m/z=90$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Enantioselectivity was observed for all amino acids on the ZWIX phases as a result of the double ion-pairing with the zwitterionic analytes [30] but was lost on most of the others, apart from the other zwitterionic phases where some enantioselectivity may be observed, even if the peak shapes were poor. An example can be seen in Fig. 8b with the chromatograms of Alanine. The zwitterionic character thus seems necessary but not sufficient to ensure enantioresolution of zwitterionic analytes. Comparing the ligand structures in Fig. 7, the QCISS ligand should be much more flexible than the ZWIX(+) ligand as the large quinoline group is not present to force the conformation in one direction. Flexibility in a chiral selector is generally recognized as an unfavorable feature.

The simplest ligand in SCXSS was totally incapable of resolving any of the pairs of enantiomers in this set. Clearly, the simple chirality borne by the cyclohexylsulfonic ligand is not sufficient to discriminate the enantiomers.

Overall, among the 22 pairs of enantiomers other than amino acids, 7 are separated on the ZWIX phases, whereas 8 pairs of enantiomers were separated on the QN-AX and QD-AX phases, 6 pairs on the QN phase and only 2 on the four other zwitterionic phases.

All structural features in the ZWIX phases then seem to play a significant role in the multi-modal enantioselective mechanism. This synergistic effect that was desired in the conception of the ZWIX ligands [5] is then proven to be completely true.

4. Conclusion

The mechanisms for retention and enantioselective separation of the Chiralpak ZWIX(+/-) columns were studied with the analysis of 161 achiral compounds and 66 pairs of enantiomers. The joint presence of a cationic and anionic charge in the ZWIX phases appears to be reducing the overall strength of ionic interactions, compared to simple anion-exchange or cation-exchange phases, probably due to intra-molecular ionic interactions, or ionic interactions between proximate ligands. The influence of the sterically constrained sulfonic group and the quinoline group were shown to be highly significant to explain the successful enantioselective separation in the ZWIX phases. Basically, all structural elements of the ZWIX ligands appeared to be significant contributors to enantioselectivity. ZWIX phases also appeared to favour small and spherical molecules, which should better fit in the chiral selector cavity, as large and/or flat molecules remained unresolved.

Compliance with ethical standards

The authors declare they have no conflict of interest.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Adrien Raimbault: Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Cam Mai Anh Ma:** Investigation. **Martina Ferri:** Investigation. **Stefanie Bäurer:** Investigation. **Pascal Bonnet:** Investigation, Resources, Writing - review & editing. **Stéphane Bourg:** Investigation, Writing - review & editing. **Michael Lämmerhofer:** Conceptualization, Resources, Writing - review & editing. **Caroline West:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2019.460689.

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***Cinchona*-based zwitterionic stationary phases: Exploring retention and enantioseparation mechanisms in supercritical fluid chromatography with a fragmentation approach**

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Supporting Information

Table S1 List of achiral probe compounds with LSER descriptors

Table S2: List of chiral probe compounds with LSER descriptors

Table S1: List of achiral probe compounds with LSER descriptors

#	Compound	E	S	A	B	V	D-(pH 5)	D+(pH 5)
1	Toluene	0.601	0.52	0.00	0.14	0.8573	0.00	0.00
2	Ethylbenzene	0.613	0.51	0.00	0.15	0.9982	0.00	0.00
3	Butylbenzene	0.600	0.51	0.00	0.15	1.2800	0.00	0.00
4	Pentylbenzene	0.594	0.51	0.00	0.15	1.4209	0.00	0.00
5	Hexylbenzene	0.591	0.50	0.00	0.15	1.5620	0.00	0.00
6	Heptylbenzene	0.577	0.48	0.00	0.15	1.7029	0.00	0.00
7	Octylbenzene	0.579	0.48	0.00	0.15	1.8438	0.00	0.00
8	Nonylbenzene	0.578	0.48	0.00	0.15	1.9847	0.00	0.00
9	Decylbenzene	0.579	0.47	0.00	0.15	2.1256	0.00	0.00
10	Undecylbenzene	0.579	0.47	0.00	0.15	2.2665	0.00	0.00
11	Dodecylbenzene	0.571	0.47	0.00	0.15	2.4074	0.00	0.00
12	Tridecylbenzene	0.570	0.47	0.00	0.15	2.5483	0.00	0.00
13	Tetradecylbenzene	0.570	0.47	0.00	0.15	2.6892	0.00	0.00
14	Allylbenzene	0.717	0.60	0.00	0.22	1.0961	0.00	0.00
15	<i>t</i> -Butylbenzene	0.619	0.49	0.00	0.18	1.2800	0.00	0.00
16	Naphthalene	1.340	0.92	0.00	0.20	1.0854	0.00	0.00
17	1-Methylnaphthalene	1.344	0.92	0.00	0.20	1.2263	0.00	0.00
18	1-Ethyl-naphthalene	1.371	0.88	0.00	0.20	1.3672	0.00	0.00
19	2-Ethyl-naphthalene	1.331	0.90	0.00	0.20	1.3672	0.00	0.00
20	Aniline	0.955	0.96	0.26	0.50	0.8162	0.00	0.30
21	N-Methylaniline	0.948	0.90	0.17	0.43	0.9570	0.00	0.32
22	N,N-Dimethylaniline	0.957	0.81	0.00	0.41	1.0980	0.00	0.51
23	N-Ethylaniline	0.945	0.85	0.17	0.43	1.0980	0.00	0.45
24	N,N-Diethylaniline	0.953	0.80	0.00	0.41	1.3798	0.00	0.88
25	Pyridine	0.631	0.84	0.00	0.52	0.6753	0.00	0.57
26	2-Ethylpyridine	0.613	0.71	0.00	0.59	0.9571	0.00	0.81
27	Benzamide	0.990	1.50	0.49	0.67	0.9728	0.00	0.00
28	Phenylurea	1.110	1.33	0.79	0.79	1.0726	0.00	0.00
29	Benzenesulfonamide	1.130	1.55	0.55	0.80	1.0971	0.00	0.00
30	<i>p</i> -Toluenesulfonamide	1.100	1.55	0.55	0.87	1.2380	0.00	0.00
31	Caffeine	1.500	1.72	0.00	1.28	1.3632	0.00	0.00
32	Theophylline	1.500	1.60	0.54	1.34	1.2223	0.00	0.00
33	Nicotinamide	1.010	1.09	0.63	1.00	0.9317	0.00	0.04
34	Nicotinic acid	0.790	1.21	0.57	0.73	0.8906	0.00	0.04
35	<i>o</i> -Picolinic Acid	0.790	1.21	0.57	0.73	0.8906	1.00	0.77
36	Indazole	1.180	1.22	0.53	0.35	0.9050	0.00	0.00
37	Carbazole	1.787	2.01	0.18	0.08	1.3154	0.00	0.00
38	Acridine	2.356	1.32	0.00	0.58	1.4133	0.00	0.93
39	Quinoline	1.268	0.97	0.00	0.54	1.0443	0.00	0.24
40	1-Naphtylamine	1.670	1.26	0.20	0.57	1.1852	0.00	0.12
41	2-Naphtylamine	1.670	1.28	0.22	0.55	1.1852	0.00	0.15

Table S1 (continued): Selection of achiral compounds for LSER characterization

#	Compound	E	S	A	B	V	D-(pH 5)	D+(pH 5)
42	1-Naphthalenemethylamine	1.566	1.25	0.10	0.73	1.3917	0.00	1.00
43	Aminodiphenylmethane	1.360	1.40	0.21	0.78	1.5649	0.00	1.00
44	N-Benzylbenzamide	1.460	1.97	0.26	0.76	1.7215	0.00	0.00
45	<i>o</i> -Toluidine	0.966	0.92	0.23	0.45	0.9570	0.00	0.23
46	<i>m</i> -Toluidine	0.946	0.95	0.23	0.55	0.9570	0.00	0.42
47	<i>p</i> -Toluidine	0.923	0.95	0.23	0.52	0.9570	0.00	0.49
48	Benzoic acid	0.730	0.90	0.59	0.40	0.9317	0.89	0.00
49	Isophthalic acid	0.940	1.46	1.14	0.77	1.1470	1.78	0.00
50	Trimesic acid	1.140	1.84	1.71	1.10	1.3623	1.92	0.00
51	1-Naphtoic acid	1.460	1.20	0.65	0.46	1.3007	0.96	0.00
52	2-Naphtoic acid	1.460	1.15	0.61	0.44	1.3007	0.91	0.00
53	1-Naphtylacetic acid	1.460	1.55	0.60	0.67	1.4416	0.64	0.00
54	2-Naphtylacetic acid	1.470	1.40	0.57	0.50	1.4416	0.65	0.00
55	2-Biphenylcarboxylic acid	1.580	1.55	0.57	0.54	1.5395	0.95	0.00
56	4-Biphenylcarboxylic acid	1.480	1.34	0.61	0.51	1.5395	0.89	0.00
57	4-Biphenylacetic acid	1.580	1.55	0.57	0.54	1.6804	0.66	0.00
58	Diphenylacetic acid	1.330	1.53	0.57	0.56	1.6804	0.79	0.00
59	3,3-Diphenylpropionic acid	1.330	1.53	0.57	0.57	1.8213	0.67	0.00
60	Benzilic acid	1.460	1.44	0.74	1.03	1.7391	0.95	0.00
61	Benzyl alcohol	0.803	0.87	0.39	0.56	0.9160	0.00	0.00
62	3-Phenyl-1-propanol	0.821	0.94	0.31	0.65	1.1978	0.00	0.00
63	4-Phenyl-1-butanol	0.811	0.90	0.33	0.70	1.3387	0.00	0.00
64	Naphthalene methanol	1.530	1.27	0.39	0.62	1.2850	0.00	0.00
65	Naphthalene ethanol	1.540	1.31	0.30	0.70	1.4259	0.00	0.00
66	Methylbenzilate	1.350	1.40	0.17	1.03	1.8800	0.00	0.00
67	Anisole	0.708	0.75	0.00	0.29	0.9160	0.00	0.00
68	Benzaldehyde	0.820	1.00	0.00	0.39	0.8730	0.00	0.00
69	Acetophenone	0.818	1.01	0.00	0.48	1.0139	0.00	0.00
70	Propiophenone	0.804	0.85	0.00	0.51	1.1548	0.00	0.00
71	Valerophenone	0.795	0.95	0.00	0.50	1.4366	0.00	0.00
72	Coumarine	1.060	1.76	0.00	0.43	1.0619	0.00	0.00
73	Benzonitrile	0.742	1.11	0.00	0.33	0.8711	0.00	0.00
74	Nitrobenzene	0.871	1.11	0.00	0.28	0.8906	0.00	0.00
75	<i>p</i> -Dinitrobenzene	1.130	1.63	0.00	0.46	1.0648	0.00	0.00
76	Chlorobenzene	0.718	0.65	0.00	0.07	0.8388	0.00	0.00
77	Bromobenzene	0.882	0.73	0.00	0.09	0.8910	0.00	0.00
78	Iodobenzene	1.188	0.82	0.00	0.12	0.9750	0.00	0.00
79	Naphtylaldehyde	1.549	1.40	0.00	0.45	1.2420	0.00	0.00
80	Naphtylacetate	1.391	1.53	0.00	0.59	1.4416	0.00	0.00
81	Cyanonaphthalene	1.472	1.51	0.00	0.39	1.2401	0.00	0.00
82	Naphtylacetonitrile	1.481	1.55	0.00	0.51	1.3810	0.00	0.00

Table S1 (continued): Selection of achiral compounds for LSER characterization

#	Compound	E	S	A	B	V	D-(pH 5)	D+(pH 5)
83	Nitronaphthalene	1.600	1.51	0.00	0.29	1.2596	0.00	0.00
84	Fluoronaphthalene	1.144	0.97	0.00	0.13	1.1030	0.00	0.00
85	Chloronaphthalene	1.417	1.00	0.00	0.14	1.2078	0.00	0.00
86	Bromonaphthalene	1.598	1.13	0.00	0.13	1.2604	0.00	0.00
87	Iodonaphthalene	1.928	1.22	0.00	0.16	1.3436	0.00	0.00
88	Benzophenone	1.447	1.50	0.00	0.50	1.4808	0.00	0.00
89	Deoxybenzoin	1.360	1.60	0.00	0.51	1.6217	0.00	0.00
90	Benzil	1.445	1.59	0.00	0.62	1.6374	0.00	0.00
91	Benzyl benzoate	1.330	1.42	0.00	0.47	1.5395	0.00	0.00
92	Phenol	0.805	0.89	0.60	0.30	0.7751	0.00	0.00
93	Eugenol	0.946	0.99	0.22	0.51	1.3544	0.00	0.00
94	Vanillin	1.040	1.33	0.29	0.69	1.1313	0.00	0.00
95	Thymol	0.822	0.79	0.52	0.44	1.3387	0.00	0.00
96	Pyrocatechol	0.970	1.10	0.88	0.47	0.8338	0.00	0.00
97	Resorcinol	0.980	1.00	1.09	0.52	0.8338	0.00	0.00
98	Hydroquinone	1.063	1.27	1.06	0.57	0.8337	0.00	0.00
99	α -Naphthol	1.520	1.05	0.60	0.37	1.1441	0.00	0.00
100	β -Naphthol	1.520	1.08	0.61	0.40	1.1441	0.00	0.00
101	Thiophene	0.687	0.57	0.00	0.15	0.6411	0.00	0.00
102	Thiophenol	1.000	0.80	0.09	0.16	0.8799	0.00	0.00
103	Thioanisole	1.063	0.68	0.00	0.32	1.0208	0.00	0.00
104	Methylphenylsulfone	1.080	1.85	0.00	0.76	1.1382	0.00	0.00
105	<i>o</i> -Cresol	0.840	0.86	0.52	0.30	0.9160	0.00	0.00
106	<i>m</i> -Cresol	0.822	0.88	0.57	0.34	0.9160	0.00	0.00
107	<i>p</i> -Cresol	0.820	0.87	0.57	0.31	0.9160	0.00	0.00
108	<i>o</i> -Isopropylphenol	0.842	0.88	0.52	0.38	1.1978	0.00	0.00
109	<i>m</i> -Isopropylphenol	0.811	0.92	0.55	0.38	1.1978	0.00	0.00
110	<i>p</i> -Isopropylphenol	0.791	0.89	0.55	0.38	1.1978	0.00	0.00
111	<i>o</i> -Nitrophenol	1.015	1.05	0.05	0.37	0.9493	0.02	0.00
112	<i>m</i> -Nitrophenol	1.050	1.57	0.79	0.23	0.9493	0.00	0.00
113	<i>p</i> -Nitrophenol	1.070	1.72	0.82	0.26	0.9493	0.01	0.00
114	<i>o</i> -Nitrobenzylalcohol	1.059	1.42	0.35	0.70	1.0900	0.00	0.00
115	<i>m</i> -Nitrobenzylalcohol	1.064	1.35	0.44	0.64	1.0900	0.00	0.00
116	<i>p</i> -Nitrobenzylalcohol	1.064	1.39	0.44	0.62	1.0900	0.00	0.00
117	<i>o</i> -Nitrotoluene	0.866	1.11	0.00	0.28	1.0315	0.00	0.00
118	<i>m</i> -Nitrotoluene	0.874	1.10	0.00	0.25	1.0315	0.00	0.00
119	<i>p</i> -Nitrotoluene	0.870	1.11	0.00	0.28	1.0315	0.00	0.00
120	2,4-Dinitrotoluene	1.150	1.61	0.00	0.49	1.2057	0.00	0.00
121	2,6-Dinitrotoluene	1.150	1.60	0.00	0.45	1.2057	0.00	0.00

Table S1 (continued): Selection of achiral compounds for LSER characterization

#	Compound	E	S	A	B	V	D-(pH 5)	D+(pH 5)
122	<i>o</i> -Methylacetophenone	0.780	1.00	0.00	0.51	1.1550	0.00	0.00
123	<i>m</i> -Methylacetophenone	0.806	1.00	0.00	0.51	1.1548	0.00	0.00
124	<i>p</i> -Methylacetophenone	0.842	1.00	0.00	0.52	1.1550	0.00	0.00
125	Methylbenzoate	0.733	0.85	0.00	0.46	1.0726	0.00	0.00
126	Ethylbenzoate	0.689	0.85	0.00	0.46	1.2135	0.00	0.00
127	Propylbenzoate	0.675	0.80	0.00	0.46	1.3544	0.00	0.00
128	Butylbenzoate	0.668	0.80	0.00	0.46	1.4953	0.00	0.00
129	Dimethylphthalate	0.780	1.41	0.00	0.88	1.4288	0.00	0.00
130	Diethylphthalate	0.729	1.40	0.00	0.88	1.7106	0.00	0.00
131	Dipropylphthalate	0.713	1.40	0.00	0.86	1.9924	0.00	0.00
132	Dibutylphthalate	0.700	1.40	0.00	0.86	2.2742	0.00	0.00
133	Methylparabene	0.900	1.37	0.69	0.45	1.1313	0.00	0.00
134	Ethylparabene	0.860	1.35	0.69	0.45	1.2722	0.00	0.00
135	Propylparabene	0.860	1.35	0.69	0.45	1.4131	0.00	0.00
136	Butylparabene	0.860	1.33	0.71	0.46	1.5540	0.00	0.00
137	Biphenyl	1.360	0.99	0.00	0.26	1.3242	0.00	0.00
138	Phenyl-naphthalene	1.950	1.20	0.00	0.34	1.6932	0.00	0.00
139	Diphenylmethane	1.220	1.04	0.00	0.33	1.4651	0.00	0.00
140	Acenaphtene	1.604	1.05	0.00	0.22	1.2586	0.00	0.00
141	Acenaphtylene	1.750	1.14	0.00	0.26	1.2156	0.00	0.00
142	Fluorene	1.588	1.03	0.00	0.20	1.3565	0.00	0.00
143	Phenanthrene	2.055	1.29	0.00	0.29	1.4544	0.00	0.00
144	Anthracene	2.290	1.34	0.00	0.28	1.4544	0.00	0.00
145	9-Methylantracene	2.020	1.28	0.00	0.23	1.5953	0.00	0.00
146	Fluoranthene	2.600	1.52	0.00	0.25	1.5846	0.00	0.00
147	Pyrene	2.600	1.52	0.00	0.25	1.5846	0.00	0.00
148	Triphenylene	2.710	1.66	0.00	0.29	1.8234	0.00	0.00
149	<i>o</i> -Terphenyl	2.000	1.18	0.00	0.30	1.9320	0.00	0.00
150	<i>p</i> -Terphenyl	2.040	1.48	0.00	0.30	1.9320	0.00	0.00
151	Acetanilide	0.900	1.39	0.48	0.67	1.1137	0.00	0.00
152	Amitriptyline	2.246	1.78	0.00	1.00	2.3996	0.00	1.00
153	Antipyrine	1.320	1.50	0.00	1.48	1.4846	0.00	0.00
154	Aspirin	0.781	0.80	0.49	1.00	1.2879	0.97	0.00
155	<i>p</i> -Chloroacetanilide	0.980	1.47	0.64	0.51	1.2361	0.00	0.00
156	Cyclobarbital	1.440	1.35	0.49	1.45	1.7859	1.99	0.00
157	Papaverine	2.190	2.76	0.00	1.47	2.5914	0.00	0.91
158	Paracetamol	1.060	1.63	1.04	0.86	1.1724	0.00	0.00
159	Phenobarbital	1.630	1.80	0.73	1.15	1.6999	0.00	0.00
160	Primidone	1.510	2.08	0.51	1.45	1.6842	0.00	0.00
161	Procaine	1.135	1.68	0.44	1.23	1.9767	0.00	1.00

Table S2: Selection of chiral compounds with molecular descriptors. The 58 first analytes served for discriminant analysis on the ZWIX phases. The analytes in bold (30 in total) served to compare the enantioseparation capabilities on the ten chiral phases. (see main text for details)

#	Compound	E	S	A	B	V	F	G	D-(pH 5)	D+(pH 5)
1	(4-Hydroxyphenylacetic)-2-propionic acid	0.908	1.46	0.96	0.68	1.2722	1.667	1.590	0.893	0.000
2	2-Phenylbutyric acid	0.750	1.07	0.57	0.48	1.3544	2.500	1.600	0.686	0.000
3	2-Phenylpropionic acid	0.730	0.97	0.57	0.68	1.2135	1.818	1.658	0.720	0.000
4	Mandelic acid	0.900	1.05	0.74	0.89	1.1313	1.818	1.700	0.947	0.000
5	Mandelic acid, methyl ester	0.800	1.01	0.17	0.89	1.2722	1.667	1.441	0.000	0.000
6	Mandelic acid, ethyl ester	0.800	1.01	0.17	0.89	1.4131	2.308	1.294	0.000	0.000
7	trans-Chlorostilbene oxide	1.570	1.39	0.00	0.45	1.6785	1.111	1.332	0.000	0.000
8	(2,3-Epoxypropyl)-benzene	0.840	0.87	0.00	0.34	1.0892	1.818	2.162	0.000	0.000
9	1,2-Epoxyethylbenzene	0.840	0.86	0.00	0.34	0.9483	1.010	2.193	0.000	0.000
10	Methylphenylsulfoxide	1.104	1.73	0.00	0.88	1.0795	1.111	1.764	0.000	0.000
11	Methyl- <i>p</i> -tolylsulfoxide	1.120	1.77	0.00	0.80	1.2204	1.000	1.594	0.000	0.000
12	Phenylvinylsulfoxide	1.220	1.89	0.00	0.85	1.1774	2.000	1.592	0.000	0.000
13	Clorazepate	2.270	2.14	1.04	1.34	2.1483	0.833	1.005	0.980	0.000
14	Chlormezanone	1.440	2.40	0.00	1.39	1.8311	0.556	1.374	0.000	0.000
15	Diazepam	2.078	1.57	0.00	1.25	2.0739	0.455	1.137	0.000	0.008
16	Diltiazem	2.420	2.55	0.00	2.12	3.1365	1.935	0.664	0.000	0.999
17	Fenoprofen	1.390	1.63	0.57	0.78	1.8800	2.105	1.307	0.916	0.000
18	Flurbiprofen	1.500	1.51	0.57	0.58	1.8389	1.579	1.190	0.792	0.000
19	Hexobarbital	1.340	1.50	0.24	1.33	1.7859	0.556	1.705	0.004	0.000
20	Ibuprofen	0.730	0.59	0.59	0.81	1.7771	2.667	1.218	0.585	0.000
21	Ketoprofen	1.560	1.97	0.57	0.87	1.9779	2.000	1.285	0.929	0.000
22	Lorazepam	2.510	1.28	0.45	1.63	2.1141	0.435	1.101	0.000	0.000
23	Mepenzolate bromide	1.350	1.41	0.17	1.08	2.7381	1.481	1.000	0.000	1.000
24	Mephobarbital	1.570	1.82	0.24	1.35	1.8408	1.053	1.620	0.004	0.000
25	5-Methyl-5-phenylhydantoin	1.370	1.58	0.44	1.06	1.4024	0.667	2.090	0.000	0.000
26	Oxazepam	2.350	1.10	0.45	1.60	1.9917	0.455	1.047	0.000	0.000
27	Secobarbital	1.160	1.20	0.49	1.31	1.8945	2.941	1.805	0.003	0.000
28	Suprofen	1.510	1.89	0.57	0.81	1.9026	2.105	1.131	0.907	0.000
29	Thiopental	1.410	1.46	0.26	1.34	1.9014	2.500	1.794	0.006	0.000
30	Warfarin	2.300	2.18	0.35	1.49	2.3077	1.600	1.101	0.216	0.000
31	Zopiclone	2.660	3.20	0.00	2.43	2.6228	1.000	0.773	0.001	0.986
32	EPN	1.990	1.91	0.00	0.91	2.2657	2.727	0.911	0.000	0.000
33	Isofenphos	1.390	1.38	0.13	1.56	2.6442	3.636	1.150	0.000	0.000
34	Isocarbophos	1.490	1.42	0.21	1.51	2.0806	2.778	1.282	0.000	0.000
35	Fonofos	1.440	0.88	0.00	0.64	1.8703	3.571	1.405	0.000	0.000
36	Fenamifos	1.020	2.05	0.13	1.29	2.3467	3.684	0.720	0.000	0.000
37	Sulprofos	1.850	1.16	0.00	0.97	2.3743	4.444	1.152	0.000	0.000
38	Cyanofenphos	1.890	1.82	0.00	0.95	2.2462	2.381	0.838	0.000	0.000
39	Tebupirimfos	1.270	1.10	0.00	1.42	2.4465	3.000	1.032	0.000	0.000
40	Mecoprop methyl ester	0.860	1.24	0.00	0.68	1.6764	2.000	1.136	0.000	0.000
41	Metalaxyl	0.850	1.96	0.00	1.60	2.2331	3.000	1.796	0.000	0.000
42	Flavanone	1.650	1.76	0.00	0.73	1.7127	0.526	1.235	0.000	0.000
43	6-Methoxyflavanone	1.720	1.89	0.00	0.93	1.9123	0.952	1.155	0.000	0.000
44	4',5,7-trihydroxyflavanone	2.230	2.19	1.30	1.14	1.8888	0.455	1.114	0.001	0.000
45	Fmoc-proline	2.310	2.36	0.57	1.15	2.4828	1.429	1.008	0.947	0.000
46	Fmoc-Threonine(tBu)	2.090	2.37	0.80	1.40	3.0728	1.935	0.908	0.956	0.000
47	Carbetamide	1.198	1.86	0.62	1.23	1.8515	2.353	0.872	0.000	0.000
48	Dichlorprop	1.050	1.40	0.57	0.61	1.5050	2.143	1.560	0.991	0.000

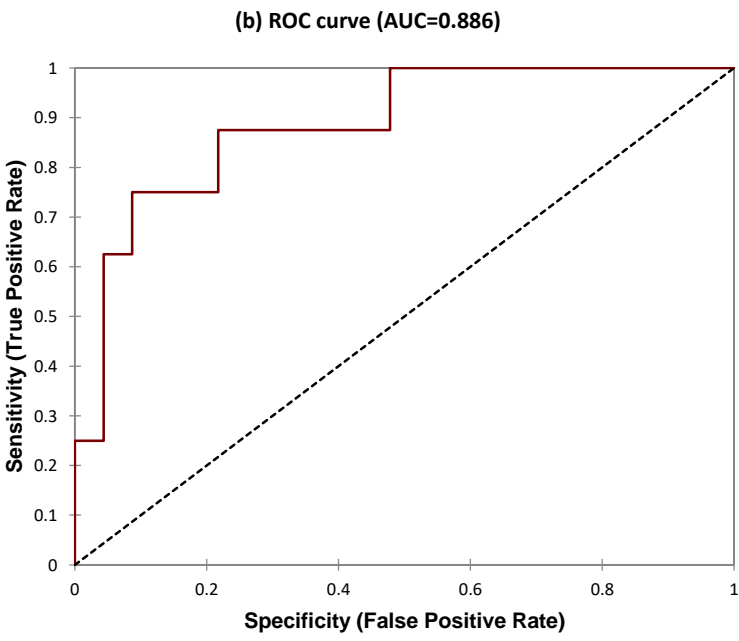
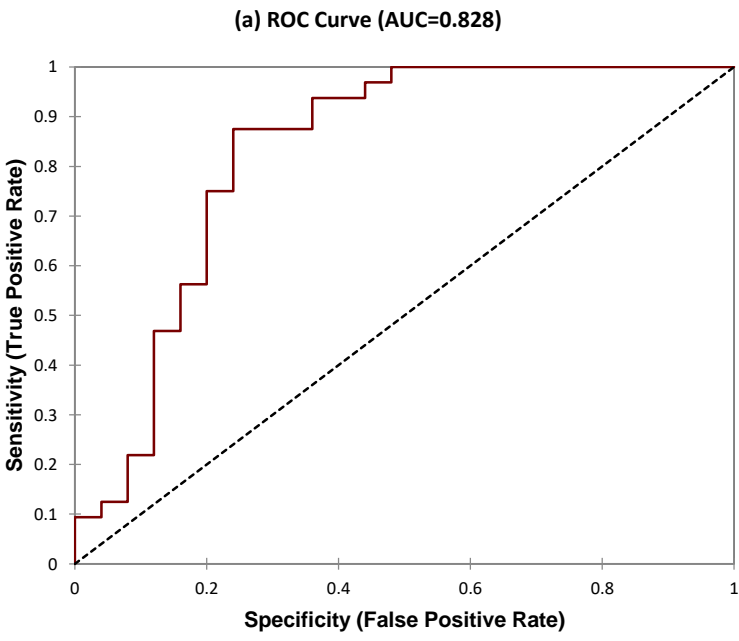
Table S2 (continued): Selection of chiral compounds with molecular descriptors. The 58 first analytes served for discriminant analysis on the ZWIX phases. The analytes in bold (30 in total) served to compare the enantioseparation capabilities on the ten chiral phases. (see main text for details)

#	Compound	E	S	A	B	V	F	G	D-(pH 5)	D+(pH 5)
49	Acebutolol	1.600	2.42	0.90	2.10	2.7556	4.167	0.409	0.000	1.000
50	Betaxolol	1.310	1.31	0.29	1.53	2.5745	4.783	0.309	0.000	1.000
51	Bisoprolol	1.140	1.37	0.29	1.77	2.7418	5.217	0.199	0.000	1.000
52	Oxprenolol	1.310	1.49	0.17	1.62	2.2174	4.737	1.261	0.000	1.000
53	Pindolol	1.700	1.65	0.30	1.48	2.0090	3.158	1.095	0.000	1.000
54	Promethazine	2.140	1.72	0.00	1.09	2.2832	1.364	1.303	0.000	1.000
55	Propranolol	1.880	1.43	0.17	1.42	2.1480	3.000	1.045	0.000	1.000
56	Sulpiride	1.910	2.78	0.72	2.15	2.5306	2.500	1.208	0.000	1.000
57	Verapamil	1.760	3.00	0.00	1.89	3.7861	3.824	0.993	0.000	1.000
58	Viloxazine	1.050	1.33	0.17	1.20	1.8700	2.778	1.441	0.000	0.999
59	Alanine									
60	Phenylalanine									
61	Methionine									
62	Lysine									
63	Agglutamic acid									
64	Serine									
65	Glutamine									
66	Proline									

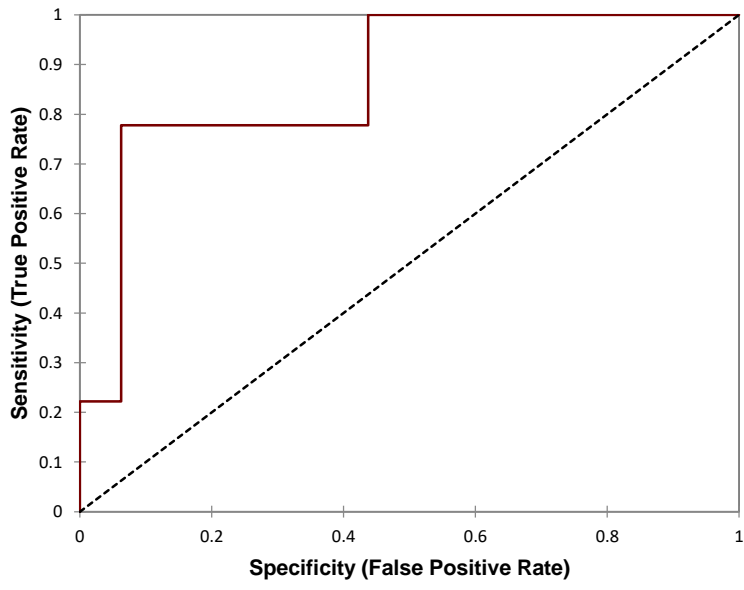
Table S3: 36 columns compared in Figure 5

Column Name	Manufacturer	Bonded ligand	Number in Figure 5
ACQUITY UPC ² HSS C18 SB	Waters	Octadecyl, non endcapped	1
ACQUITY UPC ² CSH Fluorophenyl	Waters	Pentafluorophenyl	2
Synergi Polar RP	Phenomenex	Phenyloxypropyl	3
Kinetex Biphenyl	Phenomenex	Biphenyl	4
Speedcore Diphenyl	Fortis Technologies	Diphenyl-alkyl	5
Ascentis Express Biphenyl	Sigma-Aldrich	Biphenyl	6
Ascentis Express Phenyl-hexyl	Sigma-Aldrich	Phenyl-hexyl	7
Cosmosil Cholester	Nacalai Tesque	Cholesteryl	8
Accucore Phenyl-X	Thermo	Phenyl-alkyl	9
ACQUITY UPLC BEH Shield RP18	Waters	Alkyl w ith embedded carbamate group	10
Ascentis Express F5	Sigma-Aldrich	Pentafluorophenyl	11
Accucore PFP	Thermo	Pentafluorophenyl	12
Kinetex PFP	Phenomenex	Pentafluorophenyl	13
Nucleoshell PFP	Macherey-Nagel	Pentafluorophenyl	14
Titan PFP	Sigma-Aldrich	Pentafluorophenyl	15
Kinetex XB C18	Phenomenex	Octadecyl	16
Accucore Phenyl-hexyl	Thermo	Phenyl-hexyl	17
Kinetex F5 (2)	Phenomenex	Pentafluorophenyl	18
Nucleodur C18 Gravity-SB	Macherey-Nagel	Octadecyl, endcapped	19
ACQUITY UPC ² Torus DIOL	Waters	Propanediol	20
ACQUITY UPC ² Torus DEA	Waters	Diethylamine	21
Nucleoshell HILIC	Macherey-Nagel	Sulfobetaine	22
Synchronic HILIC	Thermo	Sulfobetaine	23
ACQUITY UPC ² BEH 2-EP	Waters	2-ethylpyridine	24
ACQUITY UPC ² Torus 2-PIC	Waters	2-Picolyl-amine	25
ACQUITY UPC ² Torus 1-AA	Waters	1-Amino-anthracene	26
Accucore 150-Amide-HILIC	Thermo	Polyamide	27
Ascentis Express OH5	Sigma-Aldrich	Penta-hydroxyl	28
Accucore Urea-HILIC	Thermo	Propylurea	29
Hypersil Gold CN	Thermo	Cyanopropyl-bonded silica	30
ACQUITY UPC ² BEH	Waters	Bare hybrid silica	31
Kinetex HILIC	Phenomenex	Bare silica	32
Accucore HILIC	Thermo	Bare silica	33
Cortecs HILIC	Waters	Bare silica	34
Hypersil Gold Silica	Thermo	Bare silica	35
Titan Silica	Sigma-Aldrich	Bare silica	36

Figure S1: ROC curves related to the discriminant analyses in Figure 6.



(c) ROC curve (AUC=0.868)



IV. Conclusion

Dans ce chapitre, différentes phases stationnaires ont été caractérisées en utilisant la méthodologie des modèles LSER. C'est-à-dire que sur ces phases stationnaires ont été analysés un set de molécules achirales et un set de molécules chirales et que les rétentions ont été étudiées en fonction des changements de paramètres : la concentration d'additif pour la colonne Chirobiotic™ TAG, et l'absence de certains groupements fonctionnels sur le greffon de référence des colonnes Chiralpak® ZWIX.

Concernant la colonne Chirobiotic™ TAG, l'étude a amené à comprendre le comportement d'un peptide macrocyclique, la teicoplanine aglycone, une molécule polaire ionisable. Les analyses d'un set achiral sur cette colonne avec des conditions de phase mobile différentes (allant d'environ 1 M d'acide trifluoroacétique à environ 1 M d'isopropylamine dans le co-solvant) ont révélé les interactions que pouvait faire ce type de molécules quel que soit le pH apparent de la phase mobile. Le mécanisme de rétention a donc été mis en évidence et les variations de rétention des composés acides, basiques, et ioniques montrent bien les différents états d'ionisation des fonctions acides et amines libres d'un peptide. Nous avons également démontré l'importance des effets d'adsorption de l'additif sur la phase stationnaire.

La deuxième partie, consacrée aux colonnes Chiralpak® ZWIX démontre que pour l'énantioséparation des acides aminés, la présence des deux groupements chargés du greffon (le groupement sulfonique chargé négativement et le groupement quinuclidine porteur de l'azote chargé positivement) est nécessaire. En ce qui concerne la rétention, différents mécanismes sont mis en jeu en fonction de la phase stationnaire étudiée. Par exemple, lorsque le groupement sulfonique est absent du greffon, la colonne se comporte comme un échangeur d'anion. L'inverse est observé lorsque l'azote chargé positivement n'est plus sur le greffon étudié. En revanche la présence simultanée des deux charges affaiblit globalement le caractère échangeur d'ions de la phase zwitterionique par rapport à un simple échangeur d'anions ou de cations.

Les acides aminés étant une des familles moléculaires à laquelle ma thèse s'intéresse, un développement de méthodes ainsi que des applications concernant l'extraction d'acides aminés natifs au sein d'une plante ou encore le dosage d'acides

aminés dans des compléments alimentaires seront présentés dans le chapitre suivant. Ces développements vont utiliser les phases stationnaires Chiralpak® ZWIX, préalablement démontrées comme étant performantes dans l'énantiorésolution des acides aminés natifs en conditions HPLC.

Chapitre 3

Analyse d'acides aminés natifs par chromatographie unifiée et applications

I. Introduction

Ayant examiné en détail les phases stationnaires Chiralpak® ZWIX(+) et ZWIX(-) au chapitre précédent, une méthode d'analyse des acides aminés natifs a été développée en utilisant ces phases stationnaires. Ces colonnes sont connues pour l'énantioséparation des acides aminés natifs [109,110] en HPLC. La méthode que nous avons développée se veut être une méthode générique pour permettre l'énantioséparation de tous les acides aminés natifs protéinogéniques (en excluant la glycine qui ne possède pas de centre asymétrique). Pour réaliser l'élution complète des acides aminés les plus polaires, il a été nécessaire de réaliser un gradient d'élution large, joignant les domaines de la SFC, l'EFLC et l'HPLC. Cette méthode peut donc être qualifiée de Chromatographie Unifiée (UC).

Cette méthode, après quelques modifications, a été utilisée par la suite pour deux applications. La première application est la quantification des acides aminés natifs dans des compléments alimentaires. Des comprimés contenant 17 acides aminés protéinogéniques, des gélules contenant de la taurine et de la théanine (acides aminés naturels non protéinogéniques), et enfin des gélules contenant les acides aminés branchés (valine, leucine et isoleucine) ont été analysés. Une validation de la méthode de dosage a été effectuée sur les compléments alimentaires contenant les acides aminés branchés. Dans les compléments alimentaires, les acides aminés retrouvés sont sous une seule forme, la forme L-, qui est celle retrouvée le plus fréquemment à l'état naturel. Aussi, l'énantioséparation n'était pas recherchée dans cette application.

La deuxième application est l'extraction d'acides aminés dans une plante utilisée en médecine traditionnelle pour ses propriétés anti-infectieuses et hémostatique, la Bourse à Pasteur (*Capsella bursa-pastoris*). Deux types d'extraction ont été testées. La première est la macération, afin d'imiter l'utilisation naturelle de cette plante, puisqu'elle peut être utilisée en infusion dans de l'eau ou de l'alcool avant dégustation. Cette macération est donc employée afin de voir si des acides aminés sont extraits via cette technique. La deuxième est une technique d'extraction plus sophistiquée, l'extraction par fluide pressurisé (PFE). La méthodologie des plans d'expérience a été mise en place afin de pouvoir extraire le maximum d'acides aminés de cette plante et les quantifier par la méthode UC préalablement développée.

II. Développement de méthodes pour l'analyse des acides aminés

Une méthode générique a été développée pour l'analyse des acides aminés natifs sur la colonne Chiralpak® ZWIX(+). Trois additifs différents, un acide, une base et un sel, ont été testés à différents niveaux de concentration. De l'eau a été ajoutée à la phase mobile afin d'augmenter sa polarité. La meilleure phase mobile en termes de nombre d'acides aminés élués et énantiomériques est conservée. Le gradient d'élution allant de 10% de co-solvant à 100%, on ne peut plus considérer que nous sommes dans le domaine de la SFC. Le terme de « unified chromatography » est employé pour faire la jonction entre les techniques de SFC et d'HPLC.

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A chiral unified chromatography-mass spectrometry method to analyse free amino acids

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Abstract

In this project, we aimed at analyzing native (or free) amino acids with supercritical fluid chromatography coupled to mass spectrometric detection, with modern instruments and methods, and maintaining as simple a mobile phase as possible to ensure applicability of the method. The purpose was twofold: (i) a generic method allowing for satisfactory elution of a wide range of amino acids (acidic, basic, or neutral residue) and (ii) resolution of the enantiomeric pairs. The Chiralpak ZWIX (+) and (–) stationary phases were selected as they are well-known for the enantioresolution of amino acids in liquid chromatographic modes. A wide range elution gradient, starting with a large concentration of carbon dioxide (90%) and finishing at 100% solvent (methanol containing 70 mM ammonium formate and 7% water) allowed the elution of 18 native proteinogenic amino acids out of 19 injected. In these conditions, enantioselectivity was achieved for 16 of them. The basic amino acids (arginine, histidine, and lysine) were the most difficult to elute in these conditions, resulting in rather poor peak shapes. Cysteine was never observed in any of the conditions tested. Sample application was attempted with two food supplements, tablets containing a mixture of 17 proteinogenic amino acids and capsules containing taurine and theanine that were not present in the standards used for the method development. The sample preparation method was very simple, involving dissolution of the tablets and capsules in acidified water, filtration, and dilution with methanol. Mass spectrometric detection (electrospray ionization with single-quadrupole mass detection) allowed for unambiguous identification of most amino acids, except for the leucine and isoleucine isomers that were not separated by the generic gradient. The observation of taurine and theanine also suggests that the method should be generally applicable to other native amino acids than the proteinogenic amino acids selected for the development of this method. As peak shapes and signal-to-noise ratios could still be improved, further developments are wanted to upgrade this method. Due to the wide gradient (10 to 100% co-solvent in carbon dioxide), the method cannot truly be called either supercritical fluid chromatography (SFC) or enhanced-fluidity liquid chromatography (EFLC), but should be related to “unified chromatography” (UC), joining SFC and HPLC.

Keywords Amino acids · Enhanced-fluidity liquid chromatography · Food supplements · Supercritical fluid chromatography · Unified chromatography

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Introduction

Supercritical fluid chromatography (SFC) was first introduced in the years 1960s, but it was only in the 1980s and 1990s that it became an emerging technique. Carbon dioxide is the main fluid used in SFC because it is relatively inert with a low toxicity, it is readily available at a low price, and its critical parameters are easy to reach (31 °C and 73 bar for CO₂, as opposed, for instance, to 374 °C and 221 bar for water). In the years 2000s, SFC was mainly used for chiral separations, and especially for preparative-scale separations [1–4]. More recently, the significant progress in instruments and understanding of the fundamentals [5] have caused a resurgence of

interest with a wide development of achiral applications [6]. SFC is finally appearing as a versatile method that should be used as an interesting complementary method to high-performance liquid chromatography (HPLC). The advantages of the technique are essentially due to the use of a compressible fluid (pressurized CO₂) having properties of both a liquid and a gas, providing high efficiency at high flow rate. This is due to the low viscosity and high density of CO₂ in the supercritical state. However, CO₂ has a low polarity, rather close to hexane. Even at the highest densities that may be reached at high pressure or low temperature, its elution strength remains low, allowing for the solubilization of non-polar or moderately polar species. It is therefore necessary to mix pressurized CO₂ to a polar organic solvent, in order to increase the polarity of this mobile phase, to achieve the elution of polar molecules. In some cases, small quantities of water may also be added to the mobile phase composition, to analyze some highly polar, water-soluble compounds.

The proportion of co-solvent observed in most SFC applications remains below 50%. When the proportion of co-solvent is larger than 50%, the technique is usually called “enhanced-fluidity liquid chromatography” (EFLC) [7]. The reasoning behind the use of a low proportion of pressurized CO₂ introduced into a hydro-organic solvent is majorly to reduce the mobile phase viscosity and increase the analyte diffusion coefficients for most polar analytes. It is worth noting that most instruments currently available for SFC make it possible to use the full range of compositions, from 100% CO₂ to 100% solvent. When the whole composition range is used, the features of SFC and HPLC are combined in a form of unified chromatography (UC). While this idea had been expressed 20 years ago by Chester [8], it was demonstrated much later, notably by the group of T. Bamba for the analysis of lipophilic and hydrophilic analytes within a single run [9]. In this study, Taguchi et al. used an elution gradient ranging from 2 to 100% co-solvent at the end of the gradient. This wide gradient was necessary for the elution of fat-soluble and water-soluble vitamins within the same analysis. Another similar methodology was proposed by Desfontaine et al. [10] where non-polar and polar metabolites (including some native amino acids) were analyzed with a single gradient elution, also ranging from 2 to 100% co-solvent.

Furthermore, an additive (acid, base, or salt) can be introduced in the co-solvent to favor the elution of ionizable species. A study by West et al. [11] showed that the apparent pH of CO₂–methanol mixtures was about 4–6. With acidic additives, the apparent pH could be lowered down to 1–2 while the use of basic additives and salts (like ammonium acetate) essentially had a buffering effect of stabilizing the apparent pH in the 5–6 range, especially along a gradient analysis.

In the present study, we are interested in ionizable polar compounds, namely amino acids in their native (free) form. Because the two principal functional groups (acid and amine)

will be in the free form, they may therefore change ionization state with the apparent pH of the mobile phase. In a CO₂–methanol mobile phase comprising no additive, it is expected that most native amino acids shall be in their zwitterionic form. Therefore, they can be considered as rather difficult compounds for SFC, with a large range of polarities and ionizable groups.

The analysis of native amino acids in SFC was previously reported by Camel et al. [12] in 1992. The separation of ten amino acids was achieved on a diol-type stationary phase as no enantioresolution was desired. In this study, the mobile phase composition was however quite complex, comprising carbon dioxide, methanol, water, trimethylamine, and pyridine or ethylene glycol. Modern methods require simpler compositions to find wide applicability. Additionally, with increasing use of mass spectrometry hyphenated to SFC, the mobile phase composition should also allow for sensitive MS detection and thus should preferentially comprise volatile additives.

SFC-MS hyphenation was considered in details in a paper from Wolrab et al. [13]. In this report, the authors compared the merits of electrospray and atmospheric pressure chemical ionization to achieve sensitive analysis of native amino acids and other metabolites. Here again, a diol-type stationary phase was used as enantioresolution was not desired. The optimized mobile phase composition, offering both good peak shapes and the best MS sensitivity, comprised methanol, 50 mM ammonium formate, and 1% water. An elution gradient running from 15 to 45% co-solvent was employed as the polarity range of target analytes was not too large.

Another study by Toribio and co-workers [14] reported the SFC enantioresolution of three aromatic amino acids (phenylalanine, tyrosine, and tryptophan) on a Chirobiotic T2 stationary phase. In that case, the elution was achieved with 40% methanol–water 9:1. No other additive was necessary in this case, as the target analytes had moderate polarity (no acidic or basic group in the residue). The method was applied to SFC-UV analysis of food supplements. Most other studies relating the analysis of amino acids in SFC (for instance [15–17]) concern derivatized species where either the acid or the amine function is modified, precluding their ionization as zwitterionic species.

Successful analysis of molecules with a wide range of polarities also involves an adequate selection of stationary phase to achieve sufficient retention of both non-polar and polar analytes. For this task, a mixed-mode stationary phase, combining hydrophobic and hydrophilic structural features, is most likely to succeed. This can be exemplified in a study from Ishibashi et al. [18], where a mixture of pesticides with a wide range of polarities was adequately resolved using a polar-embedded octadecylsiloxane-bonded silica (ODS) stationary phase. While polar-embedded ODS phases are usually employed in reversed-phase HPLC where they provide

comparable retention mechanisms to those obtained with classical ODS phases, when employed in SFC, both the hydrophobic features (alkyl chains) and polar features (polar-embedded group) contribute significantly to the retention mechanism to interact with non-polar and polar analytes [19].

The purpose of this study was to find suitable analytical conditions for the elution and enantioseparation of native (or free) amino acids with a mobile phase containing carbon dioxide and a co-solvent, using the full range of possible compositions, thereby being relevant of unified chromatography. To perform this analysis, two stationary phases, Chiralpak ZWIX (+) and ZWIX (–), commercialized by Daicel or Chiral Technologies, were used. These columns are well-known for their capability to resolve amino acid enantiomers in their native [20, 21] or derivatized form [22, 23] and also peptides [20] stereoisomers in liquid mobile phases. In addition, the ligand in Chiralpak ZWIX phases comprises both hydrophobic and hydrophilic groups, suggesting that adequate retention of the least polar (like phenylalanine) and most polar (like glycine) amino acids should be possible. In order to find most generic conditions for this task, 19 racemic native proteinogenic amino acids were analyzed with a wide elution gradient from 10 to 100% of co-solvent comprising significant portions of water and additives. The focus was placed on the choice of additive, then its concentration and the percentage of water in the mobile phase to achieve elution and enantioseparation of the racemic amino acids, together with satisfying MS detection. Sample applications are proposed with food supplements containing proteinogenic or non-proteinogenic amino acids.

Material and methods

Chemicals and solvents

All racemic native amino acids were purchased from Sigma-Aldrich (Merck, France). Ultra-pure water was supplied by an Elga UHQ system from Veolia (Wissous, France). The HPLC-grade methanol used as the mobile phase was supplied by VWR (Fontenay-sous-Bois, France). CO₂, with a purity of 99.995%, was delivered by Air Liquide (Paris, France). Isopropylamine and trifluoroacetic acid were provided by Sigma-Aldrich (Merck, France). Ammonium formate was from Riedel de Haën, supplied by Fisher chemicals (Illkirch, France). Hydrochloric acid 37% was purchased from Fisher Chemicals.

Instrument

The supercritical fluid chromatography system used 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, two-position column oven compatible with 150 mm length columns, and a photodiode-array (PDA) detector. An ACQUITY QDa® single-quadrupole mass detector with electrospray ionization source was also 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 system, only a portion of the column flow enters the MS. Empower® 3 was used for integration of peaks for column efficiency measurements.

Analytical methods

All experiments have been carried out on two columns: Chiralpak ZWIX (+) and Chiralpak ZWIX (–) (150 × 3.0 mm; 3 μm) commercialized by Chiral Technologies. The mobile phase was composed of carbon dioxide and a co-solvent, with a gradient elution as follows: initial composition 10% isocratic; from 1 to 19 min, increase to 100%; 3 min isocratic step at 100% co-solvent; and from 22 to 24 min, decrease down to 10% and stabilize during 6 min. The gradient table can be seen in Table S1 in the Electronic Supplementary Material (ESM).

Three different additives were used: isopropylamine (IPA), ammonium formate (AF), and trifluoroacetic acid (TFA). They were added at different concentrations within the mobile phase to see the improvement of detection and peak shape of all native amino acids. The best one is selected for further analysis. The 19 chiral native amino acids were injected in all the tested mobile phase conditions. The flow rate was 0.5 mL/min, because of the high pressure generated at the end of the gradient, due to a most viscous fluid. The back pressure was maintained at 150 bar and the column was heated to 25 °C. The injection volume of each sample was set at 2 μL. All chromatograms were recorded with UV at 210 nm and mass detection, but only mass detection was used since most of the native amino acids have no chromophore groups. The *m/z* values observed for each amino acid can be seen in Tables S2 and S3 in the ESM.

Sample preparation

Standard solutions of all native proteinogenic amino acids (racemic mixture) were dissolved in water with 0.1 M hydrochloric acid at a concentration around 1 mg/mL. Stock solutions were diluted by 10 with methanol before injection.

Two food supplements were bought from the local supermarket in order to extract some amino acids. The first food supplement is tablets with a mixture of 17 proteinogenic amino acids in their L-form (glutamine, aspartic acid,

phenylalanine, methionine, lysine, leucine, isoleucine, histidine, threonine, alanine, valine, serine, proline, tyrosine, arginine, cystine, and glycine) and the second food supplement is capsules with two other, non-proteinogenic amino acids (taurine and theanine). These food supplements were dissolved in 10 mL of water with 0.1 M hydrochloric acid. After 1 h, they were filtrated with cotton and also with a 0.45- μm PTFE syringe filter. They were finally diluted by 10 with acetonitrile or methanol prior to injection.

Results and discussion

Method development

A method was developed to analyze chiral amino acids with a mobile phase containing carbon dioxide and a co-solvent in all proportions. Indeed, we decided to use a wide gradient elution (10 to 100% co-solvent) in order to favor the elution of all amino acids, whatever their polarity. This method could therefore be said to belong to unified chromatography (UC) as it covers the usual mobile phase domains of supercritical fluid chromatography (SFC), enhanced-fluidity liquid chromatography (EFLC), and HPLC [8].

Nature of additive

The first step in this method development was to choose which additive would allow for a most generic method, with elution, and possibly enantioresolution of all sorts of amino acids. Three different additives were used: trifluoroacetic acid (TFA), ammonium formate (AF), and isopropylamine (IPA). The 19 racemic proteinogenic amino acids were injected in the two Chiralpak ZWIX columns with a gradient elution from 10 to 100% of co-solvent. Because previous works had shown the interest of combining the acidic, basic, or salt additive to a small portion of water [24], both for chromatography and MS detection, the initial co-solvent composition was composed by a mixture of 98% methanol and 2% water. The additive was introduced at a concentration of 20 mM. The best additive was chosen based on the detection and the enantioseparation of the 19 chiral amino acids. Summary results are shown in Fig. 1 while full details can be found in Tables S2 and S3 in the ESM.

These results indicate that the best additive for a generic enantioselective method is ammonium formate, because it leads to the highest number of positive answers, i.e., the combined detection and enantioseparation of native amino acids. This is consistent with previous findings [13]. With the combination of ammonium formate and water, 14 amino acid pairs out of 19 were detected and resolved, 2 were only detected but not separated (aspartic acid and histidine), and 3 remained undetected. In the latter case, because the missing amino acids

are non-chromophoric (cysteine, lysine, and arginine), we did not determine whether they were not eluted from the column or not well ionized in the ESI-MS detector with these conditions. However, because two of them have a basic residue, it is probable that they were simply not eluted from the column.

The second additive that would be interesting to study in more details is the trifluoroacetic acid. For the purpose of separating the enantiomers, it would be a poor choice as only one racemate was resolved (phenylalanine). However, when no enantioresolution is desired, the elution strength seems reasonably good as 17 amino acids out of 19 were detected; thus, only 2 were not detected (valine and cysteine). It is possible that deprotonated TFA forms ion pairs with the cationic amine function in the amino acids, thereby favoring their elution but, in this case, preventing their enantiorecognition by the zwitterionic ligand of the Chiralpak ZWIX stationary phases.

The worst additive in this preliminary test was isopropylamine as 7 amino acids were not detected. However, it is interesting to note that, among the 12 amino acid pairs eluted, only one was not resolved (phenylalanine, which was the only one resolved with TFA). Similarly to the above comments about the effect of TFA, it is possible that protonated isopropylamine might form ionic pairs with the acidic functions of the amino acids, favoring the elution of some of them. However, in that case, the enantiorecognition process does not seem impaired, as was the case with TFA.

A typical example is shown in Fig. 2 where ammonium formate appears as the best additive to elute and resolve leucine enantiomers. A good enantioseparation is obtained with ammonium formate, while trifluoroacetic acid allows for faster elution but with limited resolution, and isopropylamine does not allow the elution (or detection) of the enantiomeric pair.

Judging from these results, ammonium formate was retained as the best choice for a generic enantioselective method for further investigations, as the method still needed improvement to detect and resolve all amino acids.

Concentration of ammonium formate and water

Having selected the best additive, it was necessary to examine the effect of concentration. To do this, two concentration levels of ammonium formate (20 mM and 50 mM) and two water levels in co-solvent (2% and 5%) were tested. Thus, four experiments were performed by combining all these parameters:

- 20 mM ammonium formate and 2% water
- 20 mM ammonium formate and 5% water
- 50 mM ammonium formate and 2% water
- 50 mM ammonium formate and 5% water

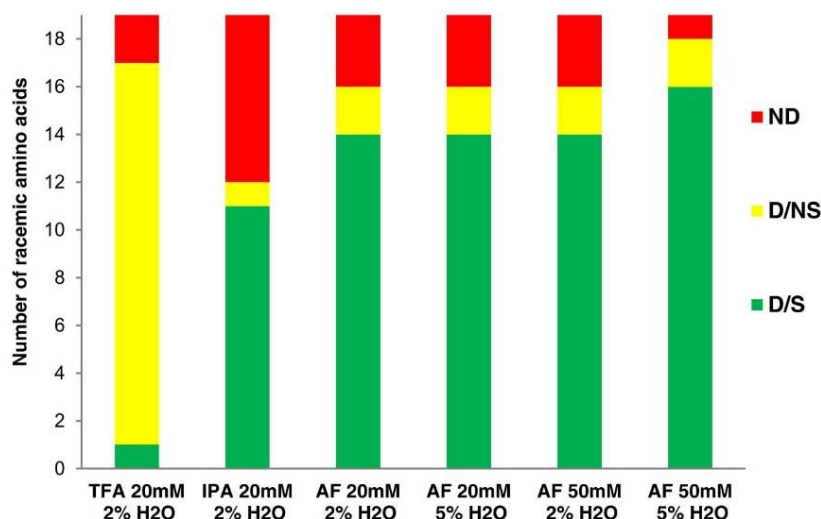


Fig. 1 Comparison of all mobile phase compositions with nature of additive (TFA, trifluoroacetic acid; IPA, isopropylamine; AF, or ammonium formate), additive concentration and water percentage in the methanol co-solvent used for the analysis of the 19 chiral native amino acids on the Chiralpak ZWIX (+) column. Green represents the most

favorable case with the detection and the separation of the enantiomers (D/S), yellow represents an average case with the detection but the co-elution of the enantiomers (D/NS), and red represents the worst case with a non-detection of the enantiomers (ND)

The same gradient program as before was performed on the 19 proteinogenic native amino acids.

The results were identical for the first three mobile phase compositions, as shown in Fig. 1, with 14 amino acid pairs out of 19 that were detected and separated, 2 that were detected but not separated, and 3 that were not detected. But when the two parameters were at their highest level, i.e., 50 mM of ammonium formate and 5% of water, the results improved with 16 amino acids detected and separated, 2 detected but not separated (aspartic acid and arginine), and only 1 amino acid not detected (cysteine). The case of cysteine was examined in more details to understand why it could never be observed. The cysteine standard solution was injected in the mass spectrometer bypassing the column but retaining all other operating parameters identical to the above experiments. In that case, cysteine could be detected, and, much more abundantly (50 times higher intensity), the dimeric form cystine. Because cystine carries twice more charges than most other

amino acids (two basic functions, two acidic functions), it is probable that it was excessively retained in the chromatographic column and hence was never detected.

The amino acids that benefited from this change in mobile phase conditions were the basic ones (arginine, histidine, and lysine) that were previously not detected with only 2% of water and/or 20 mM ammonium formate. These three basic amino acids eluted at the end of the gradient and therefore required a greater concentration of additives and a larger percentage of water to elute. The improvement was however not equivalent to the three of them. Indeed, lysine was detected and separated with these new mobile phase conditions while it was non-detected with only 2% of water and 20 mM ammonium formate. Regarding histidine, the co-elution of the two enantiomers with the lowest levels of water and additive disappeared when going to the highest levels. Meanwhile, arginine enantiomers were not detected before, while they were co-eluted with the new mobile phase conditions. The example

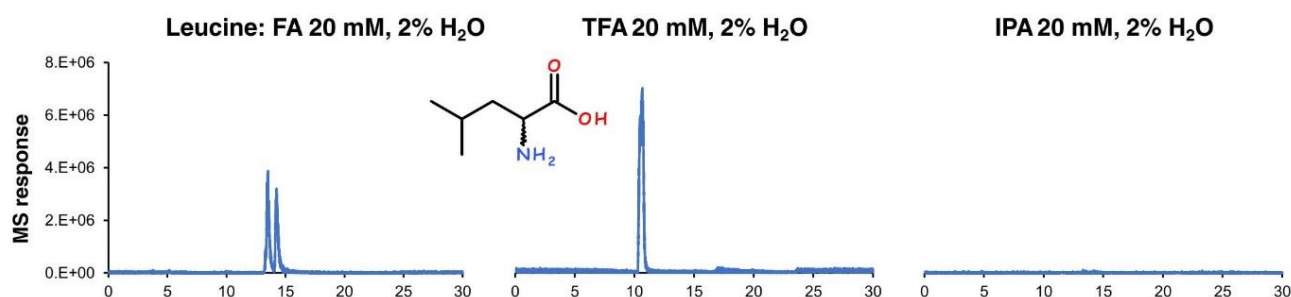


Fig. 2 Effect of the nature of the additive on leucine racemate with the three different additives tested at the same concentration level: ammonium formate on the left, trifluoroacetic acid in the middle, and isopropylamine on the right. Analytical conditions: Chiralpak ZWIX

(+) column, gradient elution from 10 to 100% co-solvent (methanol containing 20 mM of additive and 2% water), 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection ($m/z=132$)

of lysine is shown in Fig. 3 where the separation of the two enantiomers is seen when the mobile phase is constituted with 50 mM of ammonium formate and 5% of water, whereas under the conditions of the first tests, lysine was not detected and probably retained in the column.

Under the high-level condition, resolution was measurable for 13 of the amino acid pairs and was observed to vary from 1.15 (glutamic acid) to 8.3 (tryptophan). Ten of them had resolution values above 1.5.

Judging from the improvement observed at the highest levels of salt and water, an additional test was carried out with a further increase of the two values: ammonium formate and water concentrations to, respectively, 70 mM and 7%. The results are shown in Fig. 1 where only one more positive result (arginine) on the Chiralpak ZWIX (–) column is present, compared to the tests at 50 mM of additive and 5% of water. The chromatograms of arginine on Chiralpak ZWIX (–) column can be seen in ESM Fig. S1. In this chromatogram, enantioresolution is clearly not complete but some enantio-recognition seems to occur in these conditions.

Increasing the levels of salt and water from (20 mM; 2%) to (70 mM; 7%) reduced analysis time for all amino acids on both columns. This can be observed in Fig. S2 in the ESM. Concomitantly, enantioselectivity decreased slightly for all pairs of enantiomers, except for tryptophan (ESM Fig. S2). However, because peak shapes improved upon increasing the concentration of additives, resolution improved.

On the other hand, the signal-to-noise ratio (s/n) was the best for most analytes when the percentage levels of salt and water were 50 mM and 5% respectively (Fig. S3 in the ESM). For instance, the s/n of leucine was 92, 120, and 72 respectively for the (20 mM; 2%), (50 mM; 5%), and (70 mM; 7%) conditions. Phenylalanine showed values of 16, 114, and 72 respectively. While improvement of peak shape may be inferred when increasing the levels from (20 mM; 2%) to (50 mM; 5%), the reduction of signal-to-noise ratio when increasing from (50 mM; 5%) to (70 mM; 7%) is probably unrelated to peak shape, as they were not seen to degrade. Additional studies are needed to improve the MS signal-to-

noise ratio through improved peak shapes and reduction of baseline noise. It is possible that phase separation of the mobile phase occurs because the co-solvent contains a portion of water, but the make-up fluid introduced before the MS detection also contains water. As the proportion of column effluent entering the MS is unknown, it is difficult to know whether the final fluid composition (comprising carbon dioxide, methanol, water, and salt) is still existing as one phase or separating in gas and liquid. The conditions of pressure and temperature should be investigated more thoroughly to achieve improved peak shapes and baseline noise. Flow rate could also be investigated to hope for improved efficiency. With lower back pressure, larger flow rates should be possible that would be closer to the optimum flow rate. Additionally, the composition of make-up fluid should also be optimized [25].

Finally, it is important to note that the somewhat limited performance of ESI-MS observed in these experiments must be partly related to the system configuration. Because the effluent flow is split after the UV detector and prior to entering the MS, with an unknown split ratio that is bound to vary along the gradient, the sensitivity is certainly impaired. Further works shall be conducted with a different instrument where no split flow occurs prior to entering the MS. This should also reduce the uncertainties regarding the exact composition entering the MS to limit the risk of phase separation.

Depending on the objectives of the analysis (elution of single-enantiomer amino acids or enantioresolution), a different set of mobile phase composition could then be decided for. The highest level of salt and water (70 mM, 7%) could be preferred to achieve more enantio-separations, while the intermediate level (50 mM, 5%) could be preferred for improved MS sensitivity.

Sample application to food supplements

Two preliminary tests were conducted with two different food supplements: tablets containing 17 native proteinogenic amino acids (only the L-form) and capsules containing two other, non-proteinogenic amino acids that had not been included in

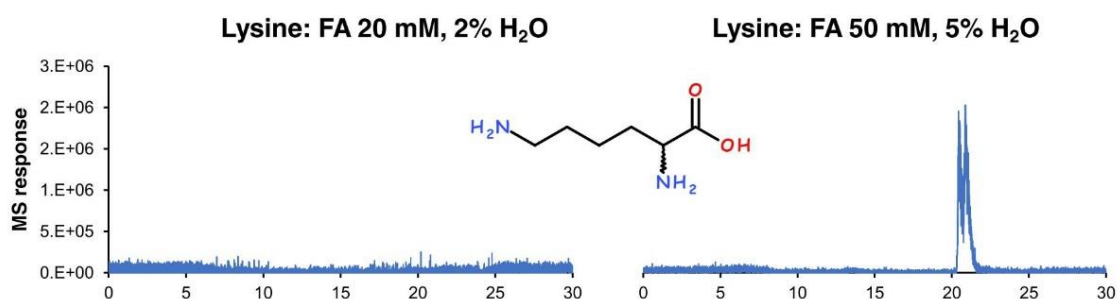


Fig. 3 Chromatograms of lysine with the two levels of ammonium formate concentration and water percentage in the methanol co-solvent: 20 mM ammonium formate and 2% of water on the left, 50 mM ammonium formate and 5% of water on the right. Analytical

conditions: Chiralpak ZWIX (+) column, gradient elution from 10 to 100% co-solvent, 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection ($m/z = 147$)

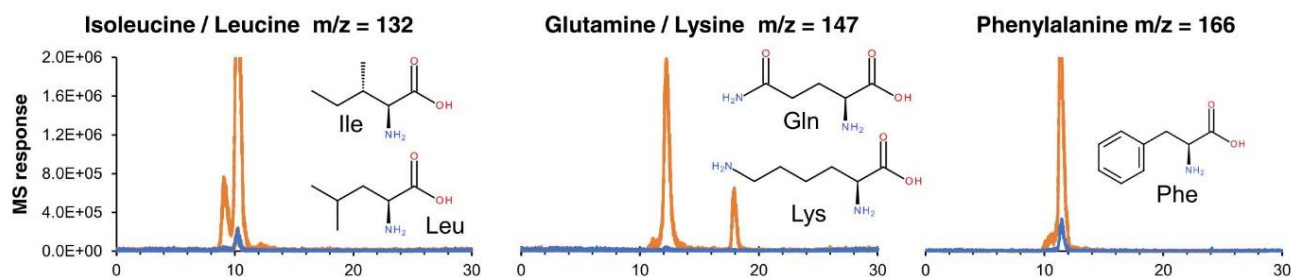


Fig. 4 Effect of the dilution solvent on the chromatograms of isoleucine and leucine on the left, glutamine and lysine in the middle, and phenylalanine on the right. The chromatogram in blue is the one with acetonitrile as injection solvent and the chromatogram in orange is the one

with methanol as injection solvent. Analytical conditions: Chiralpak ZWIX (+) column, gradient from 10 to 100% co-solvent (70 mM ammonium formate and 7% of water), 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection

the method development (taurine and theanine). Both samples were dissolved in aqueous acidic solution and filtered, then diluted by 10 in acetonitrile or in methanol. While the introduction of an organic solvent in the aqueous sample is obviously reducing the solubility of the analytes, purely aqueous solutions are not desirable for injection in the carbon dioxide-rich mobile phase used at the beginning of the gradient, because of the limited miscibility between carbon dioxide and water. Some chromatograms of free amino acids present in the tablets are presented in Fig. 4. When acetonitrile was used as dilution solvent for the tablets, no signal was observed for 8 molecules out of 17, and for the other 9, only small peaks could be observed with MS detection. When methanol was

used as dilution solvent, all amino acids were detected and, as shown in Fig. 4, for those that had been detected with acetonitrile, the peaks observed with MS detection with methanol dilution solvent were much larger. We may infer that the solubility of free amino acids in the acidic methanol–water is probably better than in acidic acetonitrile–water. In other studies related to the dilution solvent in SFC [26], methanol was generally found to provide poor peak shapes while acetonitrile provided much better results. However, in this study, generally less polar species were investigated, for which solubility in the dilution solvent and mobile phase was not an issue. Additionally, the species examined in this study had much lower retention than in the present case and it is well-known

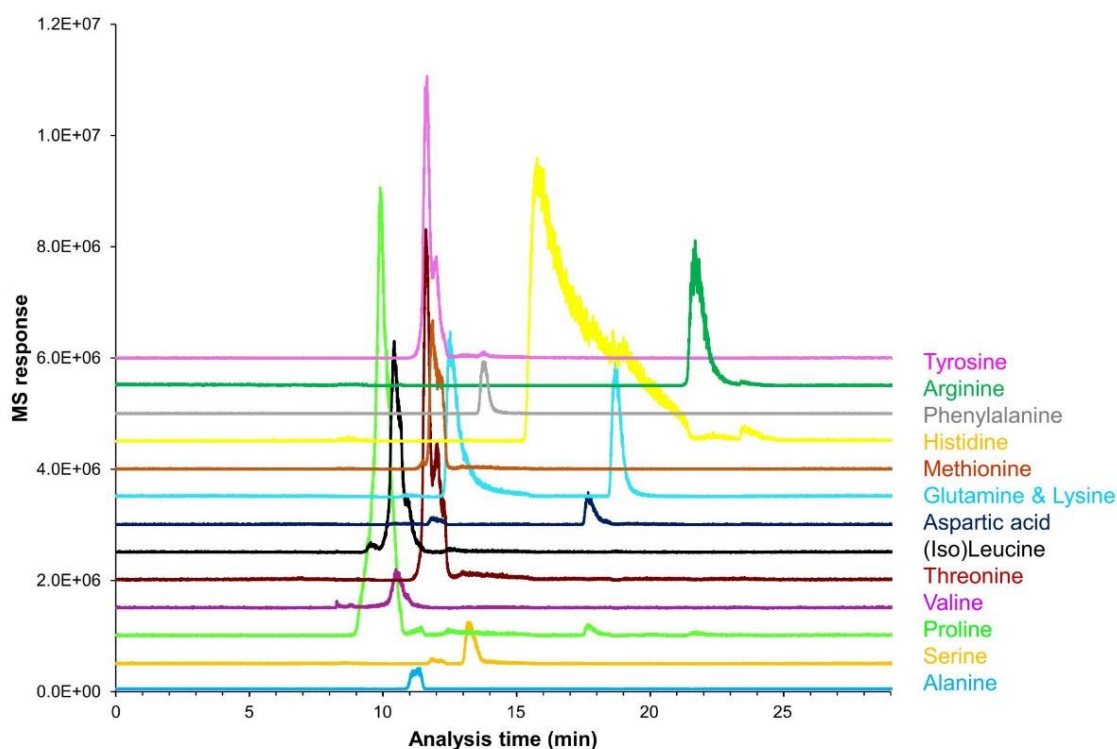


Fig. 5 Chromatograms of the amino acids observed in the tablets containing a mixture of 17 native amino acids. Analytical conditions: Chiralpak ZWIX (+) column, gradient from 10 to 100% co-solvent

(methanol containing 70 mM ammonium formate and 7% of water), 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection; 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection

that dilution solvent affects the peak shapes of low-retention analytes more significantly. In the present case, the analytes observed have a significant retention in this chromatographic system; thus, the effect of dilution solvent on peak shape should be minor and only solubility issues should prevail.

These two dietary supplements were injected into the two columns Chiralpak ZWIX (+) and ZWIX (−) with the highest levels of salt and water (70 mM ammonium formate, 7% water). Although all amino acids were detected, the chromatograms showed a very noisy baseline and poor peak shapes for some analytes (e.g., tailing peak for histidine, fronting peak for proline), as can be observed in Fig. 5.

It is worth noting that theanine and taurine were both well detected from the capsule samples when methanol was used as dilution solvent, although the theanine peak was tailing. This is however an encouraging result, indicating that other amino acids than the proteinogenic alpha-amino acids used here as standards could be analyzed with this method.

Conclusions

In this study, we have demonstrated that a simple mobile phase composition could be applied to achieve the elution and enantioresolution of native amino acids with supercritical fluid chromatography on Chiralpak ZWIX columns. The mobile phase is easy to prepare, with a 1-M solution of ammonium formate diluted with methanol used as co-solvent in a wide gradient ranging from 10 to 100% in carbon dioxide. With this method, enantioresolution was achieved for 16 amino acids out of 19.

Further improvement is wanted as (i) the isomeric species leucine and isoleucine were not separated; (ii) cysteine was never detected (nor its dimeric form cystine), possibly due to excessive retention; and (iii) some amino acids (arginine and aspartic acid) remained unresolved.

Although not fully demonstrated here but suggested by the analysis of samples containing taurine and theanine, it is expected that the method shall also elute other native amino acids (non-proteinogenic, alpha-, beta-, or gamma-amino acids).

The benefit over a liquid chromatography method may not be obvious because the peak efficiencies and analysis speed were not particularly superior to traditional HPLC methods, contrary to what is usually observed when pressurized carbon dioxide is present in the mobile phase. However, we hope to have demonstrated that the analyte polarity limit that is usually expected from SFC can be overcome by the adoption of wider mobile phase gradients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Electronic Supplementary Material

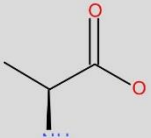
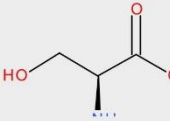
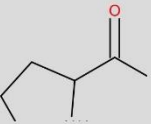
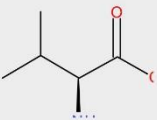
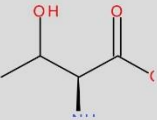
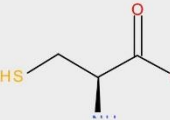
A chiral unified chromatography–mass spectrometry method to analyze free amino acids

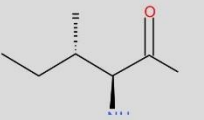
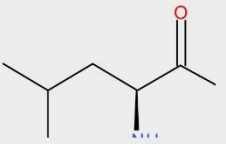
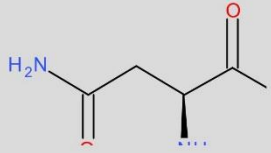
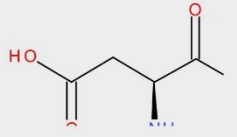
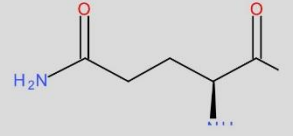
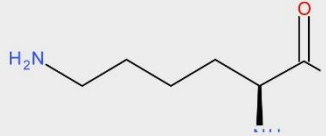
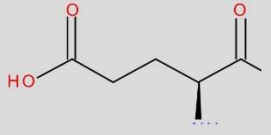
Adrien Raimbault, Magdalena Dorebska, Caroline West

Table S1 Gradient elution program used for all analyses, with flow rate set at 0.5 mL/min

Time (min)	% A (CO₂)	% B (co-solvent)
0	90	10
1	90	10
19	0	100
22	0	100
24	90	10
30	90	10

Table S2 Table showing the detection and separation of enantiomers of all native amino acids according to the nature of the additive and the percentage of water in Chiralpak ZWIX(+) (D: Detected; ND: Not Detected; S: Separated; NS: Not separated). ESI + detection with the m/z value employed for each amino acid is indicated in the first column

Amino Acids	m/z Positive mode	Structure	TFA 20mM		IPA 20mM		AF 20mM 2% H ₂ O		AF 20mM 5% H ₂ O		AF 50mM 2% H ₂ O		AF 50mM 5% H ₂ O		AF 70mM 7% H ₂ O	
			D	NS	ND	/	D	S	D	S	D	S	D	S	D	S
Alanine	90		D	NS	ND	/	D	S	D	S	D	S	D	S	D	S
Serine	106		D	NS	ND	/	D	S	D	S	D	S	D	S	D	NS
Proline	116		D	NS	D	S	D	S	D	S	D	S	D	S	D	S
Valine	118		ND	/	ND	/	D	S	D	S	D	S	D	S	D	S
Threonine	120		D	NS	ND	/	D	S	D	S	D	S	D	S	D	S
Cysteine	122		ND	/	ND	/	ND	/	ND	/	ND	/	ND	/	ND	/

Isoleucine	132		D	NS	D	S	D	S	D	S	D	S	D	S	D	S
Leucine	132		D	NS	D	S	D	S	D	S	D	S	D	S	D	S
Asparagine	133		D	NS	D	S	D	S	D	S	D	S	D	S	D	S
Aspartic acid	134		D	NS	ND	/	D	NS	D	NS	D	NS	D	NS	D	NS
Glutamine	147		D	NS	D	S	D	S	D	S	D	S	D	S	D	S
Lysine	147		D	NS	D	S	ND	/	ND	/	ND	/	D	S	D	S
Glutamic acid	148		D	NS	D	S	D	S	D	S	D	S	D	S	D	S

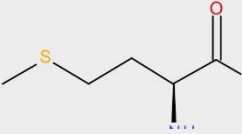
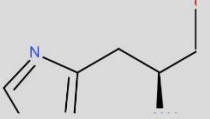
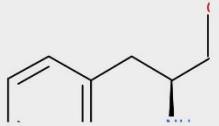
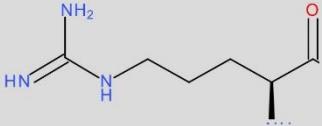
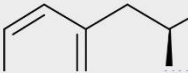
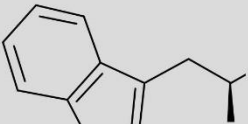
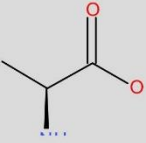
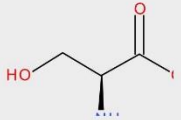
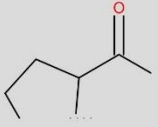
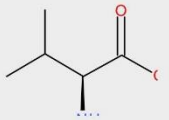
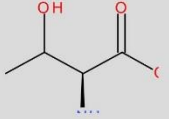
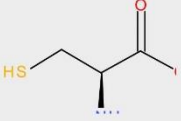
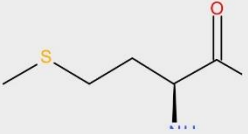
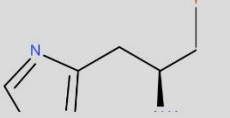
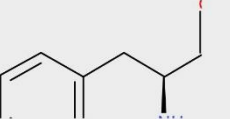
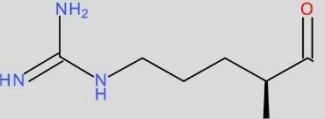
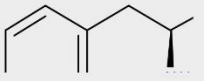
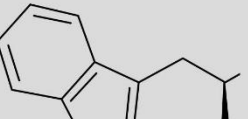
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Histidine	156		D	NS	D	S	D	NS	D	NS	D	NS	D	NS	D	S	D	NS	
Phenylalanine	166		D	S	D	NS	D	S	D	S	D	S	D	S	D	S	D	S	
Arginine	175		D	NS	ND	/	ND	/	ND	/	ND	/	D	NS	D	NS			
Tyrosine	182		D	NS	D	S	D	S	D	S	D	S	D	S	D	S	D	S	
Tryptophan	205		D	NS	D	S	D	S	D	S	D	S	D	S	D	S	D	S	
<u>Positive Answers</u> (D&S)			1		11			14			14			14			16		15

Table S3 Table showing the detection and separation of enantiomers of all native amino acids according to the nature of the additive and the percentage of water in Chiralpak ZWIX(-) (D: Detected; ND: Not Detected; S: Separated; NS: Not separated) ESI + detection with the m/z value employed for each amino acid is indicated in the first column

Amino Acids	m/z Positive mode	Structure	TFA 20mM		IPA 20mM		AF 20mM 2% H ₂ O		AF 20mM 5% H ₂ O		AF 50mM 2% H ₂ O		AF 50mM 5% H ₂ O		AF 70mM 7% H ₂ O	
Alanine	90						D	S	D	S	D	S	D	S	D	S
Serine	106						D	S	D	S	D	S	D	S	D	S
Proline	116						D	S	D	S	D	S	D	S	D	S
Valine	118						D	S	D	S	D	S	D	S	D	S
Threonine	120						D	S	D	S	D	S	D	S	D	S
Cysteine	122						ND	/	ND	/	ND	/	ND	/	ND	/

Isoleucine	132							D	S	D	S	D	S	D	S	D	S
Leucine	132							D	S	D	S	D	S	D	S	D	S
Asparagine	133							D	NS	D	NS	D	NS	D	NS	D	S
Aspartic acid	134							D	NS	D	NS	D	NS	D	NS	D	NS
Glutamine	147							D	NS	D	NS	D	NS	D	S	D	S
Lysine	147							ND	/	ND	/	ND	/	D	S	D	S
Glutamic acid	148							D	NS	D	NS	D	NS	D	NS	D	NS

Methionine	150							D	S	D	S	D	S	D	S	D	S
Histidine	156							D	NS	D	NS	D	NS	D	NS	D	NS
Phenylalanine	166							D	S	D	S	D	S	D	S	D	S
Arginine	175							ND	/	ND	/	ND	/	D	NS	D	S
Tyrosine	182							D	S	D	S	D	S	D	S	D	S
Tryptophan	205							D	S	D	S	D	S	D	S	D	S
<u>Positive Answers</u> (D&S)								11		11		11		13		15	

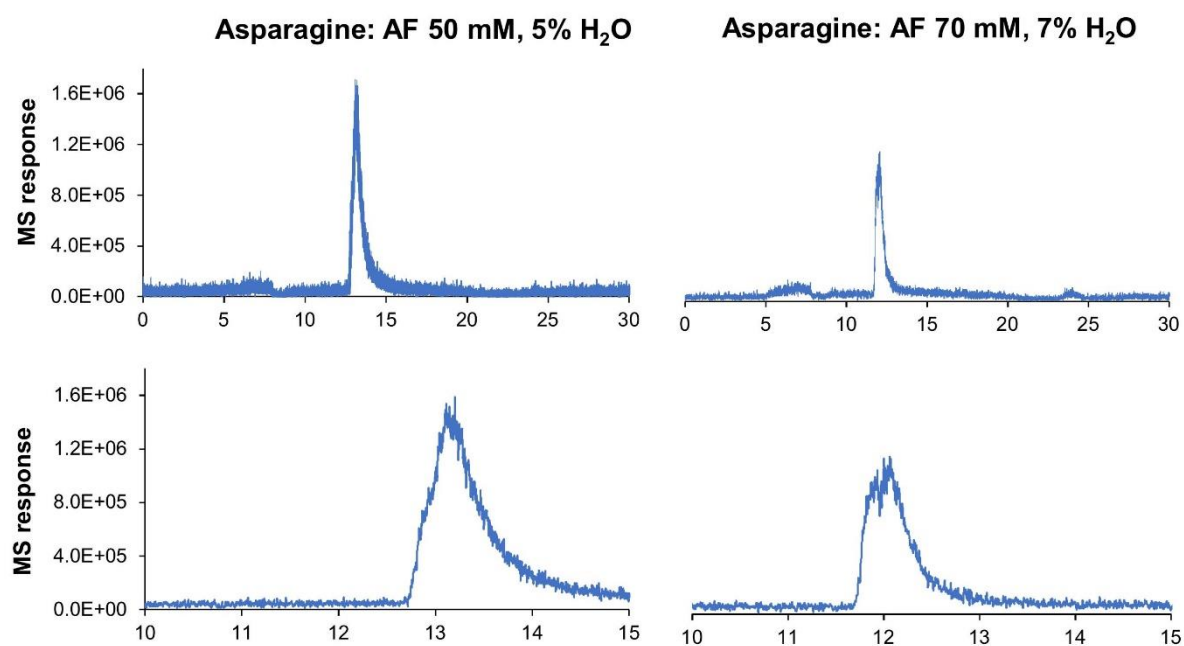


Fig. S1 Chromatograms of asparagine with the highest levels of ammonium formate and water percentage: 50 mM ammonium formate and 5% of water on the left; 70 mM ammonium formate and 7% of water on the right. Chromatograms on the bottom were zoomed between 10 and 15 minutes to see the beginning of the enantioseparation on the right figure. Analytical conditions: Chiralpak ZWIX(-) column, gradient from 10% to 100% co-solvent, 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection ($m/z = 133$)

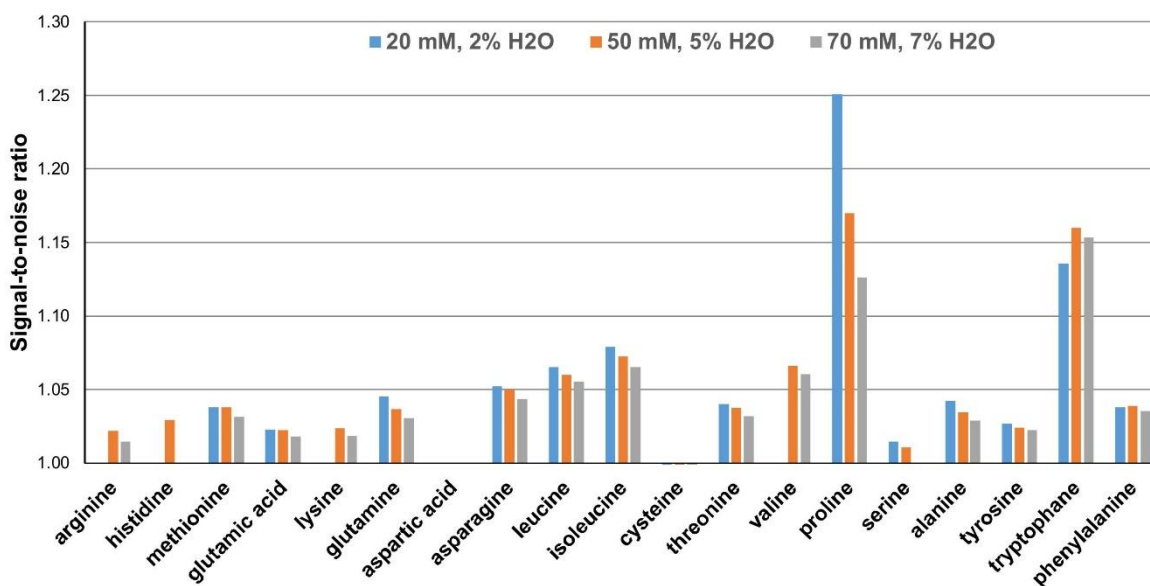
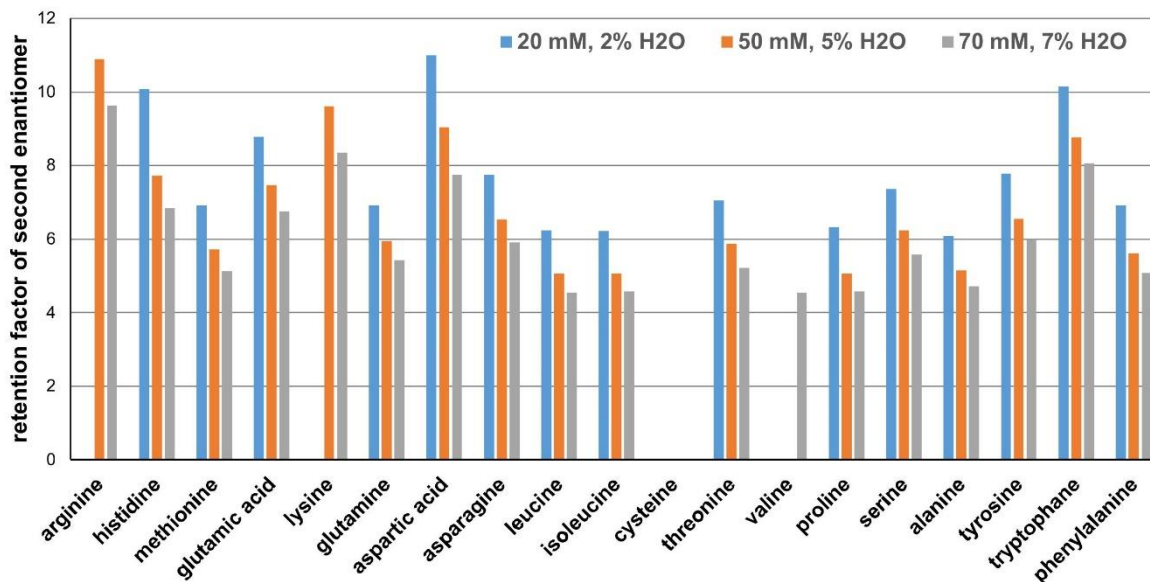


Fig. S2 Above retention factors measured for the second eluted enantiomer and below separation factors between the two enantiomers at three levels of ammonium formate and water in the co-solvent: (20 mM; 2%), (50 mM; 5%) and (70 mM; 7%) ammonium formate and water respectively. Other conditions: Chiralpak ZWIX(+) column, gradient from 10% to 100% co-solvent, 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection

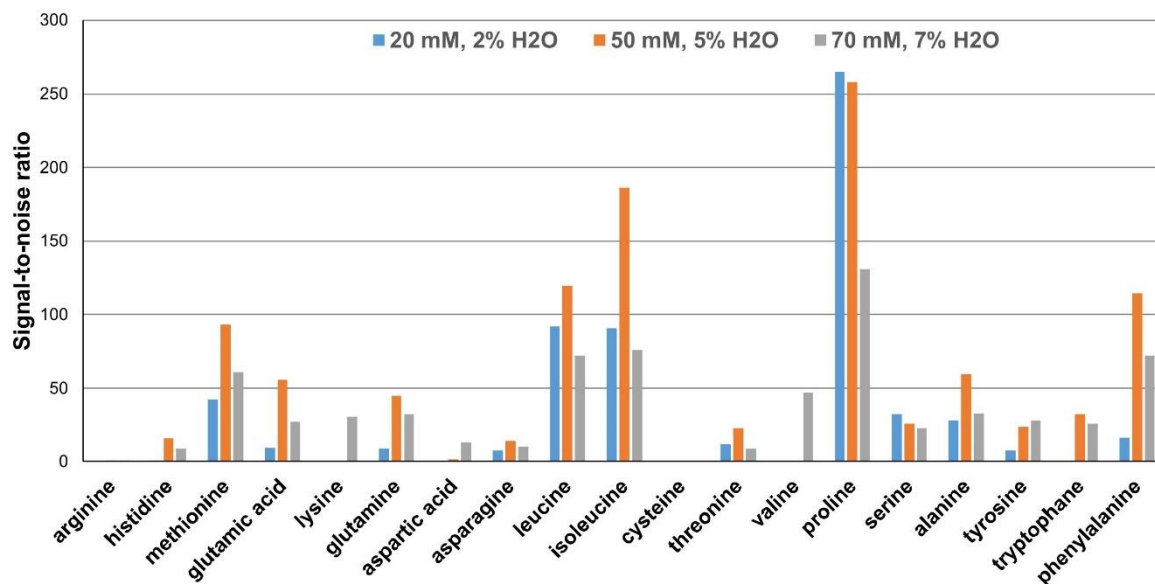


Fig. S3 Signal-to-noise ratio measured for the second eluted enantiomer at three levels of ammonium formate and water in the co-solvent: (20 mM; 2%), (50 mM; 5%) and (70 mM; 7%) ammonium formate and water respectively. Other conditions: Chiralpak ZWIX(+) column, gradient from 10% to 100% co-solvent, 25 °C, 150 bar, 0.5 mL/min, ESI (+) detection

Cette méthode développée avec du formiate d'ammonium, qui devait permettre l'énantiorésolution d'un maximum d'acides aminés protéinogéniques, a ensuite été modifiée pour améliorer encore les formes de pics, surtout pour les acides aminés basiques (histidine, lysine et arginine). Un autre additif a alors été testé, l'acide méthanesulfonique (AMS). Sa structure est représentée sur la figure 3.10.

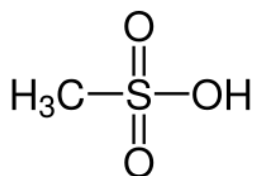


Figure 3.10 : Structure de l'acide méthanesulfonique

Le formiate d'ammonium a été remplacé dans la phase mobile par l'AMS en tant qu'additif, à la concentration de 20 mM avec toujours l'ajout de 2% d'eau, en gardant le même gradient d'élution de 10 à 100% de co-solvant. Cet acide, qui est corrosif, a entraîné une dégradation au niveau du spectromètre de masse. Une nouvelle pièce, la vanne d'exhaust, a été nécessaire afin de résister à la corrosion. Après de longs mois d'attente, l'AMS a pu être utilisé sans soucis pour l'appareillage. Les résultats furent très satisfaisants comme le montrent les chromatogrammes de la figure 3.11.

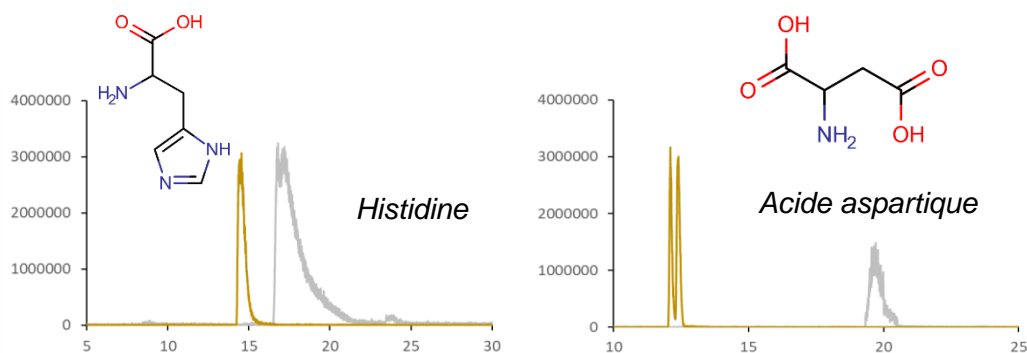


Figure 3.11 : Chromatogrammes de l'histidine et de l'acide aspartique. Additifs utilisés : formiate d'ammonium en gris et acide méthanesulfonique en ocre.

Pour l'histidine, nous voyons bien que le pic est beaucoup plus symétrique même si l'énantiorésolution n'est toujours pas observée. En ce qui concerne l'acide aspartique, les pics sont également plus symétriques, et cette fois l'énantiorésolution est obtenue. Un des principaux avantages de cet additif est la séparation de

l'isoleucine et de la leucine. Ces deux molécules sont isomères, et grâce à l'AMS, elles sont séparées. Les énantiomères de chacun des isomères sont également séparés comme le montre la figure 3.12.

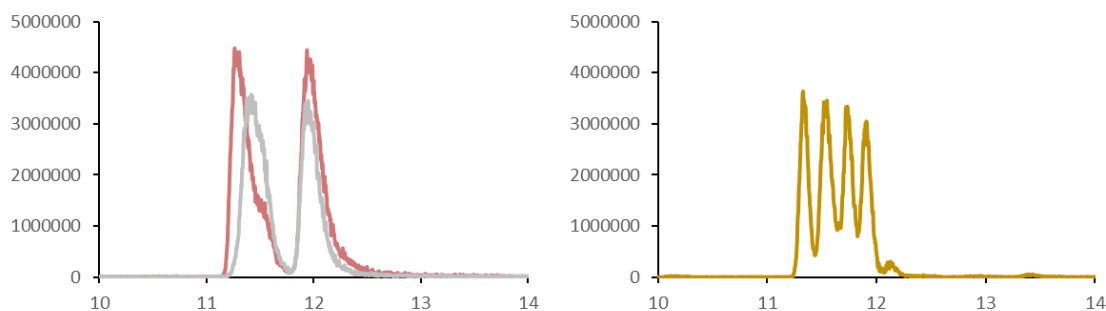


Figure 3.12 : Chromatogrammes de l'isoleucine et de la leucine en fonction de l'additif utilisé : formiate d'ammonium à gauche, acide méthanesulfonique à droite. À gauche, l'isoleucine est en bordeaux, la leucine en gris. À droite, le mélange des deux est en ocre. La leucine est éluee avant l'isoleucine.

On voit sur cette figure que les 4 isomères (deux paires d'énantiomères) sont séparés avec l'AMS en additif dans la phase mobile alors qu'ils ne le sont pas lorsque l'on utilise du formiate d'ammonium.

À la suite de l'amélioration de la méthode analytique par le changement d'additif dans la phase mobile, le temps d'analyse a également été raccourci. En effet la méthode précédente se déroulait à un débit de 0,5 mL/min en raison de la forte pression engendrée en fin de gradient lorsque le pourcentage de co-solvant atteint 100%. Une augmentation de débit a été tentée, mais la pression en tête de colonne ne peut pas dépasser 40 MPa sur notre instrument (Acquity UPC² de Waters). La méthode finale met donc en œuvre un gradient inverse de débit et de contre-pression en même temps que le gradient d'élution. Le débit évolue de 3 mL/min au début du gradient à 1 mL/min en fin de gradient. De la même façon la contre-pression évolue de 15 MPa à 11 MPa. De cette façon, la pression d'entrée ne dépasse jamais 40 MPa, et le temps d'analyse (avec rééquilibrage en fin d'analyse) passe de 30 min à 10 min. Cette méthode améliorée a été utilisée pour les applications avec les compléments alimentaires et les extraits de *Capsella bursa-pastoris* dans la suite de ce chapitre.

III. Applications

Deux applications ont été mises en place grâce à la méthode développée, la première est une validation de méthode de dosage des acides aminés natifs dans des compléments alimentaires. La seconde application est une optimisation des paramètres d'extraction des acides aminés de la plante Bourse à Pasteur (*Capsella bursa-pastoris*).

1. Validation d'une méthode de dosage des acides aminés contenus dans des compléments alimentaires

Une validation de méthodes de dosage d'acides aminés contenus dans des compléments alimentaires a été mise en place en utilisant la méthode développée dans la partie II. de ce Chapitre 3. Des gélules contenant de la L-valine, L-leucine et L-isoleucine ainsi que des gélules contenant de la taurine ont été dissoutes puis analysées en SFC. Une validation de méthode utilisant les ajouts dosés a été effectuée sur 4 jours. 4 niveaux de concentration (1 sans ajout ainsi que 3 avec ajouts) ont été testés. Pour chaque niveau de concentration, 6 répétabilités de préparation d'échantillon ont été effectuées. D'un point de vue analytique, 3 injections pour chaque préparation d'échantillon ont été faites, ce qui fait un total de 576 injections.

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Application to food supplements

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Analysis of free amino acids with unified chromatography-mass spectrometry—application to food supplements

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ABSTRACT

Amino acids are most often analyzed in reversed-phase liquid chromatography after a derivatization procedure to render them sufficiently hydrophobic and detectable with UV or fluorimetric detection. Simpler methods should be possible to avoid additional chemical reactions. We present an improved method to analyze free amino acids with unified chromatography, that is to say with a wide elution gradient starting with supercritical fluid chromatography (SFC) conditions (high percentage of carbon dioxide) and ending with high-performance liquid chromatography (HPLC) conditions (100% co-solvent). The mobile phase composition was carefully adjusted to permit the elution of 21 natural amino acids (among which 19 proteinogenic) with very good peak shapes from a zwitterionic cinchona-based stationary phase (Chiralpak ZWIX(+)). Chiral separation was not desired. The mobile phase finally selected comprised carbon dioxide and a co-solvent (methanol containing 2% water and 20 mM methanesulfonic acid), ranging from 10 to 100% in 7 min followed by 3 min re-equilibration at 25 °C. A reversed pressure gradient (15 to 11 MPa) and a reversed flow rate gradient (3 to 1 mL/min) were applied to avoid reaching the upper pressure limit of the pumping system (40 MPa) and to favor high chromatographic efficiency at every stage of the elution gradient. Detection was achieved with electrospray ionization-mass spectrometry (ESI(+)-MS). The method is then fast and straightforward as no derivatization step is necessary, and all isobaric species were chromatographically resolved.

To demonstrate the applicability of the method, it was applied to the quantitation of amino acids in food supplements commonly consumed by sportsmen, containing taurine (a common natural amino acid) or branched-chain amino acids (BCAA), namely valine, and the isobaric leucine and isoleucine. A standard addition method was examined for sensitivity, linearity, repeatability and intermediate precision.

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1. Introduction

Supercritical Fluid Chromatography (SFC) was described for the first time during the 1960s [1]. After a long period of moderate interest, in the years 2000s, SFC was mainly used for chiral separations, and especially for preparative-scale separations [2–4]. In the last decade, SFC has known a renewed interest, due to the improved instruments now available. These new instruments have yielded a significant increase in achiral applications of SFC [5]. The coupling to mass spectrometry has also led to the development of some application fields that were previously little explored in SFC, like bioanalysis and complex samples of natural products [6–8]. Supercritical CO₂, the major fluid used in SFC, is a rather non-polar solvent, with elution strength close to hexane. To elute more polar molecules, it is therefore necessary to add a polar organic

co-solvent, to increase the polarity of this mobile phase. An additive, and even in some cases small amounts of water are added to analyze highly polar compounds. The effects of additives are complex and varied, and not yet fully understood. However, it is known that, at the usual concentrations employed (typically, arbitrary concentration of about 0.1–0.5% in the co-solvent) they have a small effect on mobile phase polarity [9], can have a significant effect on the mobile phase apparent pH [9,10] and adsorb on the surface of the stationary phase, thereby changing the retention of analytes [11]. In addition, the elution of ionic or ionisable species is especially favoured by ionic or ionisable additives that may form ion pairs with the analytes. When using an ionic stationary phase, the ionic mobile phase additive may act as a displacer [12].

While SFC mostly employs solvent proportions up to 50%, enhanced-fluidity liquid chromatography (EFLC) [13] is a separation method described as an intermediate technique between SFC and HPLC: it uses co-solvent percentages greater than 50%, up to 100% co-solvent [6,14]. More recently, following the proposal of

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Bamba and co-workers [14], wide elution gradients starting in SFC conditions, going through EFLC conditions and ending with HPLC conditions have shown interesting possibilities to achieve the elution of a wide range of analytes, from the least polar to the most polar [6], in a single experiment that can be named “unified chromatography” (UC).

Free (or native) amino acids possess two ionizable functions, one acidic and one basic, thus they can be zwitterionic (deprotonated acid function, protonated basic function) in certain mobile phase compositions. Typically, this should be the case in neutral liquid mobile phases and in SFC mobile phases containing carbon dioxide and an alcoholic co-solvent. The simultaneous presence of a negative and positive charge renders them quite polar and difficult to retain in reversed-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC analysis of amino acids is thus most often done after derivatization to render them more hydrophobic and more detectable with UV or fluorimetric detection [15]. The first liquid-phase separation of underivatized amino acids on a starch column was demonstrated by Synge in 1944 [16], while the first enantioseparation was proposed by Davankov and Rogozhin in 1971 [17]. Free amino acids are now mainly analyzed in liquid-phase chromatography using ion-exchange chromatography with cation- or anion-exchange stationary phases, or with ion-pairing RP-HPLC, and detected with evaporative light scattering detection (ELSD) [18] or mass spectrometry (MS) [19]. More recently, hydrophilic interaction liquid chromatography (HILIC) also proved to be efficient [20], especially as hyphenation to MS is favoured by the high concentration of organic solvent required for HILIC retention. When enantioresolution is desired, zwitterionic chiral selectors [21,22], macrocyclic antibiotics [23] and polysaccharide phases [24] may all be used. Further details on this topic can be found in a recent review [25]. A small number of papers report SFC methods to analyze free amino acids as their zwitterionic character renders them rather difficult to elute with usual SFC mobile phases. The first such experiment was reported in 1992 by Camel et al. [26]. No enantioresolution was desired, but the method achieved the elution of several proteinogenic amino acids. However, the mobile phase used was incompatible with mass spectrometric detection. A second study focused on the enantioseparation of the three aromatic amino acids using UV detection [27]. Another one was focused on optimizing mass spectrometric detection for a small group of proteinogenic amino acids [28]. Finally, another paper reported enantioresolution of a larger selection of proteinogenic amino acids but the most basic ones were not reported [29]. Recently, we have demonstrated that the elution of all proteinogenic amino acids was possible with unified chromatography and a carefully selected mobile phase composition, while enantioresolution was achieved for most of them on a zwitterionic cinchona-based chiral selector (Chiralpak ZWIX(+)) stationary phase [30]. However, peak shapes were not totally satisfying for all amino acids, particularly the most basic ones. Building on this first attempt, in the present paper we mostly aimed at a rapid elution of all free amino acids, with improved peak shapes to hope for better sensitivity. Enantioresolution was not desired in this instance. Food supplements were selected as an interesting sample application, as many of them contain natural amino acids.

Food supplements, also called dietary supplements, are “food-stuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities” [31]. By nutrients, the legislation means vitamins and minerals. Global sales of food supplements are growing rapidly, es-

pecially those based on plant extracts, because they are considered safer and healthier than synthetic drugs. They are also thought to be free of side effects. These increasing sales attest to the great popularity of food supplements. However, this market is vulnerable, with the possibility that food supplements containing adulterants are sold [31,32]. Validated methods to achieve an efficient quality control of such samples are thus highly desirable.

In this paper, we present the development and partial validation of a fast method to quantify free amino acids in food supplements with unified chromatography hyphenated to electrospray ionization – mass spectrometry. Limits of detection and quantitation, linearity and precision are examined and compared to previously reported methods.

2. Material and methods

2.1. Chemicals and solvents

The aim of this study was to develop and validate (in part) an efficient method to quantify L-amino acids in food supplements. Food supplements analyzed were tablets of Amino Acids Complex (AAC), and capsules of Branched-Chain Amino Acids (BCAA) and Taurine and L-Theanine complex (TTC) from VITALL +® (Etik & Bio, France). Standards of L-amino acids used for method validation were all purchased from Sigma-Aldrich (Merck, France) and are described in Table 1. L-Theanine was not available and only examined in the TTC sample. L-Tryptophan, which was present in no sample, was used as internal standard (IS). Ultra-pure water was supplied by an Elga Purelab Flex station from Veolia (Antony, France). The HPLC gradient grade methanol (MeOH) used as the mobile phase co-solvent was supplied by VWR (Fontenay-sous-Bois, France). Methanesulfonic acid (MSA) was purchased from Janssen Chimica (Geel, Belgium). Hydrochloric acid 37% was supplied by Fisher Chemicals and Air Liquide (Paris, France) provided CO₂ with a purity ≥ 99.7%.

Samples and standards preparation for external calibration method:

Food supplements tablets were dissolved in 20 mL of water with 0.1 M HCl during 1 h minimum. Then, the mixture was handily agitated and filtered through 0.45 μm syringe filters. Samples were diluted by five in MeOH before injection. L-Tryptophan was chosen as internal standard and was added in stock solutions and food supplement samples at 2000 ppm. Seven mixtures of stock solutions were prepared (see Table 2), to avoid isobaric compounds in each group. From each of these mixtures, diluted solutions were prepared to build a calibration curve with six points, and with concentrations in accordance with the expected concentrations in the samples prepared from food supplements.

Samples and standards preparation for standard addition method:

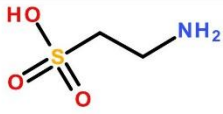
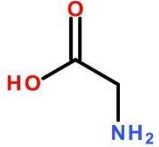
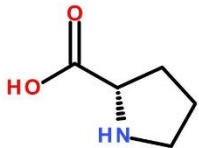
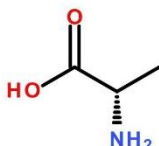
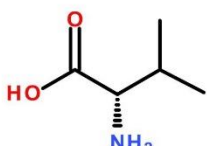
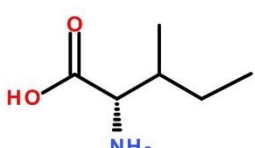
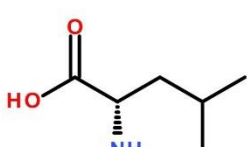
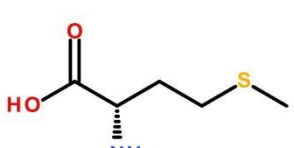
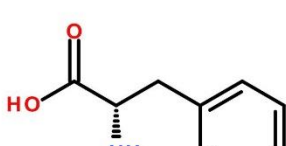
As significant matrix effects were observed with external calibration (even considering the internal standard), a standard addition method was preferred. Three successive additions were done: 100 μL of food supplement solution (in 0.1 M HCl in water) were put in each vial, a standard solution containing Taurine (for the TTC sample) or Valine, Leucine and Isoleucine (for the BCAA sample) was added in increasing amount (from 25 to 625 ppm) and MeOH was used as diluent to obtain the same final volume of 1 mL.

2.2. Instrumentation

The analytical system used for method development was an ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) from Waters Corporation (Milford, MA, USA). It was equipped with a binary solvent delivery pump, compatible with mobile phase

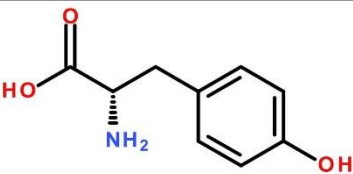
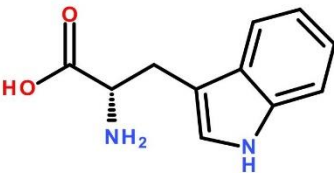
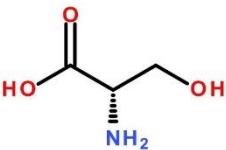
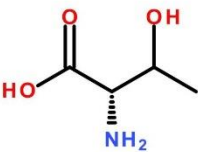
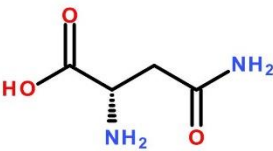
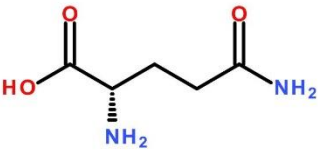
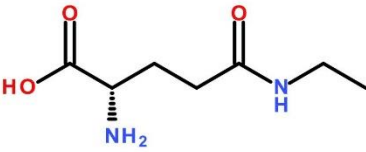
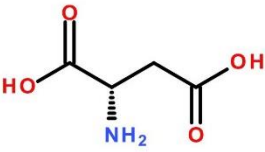
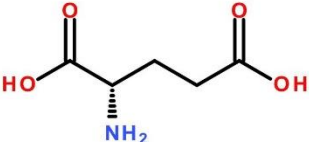
Table 1

Amino acids examined in this work.

Sample	Name	Abbreviation	Structure	Amino acids class	tR (min)	MW (g/mol)	ESI(+) m/z
TTC	Taurine	Tau		Special cases	7.69	125.15	126
AAC	Glycine	Gly		Special cases	5.41	75.07	76
AAC	L-Proline	Pro		Special cases	4.05	115.13	116
AAC	L-Alanine	Ala		Hydrophobic side chain	4.73	89.09	90
AAC / BCAA	L-Valine	Val		Hydrophobic side chain	4.92	117.15	118
AAC / BCAA	L-Isoleucine	Ile		Hydrophobic side chain	4.96	131.17	132
AAC / BCAA	L-Leucine	Leu		Hydrophobic side chain	5.14	131.17	132
AAC	L-Methionine	Met		Hydrophobic side chain	5.50	149.21	150
AAC	L-Phenylalanine	Phe		Hydrophobic side chain	5.30	165.19	166

(continued on next page)

Table 1 (continued)

Sample	Name	Abbreviation	Structure	Amino acids class	tR (min)	MW (g/mol)	ESI(+) m/z
AAC	L-Tyrosine	Tyr		Hydrophobic side chain	5.67	181.19	182
Internal standard	L-Tryptophan	Trp		Hydrophobic side chain	5.74	204.23	205
AAC	L-Serine	Ser		Polar uncharged side chain	5.23	105.09	106
AAC	L-Threonine	Thr		Polar uncharged side chain	4.89	119.12	120
X	L-Asparagine	Asn		Polar uncharged side chain	5.28	132.12	133
AAC	L-Glutamine	Gln		Polar uncharged side chain	5.32	146.14	147
TTC	L-Theanine	The		Polar uncharged side chain	4.86	174.2	175
AAC	L-Aspartic Acid	Asp		Acidic side chain	5.33	133.11	134
X	L-Glutamic Acid	Glu		Acidic side chain	5.50	147.13	148

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Table 1 (continued)

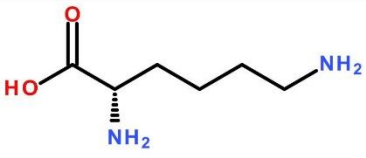
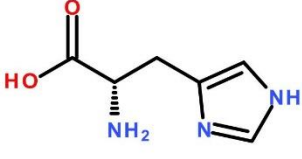
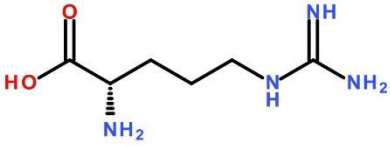
Sample	Name	Abbreviation	Structure	Amino acids class	tR (min)	MW (g/mol)	ESI(+) m/z
AAC	L-Lysine	Lys		Basic side chain	6.11	146.19	147
AAC	L-Histidine	His		Basic side chain	6.05	155.16	156
AAC	L-Arginine	Arg		Basic side chain	6.21	174.2	175

Table 2

Preparation of standard solutions for quantitation of amino acids.

Group	Amino acid	Concentration (ppm)	MW (g/mol)	C1 (ppm)	C2 (ppm)	C3 (ppm)	C4 (ppm)	C5 (ppm)	C6 (ppm)
1	Glycine	150	75.07	10	50	100	200	300	500
	L-Arginine	200	174.2	10	50	100	200	300	500
	L-Tyrosine	250	181.19	10	50	100	200	300	500
	L-Asparagine	/	132.12	10	50	100	200	300	500
2	L-Serine	400	105.09	100	300	400	500	800	1000
	L-Proline	400	115.13	100	300	400	500	800	1000
	L-Valine	400	117.15	100	300	400	500	800	1000
	L-Alanine	420	89.09	100	300	400	500	800	1000
	L-Threonine	500	119.12	100	300	400	500	800	1000
3	L-Isoleucine	400	131.17	100	300	500	700	800	1500
4	L-Leucine	1600	131.17	200	700	800	1500	1700	2000
5	L-Lysine	750	146.19	200	500	700	800	1000	1500
	L-Methionine	750	149.21	200	500	700	800	1000	1500
	L-Histidine	750	155.16	200	500	700	800	1000	1500
	L-Phenylalanine	750	165.19	200	500	700	800	1000	1500
	Acid L-Aspartic	800	133.11	200	500	700	800	1000	1500
6	Taurine	1000	125.15	500	750	1000	1250	1500	2000
7	L-Glutamine	1400	146.14	750	1000	1500	1750	2000	2500

flow rates up to 4 mL/min and pressures up to 414 bar. The system had an autosampler including a partial loop volume injection system. A backpressure regulator and 2-position column oven compatible with 150 mm length columns were also present. Two detectors were available: a photodiode-array (PDA) detector and an ACQUITY QDa® single-quadrupole mass spectrometric (MS) detector with electrospray ionization source (ESI). An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was split after the introduction of make-up fluid: a portion of the column flow went to the backpressure regulator and another (unknown) portion went to the mass detector. Empower®3 software was used for system control, data acquisition and data retreatment. Because methanesulfonic acid (MSA) was employed in the mobile phase, it was necessary to modify the exhaust valve on the QDa system. It is worth mentioning that MSA may damage some mass spectrometric systems (corrosion of certain parts) and care must be taken when employing this acid.

2.3. Analytical method

All experiments were carried out on Chiralpak ZWIX(+), (150 × 3.0 mm; 3 μm) from Chiral Technologies (Illkirch, France).

This column was previously demonstrated to be most effective in the retention and enantioresolution of amino acids in unified chromatography [30].

The co-solvent was MeOH containing 2% water and 20 mM methanesulfonic acid. Initial conditions were as developed in our previous works [30]: flow rate was set at 0.5 mL/min, oven temperature at 25 °C and back-pressure at 15 MPa. The elution was done with a gradient from 10% to 100% co-solvent for 22 min. The elution gradient was then improved in order to achieve a faster and most efficient analytical method as further described in results section. In the optimized method, the elution gradient was faster (7 min instead of 22). The oven temperature was still 25 °C but a reversed pressure gradient (15 down to 11 MPa) and a reversed flow rate gradient (3 down to 1 mL/min) were adopted to minimize analysis time and optimize efficiency along the elution gradient as is further discussed in the results section.

The injection volume of each standard and sample solution was 2 μL. As most amino acids have no chromophore, chromatograms were recorded with UV at 210 nm and with ESI-MS detection (positive ionization mode) and ion extraction from Total Ion Chromatogram (TIC). The make-up solvent to favor mass spectrometric detection and avoid analyte precipitation upon CO₂ decompression was MeOH containing 2% water and 1% formic acid at 0.3 mL/min.

Table 3

Linearity, limits of detection and quantification with external calibration method
LOD and LOQ are expressed in terms of amount detected/quantified per analysis.

Amino acid	LOD (ng)	LOQ (ng)	Linearity	
			Regression equation	r ²
Val	12.5	41.7	y = 0.00049x + 0.03419	0.9997
Ile	1.0	3.3	y = 0.00080x + 0.08880	0.9990
Leu	1.3	4.2	y = 0.00096x + 0.17094	0.9999
Ala	4.0	13.3	y = 0.00015x + 0.01043	0.9992
Ser	2.5	8.3	y = 0.00035x + 0.00775	0.9997
Gln	7.0	23.3	y = 0.00042x + 0.06272	0.9992
Thr	2.0	6.7	y = 0.00042x + 0.03137	0.9979
Asn	4.5	15.0	y = 0.00024x + 0.00032	0.9998
Phe	2.0	6.7	y = 0.00065x + 0.15310	0.9939
Asp	6.3	20.8	y = 0.00021x + 0.06617	0.9857
Met	2.5	8.3	y = 0.00067x + 0.03820	0.9976
Arg	6.3	20.8	y = 0.00079x - 0.00985	0.9976
Gly	33.4	111.3	y = 0.00004x + 0.00065	0.9987
His	6.3	20.8	y = 0.00078x + 0.04876	0.9966
Tyr	5.0	16.7	y = 0.00039x - 0.00240	0.9993
Pro	1.0	3.3	y = 0.00106x + 0.11285	0.9986
Lys	10.0	33.3	y = 0.00077x - 0.02023	0.9973
Tau	5.0	16.7	y = 0.00071x + 0.05083	0.9992
Glu	1.5	5.0	Not present in samples	
Trp	1.5	5.0	Internal standard	

ESI-MS parameters were set as follows: 600 °C for the probe temperature, 0.8 kV for the capillary voltage and 15 V for the cone voltage. The mass range was set from 75 Da to 250 Da.

2.4. Method validation

A partial method validation was carried out on the dietary supplement samples with standard addition method and with the optimized elution gradient. For this purpose, the limits of detection and quantification of each amino acid, repeatability, linearity and intermediate precision of the method were estimated, according to ICH guidelines although these are not pharmaceutical products [33]. To demonstrate the interest of the method, only the quantification of Valine, Leucine and Isoleucine in BCAA sample and Taurine in the TTC sample were validated.

2.4.1. Limit of detection and limit of quantification

The ESI-MS LOD and LOQ of 20 amino acids (all amino acids from Table 1 apart from L-Theanine) was assessed with standard solutions (see Table 3). Nine stock solutions of 5000 ppm were prepared. Successive dilutions were performed until the LOD of each amino acid was observed. LOD is considered as 3 times the baseline noise level, while LOQ is 10 times the noise level.

2.4.2. Linearity

To determine the linearity of the analysis using external calibration or standard addition method, the solutions of different concentrations of amino acids shown in Table 2 were injected. An

internal standard (Tryptophan) was added in all solutions at the same concentration. Peak areas at each concentration level were recorded and a plot of amino acid peak area divided by internal standard peak area versus amino acid concentration divided by internal standard concentration was done.

2.4.3. Repeatability

To assess repeatability, three concentration levels of doped BCAA sample were examined. Each solution was analyzed three times.

Relative standard deviation (RSD) was calculated as below:

$$(\%)RSD = \frac{\text{Standard deviation of AAA over ISA}}{\text{Mean of AAA over ISA}} \times 100$$

AAA and ISA are respectively the amino acid peak area and the internal standard peak area. In dietary supplements, RSD values inferior to 10% are considered acceptable [34].

2.4.4. Precision

The ability to reach the same value, for each sample analysis, in the same analytical conditions but with different technicians or during different days, was examined. Firstly, at each concentration level, six solutions were prepared and each of them was analyzed three times. This served to determine intra-day precision. Secondly, the previous experiments (six solutions analyzed three times) were reproduced during 4 days and relative standard deviation served to determine inter-day or intermediate precision. In this case, the RSD value should be less than 15% [34].

3. Results and discussion

3.1. Development of an optimized method to elute all amino acids

Previously, we had developed a method to elute and resolve the enantiomers of proteinogenic amino acids [30]. Chiralpak ZWIX(+) and Chiralpak ZWIX(-) stationary phases had been selected for their recognized ability to resolve enantiomeric pairs of amino acids in the liquid phase. Even without the need for enantioresolution, these stationary phases would be excellent options for amino acids due to their multimodal interactions, allowing for the retention and separation of a wide range of amino acids carrying hydrophobic, polar non-ionic, acidic or basic side-chains. In this first stage of method development, a wide elution gradient was employed (10 to 100% co-solvent in CO₂) [30], and three different mobile phase additives had been tested: one base (isopropylamine), one acid (trifluoroacetic acid) and one salt (ammonium formate). Ammonium formate had proven to be the most effective in eluting all free proteinogenic amino acids and resolving a majority of enantiomeric pairs (16 out of 19). Some improvement was however wanted as (i) peak shapes for the basic analytes (Histidine,

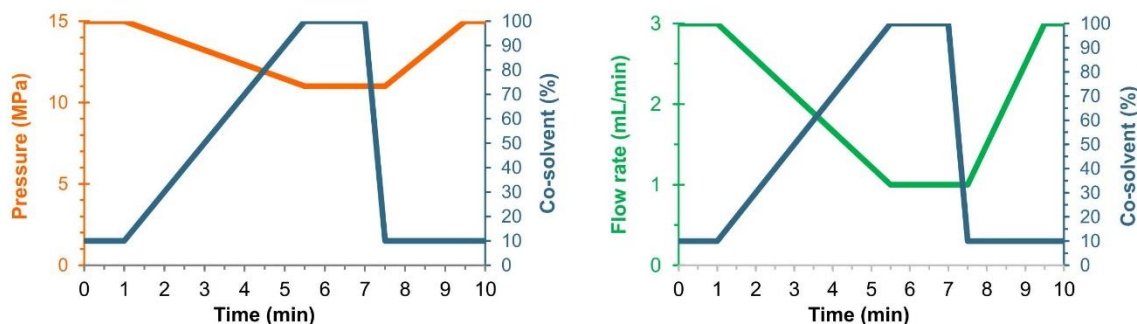


Fig. 1. Gradient profile in the optimized method showing the variation of co-solvent (blue lines), back-pressure (orange lines) and flow rate (green lines).

Lysine and Arginine) were still imperfect; (ii) the isomeric compounds Leucine and Isoleucine remained co-eluted and (iii) the method was still rather long.

Looking for some improvement in all these points, we explored other mobile phase compositions with less common additives. Methanesulfonic acid (MSA), a strong acid with aqueous $pK_a = -1.9$, appeared as an interesting alternative. MSA is rather UV transparent and fully compatible with MS detection, making it an interesting option. MSA employed in reversed-phase HPLC is known to be an excellent ion pairing agent for basic drugs, favoring their elution with excellent peak shapes [35]. In hydrophilic interaction liquid chromatography (HILIC) mode, MSA was shown to provide different selectivity and generally improved peak shapes when compared to trifluoroacetic acid [36]. In normal-phase HPLC, sulfonic acids were shown to be beneficial to the enantioresolution of free amino acids [24]. Along with the slightly less acidic ethanesulfonic acid (ESA), MSA was also employed several times in SFC to achieve the enantioresolution of acidic or basic analytes on polysaccharide stationary phases [37,38]. When compared to other acidic additives (with higher or lower pK_a values) or to basic additives, ESA and MSA both provided improved enantioresolution due to improved enantioselectivity together with improved efficiency. The benefits were mostly attributed to (i) the changes in ionization state of the analytes, impacting their interaction with the stationary phase and (ii) the formation of ion pairs between the additive and basic analytes, impacting their solubility and interaction with stationary phase. Indeed, in the carbon dioxide – methanol mobile phase, MSA should be fully deprotonated in methanesulfonate. It should also acidify the mobile phase significantly (apparent pH around 1–2 [9]), thus the amine functions of amino acids would probably be in their cationic form while acidic functions would be in their neutral form. This way, methanesulfonate should form ion pairs with cationic amino acids. On another hand, the stationary phase sulfonic group should remain unaffected by the presence of MSA, while the quinuclidine group should be fully protonated.

In the present case, the use of MSA was not so favorable to enantioresolution of free amino acid pairs on the zwitterionic stationary phase: only 12 amino acids (Asn, Asp, Cys, Gln, Glu, Ile, Leu, Phe, Pro, Ser, Tyr, Val) out of 19 could be resolved, with the notable case of aspartic acid that was well resolved with MSA while it had not been resolved with any of the additives previously tested. As enantioresolution was not an objective in the present paper, no further attention was paid to the unresolved pairs. However, MSA proved to be an excellent alternative to achieve elution of all free amino acids with good peak shapes, supporting the theory of ion-pair formation. The formation of an ion pair should reduce the overall polarity of the analyte, thereby reducing retention through the combined effects of improved mobile phase solubility and reduced interactions with the stationary phase. Whenever the protonated amino group of the amino acid analytes was taking a significant part in the enantioresolution process, its masking by the MSA anion would however be unfavorable to enantioresolution. The MSA anion may also adsorb on the stationary phase cation, thereby shielding it from the analyte. In other words, MSA somewhat reduced enantioselective binding to the stationary phase.

Our initial method was rather long, with a slow elution gradient from 10 to 100% co-solvent in CO_2 and low flow rate (0.5 mL/min) to avoid reaching the upper pressure limit of the pumping system at the end of the elution gradient, in liquid mobile phase. We then proceeded to accelerate the method in progressively increasing the slope of the elution gradient and the flow rate. To avoid the pressure limitations, a reversed pressure gradient was however designed, starting at 15 MPa and ending at 11 MPa back-pressure, together with a reversed flow rate gradient, starting at 3 mL/min and ending at 1 mL/min (Fig. 1). This needed to be optimized step

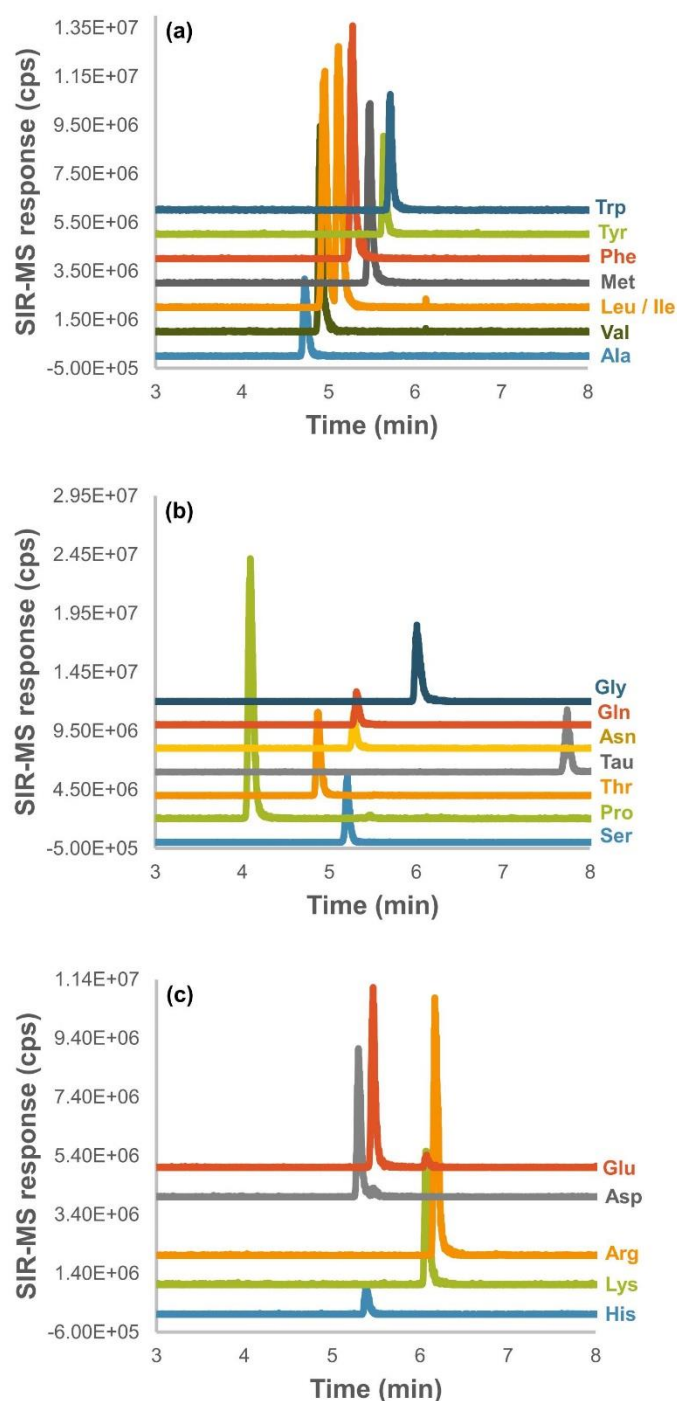


Fig. 2. Chromatograms of all amino acid standards with the optimal gradient program (defined in Fig. 1). Other conditions: Chiralpak ZWIX(+) ($150 \times 3.0, 3 \mu m$), 25 °C. ESI-MS detection with single-ion recording (see table 1). (a) Hydrophobic side chains (b) Polar side chains and special cases (c) Acidic and basic side chains.

by step, in a trial-and-error process, because it is difficult to predict the inlet pressure resulting from all operating parameters in a gradient program in SFC.

The flow rate reversed gradient also had another motive. It was shown in previous works from Desfontaine et al. [6] that the optimal flow rate (based on Van Deemter curves) should vary significantly along a wide elution gradient such as the one employed here: while the analytes eluting at the beginning of the gradient would reach optimal chromatographic efficiency at a fast flow rate (as is normally observed in supercritical fluids), the analytes

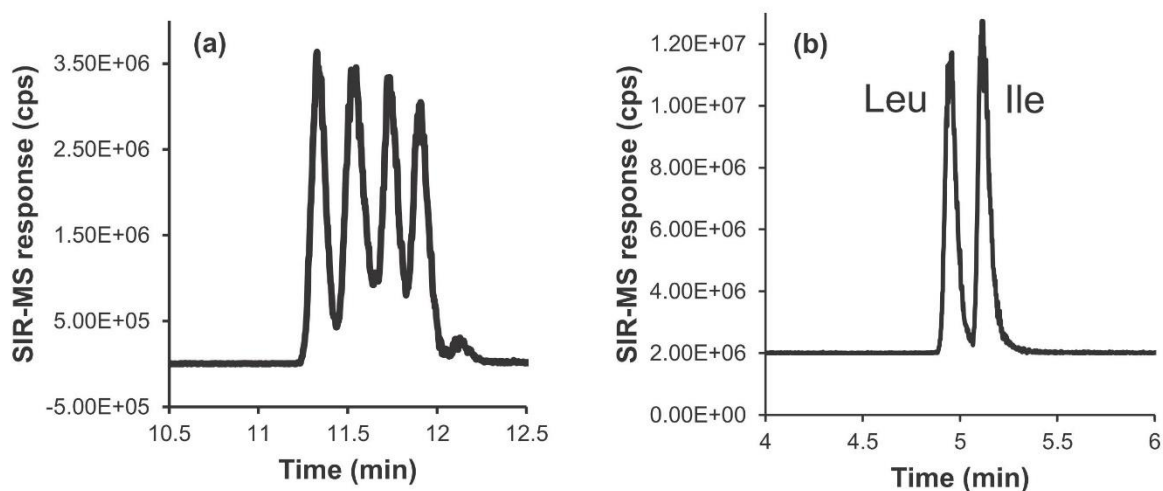


Fig. 3. Chromatograms of isobaric branched-chain amino acid standards (leucine and isoleucine) with the (a) initial and (b) optimal gradient program (see text for details). Other conditions: Chiralpak ZWIX(+) (150 × 3.0, 3 μm), 25 °C. ESI-MS detection (see Table 1).

eluting at the end of the gradient would reach optimal chromatographic efficiency at lower flow rates (as should be the case in liquids). Having made similar observations in this chromatographic system, rather than selecting an intermediate flow rate that would provide a compromise efficiency, we reasoned that the reversed flow rate gradient should be the best option to favor both early-eluting and late-eluting analytes.

The final method was a 7-minutes gradient with 3 min re-equilibration, thus cycle-time was 10 min (Fig. 1). With this method, all free amino acids analyzed (19 proteinogenic amino acids and Taurine) eluted from the column with good peak shapes, as appears in Fig. 2.

Fig. 2 is divided into three parts to see (a) the amino acids with hydrophobic side chains (Alanine, Valine, Leucine, Isoleucine, Methionine, Phenylalanine, Tyrosine and Tryptophan); (b) the amino acids with polar side chain and special cases (Serine, Proline, Threonine, Taurine, Asparagine, Glutamine, Glycine) and (c) the amino acids with acidic or basic side chains (Aspartic acid, Glutamic acid; Arginine, Histidine and Lysine).

In the first group (Fig. 2a), retention was mostly in accordance with increasing hydrophobicity. However, the isomeric Leucine and Isoleucine were very well resolved, as is more clearly visible from Fig. 3b. In these accelerated conditions, the enantioresolution was lost, but in the longer gradient program, it was possible to separate all four isobaric compounds with the two enantiomers of Leucine being separated from the two enantiomers of Isoleucine (Fig. 3a). The other isobaric pair formed by Lysine and Glutamine was also chromatographically resolved with the optimized method, thus the UC-ESI-MS method permits the full identification of all the L-amino acids examined, based on analysis time and MS single-ion recording trace.

We then proceeded to apply this optimized method to samples of food supplements containing natural L-amino acids.

3.2. Quantification of amino acids in food supplements

Three different samples were examined. The first one contained 17 proteinogenic L-amino acids (see Table 1) (Amino Acid Complex AAC). The second one contained only three “branched-chain amino acids” (BCAA, containing L-Valine, L-Leucine and L-Isoleucine), as these are usually known by sportsmen to boost muscle growth and enhance exercise performance. The third one contained two non-proteinogenic amino acids, Taurine and L-Theanine (Taurine & Theanine Complex, TTC). Taurine is thought to have anti-oxidant

properties and is often found in dietary supplements like energy drinks, while L-Theanine is believed to affect mood and stress levels favorably.

3.2.1. External calibration with internal standard

First, a simple external calibration was attempted, using L-Tryptophan as an internal standard as it was not present in any of the three samples. Limits of detection and quantification (LOD and LOQ) were determined from the signal-to-noise ratio through successive dilutions of standard solutions. The results are presented in Table 3. LOD values ranged from 1.0 to 33.4 ng (per injected solution), with the lowest LOD being obtained for Isoleucine and Proline, and the highest LOD for Glycine. The average LOD value was about 5.8 ng. LOQ values ranged from 3.3 to 111.3 ng, with an average LOQ value of 19.2 ng. In comparison with validated methods in the literature, these values are in the same range. For example, Sanchez-Hernandez et al. found that for the 3 native amino acids comprising an aromatic ring (Tryptophan, Phenylalanine and Tyrosine) LOD values ranged from 10 to 40 ng [39]. However, these results are worst compared to those found in LC-MS / MS [40], which is natural as MS/MS methods should be more sensitive than a single-quadrupole MS detection.

Excellent calibration curves were obtained as appears in Table 3, with r^2 values mostly above 0.99, with a single exception of 0.9857 for Aspartic acid. However, the calculated concentrations in the samples were significantly different from the ones expected, based on the information provided by the manufacturers. Very strong positive and negative matrix effects were responsible for the deviations observed. A second method was then explored, with standard additions and the same internal standard, to eliminate matrix effects.

3.2.2. Standard addition with internal standard

The standard addition method was carried out and validated on the BCAA sample and on Taurine in the TTC sample, to demonstrate the feasibility of the method in different samples. The results are presented in Table 4.

Repeatability of the method (expressed in %RSD) was determined with three replicate injections of each calibration solution and measured for retention time and peak areas. Repeatability of retention times was excellent, with %RSD being inferior to 0.1%. This is an interesting observation as the Acquity UPC² instrument was not necessarily designed to operate under such wide elution

Table 4

Features of the standard addition method.

Polynomial regression			Repeatability (RSD, %, n = 3)		Intermediate precision (RSD, %)		Weight in one capsule (mg)		
Amino acid	Regression equation	r ²	Retention time	Peak area	Intra-day (n = 6)	Inter-day (n = 4)	Theoretical	Determined	Recovery (%)
Val	$y = -2E-05x^2 + 0.0194x + 0.9374$	0.9927	0.05	0.9	2.3	14.6	160.0	185.7	116.1
Leu	$y = -2E-05x^2 + 0.0312x + 1.1716$	0.9974	0.03	1.0	2.5	12.3	160.0	146.4	91.5
Ile	$y = -2E-05x^2 + 0.0304x + 0.7506$	0.9970	0.06	1.5	2.4	13.8	90.0	97.2	108.0
Tau	$y = -2E-06x^2 + 0.0054x + 0.1661$	0.9998	0.02	1.1	3.6	11.0	100.0	121.7	121.7

gradients. Repeatability of relative peak areas was inferior to 2% in all cases.

Precision of the method was demonstrated for four concentration levels (no addition and three levels of standard addition). Intra-day precision was assessed using six separately prepared sample solutions at each concentration level and each of them was analyzed three times. Inter-day precision was evaluated over four days using the same protocol. In the worst cases, intra-day and inter-day precision were as high as 13 and 15% respectively, but were mostly below 6 and 12% respectively. In any case, this was still acceptable in this context. For example, intra-day and inter-day precision on the sample solutions with one standard addition is shown in Table 4. Compared to the literature, these values are equivalent. For instance, Liyanaarachchi et al. found that the intra-day and inter-day precision were below 10% with LC-MS analysis of rice samples [40].

While linear calibration curves had been obtained with the external calibration method (Table 3), the standard addition method yielded curves that were better fitted with polynomial regression (Table 4). This is not uncommon for mass spectrometric detection [41]. The determination coefficient for polynomial regression was higher than 0.992 for all studied compounds.

Finally, accuracy was largely improved with the standard addition method over the external calibration method as appears in Table 4. Deviations from the theoretical concentrations (as indicated on the sample packagings) ranged from 8 to 22%.

4. Conclusions

The use of an uncommon mobile phase additive (methanesulfonic acid) and a broad elution gradient ranging from supercritical fluid to liquid mobile phase allowed the elution of 20 free amino acids with satisfying peak shapes from the zwitterionic column. Furthermore, the application of a reversed pressure gradient and a reversed flow rate gradient permitted a fast method with total cycle time of 10 min. Application to dietary supplements proved to be difficult, due to strong matrix effects that were circumvented by a standard addition method. The proposed method permits the separation of isobaric compounds (Leucine and Isoleucine) and offers simple sample preparation (no derivatization is required) followed by a fast analysis, which should be advantageous when large quantities of samples must be analyzed. Finally, it is worth mentioning that, although no thorough study of column stability was performed, no impact of the acidic analyte on stationary phase was observed in the course of this project. It is possible that the degradation may be lessened by the very low concentration of water (2% in the co-solvent).

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

Adrien Raimbault: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Angéline Noireau:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Caroline West:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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2. Extraction par fluide pressurisé et analyse d'acides aminés contenus dans la Bourse à Pasteur (*Capsella bursa-pastoris*)

Suite à la validation de méthode de dosages des acides aminés dans des compléments alimentaires, une autre application a été testée, il s'agit d'une extraction des acides aminés natifs d'une plante commune, la Bourse à Pasteur (*Capsella bursa-pastoris*). Une optimisation des paramètres d'extraction a été effectuée grâce à la méthodologie des plans d'expériences. Cette méthodologie permet en un nombre restreint d'expériences de définir un optimum. La PFE est la technique d'extraction utilisée, et les paramètres à optimiser dans le plan d'expériences ont été la composition de la phase d'extraction, la température de la cellule d'extraction ainsi que le nombre de cycles d'extraction.

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Optimised pressurized liquid extraction of native amino acids from *Capsella bursa-pastoris* and their analysis with unified chromatography

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Optimised pressurized liquid extraction of native amino acids from *Capsella bursa-pastoris* and their analysis with unified chromatography

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1. Introduction

Shepherd's Purse (*Capsella bursa-pastoris*), belonging to the Brassicaceae or Cruciferae (Mustard) family, is considered the second-most common weed in the world and is found in most regions of the world outside the tropics. Among its many uses (food, animal feed, mosquito killer or cosmetics), it is also used as a traditional medicine and described in French Pharmacopeia, notably for hemostatic and anti-infectious properties. The dried aerial parts, flowers and fruits harvested at the end of blossoming and in the middle of fruiting period, are ingested as an infusion in water or alcohol. Homeopathic remedies are also made from the fresh plant. The monography from French Pharmacopeia describes a thin-layer chromatography (TLC) analysis to identify amino acids (proline, valine, leucine and gamma-aminobutyric acid) in the ethanolic extract. This is a very simple method with ninhydrin derivatization, providing rather vague information (the possible presence of the desired amino acids when compared to standards) but clearly not very sensitive nor selective. For instance, it is unlikely to separate leucine from its isomer isoleucine. Nor does it mention the presence of any other amino acids, apart from the four cited above.

The current wide development of natural products as medicinal remedies is calling for modern analysis methods to verify the composition and control the quality of samples. European Pharmacopeia recommends the use of high-performance liquid chromatography (HPLC) for the analysis of amino acids, mostly with reversed-phase HPLC after a derivatization procedure. Previously, we have developed a unified chromatography (UC) method to analyze free amino acids without the need for derivatization. The UC method combines supercritical fluid chromatography (SFC) and HPLC in a single elution gradient starting with a CO₂-rich supercritical fluid mobile phase and ending with a solvent-rich liquid mobile phase within 8 min. Mass spectrometric detection with electrospray ionization (ESI-MS) was used to detect and quantitate the amino acids. The method was developed on standard analytes and applied to food supplements.

Amino acids are the main part of the protein composition. They are classified into two distinct categories: essential amino acids (EAAs) and non-essential amino acids (NEAAs). EAAs are not synthesized by humans, unlike NEAAs, that is why food must provide humans the necessary amounts of EAAs. These EAAs are synthesized only by plants, so it is necessary to extract them. Plants of the Chenopodium family such as Quinoa are rich in protein [1]. This plant contains the 8 EAAs, unlike other cereals that contain only very low concentrations of lysine [2]. Other plants contain significant amounts of amino acids. The next example is the *Adenopus breviflorus* described in an article by Oshodi in 1996 where this plant is studied and demonstrated to have as many EAAs as soybeans [3]. Chickpeas, *Cicer arietinum*, are also good sources of protein as shown by de Almeida Costa *et al.* [4]. Many plants synthesize proteins, and EAAs, that it is important to know how to extract for consumption. A review reports the distribution of amino acids in plant [5].

Different amino acid extraction techniques have been used over the years. Supercritical fluid extraction has been employed on different plants, such as sugar beet and sugar cane [6], maize and soy [7] or broccoli leaves [8]. Soybeans were also used in solid phase microextraction (SPME) before amino acid derivatization and analysis in gas chromatography (GC) [9]. The extractions carried out by SPME were

the subject of an optimization by experimental plans. Microwave extractions were also used for proteins and free amino acids, found in plants such as broccoli [10].

The aim of this study is to develop and optimize a method for extracting native amino acids from the aerial parts of Shepherd's Purse and quantify them with unified chromatography-mass spectrometry (UC-MS). For this purpose, a design of experiments (DoE) was used to define an optimal extraction. The extractions are carried out by pressurized fluid and the extracts are analyzed by unified chromatography (UC) with a wide elution gradient starting in CO₂-rich mobile phase (supercritical fluid chromatography, SFC) and ending with a methanol-rich liquid mobile phase (high-performance liquid chromatography, HPLC). The studied parameters for the optimization of extractions were the composition of the extraction phase, the temperature of the extraction cell and the number of extraction cycles.

2. Material and methods

2.1 Chemicals and solvents

Dried aerial parts of *Capsella bursa-pastoris* were provided by the local pharmacist (Pharmacie au Lys, Mulhouse, France). L-Methionine was used as internal standard (IS) and was purchased from Sigma Aldrich (Merck, France). Ultra-pure water was supplied by an Elga Purelab Flex station from Veolia (Antony, France). The HPLC gradient grade methanol (MeOH) and ethanol (EtOH) absolute used as the mobile phase co-solvent and to extract the samples was supplied by VWR (Fontenay-sous-Bois, France). Methanesulfonic acid (MSA) was purchased from Janssen Chimica (Geel, Belgium). Ammonium formate (AF) was purchased from Riedel de Haën, supplied by Fisher Chemicals (Illkirch, France). Air Liquide (Paris, France) provided carbon dioxide (CO₂) with a purity $\geq 99.7\%$.

2.2 Instruments

The pressurized fluid extraction (PFE) system used for the design of experiments was a Dionex™ ASE™ (Accelerated Solvent Extraction) 150 from Thermo Scientific (Thermo Fisher). It was equipped with a delivery pump for the extraction solvent. An oven capable of accommodating extraction cells of different sizes was also present.

The analytical system used for the UC-MS analysis of extract was an ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) from Waters Corporation (Milford, MA, USA). It was equipped with a binary solvent delivery pump, compatible with mobile phase flow rates until 4 mL/min and pressures up to 414 bar. The system had an autosampler including a partial loop volume injection system. A backpressure regulator and 2-position column oven compatible with 150 mm length columns were also present. The chromatographic column was Chiralpak ZWIX(+) (150 x 3.0 mm, 3 μ m) commercialized by Chiral Technologies (Illkirch, France). Two detectors were available: a photodiode-array (PDA) detector and an ACQUITY QDa® single-quadrupole mass spectrometric (MS) detector with electrospray ionization source (ESI). An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. Thus, the main flow stream was split. With this system, a portion of the column flow goes to the backpressure regulator and another (unknown) portion goes to

the mass detector. Empower[®]3 software was used for system control, data acquisition and data retreatment.

2.3 Preliminary experiments

A first round of maceration of dried *Capsella bursa-pastoris* was done to get a first idea of the best solvent for amino acid extraction. 3 macerations were carried out with 3 different solvents: methanol, ethanol and water. Then, the macerates were analyzed to determine which solvent is the best for extraction. 3 g of *Capsella bursa-pastoris* were put in a bottle for one day containing a maceration solvent. After one day, the macerates were filtered and analysed in UC-MS. The mobile phase was composed of a mixture of pressurized carbon dioxide and a co-solvent (MeOH containing 5% water and 50 mM ammonium formate, as developed in our previous works). The analysis is carried out with an elution gradient ranging from 10% to 100% co-solvent. Initially, the flow rate was first set at 0.5 mL / min, the column temperature at 25 ° C and the backpressure was 15 MPa with a 20 minutes-gradient.

2.4 Extraction optimization by Design of Experiments

800 mg of dried plants were placed in a 5-mL cell and extracted with the Pressurized Fluid Extraction (PFE) system. A design of experiments was carried out to determine the optimal extraction parameters. The factors studied were the composition of the extraction solvent, the extraction temperature and the number of cycles. A Box-Behnken design was used to define the optimum point on these three factors. This design is defined by experiments performed in the middle of each edge of a cube. Replicate central points are also made to ensure the repeatability of the extractions. Then, each extract was evaporated to dryness under a stream of nitrogen, and then dissolved in 5 mL of an ethanol-water 70-30 mixture. The table of extractions is presented in table 1.

2.5 Extract analysis

An internal standard, methionine, was added to each extract to quantify the amino acids present. All extracts were analyzed with UC-ESI-MS as before, but the analytical method was optimized and consists of an elution gradient ranging from 10% to 100% co-solvent in 3 times less than the one used before. Ammonium formate was replaced with methanesulfonic acid (MSA) that ensured higher chromatographic efficiency and elution strength. The mobile phase was a mixture of carbon dioxide with a co-solvent consisting of MeOH, 2% water and 20 mM MSA. The major amino acids mentioned in French Pharmacopeia monograph were taken into account for method optimization, i.e. proline, valine, isoleucine and *gamma*-aminobutyric acid (GABA). Mass spectrometry detection was used to determine peak area. The area ratio between the peak of the studied amino acid and the peak area of the internal standard was then calculated for each extract. Thanks to the JMP 13.0.0 software (S.A.S Institute Inc, Cary, NC, USA), desirability functions were defined to maximize the amount of amino acids extracted.

Table 1: Design of experiments carried out for the extraction of amino acids from *Capsella bursa-pastoris*

	Solvent composition	Temperature	Nb of cycles
1	50% water	100	2
2	50% water	70	1
3	50% water	70	3
4	50% water	40	2
5	50% water	40	2
6	10% water	70	3
7	10% water	70	1
8	10% water	70	2
9	30% water	70	2
10	30% water	70	2
11	30% water	100	1
12	30% water	40	3
13	30% water	40	1
14	30% water	100	3
15	10% water	100	2

3. Results & Discussion

3.1 Preliminary experiments

Initially, three different macerations were carried out with different solvents: methanol, ethanol and water. Alcohol and water are recommended by the pharmacist to prepare an infusion of the plant for medicinal uses. The macerates were analysed with UC-MS to see which solvent would be the most promising in the extraction of amino acids in *Capsella bursa-pastoris*. The results are shown in Figure 1.

These chromatograms show that ethanol is the worst solvent for extracting the amino acids from *Capsella bursa-pastoris*. Indeed, the extracted amino acids were extracted in very small amounts when compared with methanol and water. This is confirmed by looking at the intensity of the peaks of proline which is the major extracted compound. It is only 230,000 for ethanol against 1,850,000 for methanol and 2,400,000 for water. The best solvent is therefore water which makes it possible to extract the target amino acids in greater quantity, and also to extract other amino acids which were not visible with the two alcohols.

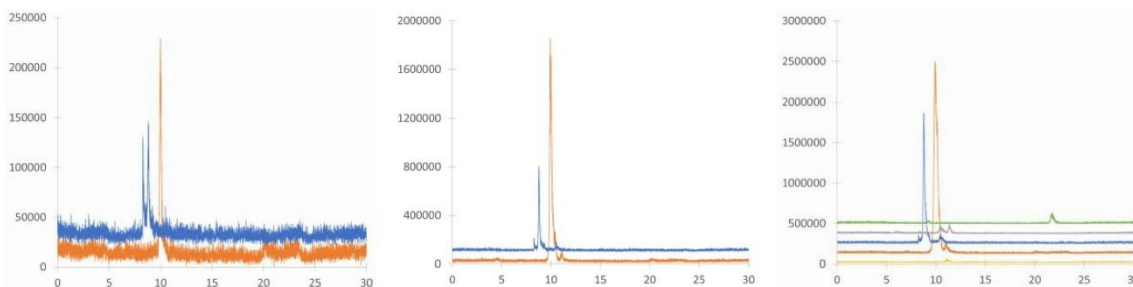


Fig. 1: Chromatograms of the 3 macerates with the 3 different maceration solvents: ethanol (left), methanol (middle) and water (right). The peaks correspond to: proline (orange), valine (blue), alanine (yellow), isoleucine (grey) and arginine (green). Chromatographic conditions: Chiralpak ZWIX (+); Elution gradient from 10% to 100% B; A: CO₂ - B: MeOH / Water (95/5) + 50mM Ammonium formate; MS detection (ESI +)

3.2 Design of experiments

Following the extractions of Shepherd's purse by pressurized fluid extraction, the extracts were dried and then dissolved in 5 mL with the phase used for the extraction. The internal standard was added just before the analysis. After the analysis, peak areas were measured and area ratios of the major compounds to the area of the internal standard were calculated. These area reports were used as answers for the experimental design. The functions of partial desirability were defined so that the maximum desirability corresponds to the greatest ratio found of the experiments. The optimum is found by maximizing global desirability, the geometric mean of partial desirability. 3 new extractions were performed at the optimum point, with 3 injections for each extraction.

The PFE method naturally extracts more amino acids than maceration, as shown in the chromatogram of Figure 2.

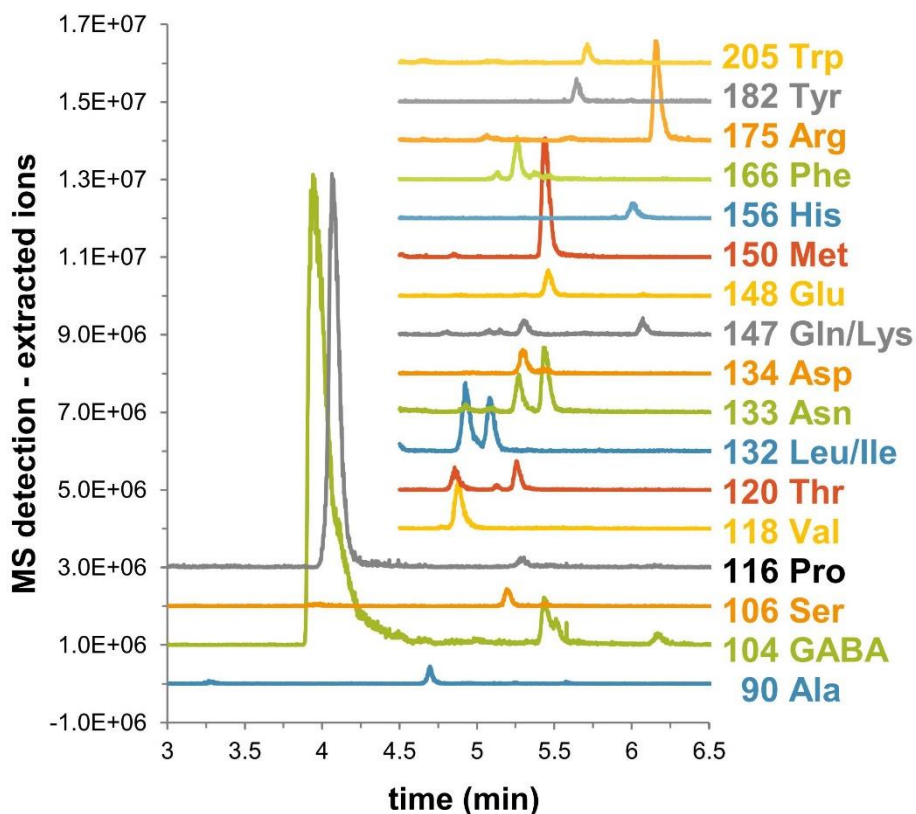


Fig. 2: Chromatogram of the optimized pressurized fluid extract of *Capsella bursa-pastoris*. MS traces of extracted ion chromatograms at the indicated m/z values and corresponding amino acids

The amino acids retained for optimization were GABA, proline, valine and isoleucine. The different ratios of the amino acid peak area by the internal standard peak area are shown in Table 2

Table 2: Maxima and minima of the amino acids of interest peak areas divided by the internal standard peak area.

	GABA	Proline	Valine	Isoleucine
min	0,9834	0,5477	0,0580	0,0387
max	1,1858	0,6868	0,0719	0,0479

The design of experiments was used to maximize all these values. The extraction optimum was defined and the experiment at this optimal point was repeated 3 times. The calculated optimum is the point of the following parameters: +1; +1; -1, which corresponds to a mixture of EtOH/H₂O 50/50; 100°C; 1 cycle. This extraction optimum was evaporated to dryness and dissolved in a mixture of EtOH/H₂O 70/30, as during design of experiments, before injecting. Each extract was analysed 3 times to see the injection repeatability. The results are shown in Table 3. In this table, some of the relative area values are slightly lower than the maxima found in the experimental design but it is a compromise for the analysis of all the targeted amino acids.

Table 3: Result of extractions at the optimal point. Mean and RSD (%) of the 4 amino acids of interest.

	GABA	L-Proline	L-Valine	L-Isoleucine
Mean	1,5166	0,7786	0,0687	0,0597
RSD % (n = 3)	3,91	1,98	1,43	3,80

4. Conclusion

The extraction of native amino acids from Shepherd's purse was optimized by a Box-Behnken experimental design. Extractions were carried out in order to find the optimum by maximizing the area of the major amino acids in these extracts (proline, alanine, valine and isoleucine). This optimum point for the major amino acids corresponded to an extraction with a solvent mixture containing ethanol-water 50/50 (v/v), an extraction cell temperature at 100 °C and 1 extraction cycle. These extracts were analysed by unified chromatography-mass spectrometry, with a wide elution gradient of a mobile phase containing a mixture of carbon dioxide and a co-solvent, containing MeOH, 20 mM of MSA and 2% of water. Proline and gamma-aminobutyric acid appeared to be the present in the sample in much larger concentrations than the other amino acids, but many minor amino acids that are not described in the French Pharmacopeia monograph could be detected.

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IV. Conclusion

Dans ce chapitre, une méthode générique par chromatographie unifiée a été développée pour l'énantioséparation de tous les acides aminés natifs protéinogéniques. Cette méthode utilise la phase stationnaire Chiralpak® ZWIX, qui a été développée précisément dans le but d'aboutir à cette énantioséparation en phase liquide. Cette méthode UC a été ensuite améliorée en changeant d'additif. L'AMS a montré de très bons résultats en termes de formes de pics, surtout pour les acides aminés avec une chaîne secondaire basique. L'utilisation de cet additif corrosif ne fut possible qu'après avoir reçu une nouvelle vanne d'exhaust sur le détecteur de masse Acquity UPC² conçue pour l'usage en contact avec des produits corrosifs. Des améliorations ont également été effectuées sur le temps d'analyse, qui a été divisé par 3, en mettant en place un gradient inverse de contrepression et de débit afin de s'assurer de ne jamais dépasser le seuil limite de la pompe (40 MPa) et d'optimiser l'efficacité.

Suite à ce développement de méthode, deux applications ont été mises en place. La première fut une validation de méthode de dosage des acides aminés dans des compléments alimentaires. Les performances de cette validation de méthode montrent que l'on obtient des résultats semblables en termes de répétabilité et de reproductibilité inter- et intra-jour que des méthodes en HPLC sur la même matrice de compléments alimentaires. La méthode UC est donc aussi performante que l'HPLC pour ces problématiques.

La deuxième application a été une optimisation de l'extraction des acides aminés de la Bourse à Pasteur, une plante très commune, ayant des applications médicinales. Les conditions optimales d'extraction ont été déterminées en utilisant les plans d'expérience : le solvant d'extraction est alors un mélange EtOH/H₂O à 50/50, une température de 100°C et 1 cycle d'extraction.

Ces différentes applications utilisant la méthode UC développée précédemment prouvent bien qu'il est possible d'analyser rapidement des composés polaires zwitterioniques avec l'utilisation d'un additif approprié ainsi qu'un ajout d'eau dans la phase mobile.

Conclusions générales & Perspectives

I. Conclusions générales

L'objectif principal de cette thèse était d'étudier des composés polaires en SFC et de trouver des conditions favorables pour les analyser. Différents aspects ont donc été traités tout au long de ce manuscrit, qui se concentre essentiellement sur l'analyse d'acides aminés.

La SFC est une technique chromatographique qui prend de plus en plus d'ampleur dans les laboratoires pharmaceutiques, même si l'HPLC reste majoritaire, par le développement d'appareils plus performants et sa complémentarité à l'HPLC. Cette technique est bien définie pour des applications avec de petites molécules peu polaires, en revanche en ce qui concerne les molécules polaires cette technique n'est pas celle de référence. Ceci s'expliquant par le fait que la phase mobile utilisée ne peut pas être très polaire en raison de la forte proportion de CO₂ pressurisé. Cependant, différentes techniques pouvant être mises en place pour analyser de telles molécules ont été utilisées lors de ces travaux de thèse.

Dans le chapitre 2, nous avons étudié trois phases stationnaires différentes, la Chirobiotic™ TAG ainsi que les deux Chiralpak® ZWIX (+/-). Un set de molécules achirales et chirales a été injecté sur ces colonnes afin de modéliser les interactions entre les analytes et les différentes phases stationnaires, grâce aux modèles LSER. Pour la Chirobiotic™ TAG, l'objectif était de voir le comportement du greffon, la teicoplanine aglycone, dans diverses conditions de phase mobile. Pour ce faire, 11 phases mobiles différentes ont été préparées à des concentrations croissantes d'additif basique, l'IPA, ou d'additif acide, le TFA. Ces études ont permis de montrer l'état d'ionisation d'un peptide lorsqu'il est soumis à diverses conditions (acides ou basiques). Pour les deux phases Chiralpak® ZWIX, l'étude était destinée à comprendre les mécanismes de rétention et d'énantioséparation liés à ces phases stationnaires. 9 autres phases stationnaires ont été utilisées afin de comparer les rétentions de molécules achirales et l'énantioséparation de molécules chirales. Ces phases stationnaires présentaient toutes des similitudes dans la composition du greffon, avec quelques dissemblances en comparaison avec les greffons de référence des colonnes Chiralpak® ZWIX. Cette étude a permis de montrer que pour l'énantioséparation des acides aminés, l'un des objectifs des colonnes ZWIX, il était préférable d'avoir la

présence à la fois du groupement quinoline, du sulfonique (porteur de la charge négative) et du quinuclidine (porteur de la charge positive).

Dans le chapitre 3, une méthode de chromatographie unifiée a été mise en place pour l'analyse des acides aminés natifs en utilisant l'une des phases stationnaires étudiées dans le chapitre précédent, la Chiralpak® ZWIX(+). L'objectif premier de cette étude était d'avoir une méthode générique pour l'énantioséparation de tous les acides aminés natifs. Différents additifs ont été testés, à différentes concentrations, afin d'avoir les meilleurs résultats possibles. Ce développement de méthode générique s'est avéré très difficile. Séparer les énantiomères des acides aminés acides et à la fois ceux des acides aminés basiques fut complexe. Une méthode de 30 minutes en gradient d'élution a été définie comme la plus satisfaisante, avec du formiate d'ammonium à 50 mM et 5% d'eau dans le co-solvant méthanol. Des améliorations en termes de forme de pics et de temps d'analyse ont été effectuées en changeant d'additif. L'AMS s'est montré plus performant mais la corrosivité de cet acide a endommagé le détecteur de masse Acquity QDa. Le temps d'analyse a été divisé par 3 en réalisant un gradient inverse de contrepression et de débit. Cette méthode a ensuite été utilisée pour deux applications distinctes : une validation de méthode de dosage des acides aminés natifs dans des compléments alimentaires. Les performances mesurées de cette validation de méthode de dosage se rapportent à celles trouvées dans la littérature pour des méthodes similaires en HPLC. La deuxième application a été une optimisation d'extraction des acides aminés de la plante *Capsella bursa-pastoris*. Par la méthodologie des plans d'expérience, l'optimum a été défini, testé et analysé. Cet optimum (mélange d'EtOH et d'eau à 50/50 ; 100°C pour la température de la cellule d'extraction ; 1 cycle d'extraction) montre des meilleurs résultats d'extraction que tous les points du plan d'expérience.

De manière générale pour l'analyse de composés polaires en SFC, l'utilisation de phases stationnaires polaires, pour avoir une certaine rétention des molécules d'intérêt, ainsi qu'une phase mobile assez polaire pour les éluer (allant parfois jusqu'au co-solvant pur) est ce qu'il y a de préférable. L'ajout d'additif et d'eau est également fortement conseillé si l'on s'intéresse à des composés de plus en plus polaires.

II. Perspectives

Dans le futur, il pourrait être intéressant de s'intéresser à d'autres familles moléculaires afin de voir si la SFC (ou l'UC) est capable d'analyser des composés aussi polaires que peuvent l'être des acides aminés natifs. Quelques familles ont été brièvement explorées par cette thèse.

1. Iridoïdes

Les premières sont les iridoïdes, qui sont des molécules ayant des propriétés curatives (cardiovasculaires, analgésiques, anti-inflammatoires, antitumorales, ...). Ces dérivés terpéniques sont très polaires, avec des $\log P$ aux alentours de -4. Les molécules étudiées dans cette thèse ont été l'aucubin, le catalpol et l'harpagide. Leurs structures sont présentées en figure 4.13.

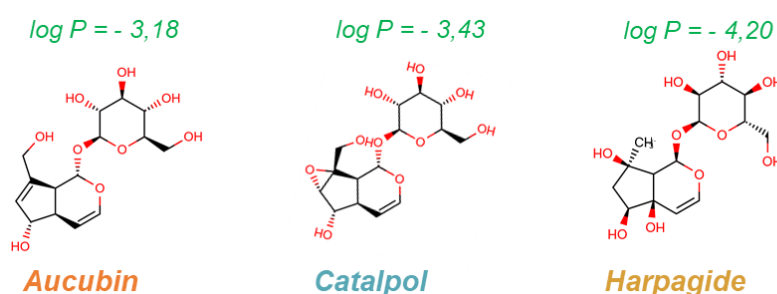


Figure 4.13 : Structures et $\log P$ des iridoïdes étudiés.

Ces molécules ont été injectées, en tant que standard, sur la colonne Acquity UPC² Torus Diol (100 x 3 mm ; 1,7 μm). La phase mobile utilisée est simple puisqu'il s'agit uniquement d'un mélange de CO₂ et de MeOH dans les proportions 70 pour 30. Le débit a été fixé à 1,5 mL/min, la température à 25°C et la contrepression à 15 MPa. L'aucubin et le catalpol sont présents dans la Véronique officinale (*Veronica officinalis*), une plante utilisée en médecine traditionnelle. L'harpagide, naturellement absent de cette plante, a été utilisé comme étalon interne. Différentes extractions sur cette plante, utilisant l'extraction par micro-ondes (MWE) ou la PFE, ont été effectuées. Les conditions d'extraction ont été déterminées pendant un stage de master avant ma

thèse, mais en se focalisant sur d'autres familles moléculaires. Ce ne sont donc pas les conditions optimales pour les iridoïdes, il faudrait optimiser cette méthode. Dans le chromatogramme ci-dessous (figure 4.14), on voit bien les 3 iridoïdes séparés en moins de 3 minutes avec des formes de pics raisonnables.

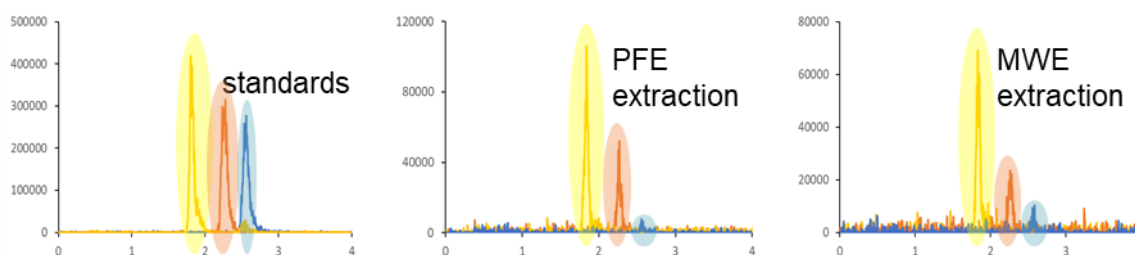


Figure 4.14 : Chromatogrammes des iridoïdes. À gauche : injection des standards ; au milieu : extraction par fluide pressurisé ; à droite : extraction par micro-ondes. En jaune : Harpagide ; en orange : Aucubin ; en bleu : Catalpol.

Des améliorations des conditions d'extraction sont nécessaires mais on voit néanmoins que cette famille est analysable en SFC de manière très simple et rapide.

2. Anthocyanes

Qu'en est-il des composés polaires ioniques ? Des anthocyanes ont été injectées sur deux colonnes différentes : l'Acquity UPC² Torus Diol, et Acquity UPC² Torus DEA (100 x 3 mm ; 1,7 µm). Les anthocyanes injectées sont représentées en figure 4.15. Nous souhaitons ici avoir trois molécules modèles ayant une génine identique mais une variation du nombre de glycosylations (0, 1 ou 2 sucres).

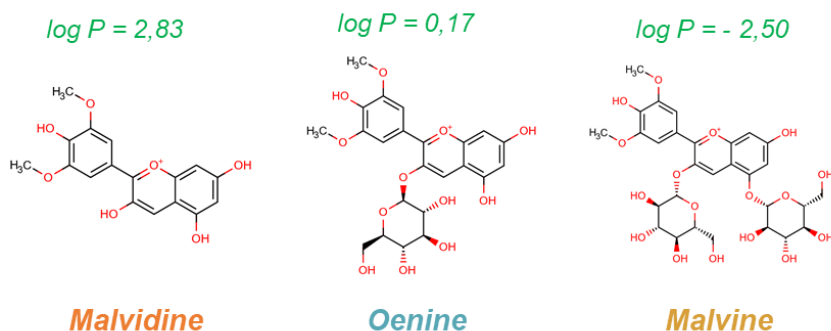


Figure 4.15 : Structures et $\log P$ des anthocyanes étudiées.

Les anthocyanes nécessitant un milieu acide sous peine de rapide dégradation, la phase mobile utilisée pour l'analyse de cette famille de molécules est, comme pour les acides aminés, un mélange de CO₂, de MeOH et d'AMS, utilisé en additif à hauteur de 0,1% dans le MeOH. La méthode consiste en un gradient générique, allant de 5% à 95% de co-solvant en 30 minutes. Le débit est fixé à 0,5 mL/min et la contrepression à 15 MPa. Seulement quelques analyses ont été faites sur ces anthocyanes, mais les résultats sont plutôt encourageants comme il est montré sur la figure 4.16 où les pics sont plutôt bien symétriques. La molécule la plus polaire, la malvine, est bien éluée, à un fort pourcentage de co-solvant. Si l'on calcule la composition à l'éluion de ce composé, elle est d'environ 60% de co-solvant pour l'analyse sur l'Acquity UPC² Torus Diol, et elle est d'environ 50% sur l'Acquity UPC² Torus DEA. Il serait intéressant de continuer à creuser dans l'analyse de ces molécules. Des applications seraient également envisageables, comme la détermination des anthocyanes dans du vin rouge ou tous les fruits rouges.

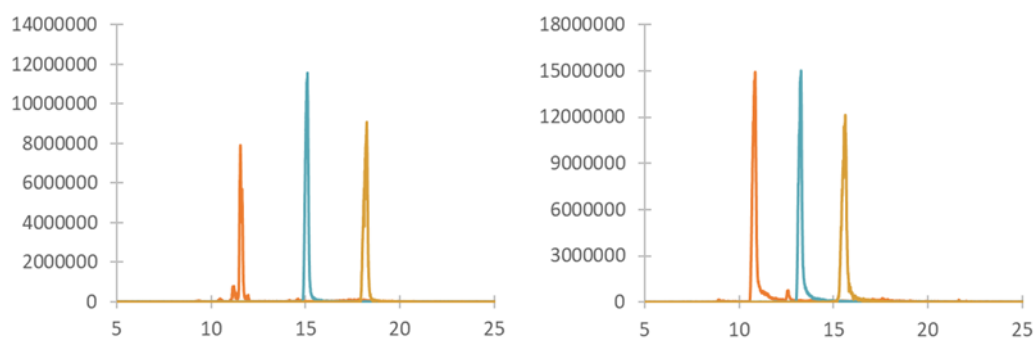


Figure 4.16 : Chromatogrammes des anthocyanes. À gauche : analyse sur l'Acquity UPC² Torus Diol ; à droite : analyse sur l'Acquity UPC² Torus DEA . En orange (élué en premier) : Malvidine ; en bleu (élué en second) : Cénine ; en ocre (élué en troisième) : Malvine.

3. Antibiotiques

La dernière famille partiellement étudiée lors de cette thèse sont les antibiotiques. Ce sont des molécules très utilisées en pharmaceutique qui provoquent une résistance lorsqu'ils sont consommés de manière non nécessaire. Les molécules analysées se trouvent dans la figure 4.17. Les log P montrent que ces molécules sont polaires, sauf l'azithromycine qui présente un log P de 2,44. En revanche cette molécule a un log D

de - 4,4 à un pH de 5, sachant que le pH apparent de la phase mobile en SFC en l'absence d'additifs (mélange de CO₂ et de MeOH) se trouve aux alentours de 5 [68].

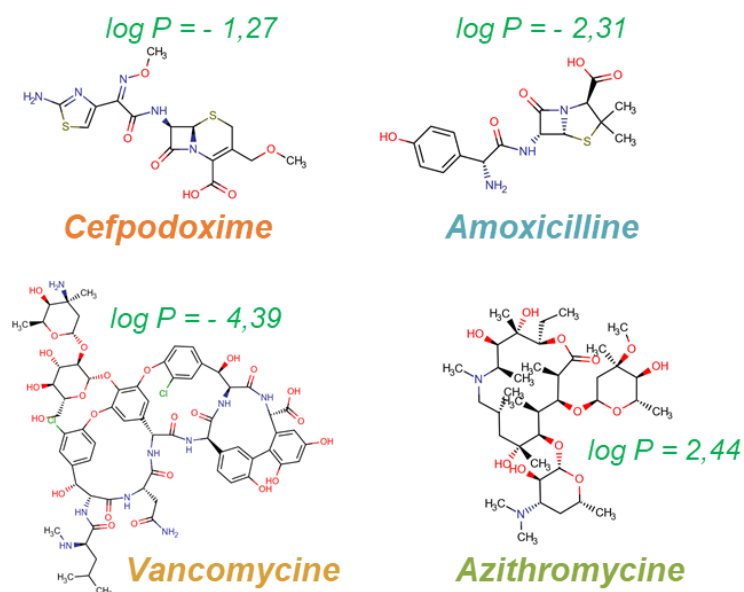


Figure 4.17 : Structures et $\log P$ des antibiotiques étudiés.

Ces antibiotiques zwitterioniques ont été analysés sur la colonne Nucleoshell HILIC, une phase stationnaire zwitterionique non chirale (sulfobetaine greffée sur silice) commercialisée par Macherey-Nagel. Les phases mobiles testées ont été un mélange de CO₂ avec 2 différents co-solvants. Les co-solvants testés ont été un mélange de MeOH dans les 2 cas avec 2% d'H₂O. La différence se fait au niveau de l'additif ajouté, de l'AMS dans un cas et de l'ammoniaque dans l'autre cas. Les premiers résultats sont plutôt mitigés, les pics ne sont pas bien définis. La vancomycine, par exemple, n'est pas détectée que ce soit grâce au détecteur UV et aussi en MS. La masse molaire de cet antibiotique est supérieure à la limite supérieure de masse détectée par le détecteur Acquity QDa, mais même les différents fragments multichargés ne sont pas vus. En ce qui concerne les autres antibiotiques, les résultats sont moins négatifs. Avec l'utilisation de l'ammoniaque comme additif, les M+1 des trois autres antibiotiques sont détectés en ESI(+) comme on peut le voir sur le chromatogramme de la figure 4.18. En revanche avec l'AMS, le M+1 de l'azithromycine n'est pas visible. Pour cette molécule, des fragments sont observés correspondant à la perte d'un des deux groupements glycosylés.

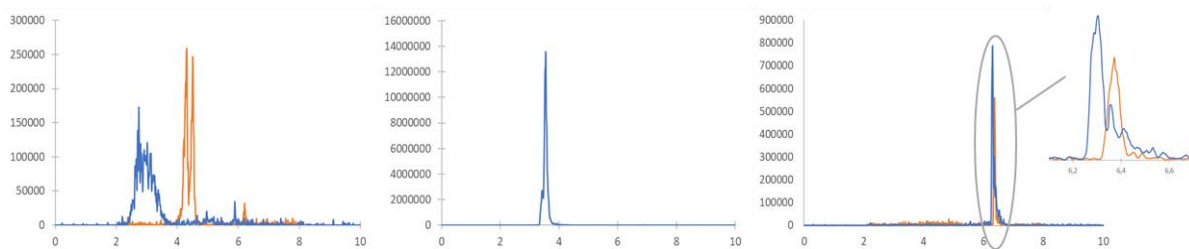


Figure 4.18 : Chromatogrammes des antibiotiques (m/z correspondant aux $M+1$ de chacun des antibiotiques). À gauche : cefpodoxime ($m/z = 428$) ; au centre : azithromycine ($m/z = 750$) ; à droite : amoxicilline ($m/z = 366$) . En orange : analyse sur la Nucleoshell HILIC avec l'AMS en tant qu'additif ; en bleu : analyse sur la Nucleoshell HILIC avec l'ammoniaque en tant qu'additif .

Toutes ces familles moléculaires ouvrent le champ des possibilités concernant l'analyse des composés de plus en plus polaires, ce qui pourrait faire l'objet de nouveaux travaux de recherche.

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Analyse de molécules d'intérêt biologique en chromatographie supercritique et chromatographie unifiée – Etudes fondamentales et applications

Résumé :

La SFC est une technique analytique ancienne qui a mis du temps à s'implanter dans les mœurs des chromatographistes, restant de longues années dans l'ombre de l'HPLC et la GC. Les petites molécules peu polaires s'analysent facilement grâce à cette technique du fait de la faible polarité du dioxyde de carbone. Qu'en est-il des composés polaires ? Plusieurs méthodes ont été mises en place afin de les analyser. Une technique intermédiaire entre la SFC et l'HPLC a été utilisée, la chromatographie unifiée.

Ce travail de recherche traite de méthodes pour analyser des molécules de polarité plus élevée, tels que des acides aminés. Des phases stationnaires ont été étudiées grâce au modèle LSER pour comprendre le comportement de telles molécules dans un milieu supercritique. Un développement de méthode a été effectué pour l'analyse d'acides aminés. Cette méthode a ensuite été utilisée pour diverses applications (compléments alimentaires, plante à intérêt pharmaceutique).

Mots clés : développement de méthode, SFC, chromatographie unifiée, applications, composés polaires

Analysis of molecules of biological interest in supercritical chromatography and unified chromatography - Fundamental studies and applications

Summary:

SFC is an old analytical technique that took time to establish itself in the morals of chromatographers, remaining for many years in the shadow of HPLC and GC. Small and non-polar molecules are easily analysed using this technique because of the low polarity of carbon dioxide. What about polar compounds? Several methods can be used to analyse them. An intermediate technique between SFC and HPLC was used, the unified chromatography.

This research deals with methods for analysing molecules of higher polarity, such as amino acids. Stationary phases were studied using the LSER model to understand the behaviour of such molecules in a supercritical environment. A method development was performed for amino acid analysis. This method was then used for various applications (food supplements, plant of pharmaceutical interest).

Key words: method development, SFC, unified chromatography, applications, polar compounds

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