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Développement de nouvelles méthodologies chromatographiques pour l'analyse de petites (bio)molécules d'intérêt pharmaceutique

Jérémy Molineau

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Jérémy Molineau. Développement de nouvelles méthodologies chromatographiques pour l'analyse de petites (bio)molécules d'intérêt pharmaceutique. Chimie organique. Université d'Orléans, 2022. Français. NNT : 2022ORLE1002 . tel-03886178

HAL Id: tel-03886178

<https://theses.hal.science/tel-03886178v1>

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UNIVERSITÉ D'ORLÉANS

ÉCOLE DOCTORALE

Santé, sciences biologiques et chimie du vivant

INSTITUT DE CHIMIE ORGANIQUE ET ANALYTIQUE

THÈSE présentée par :

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soutenue le : 3 mars 2022

pour obtenir le grade de : **Docteur de l'Université d'Orléans**

Discipline/ Spécialité : Chimie analytique

Développement de nouvelles méthodologies chromatographiques pour l'analyse de petites (bio)molécules d'intérêt pharmaceutique

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Remerciements

Cette thèse est le fruit d'un travail de longue durée. De nombreuses personnes ont contribué, de près ou de loin, à l'avancement de ces recherches et je souhaiterai à présent les remercier.

Mes premiers remerciements s'adressent au Professeur Caroline West. Merci ! Merci d'avoir partagé autant de connaissances que ce soit en réunion ou lors de discussions, mes cahiers sont remplis de nombreuses idées.

Merci d'avoir été patiente tout au long de ces travaux, de m'avoir corrigé, d'être restée calme et joignable.

Merci de m'avoir permis de suivre ou participer à autant de congrès et de journées thématiques, je conserverai toujours cette volonté de m'instruire.

Merci de m'avoir poussé à aller plus loin que ce dont je me pensais capable.

Et surtout, merci de m'avoir fait confiance pour cette aventure qu'est la thèse. Avoir la chance de travailler avec une directrice de thèse responsabilisante m'a permis de m'épanouir au quotidien.

Je tiens à remercier également Mme Maria Hideux qui a co-encadré cette thèse. Merci Maria pour la rapidité de tes réponses à mes diverses sollicitations. Echanger avec toi est toujours un plaisir, de par ta gentillesse et d'autre part, parce que tu transmets n'importe quelle information avec une grande pédagogie. Malgré les conditions sanitaires, nous avons conservé un contact régulier et mes quelques visites au sein de l'IdRS furent toujours humainement et scientifiquement enrichissantes.

Je souhaite remercier le Dr. Eric Lesellier pour son accompagnement au cours des multiples réunions. Merci d'avoir l'œil aussi aiguisé pour pointer du doigt un phénomène intrigant et de toujours conserver un recul sur la discussion en cours.

Je tiens également à remercier M. Philippe Hennig pour l'accompagnement au cours de la thèse. Merci de m'avoir permis d'encadrer des travaux pratiques avec les étudiants de l'université d'Orléans, ce fut très enrichissant.

Je remercie également Mme Sophie Bertin et M. Fabien Mauge d'avoir consacré de leur temps pour échanger régulièrement sur ces travaux de thèse. Vos remarques et conseils m'ont été d'une grande utilité au cours des réunions.

Cette thèse a été réalisée au sein de l'ICOA, anciennement dirigé par le Pr. Pascal Bonnet et actuellement par le Pr. Sylvain Routier. L'ensemble de ce projet prit place au sein de l'équipe « Stratégies Analytiques, Affinités et Bioactifs » dirigée par le Pr. Reine Nehmé. Au sein de l'institut de nombreuses personnes ont partagé mon quotidien de plus ou moins loin et je souhaite les en remercier. Merci à Sophie, Stéphane et Christophe pour leur accompagnement quotidien. Merci à Alain-Michel pour toutes les bouteilles de CO₂ que tu as changées très rapidement. Merci Sandrine et Alexandra pour les pauses café, gâteaux, ... Merci à Laetitia qui a toujours été de bon conseil pour mener des enquêtes en spectrométrie de masse. Ta passion pour la recherche est contagieuse. Je joins Cyril à ces remerciements, les quelques échanges que nous avons pu avoir ont toujours été instructifs. Merci à Florian pour la formation sur les appareils de TP et l'accompagnement au cours des séances de TP. Merci à Véronique et Jihane pour la facilité de commander tout le matériel dont nous avons besoin. Merci à M. le verrier, Pascal, regarder la réparation d'une pièce de verrerie est toujours un moment de plaisir. Merci Cédric, Laurent et Nicolas pour vos aides et réparations informatiques qui nous permettent d'avancer au quotidien. Merci Sophie pour nous fournir en solvant ainsi que les étudiants qui y ont travaillé (Shelly, Sabrina, Jonathan, Sofiane et beaucoup d'autres).

Je remercie le Pr. Emilie Destandau et le Dr. Pierre-Eric Campos pour leur confiance lors des enseignements de TP. C'est une expérience très enrichissante et leur encadrement m'a permis d'en tirer des leçons très positives. Merci au Dr. David Da Silva, au Pr. Emilie Destandau, au Pr. Reine Nehmé, au Dr. Pierre-Eric Campos, au Dr. Bérengère Claude, au Dr. Agnès Chartier ainsi que l'ensemble des enseignants-chercheurs de l'institut qui m'ont enseigné multiples leçons au cours de mon cursus.

Je tiens à remercier ceux qui m'ont accompagné quotidiennement au cours de ces années, en commençant par les « disparus » avant la fin de ces travaux. Merci Adrien, ton calme et ta réflexion ont été inspirants pour moi. J'espère pouvoir continuer à te défier aux pronostiques sportifs encore quelques années. Merci Nhi, pour ta gentillesse et ta bonne humeur contagieuse. Merci Souhila, tu es une scientifique au mental d'acier avec un cœur en or. Merci Thibault, à jamais maître du jeu et créateur de bon moments. Tu es une personne unique pour fédérer les personnes autour de toi. Merci Ghassan, ton calme et ta gentillesse font de toi une

personne avec qui on aime partager des moments de culture. Merci pour les kebabs et toutes les autres restaurations équilibrées. Merci Laurine, la gentillesse incarnée, pour tous ces mots croisés presque finis. Merci à toutes les personnes qui ont partagé le quotidien de l'équipe analytique, Vanille, Farah, Magdalena, Andra, Vanessa, Fanélie, Laurine, Cyril, Cécile, Kaï, Helena, Mohamed, Massi ...

Je tiens à présent à remercier ceux encore présents au sein de l'institut. Merci Angéline, présente depuis mes débuts, tu resteras pour toujours ma formatrice en SFC. Travailler avec toi est un vrai plaisir car tu possèdes un grand esprit d'équipe. Merci Quentin pour ta voix reconnaissable entre toutes, tu es impressionnant de dynamisme et je te souhaite le meilleur pour ta vie professionnelle. Merci Rouba, pour tes conseils et tes discussions toujours franches. Je te souhaite bon courage dans tes nouvelles occupations professionnelles. Merci Solweig, notre futur prix Nobel, pour tes conversations, tantôt profondes, tantôt lunaires. Merci Gaëlle, pour ton calme à toute épreuve et de m'avoir permis d'assister à tes matchs de handball. Merci Sirine, tu es arrivée au cours d'un stage et maintenant tu grandis au quotidien pour faire avancer ta thèse de belle manière. Bon courage à Sirine, Vanille, Solweig, Audrey et Marine ainsi que les autres étudiants de l'institut pour leurs projets de thèses, vous ne pouvez qu'en ressortir grandis.

Un remerciement appuyé à Manon, Yasmine, Anas et Farah qui ont accepté de travailler avec moi lors de leurs stages ou projets de recherche. Travailler avec vous a été enrichissant humainement et je vous remercie pour votre implication dans les divers pans du projet.

Merci à Pascal Kretzel et au Pr. Pascal Bonnet pour votre accompagnement et votre aide sur le projet de prédiction de rétention.

Une pensée aux enseignants, aux professeurs et formateurs qui ont eu impact sur mon cheminement professionnel en employant des mots justes. Merci à Mme Augis, Mme Pageard, M. Cagnon, M. Gaudin, Jordan, Vincent et beaucoup d'autres.

Merci à mon frère, tu m'as toujours impressionné par ta culture et ta détermination, je suis fier de toi. Merci à ma famille pour avoir respecté mon chemin de traverse.

Merci à mes amis champions du monde et mes « grosvengers », vous avez énormément contribué à la réussite de ce travail. La dernière ligne droite m'a un peu éloigné de vous mais je suis impatient de pouvoir vous retrouver et partager de nombreux moments de bonheur en votre compagnie. Merci à Maxime, je n'oublierais jamais que j'ai pu poursuivre mes études grâce à toi.

Enfin, merci à mon pays qui m'a permis de poursuivre mes études, même après des moments compliqués. Cette possibilité est le résultat de nos anciens et j'espère un jour leur faire honneur.

Productions scientifiques

Ce projet de recherche a été réalisé à l'Institut de Chimie Organique et Analytique au sein de l'université d'Orléans, dans le cadre d'une collaboration avec l'Institut de Recherches Servier (Suresnes et Croissy). Le sujet de ces travaux s'intitule « Développement de nouvelles méthodologies chromatographiques pour l'analyse de petites (bio)molécules d'intérêt pharmaceutique ». Ces travaux ont été supervisés par le Pr. Caroline West (ICOA) et co-encadrés par Mme Maria Hideux (Servier), avec l'accompagnement des Dr. Eric Lesellier, Dr. Sophie Bertin, Dr. Philippe Hennig et M. Fabien Mauge.

Ces travaux de thèse ont fait l'objet de publications et de communications, orales, par visio-conférence ou par affiche, lors de congrès.

Tous les articles présents dans ce manuscrit sont indépendants, ils possèdent leurs propres références bibliographiques et les figures qui les composent ne sont pas référencées dans la table globale des figures.

Publications apparaissant dans ce manuscrit

- I. Analysis of flavonoids with unified chromatography-electrospray ionization mass spectrometry – Method development and application to compounds of pharmaceutical and cosmetic interest*

Jérémy Molineau; Manon Meunier; Angéline Noireau; Laetitia Fougère; Anne-Marie Petit; Caroline West

Analytical and Bioanalytical Chemistry, volume 412, 6595–6609 (2020)

- II. Chromatographic analysis of biomolecules with pressurized carbon dioxide mobile phases – A review*

Jeremy Molineau, Maria Hideux, Caroline West

Journal of Pharmaceutical and Biomedical Analysis, Volume 193, 113736 (2021)

III. Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part I. Method development

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe, Hennig, Sophie Bertin, Fabien Mauge, Eric Lesellier, Caroline West

Journal of Chromatography A, Volume 1658, 462631 (2021)

IV. Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part II. Comparison to reversed-phase ultra-high performance liquid chromatography

Jérémy Molineau, Maria Hideux, Philippe, Hennig, Sophie Bertin, Fabien Mauge, Eric Lesellier, Caroline West

Journal of Chromatography A, Volume 1663, 462771 (2022)

Publication en préparation

V. Effect of dilution solvent and injection volume on the analysis of basic polypeptide salts with enhanced-fluidity liquid chromatography

Jérémy Molineau, Maria Hideux, Philippe, Hennig, Sophie Bertin, Fabien Mauge, Eric Lesellier, Caroline West

Participations à d'autres travaux publiés

- VI. *Characterization of stationary phases in supercritical fluid chromatography including exploration of shape selectivity*

Quentin Gros, Jeremy Molineau, Angeline Noireau, Johanna Duval, Takeshi Bamba, Eric Lesellier, Caroline West

Journal of Chromatography A, Volume 1639, 461923 (2021)

- VII. *Interlaboratory study of a supercritical fluid chromatography method for the determination of pharmaceutical impurities: Evaluation of multi-systems reproducibility*

Amandine Dispas, Adrian Clarke, Alexandre Grand-Guillaume Perrenoud, Luca Gioacchino Losacco, Jean-Luc Veuthey, Quentin Gros, Jérémy Molineau, Angéline Noireau, Caroline West, Fabio Salafia, Mariosimone Zoccali, Luigi Mondello, Amber Guillen, Jenny Wang, Kelly Zhang, Philipp Jochems, Gesa Schad, Kosuke Nakajima, Shinnosuke Horie, Jan Joseph, Maria Kristina Parr, Pierre Billefont, Antoni Severino, Sonja Schneider, Edgar Naegele, Daniel Kutscher, Rick Wikfors Regina Black, Lee Ingvaldson, Jimmy Oliveira Da Silva, Raffael Bennett, Erik L.Regalado, Thi Phuong Thuy Hoang, David Touboul, Yana Nikolova, Mariana Kamenova-Nacheva, Vladimir Dimitrov, Blair K.Berger, Kevin A.Schug, Solène Kerviel-Guillon, Fabien Mauge, Masatomo Takahashi, Yoshihiro Izumi, Takeshi Bamba, Florent Rouvière, Sabine Heinisch, Davy Guillarme, Philippe Hubert

Journal of Pharmaceutical and Biomedical Analysis, Volume 203, 114206 (2021)

Communications orales et visio-conférences

- 1) *Détermination de la composition d'un médicament commercialisé
« mystérieux », l'HIDROSMINE par SFC-MS*

Jérémy Molineau, Angéline Noireau, Anne-Marie Petit, Caroline West
Club jeune de l'AfSep, Mai 2019, Montpellier

- 2) *Development of novel chromatographic methods for the analysis and
purification of small (bio)molecules of pharmaceutical interest*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin,
Fabien Mauge, Anne-Marie Petit, Eric Lesellier, Caroline West
Soutenance à mi-thèse à l'ICOA, Octobre 2020, Orléans

- 3) *Analyse de petits peptides pharmaceutiques par Chromatographie Unifiée*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin,
Fabien Mauge, Eric Lesellier, Caroline West
Club lyonnais de l'AfSep, Mai 2021, en ligne

- 4) *Les fonctions de désirabilité de Derringer, un outil d'aide au développement
analytique*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin,
Fabien Mauge, Eric Lesellier, Caroline West
Club chromatographie Ouest-Atlantique, Juin 2021, en ligne

- 5) *Unified chromatography hyphenated to mass spectrometry for small
pharmaceutical peptide analysis*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin,
Fabien Mauge, Eric Lesellier, Caroline West
Flash-talk SEEDPODS 2021 Servier, Juin 2021, enregistrement vidéo

6) *Analyse de peptides à visée pharmaceutique par chromatographie unifiée couplée à la spectrométrie de masse*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin, Fabien Mauge, Eric Lesellier, Caroline West
33ème colloque Biotechnocentre, Octobre 2021, Chaumont-sur-Tharonne

7) *Development of novel chromatographic methods for the analysis of small (bio)molecules of pharmaceutical interest*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin, Fabien Mauge, Eric Lesellier, Caroline West
Sharing Scientific Information Servier, Novembre 2021, en ligne

Communications par posters

1) *Development of a generic method to analyze flavonoids with unified chromatography – electrospray ionization mass spectrometry*

Jérémy Molineau, Manon Meunier, Angéline Noireau, Laëtitia Fougère, Anne-Marie Petit, Eric Lesellier, Caroline West
HTC-16, Janvier 2020, Gand (Belgique)

2) *Unified chromatography hyphenated to electrospray ionization-mass spectrometry for small pharmaceutical peptide analysis*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin, Fabien Mauge, Eric Lesellier, Caroline West
SEEDPODS 2021 Servier, Juin 2021, en ligne

3) *Analyse de petits peptides pharmaceutiques par chromatographie unifiée couplée à la spectrométrie de masse*

Jérémy Molineau, Yasmine Hamel, Maria Hideux, Philippe Hennig, Sophie Bertin, Fabien Mauge, Eric Lesellier, Caroline West
SEP 2021, Octobre 2021, Paris

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Liste des abréviations

AA : acide aminé
AcN : acétonitrile
ADME-Tox : absorption distribution métabolisme excrétion - toxicité
Ag : aglycone
AMS : acide méthanesulfonique
BPR : régulateur de contre-pression
CO_{2 sc} : dioxyde de carbone supercritique
CSH C18 : surface chargée hybride C18
Da : Dalton
DdM : disubstitué d'un monosaccharide
DEA : diéthylamine
DMSO : diméthylsulfoxyde
dp : diamètre des particules
EFLC : chromatographie liquide à fluidité améliorée
EGCG : gallate d'épigallocatechine
Es : estérifié
ESI : ionisation par électrospray
FPP : particules totalement poreuses
GC : chromatographie en phase gazeuse
G : génine
Gn : GRAVY number
HETP : hauteur équivalente à un plateau théorique
HILIC : chromatographie liquide à interaction hydrophile
HPGC : chromatographie gazeuse à haute pression
HPLC : chromatographie en phase liquide à haute performance
ICOA : Institut de Chimie Organique et Analytique
IdRS : Institut de Recherches Servier
LC : chromatographie en phase liquide
LC prep : chromatographie en phase liquide préparative
log P : logarithme du coefficient de partage octanol/eau
MdD : monoglycosylé d'un disaccharide
MdM : monoglycosylé d'un monosaccharide
MdT : monoglycosylé d'un trisaccharide

MS : spectrométrie de masse
N : efficacité (nombre de plateaux théoriques)
NPLC : chromatographie liquide en phase normale
P : Pression
Patm : pression atmosphérique
Pc : pression critique
PEG : polyéthylène glycol
PLS-R : régression des moindres carrés partiels
PM : phase mobile
PS : phase stationnaire
PSA : aire de la surface polaire
QSRR : relation quantitative structure-rétention
RMN : résonance magnétique nucléaire
RPLC : chromatographie liquide en phase inverse
Rs : résolution
SEC : chromatographie d'exclusion stérique
SFC : chromatographie en phase supercritique
SFC prep : chromatographie en phase supercritique préparative
SFE : extraction par fluide supercritique
s/n : rapport signal sur bruit
SPP : particules partiellement poreuses
THF : tétrahydrofurane
UC : chromatographie unifiée
UHPLC : chromatographie en phase liquide à ultra-haute performance
UHPSFC : chromatographie en phase supercritique à ultra-haute performance
T : température
Tc : température critique
TFA : acide trifluoroacétique
TPSA : aire de la surface polaire topologique
 Ψ : pseudouridine
5-FU : 5-fluorouracile

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Introduction générale

La progression des connaissances sur un sujet augmente le nombre d'investigations à mener ainsi que les connaissances des expérimentateurs mais, quelles en sont les limites ? Derrière le terme « sujet », on peut trouver deux significations. La première correspond au manuscrit lui-même et à la réalisation de ces travaux de thèses. Plus les questionnements, que nous avons étudiés à l'aide d'expériences, nous ont apportés des connaissances et plus ceux-ci nous ont permis d'évaluer les barrières matérielles ainsi que nos limites temporelles. La seconde correspondance du terme « sujet », spécifique à ce manuscrit, est celle des biomolécules, nos familles moléculaires d'études. Ces biomolécules ont présenté des poids moléculaires et des polarités augmentant au cours de nos recherches. Il fut donc nécessaire d'adapter nos raisonnements et nos protocoles à ces évolutions. Nous tenterons de décrire les apports scientifiques de nos travaux tout en portant un regard critique sur les limites actuelles.

Dans ce manuscrit, nous nous intéressons aux biomolécules présentant des potentielles applications pharmaceutiques ou cosmétiques. Les biomolécules peuvent être produites par des organismes vivants ou obtenues par un processus de synthèse. Dans ce document, les biomolécules étudiées sont d'origines synthétiques mais les méthodes « génériques » développées fonctionnent également pour des composés d'origine naturelle. Au sein du milieu pharmaceutique, les principes actifs basés sur les biomolécules sont légion et leur nombre ne fait que croître au cours du temps. Il s'agit d'anticorps monoclonaux, de protéines recombinantes, d'hormones... Cet éventail de substances aux potentiels curatifs ou préventifs a amené l'industrie pharmaceutique à de nouveaux challenges. Chaque étape de développement d'un nouveau médicament nécessite l'identification et la quantification de toutes les impuretés. Pour ce faire, les laboratoires emploient des techniques chromatographiques couplées à la spectrométrie de masse qui vont permettre de séparer et d'identifier les différents composants du médicament. L'institut de Recherches Servier et Technologie Servier ont mis à notre disposition des biomolécules pour mener des études par chromatographie, il s'agit de peptides et de flavonoïdes.

Au sein des laboratoires Servier, la chromatographie en phase liquide (LC) et la chromatographie en phase supercritique (SFC) sont deux techniques employées pour réaliser le profil d'impuretés d'actifs pharmaceutiques. Cependant, une technique faisant le lien entre la LC et la SFC, appelée chromatographie unifiée (UC), nécessite

d'être explorée pour l'analyse de biomolécules. En effet, les biomolécules sont des composés présentant des polarités très variées et l'UC permet d'analyser une large gamme de polarité. Une analyse par UC consiste en un gradient débutant par une proportion majoritaire de dioxyde de carbone à l'état supercritique (CO_2sc) et se termine par une proportion majoritaire d'éluant liquide. La force éluante du CO_2sc que l'on emploie en SFC et en UC est généralement comparée à celle de l'hexane. L'insertion progressive d'un co-solvant liquide mélangé avec le CO_2sc va augmenter la force éluante de la phase mobile et permet en fin d'analyse l'élution de composés plus polaires.

L'objectif de ces travaux est le développement de méthode UC pour analyser des biomolécules à visée pharmaceutique et cosmétique. Nous débuterons par l'analyse d'un ensemble de flavonoïdes avec des poids moléculaires inférieurs à 1000 Da. Puis, nous développerons les analyses UC sur des peptides à courtes chaînes que nous appellerons « petits » peptides. Pour finalement, terminer par initier des analyses UC sur des peptides de plus hauts poids moléculaires jusqu'à 5000 Da, que nous appellerons « gros » peptides. L'ensemble de ces travaux est divisé en trois chapitres.

Le premier chapitre de ce manuscrit consiste en une synthèse bibliographique du sujet. Nous aborderons les techniques chromatographiques qui emploient le CO_2sc comme éluant dans la phase mobile. Un succinct voyage au cœur des découvertes et avancées des fluides supercritiques nous permettra de comprendre l'arrivée de l'UC comme technique prometteuse. Du côté des biomolécules, nous détaillerons ce que sont les flavonoïdes ainsi que les études réalisées par SFC sur ces molécules. Ensuite, nous évoquerons les peptides préalablement analysés par CO_2sc dans une revue. La thématique de cet article était plus large que seulement les peptides. Elle se focalisait sur des familles de biomolécules conçues à partir de deux types de sous-unités qui ont été analysées avec du CO_2sc . D'un côté, les acides aminés comme unité de construction des peptides et des protéines et de l'autre, les nucléobases, nucléosides et nucléotides comme brique initiale de construction des oligonucléotides.

Le second chapitre s'applique à décrire le développement et l'optimisation d'une méthode UC couplée à la spectrométrie de masse (MS) pour l'analyse de flavonoïdes. Les précédentes analyses de flavonoïdes réalisées par CO_2sc sont revues puis nous décrivons le travail de développement sur un set de standards. La méthode finale s'appuyant sur une colonne diéthylamine, ou DEA, et un co-solvant composé de

méthanol auquel s'ajoute de l'acide méthanesulfonique permettra par la suite d'investiguer des produits commercialisés dans des pays non européens. L'objectif étant d'étudier la composition par UC-MS d'un produit pharmaceutique nommé l'Hidrosmine et celle d'un produit cosmétique nommé l' α -glucosyle-hespéridine.

Le troisième et dernier chapitre traite de l'analyse de peptides pharmaceutiques par UC-MS. Les peptides mis à disposition ont été divisés en deux sous-catégories fonction de leur taille. Les « petits » peptides ont été étudiés dans un premier temps. Après avoir eu accès aux multiples résultats d'analyses des peptides Servier par chromatographie liquide en phase inverse, nous avons développé une méthode UC-MS sur un lot de 76 petits peptides (<1 000 Da). L'objectif de cette méthode était de couvrir le mieux possible la gamme de polarité et de poids moléculaires de ces composés. Puis, 43 peptides de ce lot ont été analysés par chromatographie liquide en phase inverse couplée à la spectrométrie de masse, ou RPLC-MS, au sein des laboratoires Servier, pour comparer les performances de leur méthode préexistante avec notre méthode UC-MS. De multiples critères analytiques sont comparés à travers les résultats des deux techniques.

Dans un second temps, nous avons eu accès à des peptides de plus haut poids moléculaire (de 1000 et 5000 Da) et ceux-ci nous ont apporté d'autres challenges analytiques. Tout d'abord, le premier défi était d'arriver à éluer ces biomolécules de plus haut poids moléculaire. Pour les comprendre, nous nous sommes appuyés sur une mesure physico-chimique spécifique aux peptides, appelé GRAVY number et qualifiant l'hydrophilie ou hydrophobie des acides aminés et de leurs polymères. Cet ensemble de composés comprend des paires de peptides qui se différencient par le sel additionné, celui-ci agissant en tant que contre-ion du peptide. Nous avons pour ces paires de peptides, deux produits aux structures identiques mais accompagnés de contre-ions différents, soit des sels d'acide trifluoroacétique, soit des sels d'acétate. Certains de ces « gros » peptides ont présenté des difficultés à être solubilisés et nous comparerons différents solvants de mise en solution selon le sel associé. Enfin, un regard sur le volume d'injection a été porté sur nos deux types de peptides.

La conclusion résume les apports de ces travaux de thèse ainsi que les recherches restant à réaliser sur ces biomolécules. Nous ouvrirons la discussion sur d'autres familles de biomolécules étudiées que sont les bases nucléiques. Une méthode UC-MS a été mise en place pour l'analyse d'un ensemble de standards. De plus, dans

ce manuscrit, nous avons tenté d'exprimer la rétention des différentes biomolécules en fonction de descripteurs physico-chimiques (masse moléculaire ou polarité). En parallèle de nos travaux en chimie analytique, nous avons travaillé sur la prédiction de rétention de petites molécules synthétiques. Nous exposerons nos avancées sur ce sujet à travers deux étapes de travail, la modélisation moléculaire et l'analyse de données par l'utilisation de régression en employant un carnet de programmation au langage Python.

Chapitre 1 : Synthèse bibliographique des
analyses de petites biomolécules
s'appuyant sur les fluides supercritiques

I- Introduction aux fluides supercritiques

Et si l'on regardait la question des fluides supercritiques en dehors de notre référentiel terrestre, que pourrait-on observer ?

Il est reconnu que les planètes géantes gazeuses et les planètes géantes glacées sont composées de multiples couches de différents états de la matière avec pour substance la plus abondante dans les régions les plus hautes, le dihydrogène. Jupiter, Saturne, Uranus et Neptune présentent des conditions de température et de pression correspondant aux états sub-critiques et supercritiques du diagramme d'état de l'hydrogène. Il semblerait donc que les planètes joviennes et les géantes de glace soient couvertes par des strates de dihydrogène sub-critique et/ou supercritique [1]. Venus, planète tellurique, avec une atmosphère composée à plus de 96% de CO₂ ainsi qu'une température et une pression atmosphérique très élevée se rapproche de l'état de la matière appliqué au CO₂ dans les systèmes chromatographiques décrits dans la suite du manuscrit [2].

Nous avons à portée de vue l'état supercritique de la matière dans le système solaire et plus encore du CO₂ supercritique sur la planète voisine à condition d'avoir les instruments nécessaires à l'observation.

Pendant ce temps sur Terre, il existe un exemple connu de fluide à l'état supercritique naturellement présent. Les profondeurs des océans situés à proximité de cheminées volcaniques réunissent les conditions de pression (profondeur de l'océan) et de température (chaleur du volcan) pour former de l'eau supercritique [3], [4].

Si l'on revient au référentiel humain, en 1822, Charles Cagniard de Latour est le premier instigateur autour des fluides supercritiques [5]. Il mène des expériences dont l'objectif est de vaporiser un liquide dans un récipient scellé. Le liquide est placé dans un vase clos de volume plus important. Ses essais sont menés sur de l'éther diéthylique (C₄H₁₀O), de l'alcool, de l'eau...[6], [7] Ils permettront la mise en évidence du point critique qui est le croisement où les deux états de la matière, liquide et gazeux, se confondent en un fluide. Le nom de « température critique » ou T_c a été introduit par le scientifique irlandais Thomas Andrews [8] après d'autres propositions d'intitulés avancées par Faraday en 1845 avec le « point de dé-liquéfaction » ou par Mendeleev en 1861 avec la « température d'ébullition absolue » [9].

A partir de ces découvertes expérimentales, un long cheminement de compréhension entourera les fluides supercritiques pour les mener à des applications humaines. Les points critiques de nombreux fluides vont être mesurés, ci-dessous quelques exemples communs de points critiques :

Tableau 1: Mesures de la température critique et de la pression critique pour quelques exemples de molécules, valeurs obtenues sur pubchem (<https://pubchem.ncbi.nlm.nih.gov/> consulté le 25.10.2021)

Nom	Température critique (en K)	Pression critique (en MPa)
H_2O	647	22
$MeOH$	513	8
NH_3	406	11
CO_2	304	7
CH_4	191	5
N	126	3
H	33	1

L'intérêt pour les fluides supercritiques va se porter dans des domaines aussi différents que l'extraction alimentaire d'huiles à partir de graines [10] (à partir des années 1970), les réactions enzymatiques comme alternative aux solvants organiques (à partir des années 1990) [11], la conversion de la biomasse en biogaz par de l'eau supercritique [12] ou la production de bio-éthanol [13]. Dans le cadre de la chimie analytique, deux procédés employant du CO_2 supercritique (ou CO_2_{sc}) sont appliqués de manière courante: l'extraction par un fluide supercritique (SFE)[14]–[16] et la chromatographie en phase supercritique (SFC)[17]–[19]. Ces deux techniques sont disponibles au sein de l'Institut de Chimie Organique et Analytique (ICOA) d'Orléans et sont étudiées au quotidien de manières fondamentales et applicatives. Le couplage des deux techniques est également investigué sous le format on-line qui permet l'envoi direct de l'extrait provenant de la dimension SFE vers l'analyse SFC. Deux types de modèles sont disponibles à l'institut, un conçu par le constructeur pour ce mode de fonctionnement [20] et un second mis en place via l'association de deux appareils distincts pour analyser des extraits sélectifs de composés bioactifs [21].

Ce chapitre va s'employer à décrire les techniques chromatographiques s'appuyant sur le CO₂ supercritique que sont la SFC, l'EFLC ou Chromatographie à Fluidité Améliorée et l'UC ou Chromatographie Unifiée.

II- Les techniques chromatographiques s'appuyant sur le CO₂ supercritique

a. Le CO₂ supercritique comme éluant

Le CO₂ utilisé à l'état supercritique (CO₂ sc) ou subcritique selon les conditions de température et de pression du fluide est l'un des éléments essentiels des techniques discutées par la suite. Il présente des avantages non négligeables d'un point de vue chromatographique mais avant de poursuivre sur ce point, il serait intéressant de définir ce qu'est un fluide supercritique.

Selon le dictionnaire Larousse, le terme supercritique est défini comme suit : « *Se dit de la pression ou de la température d'un fluide lorsque sa valeur est supérieure à celle qui correspond au point critique ; se dit du fluide lui-même.* »[22]. Il existe différents états de la matière pour chaque corps. Les 3 états les plus connus sont l'état solide, l'état liquide et l'état gazeux. Il existe de nombreux diagrammes d'états dépeignant les états de la matière de corps purs comme celui schématisé de la figure 1, qui représente spécifiquement les états du dioxyde de carbone.

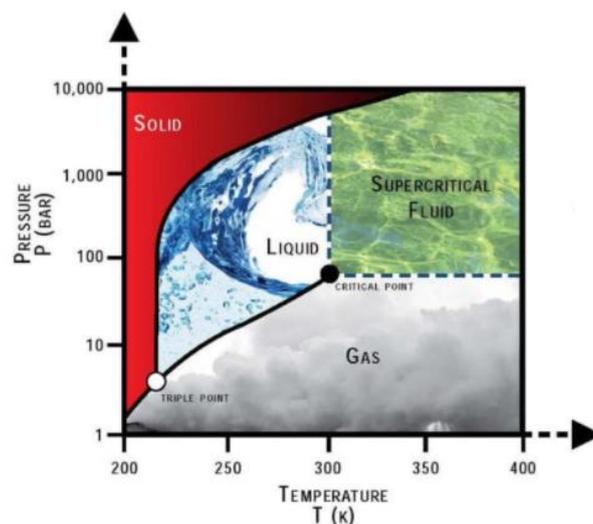


Figure 1 Diagramme d'état du dioxyde de carbone (repris sur powermag.com le 15/10/2021)

On observe sur cette figure deux points de croisements entre les états de la matière. Le point triple (T) correspond à une condition de température et de pression pour laquelle les 3 états de la matière représentés coexistent. Le point critique (C) d'un corps pur correspond au point final de coexistence de l'équilibre liquide-gaz. Lorsque l'on regarde au-delà de ce point critique du CO₂ (T_c = 304.3 K et P_c = 7.4 MPa), on se situe dans la zone dite des fluides supercritiques.

D'autres états de la matière ont été découverts au cours du XX^{ème} siècle tels que l'état plasma (matière partiellement ou totalement ionisée) [23], l'état superfluide (fluide dépourvu de viscosité à basse température) [24], l'état cristal liquide (combinaison des propriétés entre l'état liquide et le solide cristallisé) [25] et l'état de condensation de Bose-Einstein (gaz refroidit à une température très proche du zéro absolu) [26], [27].

Le CO₂ sc a une multitude d'avantages pour une utilisation en sciences séparatives. Tout d'abord, c'est un solvant non-inflammable, peu toxique chimiquement inerte, non corrosif et recyclable, des arguments intéressants pour une utilisation dans des laboratoires de chimie. D'un point de vue économique, le CO₂ sc est peu coûteux et disponible en grande quantité. Enfin les conditions critiques de T et P pour ce fluide sont simples à atteindre.

D'autres fluides supercritiques ont été testé comme éluant en chromatographie. Une étude de 1987 s'est appliquée à comparer les comportements de plusieurs fluides en tant qu'éluants SFC en travaillant sur la séparation de 4 hydrocarbures aromatiques polycycliques [28]. Le trifluorométhane, le chlorotrifluorométhane, le n-butane, l'i-butane, l'oxyde nitreux ... sont autant de phases mobiles, à l'état supercritique, testées et comparées sur deux mesures analytiques : la résolution et la sélectivité. L'oxyde nitreux est un fluide alternatif au CO₂ sc que l'on retrouve dans quelques travaux de recherches du fait qu'il ne soit pas acide comme le CO₂ sc [28]–[33]. Cependant, sa capacité oxydante relativement forte et les risques d'explosions qu'il peut présenter n'en font pas une alternative plus étudiée [34]. Le trifluorométhane est un autre exemple de phase mobile supercritique testée par l'équipe de Mme Olesik [35], [36] qui a comparé ses performances, associées au méthanol et au mélange méthanol/eau, avec le CO₂ sc lui aussi associé à des solvants liquides. Il n'en reste que le CO₂ sc est le fluide supercritique très majoritairement utilisé dans les articles publiés. Sa « facilité » d'atteindre l'état supercritique pour le CO₂ est intéressante d'un point de vue mécanique en comparaison avec d'autres fluides supercritiques. L'eau et le méthanol ont des

pressions critiques et des températures critiques bien trop élevées pour une utilisation chromatographique en l'état actuel des techniques (tableau 1).

Dans notre cas, nous nous focaliserons sur les propriétés physico-chimiques du CO₂ sc à travers l'axe de réflexion de la chromatographie. L'état supercritique du CO₂ lui confère des propriétés intermédiaires entre les états solides et gazeux (tableau 2) et nous allons tenter de les décrire.

D'un côté, la masse volumique du CO₂ sc est comparable à celle des liquides tandis que sa viscosité est similaire à celle des gaz. Le coefficient de diffusion est compris entre celui des liquides et des gaz. Ces diverses propriétés lui confèrent un avantage chromatographique puisqu'un éluant contenant une majorité de CO₂ sc permet d'analyser à hauts débits de phase mobile avec une efficacité élevée sans générer de pression excessive en tête de colonne (la pression étant un facteur instrumental limitant). Le CO₂ sc apporte une haute diffusivité aux analytes ce qui favorise l'échange rapide des composés entre la phase stationnaire (PS) et la phase mobile (PM). De plus, il présente une résistance au transfert de masse faible (terme C de l'équation de Van Deemter) ce qui implique une meilleure symétrie de pic (As) et une haute efficacité (N).

Tableau 2: Comparaison de propriétés physiques entre les fluides liquides, gazeux et supercritiques [17]

Nature du fluide	Masse volumique (kg.m ⁻³)	Viscosité (kg.m ⁻¹ .s ⁻¹)	Coefficient de diffusion (m ² .s ⁻¹)
Liquide (101 325 Pa 288 – 303 K)	600 - 1600	(0,2 – 3) .10 ⁻³	(0,2 – 2) . 10 ⁻⁹
Supercritique (T _c et P _c)	200 - 1200	(1 – 3) .10 ⁻⁵	0,7 .10 ⁻⁷
Gaz (101 325 Pa 288 – 303 K)	0,6 - 2	(1 – 3) .10 ⁻⁵	(1 – 4) . 10 ⁻⁵

Pour résumer, le CO₂ sc est le fluide supercritique employé très majoritairement en SFC et l'ensemble des résultats et discussions développés dans ce document ont été réalisés avec ce fluide. Sa force d'élution a été établie proche de celle de l'hexane [37] et selon la proportion de co-solvant ajouté dans la phase mobile on distinguera des techniques chromatographiques différentes.

b. La chromatographie en phase supercritique ou SFC

Lorsque l'on annonce le terme « chromatographie », les techniques les plus évoquées dans un premier temps sont celle de la chromatographie liquide ou LC et la chromatographie en phase gazeuse ou GC. La SFC s'est démocratisée plus tardivement que la LC et la GC. Ceci est le fait de l'avènement de l'HPLC (chromatographie liquide haute performance) qui a retardé le développement de la SFC par les constructeurs.

La première utilisation d'un fluide supercritique comme éluant analytique a été décrite par Klesper et al. avec l'utilisation de chlorofluorocarbure en 1962 [38]. L'objectif était de séparer des porphyrines thermolabiles sur colonne de polyéthylène glycol ou PEG (cf. structure sur la figure 2). Le premier nom donné à cette chromatographie était « chromatographie gazeuse à haute pression » ou HPGC.

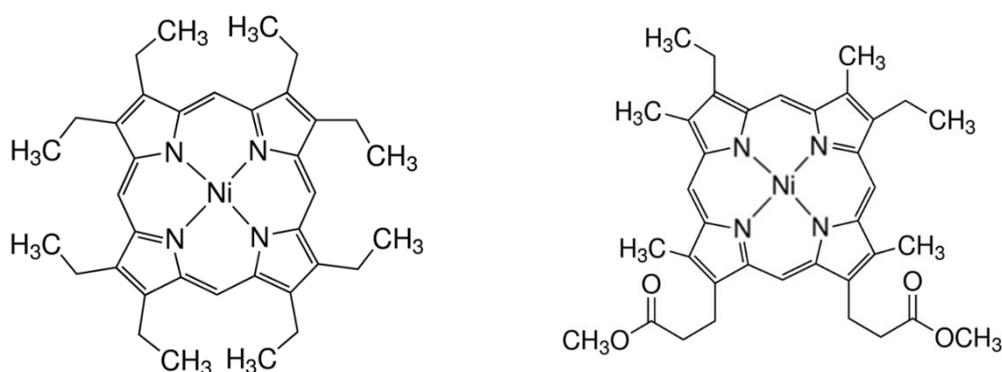


Figure 2 Structure des 2 porphyrines séparées par HPGC [38] – A gauche : Etioporphyrin II nickel et à droite : Mesoporphyrin IX dimethylester nickel

D'autres travaux sur ce concept d'HPGC ont été publiés dans les années 60. L'intérêt était porté sur la possibilité d'analyser des composés peu ou non volatils en employant des températures du fluide éluant plus faibles qu'en GC [39]–[43]. Ces publications sont également les premiers essais rapportés d'analyse avec du CO₂ sc.

La chromatographie supercritique a fait face à de nombreux contretemps. Terry A. Berger a retracé l'histoire des difficultés et des controverses qui ont retardé le développement de la SFC [44]. Cela a commencé par le nom de la technique, avec premièrement les différentes propositions de Giddings qui la référençait sous des dénominations variantes de la GC. Définir la polarité du CO₂ sc, comprendre les phénomènes physiques de dégradation des colonnes remplies (discutés dans les années 80) ou encore éluer les composés polaires avec des formes de pics

satisfaisantes sont autant de controverses référencées et discutées dans la partie rétrospective de la SFC par Berger.

La première mise au point d'un appareil SFC à partir d'un équipement de LC fut réalisée par Jentoft et Gouw. Ils ajoutèrent un régulateur de pression et séparèrent un mélange d'hydrocarbures aromatiques polycycliques ainsi que différents degrés de polymérisation du polystyrène avec un éluant composé de n-pentane et 5% de méthanol sur des colonnes de 4 mètres remplies de n-octane en plus du Poracil C (Silice poreuse) [45]. Ce premier exemple de conception ou adaptation d'un appareil dédié à la SFC à partir de la chromatographie liquide sera suivi par d'autres. Comme l'introduit Berger lorsqu'il présente l'instrumentation SFC, les formations d'utilisateurs sur la SFC sont maintenant introduites après et à partir de l'instrumentation LC. Il a donc décrit les approches, les problèmes rencontrés et les résolutions de ceux-ci en SFC à partir de connaissances et de l'appareillage LC [46]. De nombreuses adaptations sont discutées tels que les modifications de l'injection (les anciens systèmes subissaient un retour à P_{atm}), du four colonne (besoin d'être plus proche des conditions adiabatiques pour éviter des décompressions du fluide), des capillaires (serrage renforcé pour éviter les fuites du CO_2_{sc} car très peu visqueux) ou encore de la cellule du détecteur UV-visible (besoin d'une résistance élevée à la pression du fluide) ...

Alors que la SFC avec colonnes capillaires était initialement plus populaire suite aux interrogations sur la perte d'efficacité des colonnes remplies, un phénomène associé aux variations de pression non contrôlées, des travaux furent menés pour évaluer les performances des colonnes remplies selon leurs diamètres de particules (d_p) en chromatographie liquide en phase inverse ou RPLC et SFC [47]. Gere et al., tracèrent les courbes de Van Deemter du naphthalène en faisant varier le d_p des colonnes et la technique chromatographique (LC et SFC) ce qui a mis en évidence deux éléments. Le premier, sans surprise, diminuer le d_p des colonnes remplies augmente l'efficacité (N) quel que soit l'état du fluide employé comme éluant. Le second élément, à d_p égales en LC et SFC, les efficacités optimales mesurées (HETP) sont équivalentes pour les deux techniques mais l'optimum de la vitesse linéaire est bien plus élevé en SFC (≈ 5 fois plus élevé). On peut donc résumer ces informations en une phrase : la SFC présente une haute efficacité similaire à la LC tout en travaillant à des débits plus élevés. C'est un élément fondateur pour la mise en avant de la technique à l'heure où l'essor de la

LC battait son plein et que la SFC restait une technique en marge comparée à cette même LC.

Les deux formats chromatographiques pour lesquels la SFC a réussi à s'installer progressivement dans les laboratoires pharmaceutiques (à partir des années 80) sont l'analyse chirale et la chromatographie préparative.

D'un côté, les analyses chirales axées sur les séparations d'énantiomères [48]–[51] s'appuient sur des phases stationnaires chirales. Ces colonnes ont été développées pour réaliser des séparations énantiosélectives avec comme sélecteurs chiraux les plus utilisés, les polysaccharides modifiés [52]. Ces mêmes colonnes chirales peuvent être utilisées dans le cadre de séparation non chirales, par exemple pour séparer d'autres formes d'isomères. Par exemple, pour la séparation de métabolites et d'analogues réalisé par Regalado et Welch [53].

De l'autre côté, la SFC préparative (ou SFC prep) permet la purification d'un ou plusieurs composés. Elle présente deux avantages majeurs en comparaison avec la LC [54]. Le premier est le remplacement du ou des solvants organiques (potentiellement toxiques) qui sont fortement consommés à l'échelle préparative par le CO₂ sc. Le second pour l'échantillon collecté qui nécessite peu ou pas d'évaporation du solvant liquide puisque la proportion liquide est minoritaire en SFC. On obtient une fraction concentrée qui nécessite peu de traitement supplémentaire puisque le CO₂ sc se décompresse (état gazeux) en sortie du système analytique. Le système SFC prep se différencie par certains aspects de l'échelle analytique que ce soit au niveau instrumental avec une capacité de débit de phase mobile pouvant aller jusqu'à plusieurs L/min ou au niveau séparatif avec l'injection d'échantillons plus concentrés et/ou de volume d'injections plus importants [55].

Dans leur revue, Speybrouck et Lipka ont présenté conjointement les deux aspects succinctement décrits dans les paragraphes précédents à savoir la SFC prep appliquée aux composés pharmaceutiques chiraux [56]. Cette association est une option de purification utilisée à grande échelle industrielle et les fabricants d'appareils analytiques présentent eux aussi souvent un instrument SFC prep dans leur catalogue. Les travaux académiques sur la thématique préparative pour des purifications énantiomériques ou racémiques sont encore parfois présentés ou comparés avec la LC prep [57]–[60]. Il en ressort la complémentarité des deux techniques pour couvrir l'ensemble de la gamme de polarité des composés via les deux techniques.

Pour le cas de la SFC à l'échelle analytique, il fut nécessaire de développer l'instrumentation pour favoriser son intérêt et par conséquent son implantation dans les laboratoires. Tout d'abord en 1988, le groupe Jasco a développé un module de régulation de la contre-pression ou BPR (back-pressure regulator) qui contrôle cette contre-pression indépendamment du débit du fluide [61]. Ensuite, l'utilisation des colonnes remplies dans les années 90 a permis de venir concurrencer la chromatographie liquide en phase normale (NPLC) pour l'analyse de composés polaires [62], [63]. Le chapitre sur l'historique de la SFC par McClain retrace et référence de manière beaucoup plus détaillée les découvertes et avancées de la technique SFC par décennies. De son côté, Berger a revisité diverses familles de composés neutres, basiques ou acides analysés par colonnes remplies mettant en avant le potentiel déjà existant de ce type de colonne et la nécessité d'investiguer des éluants binaires et ternaires [64].

Finalement, l'arrivée et la démocratisation des colonnes remplies de faible d_p (colonnes remplies de particules poreuses en sub-2 μm ou superficiellement poreuses inférieures à 3 μm) [65] ont amené la SFC vers des performances analytiques plus avancées. L'utilisation de ces colonnes à faibles diamètres de particules a permis dans un premier temps d'améliorer l'efficacité chromatographique (augmentation du nombre de plateaux) via l'utilisation des colonnes remplies de particules totalement poreuses sub-2 μm , ou FPP (fully porous particles). Puis, dans un second temps, les colonnes superficiellement poreuses < 3 μm également appelées core-shell, ou SPP (superficially porous particles), ont permis de conserver ces efficacités en travaillant à des débits plus élevés sans augmentation notable de pression [66]. Finalement, ces mêmes colonnes SPP ont-elles aussi été développées en sub-2 μm en 2009 [67].

L'histoire récente de la SFC a vu apparaître une nouvelle dénomination de la technique : l'UHPSFC comme acronyme d'Ultra-haute performance de la chromatographie en phase supercritique. Comme l'ont écrit Grand-Guillaume Perrenoud et al. : « ... l'introduction du terme UHPSFC pour désigner la combinaison ... SFC et les colonnes remplies de particules sub-2 μm » [68].

Si l'on revient aux tracés des courbes de Van Deemter, elles furent actualisées par Grand-Guillaume Perrenoud et al. en 2012, cette fois-ci en comparant les performances des colonnes remplies toujours selon leur d_p qui correspondent dorénavant à des niveaux de performances différentes (5 μm et 3.5 μm pour l'HPLC et la SFC, 1.7 μm

pour l'UHPSFC et l'UHPLC) [69]. Ces travaux ont mis évidence les performances cinétiques supérieures de l'UHPSFC vis-à-vis de l'UHPLC. L'UHPSFC atteint une meilleure efficacité que la SFC tout en travaillant à des débits encore plus importants. Cependant, les limites de capacité de pression du système à $\approx 41,5$ MPa restent un inconvénient majeur pour travailler avec des colonnes sub-2 μm à hauts débits de phase mobile. Les hautes capacités de l'UHPSFC ne peuvent pas être exploitées en l'état actuel des instruments.

De notre côté, l'entièreté des travaux présentés dans ce manuscrit a été effectuée sur un appareil Acquity UPC² de la société Waters™. Le système est représenté de manière schématique sur la Figure 3 avec l'introduction d'un solvant liquide additionnel ou solvant make-up avant l'arrivée en MS et l'application d'une contre-pression par un BPR. La détection MS est présente sur l'appareil via un Acquity QDa [70] qui est un simple quadripôle avec ionisation douce de type électrospray (ou ESI) en mode positif et négatif et une source en Z (la plus commercialisée).

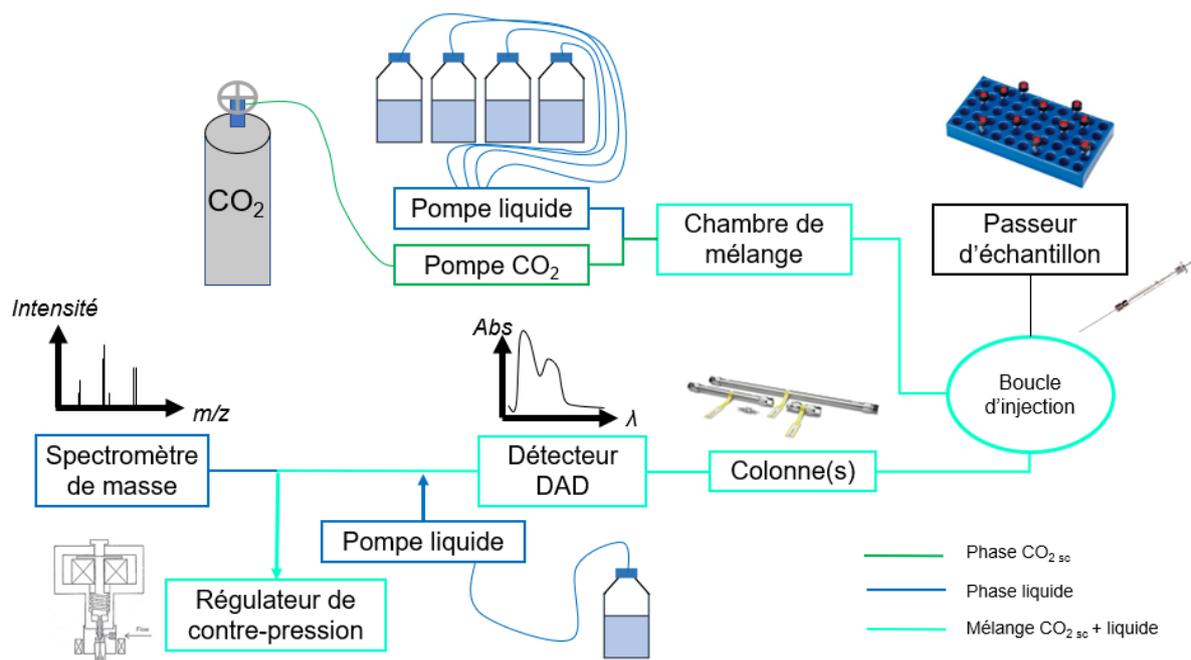


Figure 3 : Schéma du système SFC-UV-MS (UPC² Waters), adapté de [61], [71]

Le nombre de publications en lien avec la SFC a augmenté après l'apparition des appareils plus robustes [72] (début des années 2010) et le couplage SFC-MS est pour au moins 50% de cet ensemble de travaux [73]. Guillaume et al. ont représenté de manière schématique les configurations SFC-MS les plus courantes et ils ont comparé des paramètres tels que la sensibilité, la fiabilité, la souplesse et la facilité d'utilisation des instruments [71]. Losacco et al. ont également mis en évidence la complémentarité de la SFC-MS et de la LC-MS dans le cadre d'analyse de petites molécules ou de matrices complexes [74].

L'histoire de la SFC à travers ses difficultés et ses innovations a apporté de riches contributions scientifiques. Les multiples avantages du CO_2 SC combinés aux instruments performants développés récemment font de la SFC une technique attrayante pour la recherche. Autrefois essentiellement utilisée à l'échelle préparative, elle est de plus en plus retrouvée dans les laboratoires pour sa dimension analytique. Sa facilité de couplage à la MS et les performances obtenues avec l'utilisation de colonnes sub 2- μm amèneront la SFC vers une utilisation plus étendue.

c. La chromatographie liquide à fluidité améliorée ou EFLC

Là où la SFC fonctionne majoritairement avec du CO₂ sc comme solvant d'éluion, la chromatographie liquide à fluidité améliorée ou EFLC travaille avec des proportions de co-solvant liquide bien plus importantes. Elle permet l'analyse d'analytes plus polaires qu'en SFC grâce aux proportions de solvants liquides plus importantes qui accompagne le CO₂ sc. Ce co-solvant est constitué d'un solvant liquide majoritaire, auquel on peut ajouter un additif acide ou basique et un faible pourcentage d'eau. L'EFLC est donc la première connexion entre les deux techniques chromatographiques préexistantes, la SFC et l'HPLC [75].

D'un point de vue instrumental, les équipements actuels de SFC tels que l'Acquity UPC² de Waters™ [76], le Nexera UC de Shimadzu™ [77] ou le 1260 Infinity II d'Agilent™ [78] permettent de travailler avec des pourcentages de co-solvant allant de 0 à 100%. La stabilité de pression apportée par le BPR permet de travailler en EFLC à des pressions élevées dues à l'application de fortes proportions de solvant liquide. La nature de l'éluant se voit modifiée en EFLC comparée à la SFC, de ce fait on ne tend plus vers l'état supercritique ou subcritique. Cet addition de solvant organique en proportion plus importante change la viscosité, la densité et la diffusion de la phase mobile [79] et Berger avait mis en avant l'importance et le manque de données lié à la viscosité pour comprendre les modifications des coefficients de diffusion [80].

Les quelques lignes précédentes discutent de l'EFLC et de cette importante proportion de phase liquide. Cependant, quelle est la limite entre SFC et EFLC ?

A notre connaissance, il n'existe pas en 2021 de délimitation ou de consensus établi pour différencier la SFC et l'EFLC sur la question des proportions constituant la phase liquide. Pour notre part, nous établissons cette limite fictive aux alentours des 50% de co-solvant liquide, zone où le CO₂ sc n'est plus le solvant majoritaire. Enfin du côté des colonnes, comme pour la SFC, toutes les phases stationnaires développées pour l'HPLC et la SFC sont compatibles avec l'EFLC.

Le nom « Enhanced fluidity liquid chromatography » pour EFLC est peu référencé dans les titres de publications. La première apparition du terme EFL est apparu en 1991 [81] et la majorité des travaux applicatifs et théoriques avec cette dénomination sont écrits par Mme Olesik et son équipe. Cependant, des travaux en

SFC appliquent des gradients de type EFLC ou combinés SFC/EFLC sans l'évoquer [82]–[89]. Il n'en reste que ces différentes méthodes d'analyses par EFLC permettent la séparation de composés plus polaires qu'en SFC seule. La contribution du co-solvant liquide permet l'élution des molécules plus polaires comme les protéines [86], [90], les carbohydrates [91], les nucléosides [92] ... A l'échelle industrielle, l'EFLC préparative présente un potentiel encore trop peu exploité vis à vis de ses apports économiques et écologiques. Bennett et al. ont transposé une méthode EFLC analytique vers une méthode EFLC prep sur une colonne de type HILIC (BEH amide) pour purifier des glucides [93]. La transposition est faisable mais la littérature présente peu d'exemples d'analyses EFLC prep [94], [95].

Selon l'angle d'utilisation des solvants de la phase mobile, l'EFLC est perçue différemment. Si l'utilisateur travaille initialement en LC, il considère le CO_2 sc comme l'ajout minoritaire d'un fluide supercritique dans la phase mobile liquide. Dans le cadre des travaux de l'équipe de Mme Olesik, ils estiment qu'un gaz liquéfié est ajouté à leur solvant liquide [96]. A l'inverse, si l'utilisateur débute par la SFC (CO_2 sc majoritaire), la proportion liquide minoritaire devient de plus en plus importante, jusqu'à devenir majoritaire, mais le CO_2 sc reste le fluide de référence comme expliqué sur la figure 4.

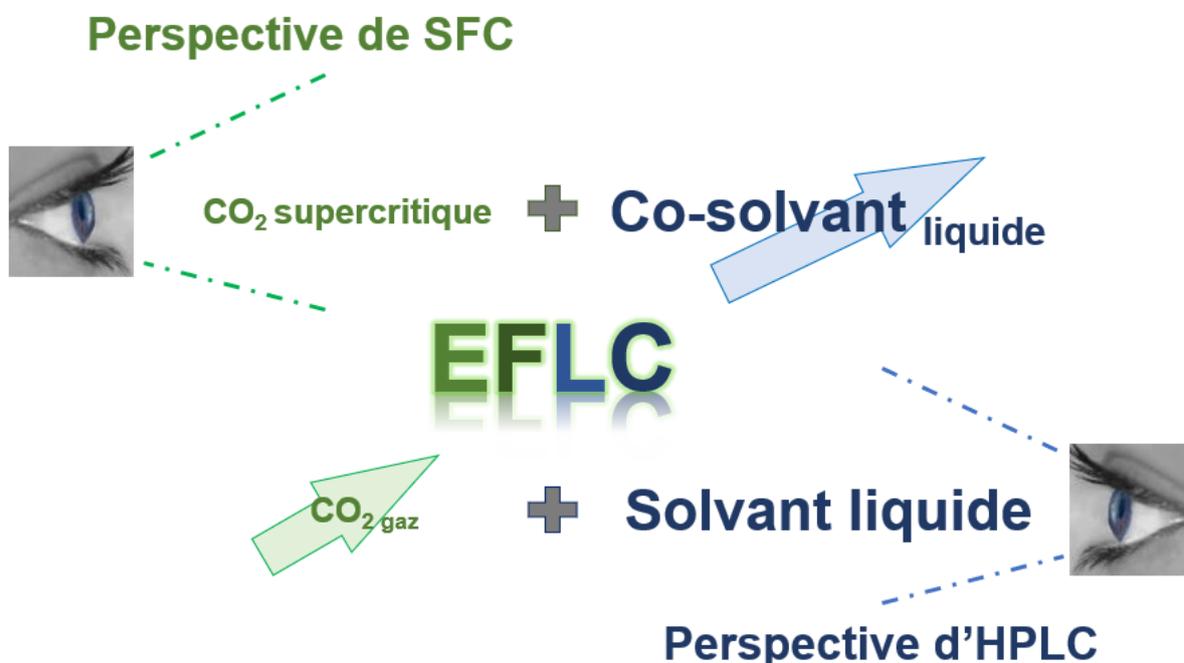


Figure 4 : Composition de la phase mobile en EFLC selon l'angle d'utilisation instrumental

L'EFLC au sens des « chromatographistes » en phase liquide a été testée sur plusieurs types de phases stationnaires à l'échelle analytique. En phase inverse sur colonne octadécyle, avec l'ajout de CO₂ SC dans le mélange de solvant liquide méthanol/eau [97] qui n'est pas le mélange le plus utilisé en RPLC. En phase normale sur colonne 3-cyanopropyle, avec un CO₂ SC combiné à de l'hexane [98]. En HILIC sur colonne de silice totalement poreuse (Rx-SIL), avec l'addition du CO₂ SC au mélange éthanol/tampon acide [99]. Enfin en chromatographie d'exclusion stérique (SEC) sur colonne octadécyle où le CO₂ SC est ajouté au THF [100]. Ces différents exemples démontrent bien le caractère polyvalent de l'EFLC (comme celui de la SFC). Avec l'EFLC, il est possible de travailler sur différents types de polarité de phases stationnaires combinés à différentes natures de solvants liquides dans lesquels on ajoute du CO₂. Cette capacité d'associer une PM majoritairement liquide à du CO₂ fait de l'EFLC une technique intermédiaire entre la LC et la SFC.

d. La chromatographie unifiée ou UC

Dans de nombreux contextes scientifiques, unifier ou concilier les lois, les techniques, les observations a toujours été un objectif majeur des chercheuses et chercheurs. En physique et mathématique, le travail sur une théorie globale nommée « Grande Unification » accapare une partie de la recherche [101]. Les découvertes d'Isaac Newton en 1687 [102] (unification des mouvements terrestres et célestes) et de James Clerck Maxwell en 1865 [103] (théorie unique de l'électromagnétisme) sont des avancées majeures de la science par l'unification de théories [104]. En sciences séparatives, le premier scientifique qui a proposé une unification des techniques est John Calvin Giddings en 1965 [105]. Ses travaux sont développés dans un livre intitulé « Unified Separation Science » où il décrit les phénomènes thermodynamiques impliqués dans les sciences séparatives pour préciser les possibilités d'unifications [106], [107]. Une première application du concept en 1995 par Tong et al. a mis en évidence la capacité de séparation de la GC-SFC sur une colonne capillaire (Silice fondue couverte de SB-méthyl) pour analyser des pesticides dans une huile végétale ou des cires [108]. Cependant, ces travaux valorisaient le terme « unifié » selon deux approches. La première, comme une combinaison des performances de la GC et de la SFC, concept similaire à celui de Giddings. La seconde approche, pour l'utilisation de l'appareil selon une seule technique chromatographique choisie entre la GC, la SFC ou

la LC. Cette deuxième possibilité deviendrait réalisable grâce à un système de basculement de vanne.

Comme l'a montré ce premier exemple, le terme unifié en chromatographie peut et doit être discuté. En 2019, Mme Lydia Kisley a mis en évidence les différences de significations du mot « unifiée » au sein des sciences chromatographiques [109]. Pour elle, cette « Unification » peut être de type instrumental [110], comme nous l'évoquerons par la suite avec l'UC, ou alors une connexion entre les approches « ... expérimentales, calculatoires et théoriques ... » [111], ou encore sous d'autres perspectives non publiées/étudiées. Dans le cas de la chromatographie à l'échelle de l'unification instrumentale, Silva et al. ont retracé le concept de chromatographie unifiée en mettant en avant des exemples d'unifications GC-SFC ou SFC-HPLC [112]. Thomas L. Chester a, lui, travaillé sur la compréhension de la phase éluante en SFC pour des unifications par la température et la pression avec la GC et la LC [113]. Il est vrai que la compréhension physique de la phase éluante améliorerait la connaissance du partage de l'échantillon entre PS et PM. L'unification des techniques chromatographiques était envisagée théoriquement et expérimentalement [114], [115] en attendant des instruments conçus dans cette optique. D'autres exemples « d'unification » en sciences séparatives se retrouvent dans le cas de la classification des colonnes remplies [116], ou pour mesurer les propriétés hydrophobes des résines échangeuses d'anions [117]. Il n'en reste pas moins que la correspondance du terme peut être à multi-usage.

L'arrivée des instruments SFC robustes combinée à ces réflexions ont amené à une configuration dite de chromatographie unifiée ou UC associant la SFC et la LC. Le premier exemple d'application, en 2014, est celui de Taguchi et al. pour l'analyse de vitamines hydrophiles et lipophiles présentes dans les aliments ou dans le corps humain [118]. Ces vitamines décrites par leurs solubilités et leurs polarités distinctes nécessitaient l'analyse de cet ensemble hétérogène par une méthode analytique unique, l'UC.

En quoi consiste l'UC selon notre utilisation à partir de l'instrumentation SFC ?

La chromatographie unifiée est une méthode employant un gradient unique qui débute avec une phase mobile majoritairement ou entièrement composée de CO₂ sc et se termine avec une phase mobile majoritairement ou totalement liquide. On applique un gradient de phase liquide (le co-solvant) qui permet de traverser trois domaines chromatographique connus, tout d'abord la SFC, puis l'EFLC, pour finir en LC. Cette

phase liquide de composition commune aux trois chromatographies se construit comme un co-solvant en SFC pour analyser des molécules de plus en plus polaires. La figure 5 met en avant deux manières d'augmenter la polarité des composés élués. La première, en changeant la nature du co-solvant et des additifs et la seconde, en augmentant les proportions de co-solvant.

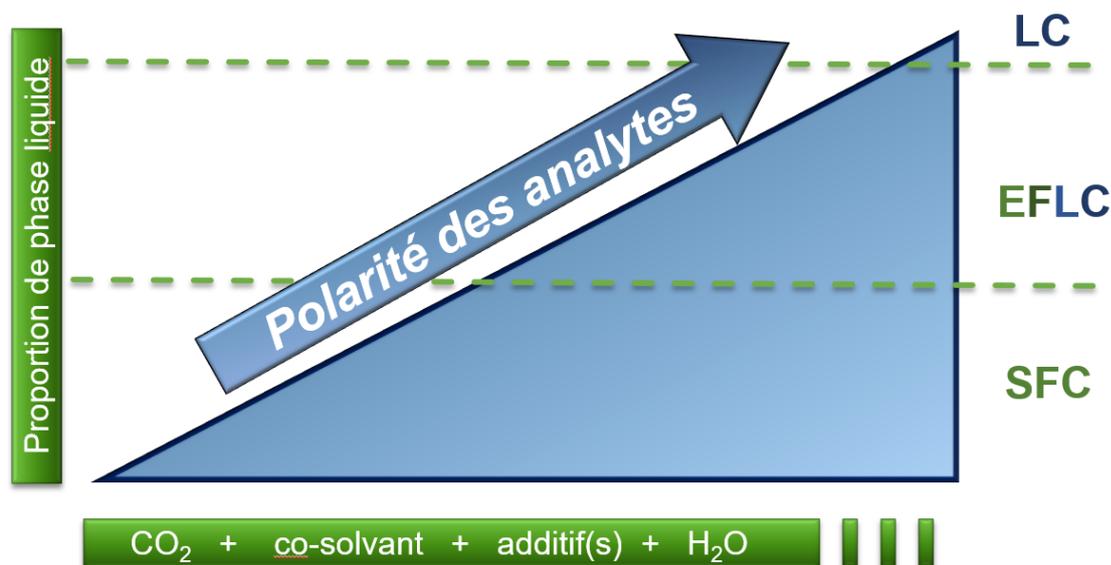


Figure 5 : Représentation des deux possibilités pour analyser des composés plus polaires, en abscisse selon la nature du co-solvant et en ordonnée selon la proportion de phase liquide.

L'UC est une méthode permettant l'analyse de composés non-polaires via sa dimension SFC et aussi l'analyse de composés très polaires via ses dimensions EFLC et LC. La polarité est une question essentielle dans le cadre des possibilités d'analyses par fluides supercritiques. Comment est-elle définie ? Comment peut-on la mesurer ? Quelles sont les limites de polarité des analytes pouvant être élués en UC ? [72]

La mesure de la polarité peut s'estimer par différentes mesures. Le coefficient de partition octanol-eau, appelé communément log P, est l'outil le plus utilisé pour exprimer la polarité d'une molécule. Ce log P correspond à la mesure de la solubilité d'une molécule entre deux solvants non miscibles, l'eau et l'octanol, et se résume comme une estimation de la lipophilicité [119]. Il peut être déterminé par mesure expérimentale, par exemple en chromatographie liquide, ou par calcul informatique à partir d'une base de données. Dans notre cas, l'ensemble des log P sont des valeurs calculées à l'aide de l'outil Moldesc (<http://moldesc.icoa.fr/>) ou du logiciel Vortex v2019.04.82972.15-s (Dotmatics Limited) de l'IdRS.

La PSA (pour Polar Surface Area) est la seconde mesurande la plus utilisée pour exprimer la polarité d'une molécule. Elle se définit comme la surface polaire d'une molécule obtenue par l'addition de ses portions polaires. Ces portions polaires sont déterminées par la combinaison entre les propriétés de solubilité, les fonctions pouvant créer des liaisons hydrogènes et la polarité intrinsèque de la molécule. Pour la majorité des molécules étudiées dans ce manuscrit, nous avons calculé la TPSA (ou Total Polar Surface Area) à l'aide de l'outil Moldesc (<http://moldesc.icoa.fr/>) ou du logiciel MOE version 2020.

Enfin, dans notre cas spécifique des peptides, le GRAVY number est un outil spécifique de mesure de l'hydrophilicité ou l'hydrophobicité. Ce GRAVY number (Gn) se calcule par addition de l'hydrophilicité de chacun des AA que l'on divise par le nombre de résidus d'AA [120], [121]. Les valeurs de Gn pour l'ensemble des peptides est généralement compris entre -2 et +2, les plus hydrophobes présentant le Gn le plus élevé. Seulement seuls les AA protéinogènes ont un indice d'hydrophilicité calculés, ce qui rend impossible la détermination du Gn de la majorité des « petits » peptides puisqu'ils contiennent pour la très grande majorité des AA modifiés. L'ensemble des Gn a été calculé à l'aide de l'outil ProtParam tool (<https://web.expasy.org/protparam/>) et vérifié sur le GRAVY CALCULATOR (<http://www.gravy-calculator.de/>) suite aux travaux de Losacco et al. [122]

D'un point de vue chromatographique, Mme West a revu les éléments attirés aux composés polaires analysés en SFC [123]. Elle en ressort « ... deux prérequis : la phase stationnaire doit-être suffisamment polaire pour les retenir et la phase mobile doit-être suffisamment polaire pour permettre une bonne solubilisation des composés. »

L'eau est un additif pouvant être ajouté à la composition du co-solvant en tant que solvant tertiaire accompagnant le CO₂ sc et un alcool. L'ajout d'eau permet d'accéder à des hautes polarités de composés mais pas seulement, elle peut augmenter l'efficacité et la sélectivité en SFC [124], [125]. Sa faible miscibilité avec le CO₂ sc en fait un solvant minoritaire du co-solvant (jusqu'à 10%) et ne permet pas de l'utiliser comme solvant liquide majeur en lieu et place des alcools ou de l'AcN dans le cas d'analyse en UC. L'impact de l'eau en SFC comme co-solvant ou additif dans le co-solvant a été revu par Roy et al. [126]. La conclusion de leur revue met en avant la différence de sélectivité et l'augmentation de l'efficacité obtenue lors de l'utilisation d'eau dans le co-solvant. Également depuis quelques années, l'eau comme additif en UHPSFC a été utilisée pour

analyser des familles de composés très variés [127]. Losacco et al. ont aussi mis en évidence les excellents résultats de ces analyses de composés très polaires (acides aminés, sucres, pesticides ...) par fluides supercritiques. La composition de la PM essentielle en UC est approfondie dans la partie IV du chapitre 1.

En 2021, on trouve déjà quelques exemples d'analyses évoquant l'UC comme méthode analytique appliquée en lieu et place de la SFC [118], [128]–[130]. Ces publications évoquent l'analyse de colorants alimentaires [130] ou d'acides aminés sans dérivations [128], [129]. D'autres travaux appliquent des gradients UC en conservant la dénomination SFC pour de plus gros peptides pharmaceutiques et dans le cas de métabolites à polarité très diverses [122], [131], [132]. Le potentiel de l'UC pour permettre l'analyse de composés à polarité et poids moléculaires très divers apporte des perspectives intéressantes à l'attention des utilisateurs de la SFC [133]–[137].

Cette mise en évidence d'un potentiel encore peu exploité de l'UC nous conduira par la suite à étudier plusieurs familles de biomolécules naturelles ou synthétiques. La montée en échelle des gammes de polarité et de poids moléculaires des familles chimiques investiguées au cours de ces travaux nous permettra de découvrir progressivement les possibilités et difficultés de l'UC. Les deux familles sur lesquelles nous exposerons des résultats obtenus par UC sont celles des flavonoïdes dans un premier temps, puis celle des peptides dans un second temps. Chronologiquement, nous avons débuté les travaux en UC sur la grande famille des flavonoïdes suite aux avancées de Raimbault et al. qui ont investigué l'analyse des acides aminés libres par UC-MS [128], [129].

Dans un second temps, les peptides seront étudiés en UC-MS mais le travail s'est déroulé en deux étapes. Tout d'abord, une focalisation sur des petits peptides (inférieurs à 1 kDa) ce qui nous a permis d'étudier des composés ionisables plus polaires que les flavonoïdes à une échelle de masse comparable. Puis, nous avons poursuivi notre cheminement et nous nous sommes dirigés vers une échelle poids moléculaire plus importante en analysant des « gros » peptides jusqu'à 5kDa.

Ce sont ces deux familles que nous allons discuter ensemble en débutant par une introduction sur la famille moléculaire puis, un état des lieux des précédents entre l'analyse par fluides supercritiques et la famille discutée.

III- L'analyse des flavonoïdes par fluides supercritiques

Les flavonoïdes (latin *flāvus* jaune, suffixe grec *-eidēs* qui marque la ressemblance)[138] sont des métabolites secondaires des plantes, c'est-à-dire des molécules liées à l'interaction entre les plantes et leur environnement. La première structure chimique d'un flavonoïde (la rutine) a été caractérisée dans les années 1930 par Albert Szent-Györgyi et ses collaborateurs. Ils avaient étudié le potentiel de certains flavonoïdes sur la santé humaine avec à la clé un prix Nobel de médecine en 1937 [139], [140]. La première dénomination de cette nouvelle famille moléculaire fut « vitamine P » pour perméabilité [141] mais le nom fut réfuté et retiré dans les années 1950. Perez-Vizcaino et al. ont mis en évidence l'intérêt grandissant des études scientifiques sur les flavonoïdes à partir des années 1990 (Figure 6) et supposent que le début de l'attrait aux flavonoïdes tient de la volonté de la population à consommer de manière plus équilibrée fruits et légumes [141]. La recherche autour du « French paradox » est un exemple de recherche populaire en lien avec la consommation de flavonoïdes et la durée de vie des populations dites méditerranéennes [142]. Une étude de grande envergure (> 50 000 participants, pendant 23 ans) a été menée au Danemark et a mis en évidence la réduction des décès liés aux cancers pour les personnes consommant des fruits et légumes [143].

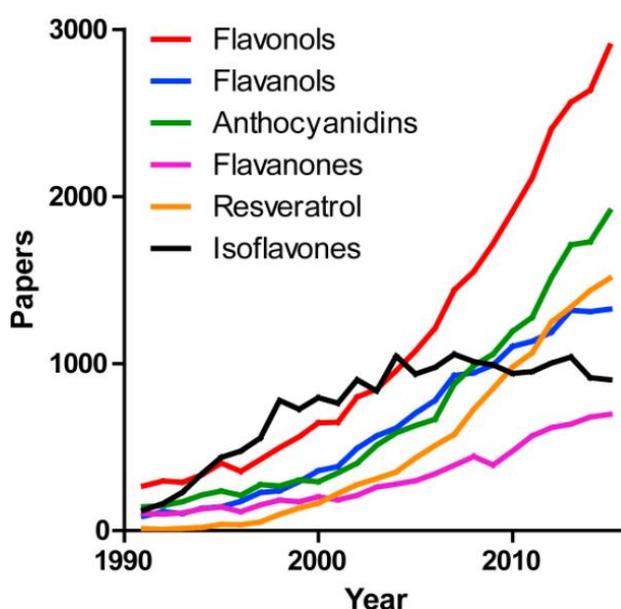


Figure 6 : Publications en lien avec les familles de flavonoïdes entre 1991 et 2016, repris de [141]

De nombreux effets bénéfiques des flavonoïdes sur la santé humaine ou animale sont à présent répertoriés : antibactériens [144]–[147], anticancéreux [148], [149], anti-inflammatoires [149]–[151], anti-ischémiques [152]–[154], antiviraux [155], [156]... Les flavonoïdes sont également des antioxydants reconnus [157], [158] et présentent des bienfaits cosmétiques contre le vieillissement et les inflammations de la peau [159]. La médecine traditionnelle chinoise est un exemple d'utilisation des flavonoïdes comme traitement à de multiples maux [160]. Cette grande famille des flavonoïdes présente un intérêt double pour des applications pharmaceutiques et cosmétiques.

On catégorise la famille des flavonoïdes au sein des polyphénols par leur squelette dyphénylpropane (C6-C3-C6) décrit sur la structure centrale de la Figure 7.

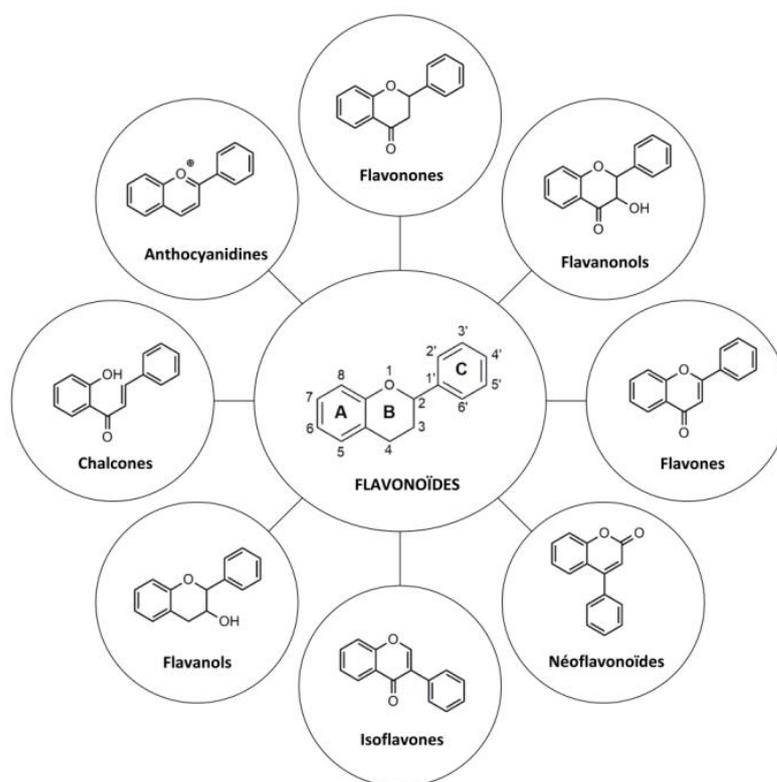


Figure 7 : Familles de flavonoïdes dérivées de la structure centrale, repris de [161]

Il existe beaucoup de positions de substitutions lorsque l'on regarde les différentes structures de flavonoïdes. Celles-ci peuvent être sous formes phénoliques via les hydroxylations mais aussi glycosylées avec un ou plusieurs sucres et sur une ou plusieurs positions. Les formules observées sur la figure 7 correspondent à la partie dite génine (aglycone) d'un hétéroside, c'est-à-dire la partie non-sucre. Un hétéroside est donc l'association d'un ou de plusieurs sucre(s) avec un motif organique (ici les motifs

flavonoïdes). Dans le chapitre 2.2, les flavonoïdes investigués par UC-MS ont des structures hydroxylées avec et sans sucres au sein de mêmes familles.

La RPLC est la méthode de référence employée pour l'analyse de flavonoïdes [162]. Malgré ses performances séparatives démontrées, la SFC n'est pas toujours envisagée pour l'analyse de flavonoïdes, même comme méthode de deuxième ou troisième intention [163]–[165]. Initialement, les travaux publiés par SFC n'étaient appliqués que pour des flavonoïdes non glycosylés. La première séparation, visible sur la figure 8, s'attelait à séparer des flavones polyméthoxylées provenant d'huiles d'oranges et de mandarines siciliennes [166].

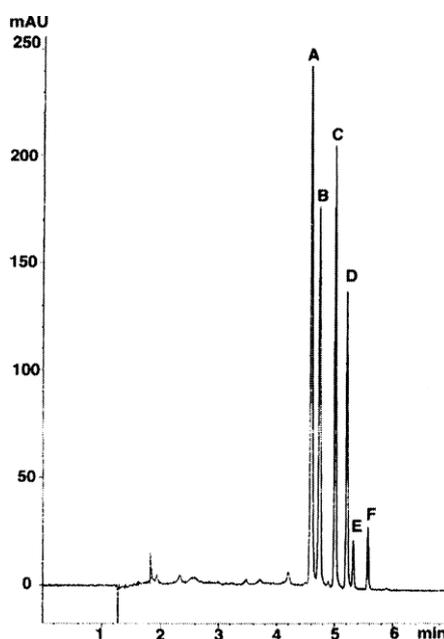


Figure 8 : Séparation par SFC de 6 flavones avec un gradient de co-solvant de 10 à 30%, repris de [166]

La possibilité de travailler en UC avec des proportions de co-solvants plus importantes, en plus des possibilités de combiner additifs acides ou basiques avec de l'eau, a permis d'étendre la gamme de polarité des flavonoïdes analysables. Un ensemble de 20 publications focalisées sur les flavonoïdes analysées par CO_2 SC a été décrit dans le chapitre 2 à l'intérieur duquel nous analyserons un ensemble de standards de flavonoïdes sous formes hydroxylées ou glycosylées ainsi que sur deux produits commercialisés eux aussi sous formes glycosylées, par UC-MS.

IV- Le CO₂ pressurisé pour l'analyse de peptides

Les peptides (du grec « pépsis » signifiant cuisson, maturation, digestion avec le suffixe -ide)[167] sont des chaînes d'acides aminés (AA) liés entre eux par liaisons amides (dites aussi liaisons peptidiques). Ils peuvent être des métabolites primaires, c'est-à-dire des molécules essentielles d'un point de vue physiologique, constitués à partir des acides aminés protéinogènes. On parle d'oligopeptides lorsque le nombre d'unités d'acides aminés est faible (< 10 AA) et on les appelle polypeptides lorsque le nombre d'acides aminés est important (> 10 AA). La limite entre peptides et protéines est estimée aux alentours de 100 AA. Cependant, les dénominations des composés sont discutées également en fonction de la structure 3D de la molécule. L'exemple de l'insuline est très parlant (association de 2 chaînes de peptides de 21 AA et 30 AA par ponts disulfures), parfois appelée polypeptide et parfois protéine, elle démontre encore l'absence de consensus autour de cette question de la dénomination [168].

La suite de ce chapitre revisite l'analyse de biomolécules par chromatographie s'appuyant sur le CO₂ sc. Nous avons focalisé cette recherche sur deux types de biomolécules. La première reprend les composés dont l'unité de base est l'acide aminé, ce qui inclut les peptides et les protéines. La seconde s'appuie, elle, sur les nucléobases et retrace aussi l'analyse de nucléosides et nucléotides par chromatographie avec du CO₂ sc.

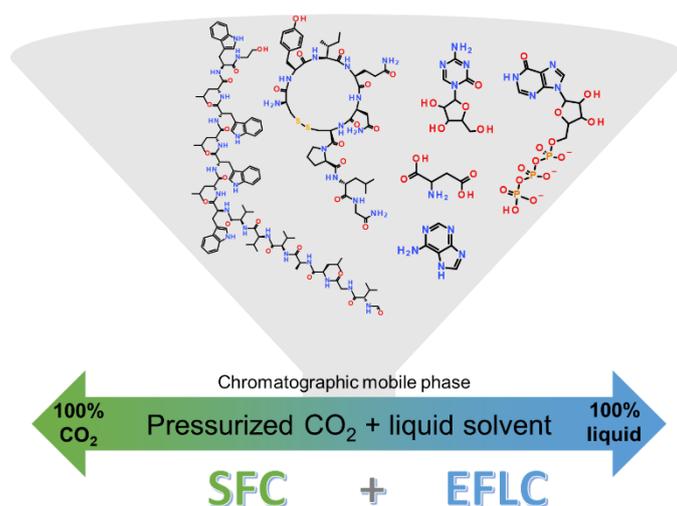


Figure 9 : Schématisation de l'analyse par UC des biomolécules étudiées, repris du résumé graphique de [169]



Chromatographic analysis of biomolecules with pressurized carbon dioxide mobile phases – A review



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ARTICLE INFO

Article history:

Received 13 July 2020

Received in revised form 24 October 2020

Accepted 24 October 2020

Available online 1 November 2020

Keywords:

Amino acids

Enhanced fluidity liquid chromatography

Nucleosides

Nucleotides

Peptides

Proteins

Supercritical fluid chromatography

ABSTRACT

Biomolecules like proteins, peptides and nucleic acids widely emerge in pharmaceutical applications, either as synthetic active pharmaceutical ingredients, or from natural products as in traditional Chinese medicine. Liquid-phase chromatographic methods (LC) are widely employed for the analysis and/or purification of such molecules. On another hand, to answer the ever-increasing requests from scientists involved in biomolecules projects, other chromatographic methods emerge as useful complements to LC. In particular, there is a growing interest for chromatography with a mobile phase comprising pressurized carbon dioxide, which can be named either (i) supercritical (or subcritical) fluid chromatography (SFC) when CO₂ is the major constituent of the mobile phase, or (ii) enhanced fluidity liquid chromatography (EFLC) when hydro-organic or purely organic solvents are the major constituents of the mobile phase. Despite the low polarity of CO₂, supposedly inadequate to solubilize such biomolecules, SFC and EFLC were both employed in many occasions for this purpose. This paper specifically reviews the literature related to the SFC/EFLC analysis of free amino acids, peptides, proteins, nucleobases, nucleosides and nucleotides. The analytical conditions employed for specific molecular families are presented, with a focus on the nature of the stationary phase and the mobile phase composition. We also discuss the potential benefits of combining SFC/EFLC to LC in a single gradient elution, a method sometimes designated as unified chromatography (UC). Finally, detection issues are presented, and more particularly hyphenation to mass spectrometry.

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1. Introduction

Biomolecules or biological molecules are molecular species present in living organisms of the fauna, the flora and microorganisms. Four principal categories of macromolecular biomolecules are usually distinguished: carbohydrates, lipids, proteins and nucleic

acids. Smaller molecules include primary and secondary metabolites. In this paper we will focus on amino acids, their oligomers and polymers (peptides and proteins), and on nucleic acid constituents like nucleobases, nucleosides and nucleotides (Fig. 1). Peptides may be linear or cyclic, where the ring structure can be formed by an amide bond ("peptide bond") or other stable bonds like disulfide [1]. Peptides and proteins differ in size and structure, in that peptides are usually smaller, with an undefined three-dimensional structure, while proteins are larger and with a defined, rather stable folding. However, there is no universally accepted limit value in

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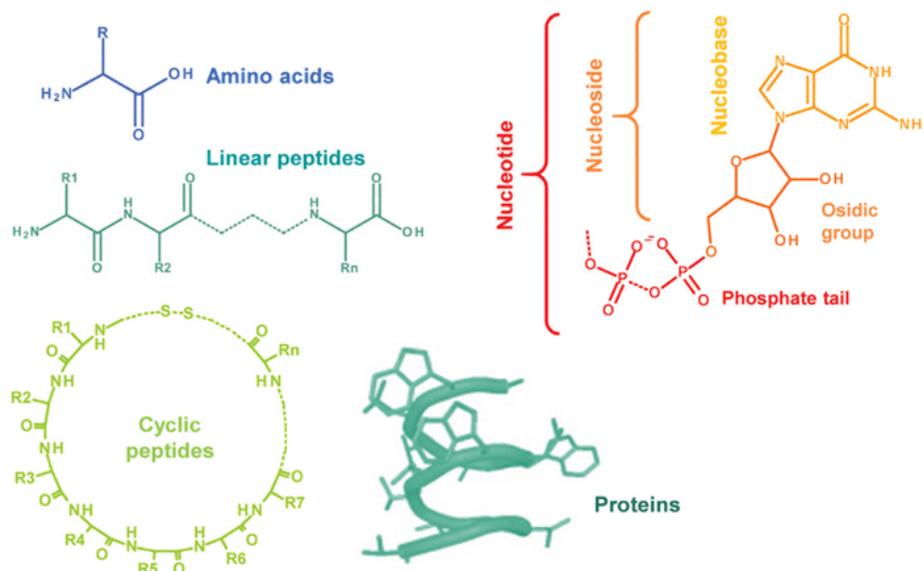


Fig. 1. Generic and representative structures of the target molecules reported in the literature reviewed in this paper.

the number of amino acid residues to distinguish between peptides and proteins. Nucleotides are the basic constituents of nucleic acids like DNA and RNA, and may be decomposed in (i) a nucleobase (purine or pyrimidine) and (ii) an osidic group, together forming a nucleoside, and (iii) a phosphate tail containing one, two or three phosphate groups linked to the 5' oxygen of the osidic group. The structures of the natural biomolecules may also be modified for specific purposes with other functional groups, as is the case with nucleoside antiviral drugs [2]. Indeed, in the recent years, peptide (linear or cyclic [1,3]), protein and nucleic acid biomolecules have largely emerged in the pharmaceutical arena, either as synthetic active pharmaceutical ingredients (API) [4,5] or from natural ingredients [6,7] as food products [8] or traditional Chinese medicine (TCM) [9]. The fast emergence of these types of bioactive molecules is setting new challenges to analytical chemists in general and chromatographers in particular.

Liquid chromatography is the most common method to analyze and purify such biomolecules [7,10,11], preferably in the reversed-phase mode (RPLC) but also in the hydrophobic interaction (HIC) and hydrophilic interaction (HILIC) modes, ion-exchange chromatography (IEX) and size-exclusion chromatography (SEC). Beside liquid chromatography, capillary electrophoresis (CE), and microfluidics are other favorite analytical methods for biomolecules. Supercritical fluid chromatography (SFC), employing pressurized carbon dioxide as a major constituent of the mobile phase, is another possible alternative that would deserve more attention in this field [12]. In fact, the low viscosity and high analyte diffusivity allow for high flow rates together with high efficiency, thus the resolution per unit time is usually excellent with SFC. In addition, SFC is also advantageous at the preparative scale as it allows significant reductions in solvent consumption compared to liquid-phase preparative separations, and produces fractions that are more concentrated. As a result, the solvent cost, solvent evaporation time and waste disposal costs are all reduced compared to LC methods. For this reason, preparative SFC is considered a green purification method. Interestingly, the benefits of pressurized carbon dioxide in the mobile phase are maintained whatever the proportions introduced in a liquid mixture. Traditionally, when the proportion of CO₂ is the largest, the method is called subcritical or supercritical fluid chromatography (SFC). When the proportion of CO₂ is the smallest, the method is rather called enhanced fluidity

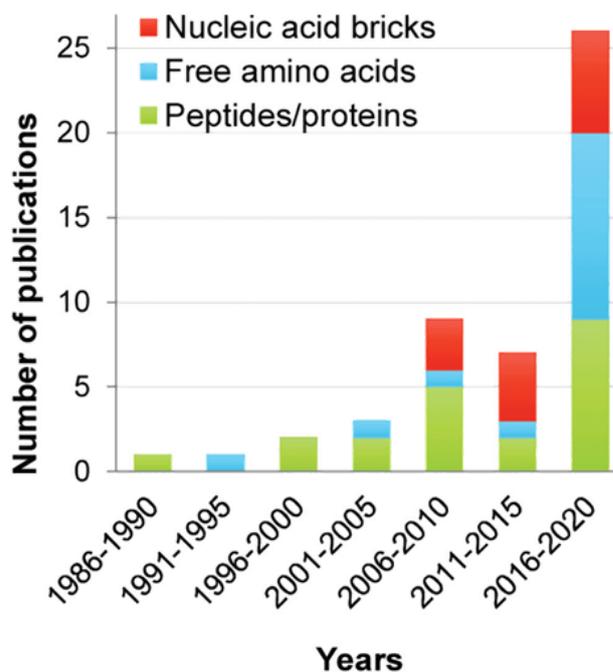


Fig. 2. Published papers related to SFC or EFLC analysis of free amino acids, peptides, proteins and nucleic acid bricks (nucleobases, nucleosides and nucleotides).

liquid chromatography (EFLC). Aside habits, there are no fundamental reasons to dissociate the two methods [13] and they will both be discussed in the following. While already an old technique, with the first experiments dating from 1962 [14], SFC has faced a rapid surge of interest in the last decade, thanks to the introduction of improved instruments by several manufacturers. Before that time, the benefits of SFC were principally recognized at the preparative scale and for enantioresolution [15–17]. A variety of application fields that were previously little investigated with SFC are now being re-explored [18]. Biomolecules analysis and purification to serve pharmaceutical and clinical research is one such field. As appears in Fig. 2, the number of papers published on this topic

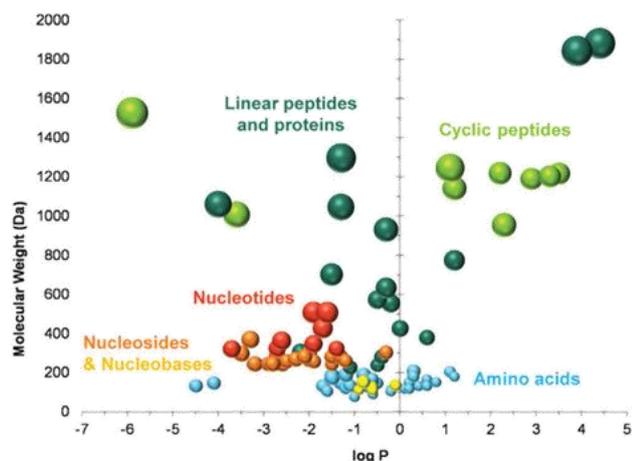


Fig. 3. Molecular weight and polarity features related to biomolecules previously analyzed with SFC or EFLC. The bubble size reflects the topological polar surface area (TPSA). The three descriptors were computed with MolDesc freeware (<http://moldesc.icoa.fr/>).

may have been scarce during several decades but has been rising in the recent years. Although the total number of them remains modest, there is clearly an interest for alternative methods in this field. When analyzing biomolecules with SFC, difficulties are expected due to solubility issues of potentially large and polar species (see section 2 below). In addition, conformational changes may occur for proteins when exposed to carbon dioxide, as will be further detailed below. The changes occurring nowadays in the way the technique is operated, typically with increasing portions of co-solvent and water in the pressurized carbon dioxide mobile phase are however raising new expectation.

The first example of peptide analysis in SFC was published in 1988 [19], where a cyclic undecapeptide Cyclosporin A (1201.8 Da), was analyzed with SFC hyphenated to mass spectrometry (MS). Further attempts at analyzing such biomolecules initially concentrated on lipophilic peptides and proteins, while recent attempts also concern more polar species, as will be detailed in Section 2.

This paper reviews most significant articles related to SFC and EFLC analysis and purification of free (underivatized) amino acids, peptides, proteins, and building blocks of nucleic acids. It is not intended to be exhaustive but rather to spotlight trends and specific features. First, the polarity and size issues related to these biomolecules is discussed, to better relate them to the choice of stationary and mobile phases that were employed for their analysis. Detection issues are also discussed, with a particular focus on MS hyphenation.

2. Polarity and size features of the target biomolecules

To understand the chromatographic features of biomolecules analysis, it is useful to examine their structures first. In Fig. 1, the generic structures of the target molecules related in this paper are presented. In addition, we have examined the structures of the molecules previously analyzed with SFC and EFLC in terms of size and polarity. For this purpose, structural descriptors were computed for the molecules mentioned in the literature reviewed with MolDesc, an open-source software (<http://moldesc.icoa.fr/>). Three descriptors were retained to discuss the relation to chromatographic analysis: molecular weight (MW), octanol-water partition coefficient (log P) and the topological polar surface area (TPSA). Note that, as any computed descriptors, they may not be entirely accurate, but they should be good enough to compare the features of the target molecules. They are summarized in Fig. 3, where MW is

plotted versus log P value, and bubble size is related to TPSA. Only the larger proteins and peptides are not represented in this figure, as it would have flattened all other molecules in an apparently small area at the bottom of the figure and because the computation of polarity features for such molecules has little meaning, as will be further discussed below. For instance, reports of large peptides and proteins with molecular weights as high as 3.7 kDa (issued from peptic digestion of myoglobin [20]), 5.8 kDa (biosynthesized human insulin [21]), 13.7 kDa (ribonuclease A [22]), 17.6 kDa (myoglobin [23]), 66.5 kDa and 80.3 kDa (bovine serum albumin and transferrin [24]) were excluded from this figure but are however discussed in the following sections.

Amino acids are all small molecules and are thus placed at the bottom of Fig. 3 (small blue bubbles). Apart from the acid and amine functions, which may both be ionized depending on operating conditions, most amino acids possess a side chain (residue), which may be hydrophobic (hydrocarbon chain or aromatic group), hydrophilic bearing no ionizable function (like hydroxyl, thiol or amide functions), acidic thus possibly bearing a negative charge, or basic thus possibly bearing a positive charge. According to these varied residues, amino acids may be slightly hydrophobic (on the right of the figure) or hydrophilic (on the left of the figure). Note that several papers relating the analysis or purification of derivatized amino acids (for instance methyl esters, carboxybenzyl esters [25,26] or dansyl esters [27]) will not be described in this review. Derivatization is usually facilitating the elution of amino acids in reducing their polarity, and often facilitating their chiral resolution.

When linear peptides are considered, some ionizable functions should remain at both ends, if the terminal amine and carboxylic functions are unmodified with protective groups. When cyclic peptides are considered, there are not necessarily any pendant amine or carboxyl functions as they may have served to form the ring, unless some side chains are attached to the ring. Thus cyclic peptides are often observed to be less polar than linear peptide chains. This can be observed in Fig. 3. Most cyclic peptides (light green bubbles) are situated in the right portion of the figure, with positive log P values, while most linear peptides (dark green bubbles) are situated in the left portion of the figure, with negative log P values. According to log P values, two polar cyclic peptides were reported, Linaclotide and Oxytocin (on the left of Fig. 3), and two non-polar linear peptides or proteins were reported: the membrane proteins Gramicidin A and B.

However, as in any other molecule, the overall polarity of a peptide expressed by log P value is the result of the positive contributions of hydrophobic residues and the negative contributions of hydrophilic residues. When the two are counterbalanced, the overall log P value may be close to zero, although a significant number of polar groups could be present. It is thus useful to observe a second polarity parameter, like the topological polar surface area (TPSA), which sums the individual contributions of all polar groups in a molecule, and is not counterbalanced by hydrophobic groups. In Fig. 3, TPSA is represented by bubble size. Then it appears that all peptides reported, whatever their log P values, have significant TPSA values that should at least result from the peptide bonds (amide functions), whatever the residues. For instance, the membrane proteins Gramicidin A and B have some of the largest TPSA values (large bubbles), even though the overall polarity (expressed by log P) is low (around 4). In addition, in their stable conformation, the linear gramicidins are folded in helices with hydrophobic rings directed outside the helix, with little exposed polar surface. The stable conformation of proteins may or may not be preserved during SFC and EFLC analysis [22,23]. It is thus difficult to conclude on the exposed polarity of such molecules during the analysis. Actually, chromatographic retention on a polar stationary phase may be the best indicator of the exposed polarity, as was demonstrated by Goetz et al. [28] for cyclic peptides: longest retention of peptides

on a polar stationary phase in SFC conditions was indeed observed to be related to lower membrane permeability. Clearly, log P and TPSA are both insufficient to assess the polarity of large flexible molecules [29].

Nucleotides are also visible in Fig. 1, with a representative structure based on a guanine nucleobase. The purine and pyrimidine structure of the five nucleobases (adenine, guanine, cytosine, thymidine and uracil) are all small and polar as they appear at the bottom of Fig. 3, with negative log P values. Adding the pentose ring to form a nucleoside is slightly increasing molecular weight but also significantly increasing polarity, as the bubbles representing nucleosides are slightly higher and on the left. Finally, introducing a phosphate tail is also causing increased molecular weight, is not greatly affecting log P values but significantly increasing TPSA values (bigger red bubbles in Fig. 3). Nucleic acid chains should then be most polar structures, in terms of log P or TPSA.

From Fig. 3 and the above discussion, we can conclude that the target molecules reviewed in this paper constitute a very heterogeneous group, with a variety of polarities and sizes, mostly from 100 to 2000 Da, or even higher for some bigger peptides and proteins not represented in the figure. While all target molecules bear polar functional groups (expressed by TPSA), be they charge-neutral or ionizable, their overall polarity expressed by log P varies in a great range. As a consequence, it is unlikely that one generic chromatographic method would work for all of them. The different choices of stationary phases and mobile phases previously selected to achieve their analysis or purification, presented in Tables 1, 2 and 3, will be detailed in the following sections.

3. Mobile phase composition

CO₂ has little polarity and is usually compared to hexane. This comparison is not entirely fair as CO₂ has some polarity features (local dipole moments, polarizability) and reactivity. However, a neat CO₂ mobile phase in usual SFC operating conditions (temperature around 30 °C, back-pressure of 11–18 MPa) is unlikely to solvate any of the target biomolecules. When charge-neutral species are considered, mixing pressurized CO₂ to a polar organic solvent, like the most frequently used short-chains alcohols [18], is sufficient to achieve good solubility. Methanol is usually the preferred co-solvent observed in SFC literature, and the specific papers related to our target molecules are no exception, as methanol was observed in above 80 % of the articles. A few papers mention other co-solvents, at least at the stage of method development: ethanol [30–32], isopropanol or acetonitrile [20,23,33–35], the latter being often introduced in mixtures with methanol. The co-solvent increases the mobile phase polarity [36] and viscosity, modifies the mobile phase density [37], introduces other possible interactions between the mobile phase and the analytes and adsorbs onto the stationary phase [38], thereby also changing the possible interactions between the analytes and stationary phase [39].

For the most polar charge-neutral or ionized species, two options are usually considered.

First, (one or more) acidic, basic or salt additives are frequently employed. For instance, the second oldest example of SFC analysis of our target biomolecules (1992) reported the achiral analysis of free proteinogenic amino acids with SFC-UV [40], and was mostly focused on adjusting the mobile phase composition with additives mixtures to elute the analytes with satisfying peak shapes. In general, additives nature and concentration is explored early on in method development, to determine adequate composition to achieve elution of the analytes with good peak shapes, taking account of detector issues. Unlike liquid-phase chromatography, the mobile phase in SFC and EFLC cannot be easily buffered because

it is not possible to measure pH* precisely. As a result, the acid and base ionizable functions are not easily controlled. It was shown that the combined presence of CO₂ and an alcohol co-solvent induces some acidic character in usual SFC [41,42] and EFLC [43] conditions due to the formation of methoxycarbonic acid, typically between pH* 4 and 6, depending on the proportion of water in the mobile phase, which is further acidifying the fluid through the formation of carbonic acid. It was also shown that the introduction of additives could modify the apparent pH*, with strong acids further reducing the apparent pH* down to 1–2, and bases only moderately increasing it to pH* 6–7 [41,42]. Salts like ammonium acetate could act as stabilizers in gradient elution modes. However, we can only conjecture on the exact ionization state of the target molecules as their exact pK_a values in such conditions are unknown. Whenever ionic groups are present, the use of additives serves not only in changing the apparent pH* and ionization state of the analytes, but may also contribute to the formation of ion pairs between the analyte and additive ions to favor their elution. Furthermore, the additive can adsorb on the stationary phase to reduce strong interactions between an ionic analyte and polar groups of the stationary phase [42,44]. Other benefits of the co-solvent and additives may be found in detection issues, particularly when hyphenation to mass spectrometry is desired [34,45]. This will be further detailed in the section related to detection issues. From a practical point of view, it is customary to use acidic additives for acidic analytes and basic additives for basic analytes, but the contrary is also observed when ion-pairing may help in favoring the elution [46]. When the analytes are large and complex, and possess several ionizable groups with acidic and basic functions, an acid and a base are sometimes used together, or salts like ammonium acetate. Examining the literature related to SFC/EFLC analysis of our target biomolecules, the most often cited additive to elute the target biomolecules was trifluoroacetic acid (TFA) [21,23,24,30,40,42,46–57]. Other acids like formic acid [20,33,40,58,59] or methane- and ethane-sulfonic acids [31,53,60,61] were also mentioned in several occasions. Isopropylamine, a basic additive that is frequently used in SFC analysis of basic drugs, appeared only in a few papers [30,46,51,62], suggesting that a basic additive may not be the best in this case. Ammonium acetate [22,34,48,51,54,56,58,63–65] and ammonium formate [33,34,52,53,59,66,67], which were both frequently employed in recent SFC methods [18], were mentioned many times. More recently, ammonium hydroxide has become more and more popular in SFC analysis of basic drugs and was found to be equally useful for amino acids, peptides and proteins [45,52,58,68]. In older papers, several other exotic additives were employed, while in the past decade, the additives employed in SFC/EFLC analyses have mostly been standardized, largely favoring the additives that are compatible to mass spectrometric detection. Still a few original proposals can be found, like ammonium fluoride to achieve the elution of most polar amino acids in SFC [66], or sodium phosphate for nucleotides in EFLC [69].

Secondly, some portion of water can also be introduced in the mobile phase. Although already an old practice [70,71], this was somewhat forgotten for several years, only to reappear most significantly in the last decade. Water may be present in small concentrations – typically 2–5% – and then acting like a polar additive [72], or in large concentrations (typically above 10%) and then acting as a modifier [73]. Because CO₂ and water are not miscible in all proportions, large concentrations of water can only be introduced in ternary compositions of CO₂-solvent-water. Such mobile phase compositions are most often encountered in EFLC experiments. Note that an EFLC method may be developed by introducing carbon dioxide in any sort of liquid mobile phase: either from organic solvents used in a normal-phase LC method [74] or SEC [75], or from buffer-organic mixtures used in RPLC [76], HILIC [35,69,77], HIC [22] and IEX [33] modes. The proportion of water possibly

Table 1
Published SFC/EFLC methods to analyze free amino acids.

Reference	Year	Samples	Stationary phase nature	Mobile phase co-solvent	Detection
[40]	1992	Standards	Diol	Methanol with water, triethylamine and pyridine	UV (225 nm), ELSD
[60]	2005	Standards	Chiral polysaccharides	Ethanol with 0.1 % ethanesulfonic acid	UV
[80]	2008	Standards	Polystyrene-divinylbenzene	Methanol-isopropanol-water	UV
[33]	2015	Standards	Polysulfoethyl	Acetonitrile or methanol, with 20 % buffer (15 mM ammonium formate / formic acid, pH 3)	ELSD
[67]	2016	Standards and human urine	Diol	Methanol with 20 mM ammonium formate	DAD and ESI(+/-)-MS/MS
[82]	2016	Standards & plant extracts	Diol, C18, 2-ethylpyridine	Methanol with 2% water and 10 mM ammonium formate	ESI(+/-)-MS/MS
[97]	2016	Standards and food supplements	Diol and chiral macrocyclic glycopeptide	Methanol with 10 % water	UV (200 & 220 nm)
[34]	2017	Standards and human serum	Diol	Methanol with 1% water and ammonium formate	ESI(+)-MS/MS & APCI(+)-MS/MS
[66]	2018	Standards	Silica and sulfobetaine	Methanol with 5% water, 50 mM ammonium formate and 1 mM ammonium fluoride	ESI(+/-)-MS/MS
[54]	2019	Standard and tea leaves	Amide	Methanol with 5% water, 5 mM ammonium acetate and 0.4 % trifluoroacetic acid	ESI(+)-MS
[53]	2019	Standards and plant extract	Chiral zwitterionic <i>Cinchona</i> alkaloid	Methanol with 5% water and 50 mM ammonium acetate	ESI(+)-MS
[61]	2019	Standards and food supplements	Chiral zwitterionic <i>Cinchona</i> alkaloid	Methanol with 2% water and 20 mM methanesulfonic acid	ESI(+)-MS
[55]	2020	Standards	Chiral crown-ether	Ethanol with 5% water and 0.8 % trifluoroacetic acid	ESI-MS
[45]	2020	Standards and human urine	Diol	Methanol with 10 mM ammonium hydroxide	ESI(+/-)-MS/MS

introduced in a CO₂-solvent mixture depends on (i) the operating conditions of temperature and pressure, (ii) the proportions of CO₂ and solvent and (iii) the nature of the solvent. Ternary diagrams of such mixtures are scarcely available [78,79] but would be most useful to develop further analytical and preparative methods for the mostly polar target biomolecules. For instance, Li and Thur-bide [80] showed that using some isopropanol in conjunction with methanol as co-solvent was favorable to the introduction of larger proportions of water, thereby allowing the elution of most polar species like free amino acids without any other additive.

Additives and water may also have combined effects. For instance, Taylor had demonstrated that the combined use of water and ammonium acetate was more powerful in achieving the elution of nucleobases with good peak shapes than the single use of either one or the other [81]. Recently, Regalado and co-workers discussed the chaotropic effects of ammonium hydroxide combined with water in a CO₂-methanol mobile phase when eluting peptides [68]. In fact, in many papers, the additives mentioned above were used in conjunction with water [21,23,33,46,54–56,61,64–66,68,69,82]. Taking aside the synergistic effect that water may have with an additive, it may also favor the additive solubility, both in the pressurized CO₂ mobile phase and after the decompression to avoid additive precipitation in the instrument.

Whenever a wide range of analyte polarities is encountered, the analyses are preferably conducted in gradient elution mode. With the evolutions in SFC practice, the proportion of co-solvent introduced in the carbon dioxide mobile phase has been increasing more and more. In the early years, only neat fluids were employed (not only CO₂ but also ammonia, fluorocarbons, etc.) Back in the years 1980s, when it was found that introducing a co-solvent brought more benefits in expanding the range of analytes amenable to the technique than the expected drawbacks of increased viscosity, only a few percents (1–5 %) of solvent were employed. In the years 1990s,

with the advent of packed column SFC over capillary SFC, the typical range of solvent proportion extended to 1–20 %, then increased further in the years 2000s. In the years 2010s, the maximum amount of co-solvent encountered in most SFC papers was rather around 60 %, which makes the fluid closer to a liquid than a supercritical fluid, although it does not change the way to operate the system.

As appears in Fig. 4, SFC is usually operating in the low portion of solvent area, while EFLC usually works in the large portion of solvent area. While no fundamental nor practical limit exists between the two [13], the distinction is rather issuing from different approaches: SFC historically started with neat fluids, among which was neat CO₂. A co-solvent was later introduced to expand the polarity limits of soluble analytes. At the other end of the solvent axis, EFLC started with LC conditions, where CO₂ was introduced to reduce the viscosity of the mobile phase, thereby providing improved efficiencies and the possibility to use higher flow rates. In addition, the EFLC mobile phase should be a greener technique compared to a fully liquid mobile phase as the consumption of solvent should be reduced [24]. Olesik and co-workers are the main explorers of the EFLC concept, particularly at the analytical scale. Specifically on biomolecules, they have successfully applied the method to nucleotides and nucleosides [35,69,77], amino acids [33] and proteins [22,24]. In addition, Bennett and co-workers [83] have brought EFLC to purification settings by introducing a guide to scaling up from analytical to preparative separations.

In 2014 [84], Bamba and co-workers proposed to use the full range of co-solvent proportion, starting with a mobile phase containing nearly 100 % CO₂ and ending the gradient elution at 100 % hydro-organic composition. Because the first portion of the chromatogram was SFC, then crossing the EFLC area and ending in liquid chromatography conditions, the method was called “unified chromatography” (UC), a term previously used in similar experiments joining gas chromatography, SFC and LC [85,86]. Most modern

Table 2
Published SFC/EFLC methods to analyze peptides and proteins.

TFA is trifluoroacetic acid; TFE is trifluoroethanol							
Reference	Year	Biomolecule type	Example biomolecules with molecular weight	Samples	Stationary phase nature	Mobile phase co-solvent	Detection
[19]	1988	Cyclic peptide	Cyclosporin A (1202 Da)	Standards	C18	Methanol	CI(+)-MS
[31]	1999	Linear & cyclic peptides	Leucine enkephalin (555 Da); Ocytocin (1006 Da); Bradykinin (1060 Da)	Standards	Divinylbenzene	Ethanol with 50 mM heptadecane-sulfonic acid	UV (210 & 254 nm)
[62]	2000	Cyclic depsipeptides	Sulfomycin (1244 Da); Promothiocin (953 Da)	Standards and animal feed	Diol	Methanol with 0.2 % isopropylamine	UV (254 & 320 nm)
[63]	2004	Linear peptides and proteins	Gramicidin D mixture (\approx 1800 Da); Met-Enkephalin (573 Da); Angiotensin II (1045 Da)	Standards	Diol	Methanol with 0.5 % TFE, or with 0.2 % water, 10 mM ammonium acetate and 0.4 % isopropylamine	ESI-MS
[47]	2005	Linear proteins	Gramicidin D mixture (\approx 1800 Da)	Standards	Polystyrene-divinylbenzene	Methanol	UV (220 & 280 nm)
[48]	2006	Linear peptides	Bradykinin (1060 Da); Sauvagine (4600 Da); Urotensin II (1389 Da)	Standards	2-Ethylpyridine	Methanol with 13 mM TFA	ESI(+)-MS
[101]	2006	Linear proteins	Gramicidin D double helices	Standards	Polystyrene-divinylbenzene	n-Pentanol	UV (220 nm)
[20]	2006	Linear peptides	Myoglobin fragments (longest fragment 3742 Da)	Myoglobin pepsin digest	Silica	Methanol-Acetonitrile-Water-Formic acid (40:40:19:1)	ESI-MS
[49]	2008	Linear peptides and proteins	Cytochrome C tryptic digest (longest fragment 965 Da); Bacteriorhodopsin (24 kDa); PS II Core proteins (up to 39 kDa)	Standards and spinach leaves	Cyano	Methanol with 0.5 % TFE, or with 0.1 % TFA; or methanol-chloroform-formic acid (4:4:1)	UV (220 & 280 nm), ESI(+)-MS
[50]	2010	Linear peptides	Peptides with 2–8 residues (1042 Da)	Standards	2-Ethylpyridine	Methanol with 0.2 % TFA	UV
[51]	2011	Linear peptides	12-mer peptides (\approx 1200 Da)	Synthetic peptides	Pyridine, Diol, Amino	Methanol with 0.2 % TFA, or with 0.2 % isopropylamine, or with 10 mM ammonium acetate	ESI-MS
[46]	2012	Linear peptides	12-mer peptides (\approx 1200 Da)	Synthetic peptides	Silica, Pyridine, Diol, C18	Methanol with 5% water and 0.2 % TFA	ELSD, ESI-MS
[32]	2016	Cyclic peptides	Cyclosporins (\approx 1200 Da)	Standards	Silica	Ethanol with water	UV (220 nm)
[24]	2017	Proteins	Ribonuclease A (13.7 kDa); Bovine serum albumin (66.5 kDa); Transferrin (80.3 kDa)	Standards	Amide	Methanol-water (80:20) with 0.1 % TFA	UV (215, 254 & 270 nm), ESI-MS/MS
[96]	2017	Cyclic peptides	Macrocyclic peptides	Synthetic peptides	2-Ethylpyridine	Methanol with 0.1 % diethylamine	UV (220 nm)
[72]	2018	Linear proteins	Gramicidin D mixture (\approx 1800 Da)	Standards	Silica	Methanol with water (up to 8.7 w/w%)	UV, ESI-MS
[68]	2019	Linear and cyclic peptides, nucleosides	Gramicidin S (1141 Da); Linacotide (1525 Da)	Standards and synthetic peptides	Pyridine	Methanol with 5% water and 0.2 % ammonium hydroxide	UV, ESI-MS
[23]	2019	Proteins	Insulin (4679 Da); Ubiquitin (8565 Da); Myoglobin (17.6 kDa)	Standards	2-Picolylamine	Methanol-Acetonitrile (3:1) with 5% water and 0.2 % TFA	circular dichroism UV (220 nm)

Table 2 (Continued)

TFA is trifluoroacetic acid; TFE is trifluoroethanol							
Reference	Year	Biomolecule type	Example biomolecules with molecular weight	Samples	Stationary phase nature	Mobile phase co-solvent	Detection
[22]	2019	Proteins	Ribonuclease A (13.7 kDa); Lysozyme (14.3 kDa)	Standards	Polybutyl, Polypentyl	Methanol-water with 1 M ammonium acetate	UV (280 nm), ESI-MS/MS
[52]	2020	Linear peptides	Leucine enkephalin (555 Da); Angiotensin II (1045 Da); hydrophilic peptides up to 41-mer	Standards and synthetic peptides	Pyridine, Diol	Methanol with 0.1 % TFA and 0.1 % ammonium hydroxide	ESI-MS
[21]	2020	Proteins	Human insulin (5808 Da)	Standard and biosynthesized	Pentafluorophenyl	Methanol with 5% water and 0.2 % TFA	UV (220 nm)

Table 3

Published SFC/EFLC methods to analyze nucleic acid bricks.

DBN is 1,5-diazabicyclo [4.3.0] non-5-ene							
Reference	Year	Biomolecule type	Samples	Stationary phase nature	Mobile phase co-solvent	Detection	
[64]	2007	Nucleoside	Standards and mouse plasma	Silica	Methanol with 1% water and 11 mM ammonium acetate	APCI(+)-MS/MS	
[59]	2010	Nucleobases	Standards	Silica	Ethanol with 20 mM ammonium formate - formic acid buffer (pH 3)	UV (254 nm)	
[56]	2010	Nucleobases	Standards	Pyridine	Methanol with 5% water and 5 mM ammonium acetate	UV	
[35]	2011	Nucleosides	Standards	Amide	Methanol-buffer or acetonitrile-buffer (90:10) with 20 mM sodium acetate - acetic acid (pH 4.4.)	UV (262 nm)	
[77]	2011	Nucleosides and nucleotides	Standards	Amide	Methanol-buffer (90:10) with 75 mM ammonium phosphate, 5 mM DBN and 0.2 M NaCl	UV (262 nm)	
[103]	2013	Nucleoside	Standards and pharmaceutical formula	2-Ethylpyridine	Methanol	UV (254 nm)	
[57]	2015	Nucleoside	Standards and pharmaceutical formula	Chiral polysaccharide	Methanol with 0.25 % TFA	UV	
[69]	2016	Nucleosides and nucleotides	Standards	Amide	Methanol-buffer with 40 mM sodium phosphate (pH 2.65)	UV (270 nm)	
[58]	2017	Nucleobases and nucleosides	Standards and plant extracts	Silica	Methanol with 5 mM ammonium acetate	ESI(+)-MS	
[65]	2018	Nucleosides	Standards and digested tRNA of <i>E.coli</i>	2-Picolylamine	Methanol with 2% water and 10 mM ammonium acetate	ESI(+)-MS/MS	
[111]	2018	Nucleosides	Synthetic drugs and their impurities	Chiral polysaccharide	Ethanol	ESI(+/-)-MS	
[30]	2019	Nucleosides	Synthetic deazapurine nucleosides	Diol	Ethanol	UV (235 & 254 nm)	

instruments are perfectly capable to operate these wide gradients, with some instruments being clearly designed for easy switching between SFC and LC operation. UC was found most useful to achieve the elution of all proteinogenic free amino acids, from the most hydrophobic to the most hydrophilic [53,61]. Guillaume and co-workers also used the same method to elute metabolites with a wide range of polarities, including free amino acids [66] and nucleosides [87]. EFLC and UC approaches might be a part of the answer to achieve the analysis of a wider range of biomolecules.

Finally, because many of the target molecules possess amine functions (amino acids, peptides, proteins), a few words of caution

should be said about the possible reactions occurring between CO₂ and amines. Indeed, CO₂ is believed to react with some amines, possibly forming carbamic acids or ammonium carbamates [88]. However, this reaction is reversible so the original amine is usually found after CO₂ depressurization [89]. This may not be an issue for smaller, unstructured molecules like amino acids and peptides, but may be a problem for 3D-stable structures like proteins, if the amine reaction causes permanent unfolding of the protein. In a paper from Regalado and co-workers [68], a co-solvent comprising methanol, 5% water and 0.2 % ammonium hydroxide was employed, with gradient ranging from 5 to 50 % co-solvent. The combined use of water

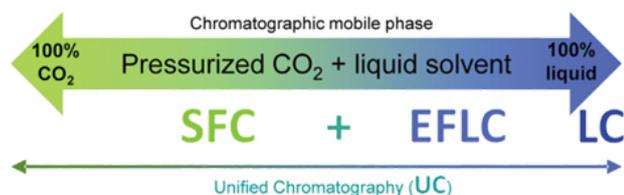


Fig. 4. Usual operating domains of SFC, EFLC and LC. Unified chromatography (UC) operates with large elution gradients covering a wide range of mobile phase compositions.

and NH_4OH were suggested to cause chaotropic effects favoring the elution of a variety of analytes including nucleobases, nucleosides, nucleotides and some peptides (linaclotide 1523 Da and gramicidin S 1882 Da). In a separate study from the same group [23], the authors found that some proteins like bradykinin and insulin could be recovered to their original higher-order structure after SFC purification, while others like ubiquitin, cytochrome C and apomyoglobin had permanently lost their higher-order structure. The co-solvent was a 3:1 mixture of methanol and acetonitrile, added with 5% water and 0.2% trifluoroacetic acid. The elution gradient ranged from 35 to 70% co-solvent in CO_2 , which is covering both the usual SFC and EFLC domains. Another recent paper from Govender and co-workers [21] presented the purification of biosynthesized human insulin with SFC. The co-solvent was methanol comprising 5% water and 0.2% trifluoroacetic acid, thus identical to the Schiavone et al. [23] mobile phase. The purified insulins retained their bioactivity, suggesting that the higher-order structure was indeed retained.

On another hand, Wang and Olesik also relate the elution of intact proteins [22] in EFLC mode starting from a hydrophobic interaction chromatography (HIC) method, and progressively introducing CO_2 in the 70:30 methanol-ammonium acetate buffer mobile phase. In addition to improved chromatographic efficiency, the introduction of CO_2 was shown to improve ESI-MS detection through facilitated desolvation. However, the claim was on intact molecular weight, suggesting that no permanent reaction had damaged the primary structure, but no information was given on preservation of higher-order structure.

4. Stationary phase nature, particle type and dimension

The range of stationary phases available for packed column SFC and EFLC was always large, as any stationary phase designed for liquid chromatography can also be used with CO_2 in the mobile phase. The nature of stationary phases has however greatly expanded in the recent years as not only reversed-phase [90,91] and normal-phase [92] stationary phases are possible but also the most recent stationary phases designed for HILIC mode [93] and for mixed-mode liquid chromatography [94] and several novel stationary phases especially designed for SFC use [95]. The diversity of available stationary phases is an interesting feature of SFC and EFLC because it means that different selectivities are available to achieve a given separation. The role of a stationary phase is twofold: it must interact with the target analytes to (i) retain them and (ii) separate them. It is customary in SFC method development to start developing a method by screening several stationary phases with complementary selectivities, to identify a good starting point for optimization. Judging from the above discussion on the biomolecules polarity, it appears that many different stationary phase chemistries could be useful as our target molecules comprise both polar and non-polar molecules. In addition, the molecules requiring some separation may have very different structural differences. For instance, when nucleobases must be resolved, polar interactions are necessary to distinguish between small polar ana-

lytes with subtle differences, thus a polar stationary phase is necessary. When large hydrophobic peptides must be separated, a non-polar stationary phase may be a best candidate.

As most of them possess a chiral center, underivatized amino acids were often analyzed on enantioselective stationary phases. The most cited phases were polysaccharide-based stationary phases [60,96], which are most popular in enantioselective SFC. Other possible enantioselective phases are *Cinchona* alkaloid-based zwitterionic stationary phases, which were specifically designed for amino acid enantioresolution in LC [53,61], macrocyclic glycopeptide stationary phase [42,97] and crown-ether stationary phase [55], which are both also known to perform well for amino acids in liquid phase.

When no enantioresolution was desired and only intra-class separation of free amino acids was looked for, a variety of achiral stationary phases were used. Diol-type stationary phases are most often cited [34,45,67,82], starting from the seminal paper from Camel and co-workers [40]. Toribio and co-workers proposed to couple a chiral glycopeptide stationary phase to an achiral diol phase to resolve two pairs of enantiomers (phenylalanine and tyrosine) in a single analysis with SFC-UV [97]. *Cinchona* alkaloid-based zwitterionic stationary phases also allowed a most complete separation of all proteinogenic amino acids from each other, together with enantioresolution for most of them [53]. Interestingly, the four isobaric leucine enantiomers and isoleucine enantiomers could all be resolved [61]. Finally, other achiral stationary phases cited for the SFC/EFLC analysis of free amino acids are phases normally used in HILIC mode, like the anionic polysulfoethyl stationary phase [33], the zwitterionic sulfobetaine-bonded stationary phase [66], bare silica gel [66] or amide-bonded stationary phase [54].

For peptides and proteins, where chiral separation is rarely desired, achiral stationary phases with pyridine ligands were the most often cited stationary phases [21,23,46,48,50–52,72,98]. 2-Ethylpyridine is a stationary phase that was designed specifically for SFC at the beginning of the years 2000s by Princeton Chromatography, and found an immediate success for basic drugs because it provided nice peak shapes without the need of mobile phase additives, supposedly due to hydrogen bonding between residual silanol groups and the pyridine nitrogen atom [99]. Other variations include a 4-ethylpyridine ligand [51,52] or pyridyl-amide. A polymeric version of 4-ethylpyridine, poly(4-vinylpyridine) (P4VP) was also cited [68]. 2-Picolylamine, appearing in the latest publications [23,72], is a most recent version of 2-ethylpyridine, which was introduced to limit retention loss through silyl ether formation [100]. Other polar stationary phases used for peptides and proteins include again diol-type stationary phases [46,51,52,62], bare silica gel [20,32,46,72] and amide bonding [24], but also cyanopropyl-bonded silica [49,63]. Non-polar and moderately polar phases, which are less frequently encountered in SFC applications, also appear in a few instances. For example, aromatic phases like pentafluorophenyl (PFP) [21] and polystyrene-divinylbenzene (PS-DVB) [31,47,80,101] were mentioned in a few occasions, starting with the first paper reporting SFC analysis of both cyclic and linear peptides [31]. Finally, alkyl-type stationary phases were also mentioned: octadecylbonded-silica (ODS) was used in the first report of cyclic peptide analysis (Cyclosporin A) [19] and later to resolve isomeric 12-mer peptides [46]. Polybutyl and polypropyl phases normally used in HIC mode (hydrophobic interaction chromatography) were recently employed to analyze intact proteins in EFLC [22].

To those less familiar with the technique, it may be surprising to observe that there seems to be little rationale behind the use of one column or another as very different stationary phases were found to be useful for the same purpose by different users. For instance, the cyclic peptides of cyclosporins were analyzed on an ODS phase [19] or on bare silica gel [32]. Gramicidin, a membrane protein compris-

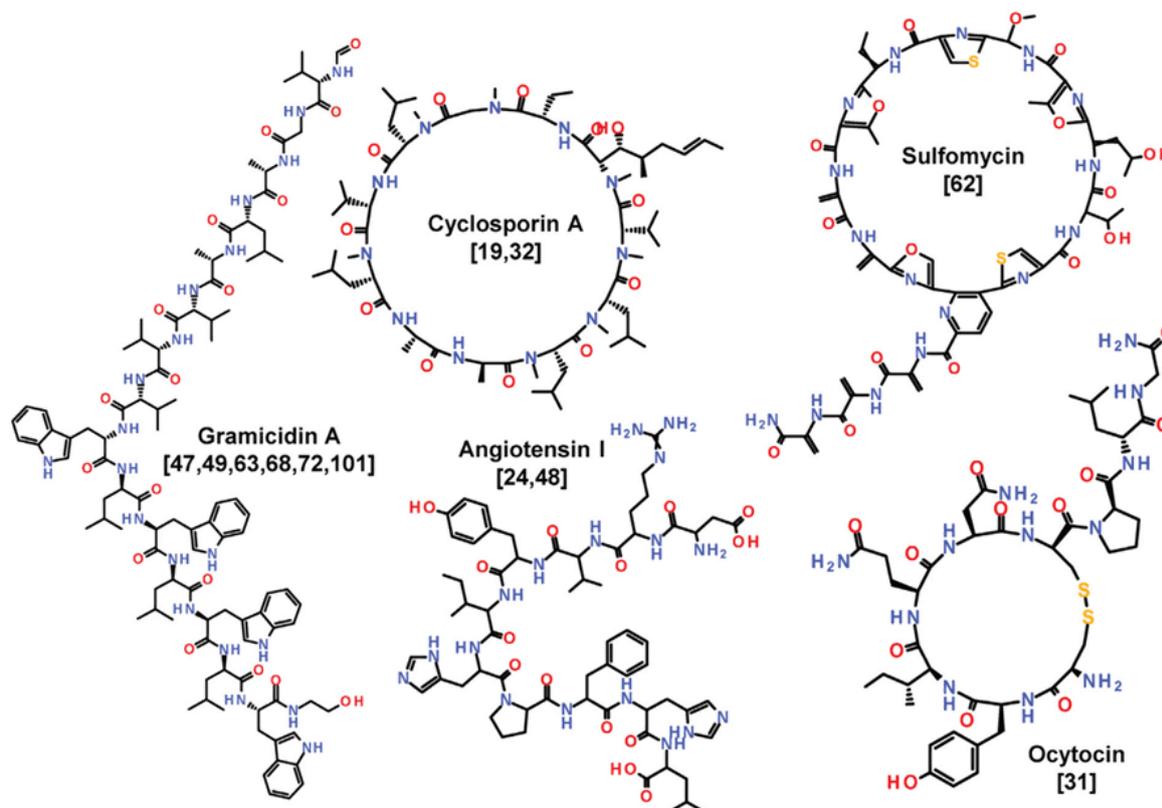


Fig. 5. Sample peptides analyzed with SFC and EFLC.

ing several members, was also analyzed by several different authors on different stationary phases: cyanopropyl-bonded silica [49,63], PS-DVB [47,101], bare silica gel [72] or pyridine phases [68,72]. Different pairs of 12-mer isomeric peptides (about 1100 Da) were successfully resolved on a variety of stationary phases including ODS, bare silica gel or 2-ethylpyridine [46]. Actually, it is often observed in SFC that several very different stationary phases can be successful for the same separation problems, although different elution orders would be observed [102].

The case of nucleic acid bricks is different because, as appeared in Fig. 3, nucleobases, nucleosides and nucleotides are all polar, with negative log P values. Thus retention and selectivity in that case is favored by polar stationary phases with bare silica [58,59,64], diol [30,56,58,67], amide [35,58,69,77] and pyridine-type [30,56,65,103] being equally cited.

The improved instruments for analytical SFC introduced at the beginning of the years 2010 tended towards higher efficiency through reduced dead volumes and detector cells. Concomitantly, there has been a trend to high efficiency packed columns, either through small fully porous particles (FPP) with sub-2 μm diameter, or through superficially porous particles (SPP) with sub-3 μm diameter. In the recent papers, sub-2 μm FPP stationary phases appear [24,65,103]. However, when a large elution gradient is desired, the viscosity of the mobile phase at the end of the gradient is high. Because small particles also generate high pressure drops, not all SFC instruments can sustain the high pressure. Indeed, some instruments are still limited to 400 bars inlet pressure (Waters, Jasco), while others can reach 600 bars (Agilent) or 660 bars (Shimadzu). In that case, the flow rate and/or back-pressure must be reduced to avoid reaching the upper pressure limit. Another option is to favor columns packed with SPP stationary phases [66].

5. Detection issues and hyphenation to mass spectrometry

Packed-column SFC and EFLC make use of the same detectors as LC, namely UV-vis or diode-array detection (DAD), evaporative light scattering detection (ELSD), Corona charged-aerosol detection (CAD) and mass spectrometry (MS). The latter is preferably equipped with LC-type ionization source, namely electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI).

Among all these detectors, not all are equally useful for the target biomolecules. Amino acids, their oligomers and polymers mostly lack of chromophores, thus UV and DAD detection are of little use, unless the desired detection limit is not too low [54]. Bigger peptides and proteins and those comprising aromatic residues may however be easier to detect with UV at 210 or 280 nm. Because the nucleobases are good chromophores, UV and DAD detection is appropriate for the nucleobases, nucleosides, nucleotides and nucleic acids.

While UV detection is placed before the back-pressure regulator, ELSD and CAD are placed after the BPR. Only a few studies explored ELSD possibilities in SFC [40,46,96] or in EFLC [33] for underivatized amino acids and small peptides.

Because it provides additional information on analyte structure, mass spectrometry is certainly the most desirable detection mode hyphenated to SFC/EFLC instruments, especially now that it is easier to access in most laboratories, with simpler instruments available at a moderate cost. Hyphenation of SFC/EFLC to MS may be done several ways [104,105]. First, flow splitting may be done – or not – prior to entering the MS, with one portion of the column effluent entering the MS and the other being directed towards the back-pressure regulator (BPR) and waste or fraction collection [106]. When the chromatographic flow rate is high, the flow is preferentially divided

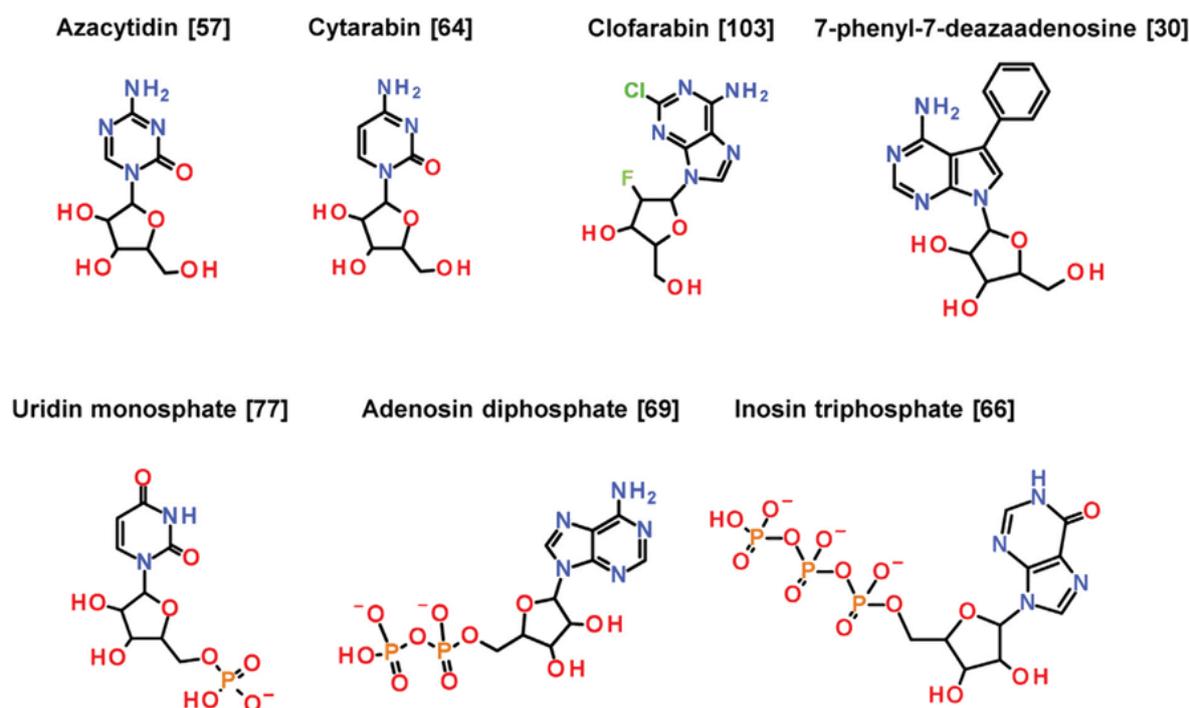


Fig. 6. Sample nucleosides and nucleotides previously analyzed with SFC and EFLC.

because the ionization sources cannot all cope with the large volume expansion caused by CO₂ depressurization. However, this can affect sensitivity for mass-sensitive detectors. As the largest portion of SFC instruments sold are based on flow-splitting configuration, this is quite logically the most often cited in the literature related to biomolecules. However, when the chromatographic flow rate is low, the whole effluent can be directed to the MS, which is then placed right after the BPR. Second, a make-up fluid may be introduced prior to entering the MS, and prior to the flow splitting, when flow splitting is desired. The interest of a make-up fluid is to avoid analyte precipitation upon the cold CO₂ depressurization and to introduce ionization enhancers prior to entering the MS source. However, the make-up fluid may cause further dilution of the column effluent, thereby may be reducing sensitivity for concentration-sensitive detectors. Deconvoluting the positive and negative effects of the make-up fluid is difficult, so the variations observed are not always in accordance with intuition [107].

The composition of make-up fluid is most often methanol, comprising a small proportion of water (1–10%) and one acidic or salt additive. This additive is sometimes the same as the one employed in the mobile phase [45,54], sometimes different [55,61] because the composition favoring good chromatography is not necessarily the same as the composition favoring good ionization. A supposedly generic composition and flow rate are often adopted and their impact was rarely studied [45]. When large elution gradients are employed, especially in UC experiments, a constant flow rate of make-up fluid could be inadequate.

The choice of ionization source, which depends on analyte size and polarity, has a significant impact on the intensity of molecular and fragment ions detected. ESI and APCI were employed in almost all studies. Wolrab et al. [34] compared ESI and APCI sources specifically for SFC-MS/MS analysis of amino acids and related compounds. They concluded that ESI seemed to be the best choice for the least polar amino acids. However, for polar amino acids and non-polar amino acids with an additional heteroatom as well as

acidic amino acids, APCI seemed the best choice. In other words, the preferred ionization source will be greatly dependent on analyte, even within one molecular family.

In terms of analyzers, some recent investigations were done with triple-quadrupole [34,45] or quadrupole-time-of-flight [22,24,65,82] analyzers, which can provide precise structural information the target analytes. When complex matrices are present, or high molecular weight biomolecules are considered, high resolution is necessary.

6. Applications areas

Due to the limited number of papers published to date, applications only articulated around three themes. First, food products were the topic of a few papers. Sánchez-Hernández et al. [97] coupled chiral and achiral stationary phases to control the quality of food supplements and confirm the absence of enantiomeric impurities of free amino acids with UV detection. Raimbault et al. [61] used a single chiral stationary phase for quantitative assessment of free branched amino acids in food supplements with single-quadrupole MS detection. Huang and co-workers [54] determined 11 free amino acids in several varieties of tea with an achiral stationary phase and single-quadrupole MS detection. The method was fully validated. Ashraf-Khorassani et al. [62] focused on depsipeptides, which are peptides with some amides functions replaced by esters. The authors developed a quantitative extraction method for some growth-promoting animal feed additives, and analyzed them with SFC-UV. The target molecules were the major components of alexomycin like sulfomycin (see structure in Fig. 5) and promothiocin.

Secondly, human/animal [34,45,66,67,87] or plant [82] metabolomics studies are also concerned with biomolecules separations in SFC/EFLC or UC. Liquid-phase chromatography hyphenated to mass spectrometry is currently dominating the metabolomics scene. However, because the samples are complex and the metabolites are extremely varied in terms of structures and

polarities, the potential of other methods to offer complementary information to other existing techniques is of great interest, as exposed by Shulaev and Isaac [108]. Wolrab et al. [34] focused on MS detection of free amino acids and related compounds in human serum. Amino acids, peptides, and nucleosides were all included in SFC-MS methods from Sen and co-workers [67], and from Akbal and Hopfgartner [45]. The latter compared the results to those of RPLC and HILIC. Many metabolites previously included in the method development were identified in human urine within fast SFC-MS runs. In a different study, Desfontaine et al. [66] focused on a most generic method to analyze a wide range of metabolite polarities, including most polar amino acids as well as non-polar lipids. For this purpose, a wide UC elution gradient was favored, and was later applied to real samples of plasma and urine [87]. Similarly, Grand-Guillaume and co-workers [82] developed a widely applicable SFC-MS/MS method including a great variety of plant metabolites, from the most polar free amino acids to the least polar lipids and terpenes. They later demonstrated the interest of combining LC-MS/MS and SFC-MS/MS to acquire a most complete knowledge on plant extracts [109].

Finally, analysis and purification of biomolecules for pharmaceutical and clinical applications were the most abundant among applied studies. Therapeutic peptides may contain a large number of impurities requiring identification and elimination, where chromatographic and electrophoretic methods are most useful [110]. Method development for peptides and proteins were well exposed in several papers [21,23,28,52,63,68,72,98]. Typical examples of therapeutic peptides are presented in Fig. 5, like gramicidin, cyclosporin, angiotensin and oxytocin. As mentioned above, proteins set particular problems in that the ternary structure should be preserved in purification methods. Free amino acids were analyzed in a traditional medicinal plant with SFC-MS [53]. Nucleobases and nucleosides were also searched in ginseng, a traditional Chinese medicine [58]. Other synthetic nucleosides were the topic of clinical studies: azacitidine, clofarabine and cytarabine (see structures in Fig. 6) were analyzed with SFC along with their impurities following ICH guidelines [57,64,103]. For instance, cytarabine was quantified in mouse plasma and the SFC-MS/MS method was compared to LC-MS/MS [64]. Finally, Pirrone et al. showed an interesting application of SFC-MS for impurity fate mapping of a nucleoside drug substance, where reversed-phase liquid chromatography failed to deliver the desired answers due to keto-enol tautomerization in aqueous conditions [111].

7. Conclusion and perspectives

As appeared in this review, amino acids, peptides, proteins and nucleic acid bricks are widely diverse molecules in terms of sizes and polarities. It comes as no surprise that no consensus stationary phase and mobile phase compositions have emerged for their SFC/EFLC analysis. Significant diversity in stationary phase chemistry and mobile phase composition was observed, although recent papers mostly cite polar stationary phases and wide elution gradients comprising some portion of water in the co-solvent, along with additives. Rather than a source of confusion, this diversity should be perceived as a great potential for successful method development and optimization. MS proves to be an essential tool for structure identification but UV detection still comes in handy for quantitative analysis of samples with low complexity, or when sensitivity is not an issue. This is typically the case in quality control of pharmaceuticals, food products or extracts of natural products.

Applications of biomolecules with SFC/EFLC clearly deserve more attention than is currently the case. At the analytical scale, the complementary information that can be obtained when comparing to most often used RPLC methods should justify the investment. At

the preparative scale, the time and solvent economy are well worth the effort. Finally, unified chromatography (UC) slowly emerges with very promising capacities shared from both LC and SFC/EFLC technologies.

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.

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V- Conclusion des recherches bibliographiques

Nous avons traversé, au fil de ce premier chapitre, une partie de l'histoire des méthodes chromatographiques s'appuyant sur le CO₂ sc. Cet état de la matière utilisé comme éluant a permis d'analyser des composés peu polaires dans un premier temps. L'évolution technologique autour de l'instrument de SFC et des colonnes remplies SPP ou FPP a contribué à la forte amélioration des performances analytiques. La possibilité d'augmenter les proportions de co-solvants, jusqu'à permettre l'analyse par UC, amène l'application du CO₂ sc en chromatographie vers de nouveaux horizons qui ne sont pas encore totalement exploités. Cette nouvelle application chromatographique qu'est l'UC accroît les capacités d'analyse vers des composés toujours plus polaires sans se départir des molécules moins polaires préalablement analysables par SFC. La figure 10 de Losacco présente la couverture de polarité des techniques chromatographiques liquides ou supercritiques selon la polarité des analytes. Elle permet de mettre en avant la large gamme applicable de l'UHPSFC et encore plus celle de l'UC.

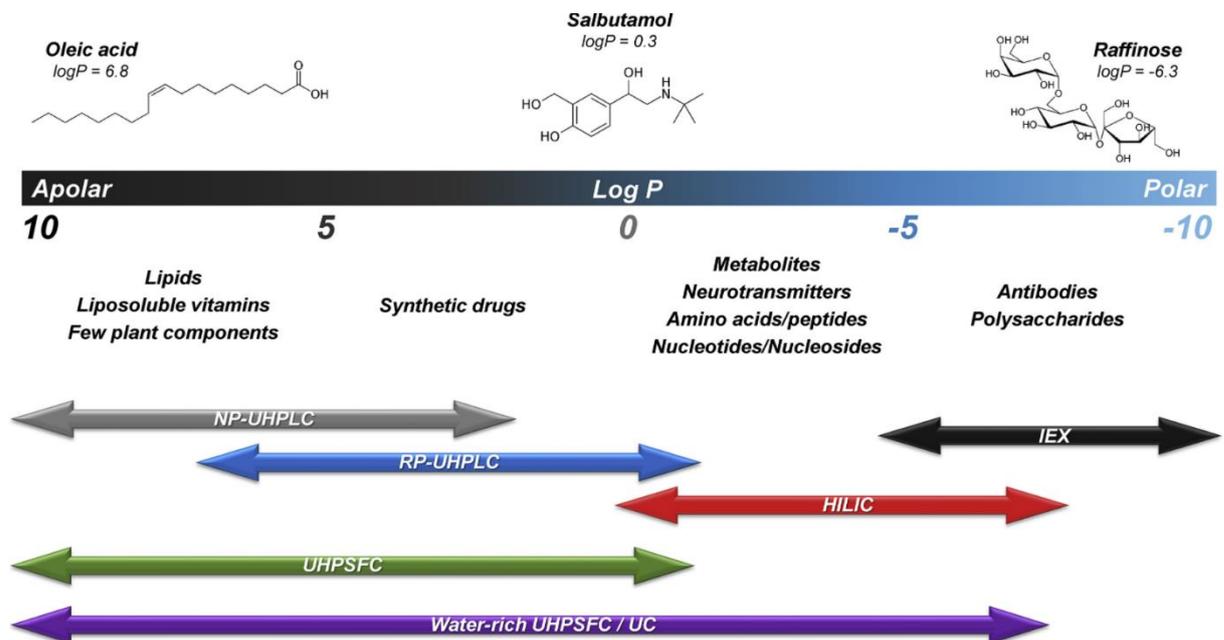


Figure 10 : Gamme de polarité des composés analysables selon la technique chromatographique, repris de [127]

Pour étudier les possibilités de cette méthode UC appliquée à des familles de biomolécules à polarité variée, nous avons discuté des flavonoïdes et des peptides, deux exemples à étudier. La variabilité des structures des deux familles nous permettra de mettre en perspectives les réussites et difficultés rencontrées lors de développement UC-MS. C'est dans cette direction que nous souhaitons vous emmener lors des prochains chapitres.

Chapitre 2 : La chromatographie unifiée pour l'analyse de flavonoïdes à visée pharmaceutique ou cosmétique

I- Introduction

Nous avons introduit les flavonoïdes dans le chapitre précédent en indiquant que l'analyse de flavonoïdes par CO_2 SC s'effectuait sur des molécules de plus en plus polaires. Nous souhaitons à travers ces travaux de thèses développer des méthodologies en chromatographie unifiée pour l'analyse de petites biomolécules. De ce fait, la famille des flavonoïdes est une porte d'entrée vers cet objectif.

En tant que biomolécules, les flavonoïdes recensés ont un poids moléculaire inférieur à 1 kDa et une gamme de polarité allant de génines (motif racine non glycosylé) apolaires jusqu'à des formes poly-glycosylées donc polaires [170]. Pour mettre en évidence la diversité relative des flavonoïdes, nous avons repris les propriétés physico-chimiques de 64 flavonoïdes auxquels nous ajoutons les 9 standards et les 2 échantillons commercialisés discutés par la suite, dans la partie II de ce chapitre. Toutes les propriétés physico-chimiques ont été calculées à l'aide de l'outil MolDesc (<http://moldesc.icoa.fr>). La figure 11 met en avant la distribution de l'ensemble des 75 flavonoïdes. Tous ces flavonoïdes sont divisés en sous catégories selon le nombre de glycosylation et le nombre d'unités saccharides. On retrouve les aglycones (Ag), les monoglycosylés d'un monosaccharide (MdM), d'un disaccharide (MdD) ou d'un trisaccharide (MdT), les disubstitués d'un monosaccharide (DdM) ou un exemple de flavonoïde estérifié (Es). Cette sélection de molécules se compose en très grande majorité de flavonoïdes non susceptibles d'être ionisés dans nos conditions analytiques. L'ensemble des structures est disponible en annexe 1. Seuls quelques anthocyanes, qui sont des flavonoïdes ionisés, ont été inséré dans la sélection mais aucun n'a été inclus dans nos standards pour développer la méthode UC-MS. L'ensemble des bulles entouré de rouge correspond aux standards analysés dans la partie II de ce chapitre tandis que les bulles entourées de bleus concernent les deux échantillons commerciaux. Cette figure permet de mettre en évidence la couverture de nos standards en termes de polarité et de poids moléculaires sur cet ensemble de flavonoïdes. Les deux produits commerciaux étudiés sont des hétérosides, le plus polaire étant un flavonoïde monoglycosylé d'un trisaccharide. La figure 11 permet également de visualiser les flavonoïdes selon le nombre d'unité saccharide fixée sur la génine. Plus ce nombre d'unité saccharide est important et plus le flavonoïde est polaire.

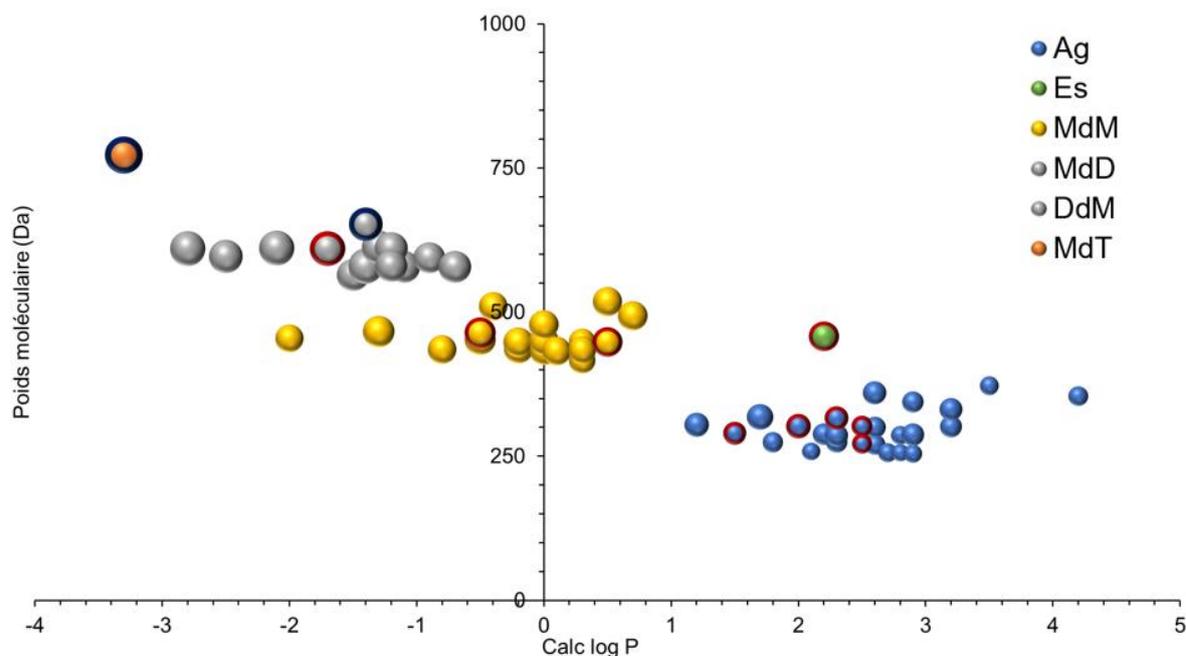


Figure 11 : Répartition des flavonoïdes sous formes d'hétérosides ou aglycones selon le poids moléculaire et la polarité, la taille des bulles représente la TPSA ou surface totale polaire.

Les flavonoïdes permettront la mise en place d'une première méthode UC-MS sur un ensemble de biomolécules à polarité variée avec des log P variant principalement de -4 à +4 ce qui correspond à des variations de TPSA allant de 60 à 300.

Premièrement, nous avons réalisé un développement de méthode en UC-MS. L'étape de screening sur de multiples colonnes a mis en évidence les performances séparatives de deux colonnes (Torus Diol et Torus DEA). Par la suite, l'étude de la composition de la phase mobile nous a permis de tester plusieurs additifs dans le méthanol (acide formique, acide citrique, acide phosphorique, acide méthanesulfonique et hydroxyde d'ammonium). Cependant l'acide citrique et l'acide phosphorique ne sont pas volatils ce qui fait que ces deux acides sont incompatibles avec une détection en MS. La nécessité d'un gradient de co-solvant liquide allant jusqu'à 100% pour éluer le gallate d'épigallocatechine, nous a contraint à la mise en place d'un gradient inverse de contre-pression et d'un gradient inverse de débit, comme décrit sur la figure 12. Ces deux gradients inverses vont compenser la montée en pression de la pompe à cause du gradient de co-solvant et éviter d'atteindre la limite supérieure de la pression du module à 414 bars.

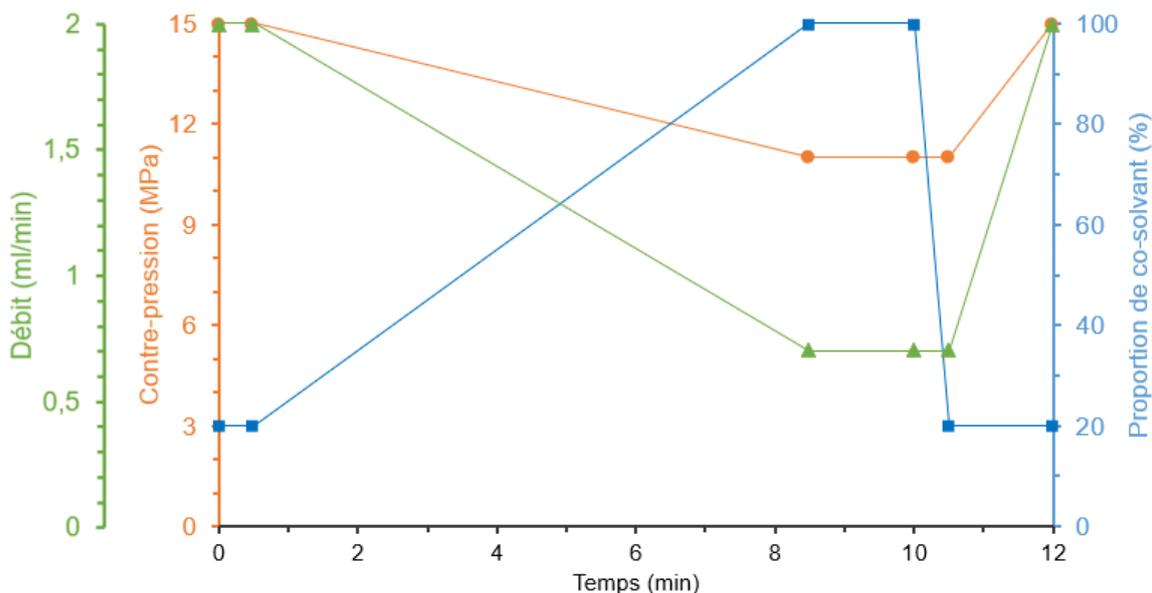


Figure 12 : Application d'une méthode UC-MS avec un gradient de pression inverse et un gradient de débit inverse, repris de [171]

Du côté de la détection en MS, nous avons étudié les paramètres de source du détecteur QDa. Ce module d'ESI-MS présente un nombre limité de paramètres modulables pour optimiser la réponse, une large part des paramètres ayant été fixée par le fabricant. Ce travail s'est appuyé sur un plan factoriel complet sur 3 paramètres à 3 niveaux d'études. De plus, à la suite de ce plan, l'additif dans la composition du solvant additionnel ou « make-up » a été optimisé en ESI. L'acide formique, l'acide méthanesulfonique ainsi que l'hydroxyde d'ammonium dans l'eau comme additifs mélangés au méthanol sont comparés sur différents rapports signal-sur-bruit (s/n) d'ions spécifiques.

Ce développement de méthode adapté à notre sélection de standards permet par la suite d'investiguer deux produits commercialisés, l'un à visée cosmétique, l'autre à visée pharmaceutique. L'objectif numéro 1 était de vérifier la pureté des produits. Le second objectif était d'identifier les composés majeurs détectés en croisant les informations des analyses RMN et MS.

II- Développement d'une méthode « générale » en UC pour des composés pharmaceutiques et cosmétiques

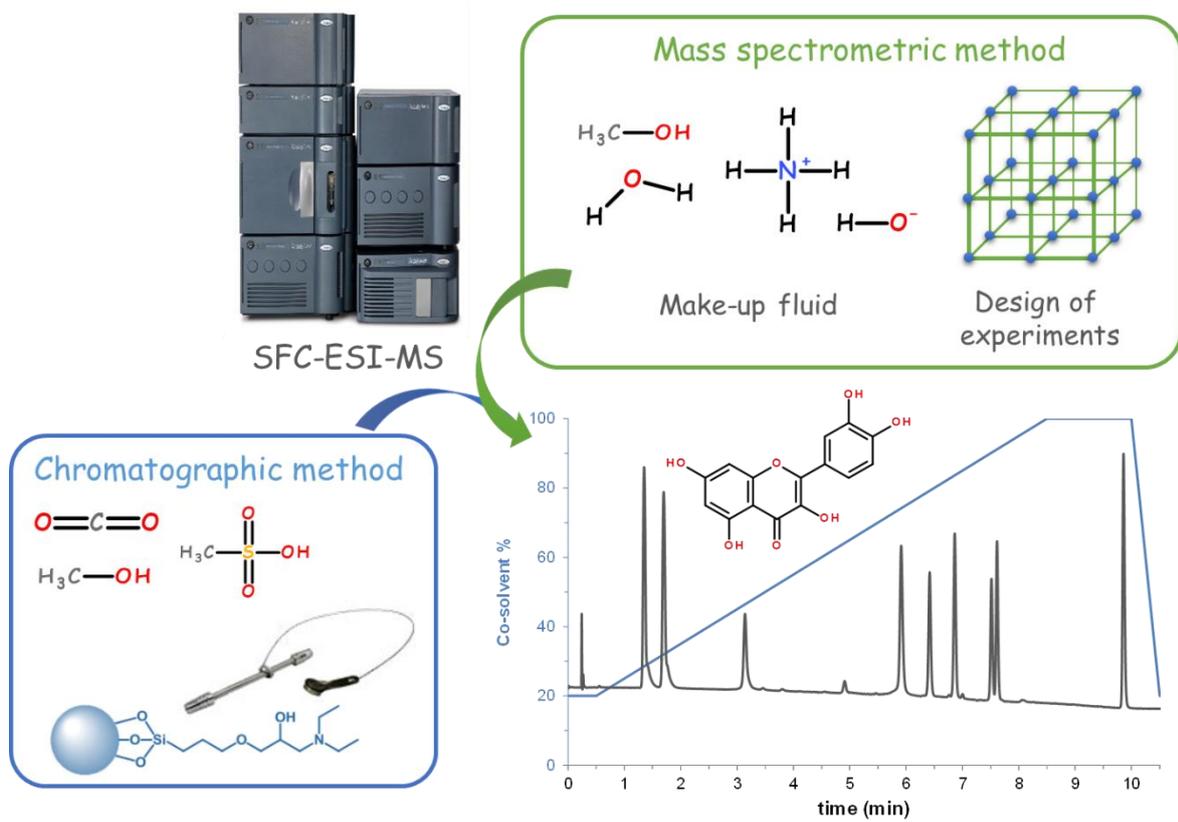


Figure 13 : Schématisation du développement de la méthode et de l'analyse de flavonoïdes par UC , repris du résumé graphique de [172]



Analysis of flavonoids with unified chromatography-electrospray ionization mass spectrometry—method development and application to compounds of pharmaceutical and cosmetic interest

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Received: 15 January 2020 / Revised: 26 June 2020 / Accepted: 1 July 2020 / Published online: 10 July 2020
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Abstract

In this project, we aimed at analysing flavonoid-type compounds with unified chromatography (joining supercritical fluid chromatography and enhanced fluidity liquid chromatography with carbon dioxide-methanol mobile phases covering a wide range of compositions) and diode-array and electrospray ionization mass spectrometric detection (UC-DAD-ESI-MS). First, the chromatographic method was developed for 9 standard flavonoid molecules from three different families (flavanols, flavanones and flavonols, glycosylated or not), with a strong focus on mobile phase composition to achieve the elution of a wide range of flavonoids with good chromatographic quality (efficiency and resolution). For this purpose, two stationary phases were selected (ACQUITY UPC² DEA and Diol), and five different additives (formic acid, citric acid, phosphoric acid, methanesulfonic acid and ammonium hydroxide) were successively introduced in the methanol co-solvent. The composition containing 0.1% methanesulfonic acid in methanol was retained as it provided the best chromatographic quality together with the possibility of hyphenating the chromatography to mass spectrometry. The DEA column appeared to provide the best efficiency and was retained for further method development. The gradient method was then optimized to achieve a fast analysis, which involved elution with a wide range of mobile phase composition (from 20 to 100% co-solvent in methanol) together with reversed flow rate and reversed pressure gradients at fixed temperature. The final gradient lasted 10 min, followed by 2.5 min of re-equilibration. Then, ESI-MS detection was optimized. Because the single-quadrupole mass spectrometer employed (ACQUITY UPC² QDa) allowed the variation of only a few parameters, a design of experiments was used to define the best compromise for three parameters (probe temperature, cone voltage and capillary voltage). The make-up fluid introduced before entering the MS was also varied: different compositions of methanol-water containing either formic acid, ammonium hydroxide or sodium chloride were tested. The best results in terms of signal-to-noise ratio were obtained with methanol containing 20 mM ammonium hydroxide and 2% water. The optimal UC-DAD-ESI-MS method was then applied to two different flavonoid formulation ingredients. The first one, hidrosmin (5-*O*-(β -hydroxyethyl)diosmin), is known for its vasoprotective properties and therefore employed in pharmaceutical formulations. The second one, α -glucosyl-hesperidin (sometimes referred to as vitamin P), is employed in cosmetic formulations. Identification of the major compounds in each sample was achieved with the help of MS detection.

Keywords Cosmetics · Flavonoids · Enhanced fluidity liquid chromatography · Pharmaceuticals · Supercritical fluid chromatography · Unified chromatography

Published in the topical collection featuring *Female Role Models in Analytical Chemistry*.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00216-020-02798-z>) contains supplementary material, which is available to authorized users.

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Introduction

Flavonoids are naturally occurring compounds comprising different structural families like flavones, isoflavones, flavanones, flavanols, flavonols, chalcones and anthocyanidins [1]. As appears with some examples in Fig. 1, the genin or aglycone (root hydrocarbon structure) bears several oxygen atoms and may be glycosylated (with one or several sugar moieties)

or not (aglycone form). Some of them, like flavanols and flavanonols, also display some chirality in the aglycone structure. They may be monomeric or polymeric (flavanols). They are all secondary metabolites produced by plants and a great variety of them (several thousands) have been described. They have recognized antioxidant properties and several health-promoting effects [2], including anti-inflammatory, anti-bacterial, anti-allergic, anti-cancer, cardio-protective, promotion of the immune system, or protection against pulmonary diseases. Applied on the skin, they can prevent ageing caused by free radicals and UV and protect the skin against cutaneous inflammation [3]. Due to these numerous interesting bioactivities, their presence in the plant kingdom has been largely used through medicinal plants [4, 5] or food supplements [6]. They have also been introduced in pharmaceutical and cosmetic formulations in their natural or chemically modified forms. They may be used as active ingredients or as excipients for their antioxidant properties [3] to protect the other ingredients from oxidation.

Due to their numerous bioactivities, the research on flavonoids has greatly expanded in the past decades. Quite logically, the number of separation methods to be applied for the analysis or purification of flavonoids has also been abundant. Most chromatographic analyses of flavonoids are conducted in high-performance liquid chromatography (HPLC), principally in the reversed-phase (RP) mode [1], with diode-array detection (DAD) or hyphenated to mass spectrometry (MS). The latter is a powerful tool to identify the molecules but is not always capable to differentiate all isomers, which are frequently encountered in natural products. Supercritical fluid

chromatography (SFC) has been demonstrated to be an interesting alternative to RP-HPLC as it can offer complementary selectivity. Indeed, when it is conducted on a polar stationary phase with carbon dioxide-alcohol mobile phase, SFC operates in normal-phase (NP) chromatographic mode and thus provides opposite elution order from RP-HPLC [7, 8], together with different capability for the separation of isomeric compounds [9, 10]. As flavonoids can have many isomeric species, a separation method that can effectively resolve them should be of interest.

While early works reporting SFC analyses of flavonoids were rather related to the most hydrophobic polymethoxylated flavones found in citrus essential oils [11, 12], the recent works concern more polar, hydroxylated and glycosylated species. Indeed, SFC was employed several times for flavonoid analyses in the recent years, mostly for their profiling in medicinal plants [9, 13, 14] and particularly for traditional Chinese medicine [4, 7, 8, 15–19], and also for food products [6, 20] and biological samples [21, 22].

Table 1 summarizes 20 papers relating SFC analyses of flavonoids. Different structural families were analysed with older papers reporting solely on aglycone flavonoids, while more recent papers included both aglycone and glycosylated species. As appears from Table 1, the stationary phases employed for this task were mostly polar like bare silica gel, 2-ethylpyridine-bonded silica (or 2-picolylamine, a modern version of 2-ethylpyridine) and propanediol-bonded silica. To elute aglycone and glycoside flavonoids in a single analysis, gradient elution was usually preferred, with increasing proportion of solvent (most often methanol) in carbon dioxide.

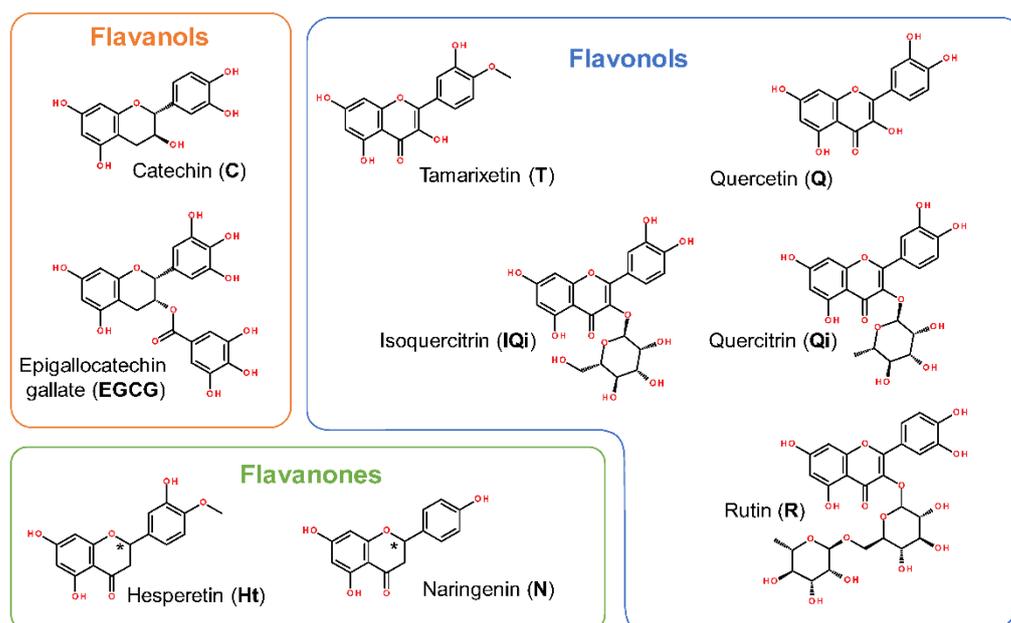


Fig. 1 Structures of the standard molecules used to develop the UC-DAD-ESI-MS method

Table 1 Previous supercritical fluid chromatographic methods to analyse flavonoids and some of their characteristics (full details in ESM Table S1)

Reference	Year	Flavonoid type	Glycosides	Sample	Stationary phase	Mobile phase co-solvent	MS detection	Make-up solvent for MS
[11]	1991	Polymethoxylated flavones	Aglyca	Citrus essential oils	Silica	MeOH; 10% isocratic		
[12]	1996	Polymethoxylated flavones	Aglyca	Citrus essential oils	Silica	MeOH; 10–30% gradient		
[23]	1999	Flavonols	Aglyca	Standards	Phenyl	EtOH + 0.1% phosphoric acid; 10% isocratic		
[24]	2002	Flavanols	Aglyca	Grape seed extracts	Propanediol-bonded silica	MeOH + 0.25% citric acid; 7–45% gradient		
[13]	2014	Flavones	Aglyca and glyco-sides	Chamomile extracts	2-Ethylpyridine	MeOH-iPrOH 1:1 + 0.5% formic acid; 5–20% gradient	ESI(+)-MS	MeOH + 0.1% formic acid
[6]	2015	Isoflavones	Aglyca and glyco-sides	Dietary supplements	Hybrid silica	MeOH + 0.05% phosphoric acid; 2–25% gradient		
[14]	2016	Unidentified		<i>Ganoderma lucidum</i> mushroom extracts	Propanediol-bonded silica	MeOH + 20 mM ammonium acetate; 5–50% gradient	ESI(+/-)-MS	na
[9]	2016	Flavones, flavanols and flavonols	Aglyca and glyco-sides	Standards and different plant extracts	Propanediol-bonded silica, octadecylsiloxane-bonded silica and 2-ethylpyridine-bonded silica	MeOH + 2% H ₂ O and 10 mM ammonium formate; 2–40% gradient	ESI(+/-)-MS/MS	EtOH, 0.8–0.3 mL/min reversed gradient
[25]	2016	Flavones and isoflavones	Aglyca	Standards	Cyanopropyl-bonded superficially porous silica	MeOH + 20 mM ionic liquid		
[8]	2017	Flavones and flavonols	Aglyca and glyco-sides	<i>Chrysanthemum morifolium</i> extracts	Silica	MeOH + 0.1% phosphoric acid; 10–20% gradient		
[15]	2017	Isoflavones	Aglyca and glyco-sides	<i>Radix astragali</i> extracts	Pentafluorophenyl	MeOH; 5–40% gradient		
[21]	2017	Isoflavones	Aglyca and glyco-sides	Human serum	Diethylaminopropyl-bonded silica	MeOH + 0.5% formic acid; 15–40% gradient		
[20]	2018	Flavanones, flavones and flavonols	Aglyca and glyco-sides	Garlic extracts	Propanediol-bonded silica	MeOH + 0.1 mM oxalic acid + 1 mM ammonium formate; 0–50% gradient elution	ESI(-)-MS/MS	na
[26]	2018		Aglyca	Bee pollen	Polybutylene-terephthalate	MeOH + 0.1% TFA; 5–50% gradient		

Table 1 (continued)

Reference	Year	Flavonoid type	Glycosides	Sample	Stationary phase	Mobile phase co-solvent	MS detection	Make-up solvent for MS
		Flavanones, flavanols and flavonols						
[22]	2018	Isoflavones	Aglyca	Rat plasma	2-Ethylpyridine	MeOH + 0.1% formic acid; 10–40% gradient	ESI(+)-MS/MS	MeOH, 0.2 mL/min
[16]	2019	Flavanone	Aglyca and glycosides	Licorice roots extracts	2-Picolylamine	MeOH + 0.2% formic acid; 15–30% gradient		
[17]	2019	Isoflavones	Aglyca and glycosides	<i>Radix puerariae</i> extracts	Propanediol-bonded silica	MeOH + 1 mM oxalic acid; 20–40% gradient	ESI(+)-MS/MS	MeOH + formic acid, 0.2 mL/min
[7]	2019	Polymethoxylated flavones and flavanones	Aglyca and glycosides	<i>Citri reticulatae</i> pericarpium	Silica	MeOH; 8–36% gradient		
[18]	2019	Flavones and flavonols	Aglyca	Sweet potato leaf extracts	2-Ethylpyridine	MeOH + 0.05% formic acid; 5–40% gradient	ESI(-)-MS/MS	<i>na</i>
[19]	2019	Prenyl-flavonoids	Aglyca and glycosides	<i>Herba epimedii</i> extracts	Propanediol-bonded silica	MeOH-AcN (1:1) + 5 mM oxalic acid and 3% H ₂ O; 10–50% gradient	ESI(+)-MS/MS	MeOH, 0.3 mL/min

In all cases, the maximum amount of solvent did not exceed 50%; thus, according to general practice, the method employed can still be called supercritical or subcritical fluid chromatography. The mobile phase also comprised an additive in most cases, with acidic additives dominating the scene: formic acid [13, 16, 18, 21, 22], trifluoroacetic acid [26], phosphoric acid [6, 8, 23], citric acid [24] or more recently oxalic acid [17, 19, 20]. One example explored the use of ionic liquids in the mobile phase [25]. In many cases, a small percentage of water was also present, as was often advised in recent SFC experiments [27]. As shown with the full data in Table S1 (see Electronic Supplementary Material, ESM), temperature and pressure conditions varied a great deal (respectively 30 to 60 °C and 9.5 to 25 MPa), but these parameters were not always optimized, thus comparison would not mean much.

Regarding the detection mode, most analyses were done with UV or DAD, and a significant portion of them included MS or tandem MS. In such cases, electrospray ionization was always employed, in positive or negative mode. Some papers mention the use of a make-up fluid to enhance the ionization and perhaps also limit solubility issues upon depressurization of carbon dioxide. This make-up fluid sometimes also comprises an acidic additive (formic acid [13, 17]) possibly to assist the ionization process in providing a supplementary source of protons.

Analysis time also varied a great deal among the previous works (from 3 to 25 min), but no fair comparison can be done when the purpose of the analysis varies from quantitation of a single analyte in a serum sample with tandem mass spectrometric detection (MS/MS) allowing for incomplete chromatographic resolution [22] to quantitation of several species in a complex natural product with multiple-wavelength UV detection requiring higher chromatographic resolution [26].

In the present work, we aimed to develop a largely applicable method to analyse monomeric flavonoids with pressurized carbon dioxide-based mobile phases and detect them with DAD and ESI-MS. Early in this method development process, it appeared that the most polar flavonoids (comprising disaccharide moieties or gallic acid substitution) could not be eluted when the co-solvent proportion was limited to 50%. It was then decided to increase this proportion close to 100% at the end of the elution gradient. In such conditions, the chromatographic method can no longer be called supercritical or subcritical fluid chromatography (SFC). At the end of the gradient, the mobile phase composition is more relevant of enhanced fluidity liquid chromatography (EFLC) or can even reach pure liquid chromatography conditions. A wide elution gradient starting with SFC and ending with EFLC or HPLC conditions can be defined as unified chromatography (UC), as was proposed by Bamba and co-workers [28]. The term of unified chromatography may be applied to other concepts but, for want of a better (more precise) name, we will use it in this paper to qualify a broad composition gradient where the fluid state varies

during the analysis. ESI-MS detection was thoroughly optimized with variation of the ionization parameters and make-up fluid. The method was then applied to analyse two flavonoid ingredients employed in pharmaceutical or cosmetic formulations.

Material and methods

Chemicals and solvents

Ultra-pure water was supplied by an Elga UHQ system from Veolia (Wissous, France). The HPLC-grade methanol and acetonitrile used as the mobile phase and sample diluent were supplied by VWR (Fontenay-sous-Bois, France). CO₂, with a purity of 99.995%, was delivered by Air Liquide (Paris, France). Formic acid (FA), citric acid (CA), phosphoric acid (PA), trifluoroacetic acid (TFA) and ammonium hydroxide were obtained from Sigma-Aldrich (Merck, France). Methanesulfonic acid (MSA) was purchased from Janssen Chimica (Geel, Belgium). Nine standard molecules (Fig. 1) were provided by Extrasynthese (Genay, France), Carbosynth (Eching, Germany) and Sigma-Aldrich. The sample of hidrosmin (5-O-(β -hydroxyethyl)-diosmetin-7-O-rutinoside) was obtained from BOC Sciences and the sample of α -glucosyl-hesperidin was bought from Sigma-Aldrich.

Standard and sample solutions of all flavonoids were prepared in methanol at a concentration around 1 mg/mL. The nine standard stock solutions were mixed with a final concentration of about 100 μ g/mL for method development and optimization.

Instrument

All experiments were done on a Waters Corporation (Millford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) system equipped with a diode-array (ACQUITY PDa®) detector and a single-quadrupole mass spectrometer with an electrospray ionization source (ACQUITY QDa®). 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. The exact proportion is unknown and likely to vary along the gradient elution program. Nitrogen was used as the nebulizing gas.

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. Empower® 3 was used for chromatographic acquisition and integration of peaks.

Unified chromatography method

The experiments were carried out on two columns: ACQUITY UPC² Torus Diol and DEA (100 × 3.0 mm; 1.7 μm) commercialized by Waters. For method development experiments, the mobile phase was composed of carbon dioxide and methanol as a co-solvent, comprising additives and with a gradient elution as described in Table 2a. The column oven was heated to 25 °C and the sample compartment was maintained at 10 °C.

The injection volume of each sample was set at 5 μL. All chromatograms were recorded with DAD in the 190–400-nm range, with 1.2-nm resolution. Scan rate was 20 pts/s. Visualization and peak integration was done at 280 nm for standards and α-glucosyl-hesperidin, and at 330 nm for hidrosmin.

Mass spectrometry method

Polarity switching was employed to record both positive and negative ions at all stages of method development and for application experiments. The *m/z* values looked for and/or observed with single-ion recording (SIR) for each flavonoid in positive and negative ionization modes can be seen in Table S2 in ESM. Specifically, we looked for [M+H]⁺ and [M-H]⁻ ions, but also adducts like [M+Na]⁺ and [M+NH₄]⁺ in the positive mode, [M+MSA-H]⁻ in the negative mode. In positive mode, for the glycosylated analytes, fragment ions of the aglycone structure [Genin+H]⁺ were also looked for. In

negative mode, possible dimer forms were observed for some analytes, as will be further discussed below.

The parameters of the MS detector used were pre-optimized by the manufacturer. Some of the parameters have little influence on the MS detection of flavonoids or can simply not be changed, so the default settings were retained. MS optimization in the positive ionization mode was then done on the 9 standards with a design of experiments focusing on three parameters at three different levels: probe temperature (200, 400, 600 °C); cone voltage (10, 15, 20 V); capillary voltage (+0.3, +0.8, +1.5 kV). A full factorial design was prepared and interpreted with JMP Pro (version 13, SAS Institute Inc.), comprising 29 randomized experiments. The full list of experiments can be found in Table S3 in ESM.

The composition of the make-up flow was also thoroughly examined with three different additives in methanol: formic acid 1%; sodium chloride 0.1 g/L with 1% water; ammonium hydroxide 20 mM with 2% water. The flow rate was always 0.4 mL/min.

Optimized UC method

The column finally selected for applications was ACQUITY UPC² Torus DEA. The final mobile phase composition was a gradient elution of carbon dioxide and methanol comprising 0.1% MSA. The gradient program is described in Table 2b. Because of the high inlet pressure generated at the end of the gradient, due to the highest viscosity of the mobile phase containing large modifier percentages, a reversed pressure gradient was applied together with a reversed flow rate gradient. The column oven was heated to 25 °C and the sample compartment was maintained at 10 °C. The optimal (ESI⁺)-MS parameters were as follows: make-up fluid methanol comprising 20 mM ammonium hydroxide and 2% water, pumped at 0.4 mL/min; probe temperature 600 °C; ion source temperature 120 °C; cone voltage 10 V; capillary voltage +0.3 kV; scan rate 2.6 pts/s. The *m/z* range for total ion recording was set from 100 to 850 Da, with SIR *m/z* values as indicated in Table S2 (see ESM).

Liquid chromatography purification method

Six hundred milligram hidrosmin sample was solubilized in 100 mL water and purified on a Nucleodur C18 HTec column (300 g of 10-μm stationary phase) from Macherey-Nagel (Hoerd, France). Water (A) and acetonitrile (B) were used in a gradient program as follows: 0 min 15% B; 6 min 18% B; 10 min 20% B; 15 min 25% B; 25 min 60% B; 30 min 95% B. The flow rate was 70 mL/min. Three fractions were collected and reanalysed with the UC-MS method to assist in analyte identification. The chromatogram can be seen in ESM (Fig. S4).

Table 2 Gradient profiles

Time (min)	Co-solvent %	Back-pressure (Mpa)	Flow rate (mL/min)
a. Used throughout the mobile phase and MS optimization process			
0	5	15	1
0.5	5	15	1
7	50	15	1
7.1	50	15	0.5
15.5	95	15	0.5
16.5	95	15	0.5
17	40	15	0.5
18	40	15	1
18.5	5	15	1
b. Final optimized gradient			
0	20	15	2
0.5	20	15	2
8.5	100	11	0.7
10	100	11	0.7
10.5	20	11	0.7
12	20	15	2
12.5	20	15	2

NMR analysis

The hidrosmin sample was analysed with ^1H NMR. The results can be seen in ESM (Fig. S5).

Results and discussion

Method development

Nine standards (Fig. 1) were selected to provide some diversity in the aglycone parent structures, including flavanones, flavanols and flavonols, with some structural similarities in the hydroxyl group substitution pattern (like hesperetin and tamarixetin, or quercetin and catechin). The molecules were also selected to comprise aglycone and glycosylated species, with glycosidic groups of different sizes (like the aglycone quercetin and the glycosylated congeners: quercitrin, isoquercitrin bearing a monosaccharide, and rutin bearing a disaccharide). Finally, they also comprise the typical variations that can be found among such analytes like the replacement of one hydroxyl group by a methoxyl group (quercetin compared with tamarixetin).

A UC-DAD-MS method was first developed to analyse these flavonoid standards. The objective was to obtain a largely applicable method for this family of compounds, which could later be applied to a variety of samples where flavonoids may be looked for.

Selection of stationary phases

Preliminary tests based on a large selection of stationary phases, comprising polar, non-polar and aromatic ligands, had indicated that polar stationary phases were more suitable for the analysis of flavonoids in SFC. This observation was also consistent with the literature (Table 1). Two stationary phases appeared to provide generally improved retention, selectivity and peak shapes over the other phases tested: a propanediol-bonded silica (ACQUITY UPC² Torus Diol) and a diethylaminopropyl-bonded silica (ACQUITY UPC² Torus DEA). As pointed out in the “Introduction” section, propanediol-bonded silica phases had also been selected in a significant portion of previous works (about a quarter of the published papers). Diethylaminopropyl-bonded silica had been selected in only one previous publication. However, it is perhaps a less common stationary phase, being available from a limited number of column manufacturers, than propanediol-bonded silica that is more widely available from manufacturers offering columns for normal-phase (NP) or hydrophilic interaction (HILIC) chromatographic modes. The two columns were retained for further method development.

Gradient range

Early on in this method development, it appeared that a wide elution gradient starting with a small percentage of co-solvent (5%) and ending with very large proportions of co-solvent (near 100%) would be necessary in order to favour both the retention of the least polar compounds and the elution of the most polar ones. Indeed, among flavonoids, the polarity range is moderately wide, typically ranging in the 0–4 log *P* values [29]. Note that the most retained analyte (epigallocatechin gallate) could not be eluted from any column with proportions of co-solvent lower than 90%.

A chromatographic method covering such a wide range of mobile phase compositions is therefore more relevant of unified chromatography (UC), as it starts in typical SFC conditions and ends in typical enhanced fluidity liquid chromatography (EFLC) conditions. At the end of the gradient, the inlet pressure is a limiting factor as the mobile phase is much more viscous than at the beginning of the gradient. One simple solution to avoid reaching the pump upper pressure limit is to apply an inverse flow rate gradient. This was done here for the first stages of method development (mobile phase composition and MS optimization) by simply reducing the flow rate from 1 to 0.5 mL/min, knowing that further optimization would be necessary when all other parameters of the method would be settled. This simple gradient method, lasting 18.5 min, is described in Table 2a.

Mobile phase composition

It also appeared in preliminary tests that a mobile phase additive would be necessary to ensure elution in reasonable analysis time, together with acceptable peak symmetry. As mentioned in the “Introduction” section, in most previous studies, an acidic additive had been employed. In our recent works on UC-MS analysis of free amino acids, methanesulfonic acid (MSA) had proven to be a most interesting additive, allowing for improved peak symmetry and chromatographic efficiency for most proteinogenic amino acids. MSA was thus an interesting candidate for the present study. On the other hand, other additives that are often employed in SFC for diverse analytes were rarely or not mentioned in previous SFC analyses of flavonoids: ammonium acetate [14, 30], ammonium formate [9, 20] and ammonium hydroxide. In addition to several mentions in the recent SFC literature [27], ammonium hydroxide was recently cited as a most desirable additive to elute polar biomolecules in SFC [31]. Thus, we initially selected five acidic additives, formic acid, citric acid, phosphoric acid, methanesulfonic acid and ammonium hydroxide, to investigate their effects on retention and peak shapes.

As mentioned in the “Introduction” section, formic acid was often employed in previous SFC analyses of flavonoids and is also often used in RP-HPLC analyses of flavonoids [1],

but was found rather unsatisfactory here, with tailing peaks and poor efficiency observed for many analytes. Citric acid and phosphoric acid, which had both been mentioned as most desirable in previous SFC papers [6, 8, 23, 24], were both much more satisfactory than formic acid in terms of chromatographic quality, as the peaks were more symmetric and sharpened. However, citric acid and phosphoric acid are both undesirable when mass spectrometric detection is desired, due to their low volatility and possible corrosion effects (phosphoric acid). Still, they could be retained when UV or DAD only is employed. Indeed, as appears in Table 1, in many cases, SFC analysis of natural products is conducted with UV or DAD only. For instance, for quality control of phytotherapeutic plants, retention time and peak purity assessed with UV spectra are considered sufficient for analyte identification [7, 8, 15, 16].

To the best of our knowledge, methanesulfonic acid (MSA) was never used for the analysis of flavonoids in SFC. However, it was mentioned in a few publications from Blackwell and Stringham [32, 33] related to the analysis of polar ionizable analytes in SFC. More recently, we have used MSA to analyse free amino acids in unified chromatography hyphenated to mass spectrometry with very good results [34]. In the present case, MSA appeared as the best of the acids tested as it provided excellent efficiency and good peak symmetry together with MS compatibility, provided that some care is taken to adapt the exhaust valve (as mentioned in the “Material and methods” section).

The only non-acidic additive tested, ammonium hydroxide provided contrasting results, with some analytes showing very sharp peaks while others showed distorted peaks, or were simply undetected. Sample chromatograms can be seen in Fig. 2.

Finally, the mobile phase comprising 0.1% methanesulfonic acid was retained for further method development.

Following these experiments, the DEA column appeared as the best one. This is visible in the chromatograms in Fig. 2, where chromatographic efficiency is visibly much better on the DEA column than on Diol. Supporting data (EP apparent plate count) can be found in the ESM (Fig. S1). Thus, the DEA column together with MSA additive in the mobile phase was retained for MS response optimization.

Optimization of MS source parameters

While optimizing gradient profile and mobile phase composition, the impact on MS response was always kept in mind. When varying the additive, the MS response had been observed to vary a great deal. In all cases, both positive and negative ionization modes in electrospray had been examined, looking for protonated/deprotonated molecular and fragment ions, or possible adducts formed with sodium or ammonium.

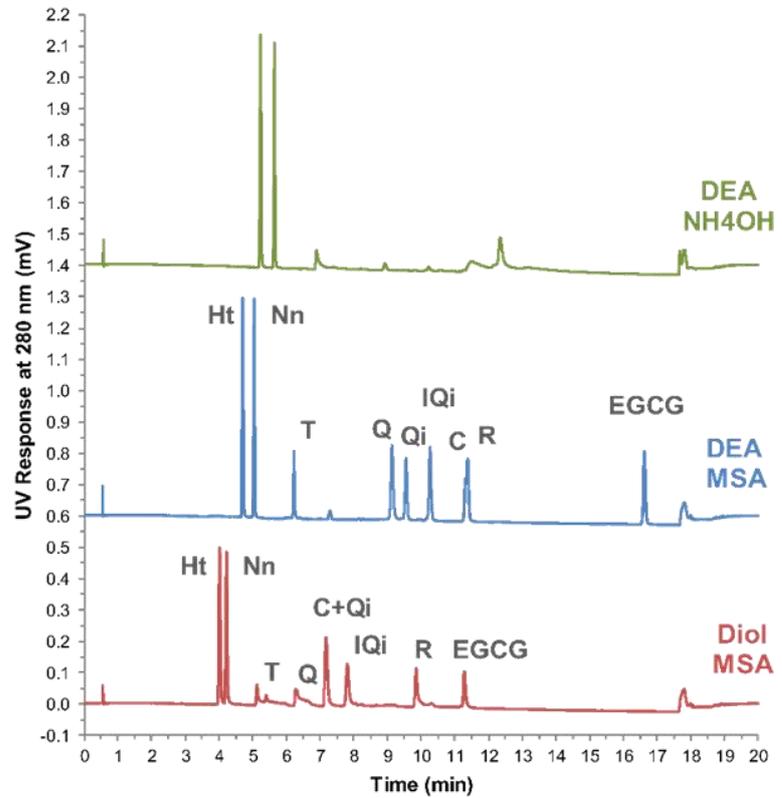
Positive ionization appeared to be more sensitive in most cases; thus, only this mode was retained for further optimization. Negative ionization mode could also have been optimized, especially for abundant adducts formed with methanesulfonic acid. The ionization parameters in ESI(+)-MS were then optimized with a design of experiments, as had been previously proposed by Bieber and co-workers [35]. The single-quadrupole mass spectrometer used here (ACQUITY UPC² QDa) allows for only limited parameter optimization, as the source parameters were initially optimized by the manufacturer to facilitate accessibility to mass spectrometry to non-specialists. As a result, only three ionization source parameters were examined: three levels of probe temperature (200, 400 and 600 °C), three levels of cone voltage (10, 15 and 20 V) and three levels of capillary voltage for positive ionization (0.3, 0.8 and 1.5 kV). A full factorial design was employed, resulting in 29 experiments, conducted in a random fashion, presented in Table S3 (see ESM). For these experiments, the standards were analysed in mixtures with the gradient elution as defined in Table 2a on the DEA column. Single-ion recording (SIR) traces were examined for each analyte, considering all possible ions as described above (ESM Table S2). Selected results for epigallocatechin gallate (EGCG) can be observed in Fig. 3. This was an interesting case because, with default settings, EGCG had remained undetected in ESI-MS. In Fig. 3a, it appears that the probe temperature is the most significant parameter to optimize the MS response, followed by the cone voltage. The high probe temperature could be expected to be favourable to desolvation from the methanol-rich mobile phase that is necessary to elute EGCG at the end of the gradient. In general, the large quantity of solvent required to elute analytes in the unified chromatography gradient may be perceived as a drawback of the method, in terms of MS sensitivity. This perception is however contradicted by other observations from Akbal and Hopfgartner [36], where increasing quantities of make-up fluid introduced between the SFC outlet and the ESI-MS appeared to cause insignificant changes in signal intensities in most cases, or 10-times increase in MS signal intensity in some cases. In another experiment, the same authors mentioned that “no correlation was observed in the MS signal response and the retention time” (in gradient mode). The effects of mobile phase and make-up fluid composition on MS response were concluded to be largely compound-dependent.

Considering all analytes, the optimal source parameters were probe temperature 600 °C, cone voltage 10 V, and capillary voltage 0.3 kV.

Optimization of the MS make-up fluid

Secondly, the make-up fluid introduced before entering the ESI-MS was also examined. Apart from rare exceptions [36], in most SFC-MS works, this make-up fluid is not

Fig. 2 Chromatograms of the nine standard flavonoids on ACQUITY UPC² Diol and DEA columns with the gradient program in Table 2a. Co-solvent: methanol containing 0.1% methanesulfonic acid (MSA) or 20 mM ammonium hydroxide and 2% water (NH₄OH) as indicated. Oven temperature, 25 °C; UV detection, 280 nm



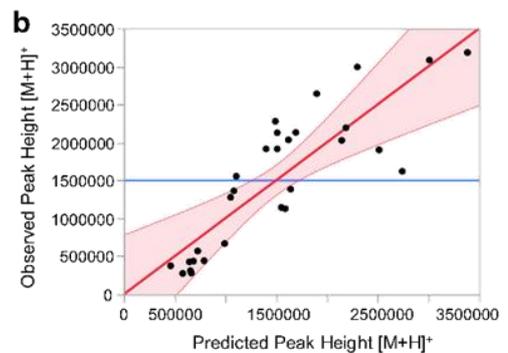
addressed and the same, supposedly generic composition is used, typically comprising methanol and formic acid. The interest of introducing a low amount of methanol at this stage

is well recognized, especially at the beginning of the gradient where CO₂ depressurization may cause analyte precipitation in the transfer line or in the MS ionization source. Admittedly,

Fig. 3 Results of the design of experiments for MS response optimization on epigallocatechin gallate. **a** Effects of the three selected parameters. **b** Observed vs. predicted peak heights of the [M+H]⁺ ion

a

MS parameters	LogWorth	p-value
Probe Temperature	5.926	0.00000
Cone voltage	2.626	0.00237
Probe Temperature*Cone voltage	1.239	0.05764
Cone voltage*Capillary voltage	0.432	0.36990
Capillary voltage	0.419	0.38087
Probe Temperature*Capillary voltage	0.241	0.57449



at the end of such a wide elution gradient, the make-up fluid is not so necessary and is mostly causing dilution of the sample that could reduce detection sensitivity. Again, this expected behaviour was somewhat contradicted by previous studies [36]. Thus, a reversed flow rate gradient of make-up fluid could be desirable but was not explored in this case. The acidic additive most often employed is normally intended to favour ionization in positive ionization mode, in providing protons to form $[M+H]^+$ ions. In the present case, when the mobile phase already contains methanesulfonic acid, the interest of introducing another, weaker acidic additive is questionable. On the other hand, introducing a sodium chloride solution that would favour sodium adducts ($[M+Na]^+$ ions), or introducing some ammonium hydroxide, hoping to favour ammonium adducts ($[M+NH_4]^+$) or negative electrospray ionization ($[M-H]^-$ ions) were interesting options. Akbal and Hopfgartner [36] also observed a significant improvement of the ESI-MS response for a diversity of molecules in positive ionization mode with ammonium ions (introduced as ammonium hydroxide or ammonium acetate).

In the present case, no positive effect related to sodium chloride could be observed. In most cases, the $[M+H]^+$ ions disappeared while the sodium adducts were not improved. When sodium adducts were observed, the sensitivity was not improved compared with the signal observed with acidic make-up fluid.

Comparing the other two make-up compositions (formic acid or ammonium hydroxide), ammonium hydroxide appeared to be very favourable as the MS responses were improved for all ions examined. This can be observed in Fig. 4, where the signal-to-noise ratios measured for the ions of epigallocatechin gallate and isoquercitrin are compared. With the set parameters, only little fragmentation occurred for EGCG, splitting the aglycone root and gallic acid moiety, so the $[M+H]^+$ ion was the most abundant. The $[M+H]^+$ ion was also the one providing the best sensitivity for all aglycone species. For the glycosylated species (quercitrin, isoquercitrin and rutin), the genin (aglycone) protonated cation $[G+H]^+$ (having fragmented the sugar moiety) was the most abundant, suggesting in-source fragmentation caused by the high cone voltage. However, in these cases, the methanesulfonate adduct anion of the intact molecule $[M+MSA-H]^-$ observed in negative mode was also most abundant, which would serve the purpose of identifying an unknown analyte as a glycosylated molecule. In addition, the chromatographic retention should also support analyte identification as the aglycone and glycosylated species were well resolved with this method, as can be observed with the example of quercetin, quercitrin, isoquercitrin and rutin.

Some analytes (quercetin, tamarixetin and hesperetin) also showed possible dimer forms, especially as MSA adducts in the negative ionization mode. Naturally occurring dimers of quercetin have been described in the past [37], which may result from an oxidative process.

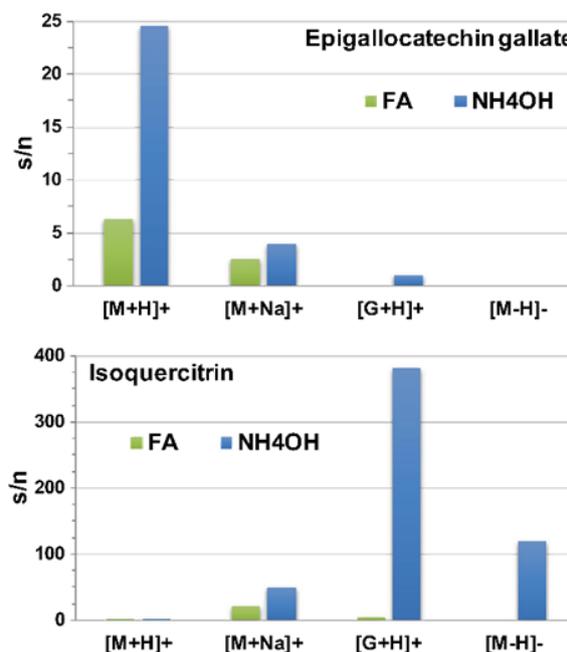


Fig. 4 Comparison of signal-to-noise (s/n) measured on the DEA column with the gradient method in Table 2a and mobile phase co-solvent containing 0.1% MSA. Make-up fluid contains either formic acid (FA, in green) or ammonium hydroxide and water (NH_4OH , in blue)

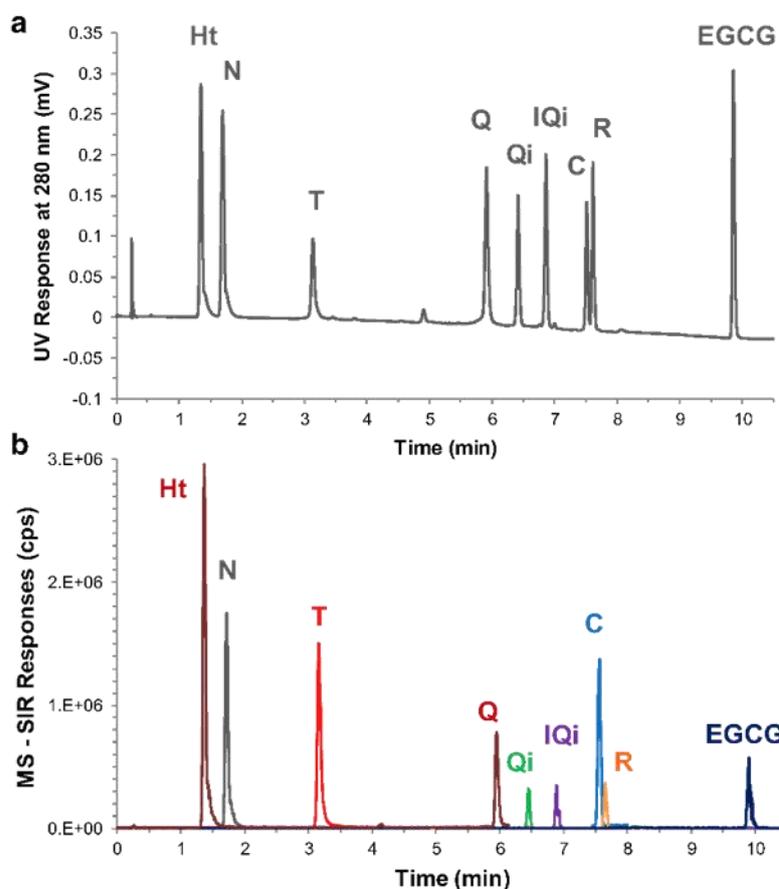
The MS spectra observed in positive and negative ionization modes can all be observed in Fig. S2, provided in ESM.

Finally, the make-up fluid selected was methanol containing 20 mM ammonium hydroxide and 2% water, pumped at 0.4 mL/min.

Final gradient optimization

At this stage, the chromatographic elution gradient could still be further optimized, to make the method both faster and more efficient. First, it appeared that the initial composition could be a stronger eluent as the first peak eluted only after 4 min. It was then changed from 5 to 20% co-solvent. As mentioned in the “Gradient range” section, the mobile phase viscosity is much higher at the end of the gradient than at the beginning. While the inverted flow rate gradient is helpful, it is also possible to use an inverted pressure gradient, as was proposed in the seminal paper from Bamba and co-workers [28] where the first UC experiment was demonstrated. Recently, we have also proposed to superimpose the inverse pressure and flow rate gradients [34]. One obvious advantage is to decrease analysis time, using the fastest mobile phase velocity at the beginning of the gradient program, when mobile phase viscosity is low, and a slower velocity at the end of the gradient when mobile phase viscosity is high. The second advantage should be in improved chromatographic efficiency. Indeed, as demonstrated by Desfontaine et al. [38], van Deemter curves

Fig. 5 Optimized UC-DAD-MS chromatograms of the nine standard flavonoids on ACQUITY UPC² DEA with the gradient program in Table 2b. Co-solvent: methanol containing 0.1% methanesulfonic acid. Make-up fluid: methanol with 20 mM NH₄OH and 2% water. Oven temperature, 25 °C. **a** UV detection, 280 nm. **b** Optimized MS responses with single-ion recordings of [M+H]⁺ ions



are varying significantly when the percentage of co-solvent varies in such a wide range. Specifically, the optimum flow rate is moving from high velocities (typically observed in SFC conditions) to low velocities (typically observed in HPLC conditions), and the C-term is increasing, indicating that any deviation from the optimum flow rate at the end of the gradient would affect chromatographic efficiency more significantly. Desfontaine et al. [38] had proposed to use a compromise, intermediate flow rate throughout the gradient elution, while we would rather modify the flow rate during the analysis to favour high chromatographic efficiency at every stage of the gradient elution, together with fast elution [34]. There is no simple way to optimize such a three-variable gradient profile except through trial and error processes. Indeed, depending on column packing and the way mobile phase components adsorb on the stationary phase, the pressure drop can vary to some extent when replacing one column by another, even when column dimensions and particle size are identical. The gradient limits and slopes were then optimized step by step to avoid overpressure.

Finally, it is worth mentioning that a temperature gradient would also be useful in decreasing mobile phase density along the elution gradient, but as column oven temperature cannot

be accurately adjusted as fast as back-pressure and flow rate, it was preferable to maintain it constant during the analysis.

The optimized gradient was as described in Table 2b to achieve faster analysis (12.5-min cycle time).

The elution order and analysis times can be observed in the chromatograms in Fig. 5. Basically, as could be expected from the normal-phase retention mode on the polar stationary phase:

- (i) Glycosidic flavonoids eluted later than their aglycone forms, e.g. quercitrin later than quercetin.
- (ii) Larger glycosidic groups elute later than smaller ones; e.g. rutin elutes later than quercitrin.
- (iii) Hydroxyl groups caused more retention than methoxyl groups; e.g. quercetin was more retained than tamarixetin.
- (iv) Isobaric species hesperetin and quercetin were largely resolved.

Although not perfectly fitting, the elution order is mostly in accordance with decreasing log *P* values or increasing polar surface area. Epigallocatechin gallate is a significant

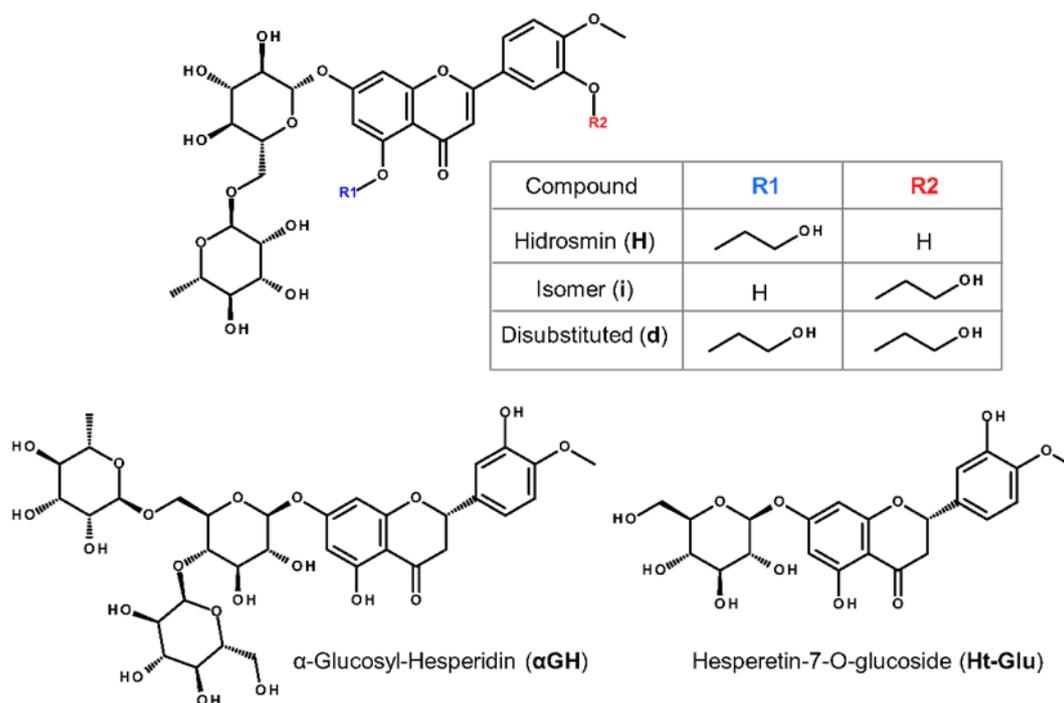


Fig. 6 Structures expected and observed in the pharmaceutical and cosmetic samples

exception, being much more retained than would be expected based on $\log P$ or polar surface area. Similarly, catechin was significantly more retained than quercetin although its polar surface area is lower.

The quality of the chromatogram is very good, with thin and symmetrical peaks. Asymmetry varied from 1.02 to 1.50, with the worst values observed for the three least retained analytes, where the relatively large volume injected (5 μ L) may have caused some deformation. Repeatability of the method was assessed with triplicate analysis of the standard mixture. The relative standard deviation (RSD%) on retention times varied from 0.02 to 0.37%, with higher values observed for the least retained analytes. RSD% on peak area varied from 0.29 to 3.04%, with no relation to retention time observed.

Sample applications

Two sample applications are presented here, hidrosmin and α -glucosyl-hesperidin. The first one is relevant in pharmaceutical formulations while the second one is a cosmetic ingredient. The structures of the two molecules are presented in Fig. 6.

Hidrosmin

Hidrosmin (5-O-(β -hydroxyethyl)-diosmetin-7-O-rutinoside) is a semi-synthetic flavone and is destined to pharmaceutical

formulations for the treatment of blood circulation diseases. A commercial sample was analysed with the optimized method to determine the presence of impurities. The chromatograms obtained with the optimized UC-DAD-MS method can be observed in Fig. 7a, b while the MS spectra can be seen in Fig. S3 provided in ESM. The m/z traces examined are indicated in Table S4 (see ESM). A first observation of the UV trace indicated that the sample had a low purity, with two major peaks and about 15 minor peaks observed. Examination of the MS traces indicated that the major peak had the expected mass of 653 Da, while the second major peak had a higher mass of 697 Da, possibly corresponding to a dihydroxyethyl-diosmin. NMR analyses (Fig. S5 in ESM) and UC-MS analyses of purified species (Fig. S4 in ESM) later confirmed that the major peak was actually not the expected hidrosmin molecule but an isomer (3'-O-(β -hydroxyethyl)-diosmetin-7-O-rutinoside), as described in Fig. 6. The expected molecule, hidrosmin, was only present in very low concentration, as pointed on the chromatogram in Fig. 7a. The disubstituted structure proposed (5,3'-di-O-(β -hydroxyethyl)-diosmetin-7-O-rutinoside) was also confirmed with NMR analysis. Note that there seems to be a confusion as to the exact identity of hidrosmin: the patents describing its synthesis [39, 40] both give the 5-O-(β -hydroxyethyl)-diosmetin-7-O-rutinoside chemical name but show the structure of the 3'-O-(β -hydroxyethyl)-diosmetin-7-O-rutinoside isomer. It is likely that the 3'-isomer is not always identified

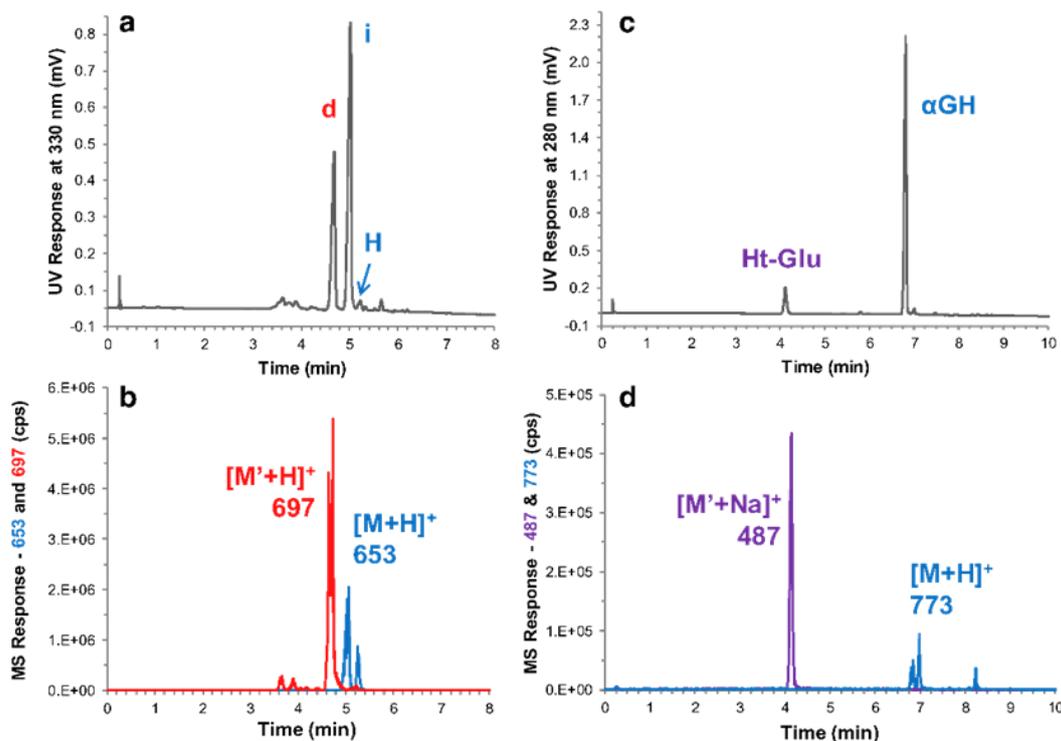


Fig. 7 Chromatograms of (a, b) hidrosmin sample and (c, d) α -glucosyl-hesperidin sample (structures described in Fig. 6) in the optimized conditions described in Table 2b and Fig. 5. a UV detection 330 nm. c UV detection 280 nm. b, d MS detection SIR as indicated

when a reversed-phase HPLC analysis is performed for quality control of the bulk synthesized drug, as it may co-elute with the desired 5-isomer, and because the UV spectra and MS spectra should be most similar.

α -Glucosyl-hesperidin

α -Glucosyl-hesperidin is a semi-synthetic flavanone derived from hesperidin to make it more soluble in aqueous formulations and destined to cosmetic formulations to improve skin tone through increased surface blood circulation. A commercial sample was again analysed with the optimized method to determine the presence of impurities. The chromatograms obtained with the optimized UC-DAD-MS method can be observed in Fig. 7c, d and the MS spectra in Fig. S3 (see ESM). The m/z traces examined are indicated in Table S4 (see ESM). A much cleaner sample is observed here, with the major peak at 6.8 min corresponding to the expected structure (773 Da), and the minor peak eluted around 4 min corresponding to the sodium adduct of hesperetin-7-O-hexoside (487 Da). The mass spectra showed significant in-source fragmentation in the positive mode, but the MSA adducts in the negative mode confirmed the expected masses. As only single-quadrupole MS analysis was available, we can only hypothesise on the identity of the minor peak: the aglycone is more likely hesperetin than any other isomer. In addition, according to

the patent related to α -glucosyl-hesperidin manufacturing [41], a significant portion of hesperidin remains at the end of the process. Because its aqueous solubility is low, the residual hesperidin is converted to hesperetin-7-O-glucoside, which has a higher aqueous solubility than hesperidin. This process is in accordance with our observations on this sample.

Conclusions

A unified chromatography-mass spectrometry method was developed for flavonoids based on 9 standard molecules with varied structures with or without glycosylation. The chromatographic method was carefully optimized on a diethylaminopropyl-bonded silica stationary phase, with a three-parameter gradient (mobile phase composition comprising carbon dioxide, methanol and methanesulfonic acid, back-pressure and flow rate) to achieve fast elution of all analytes, including the most polar ones, with good symmetry and efficiency. The electrospray ionization was optimized through a design of experiments and attention paid to the composition of make-up fluid, comprising ammonium hydroxide and water in methanol. Then, the two sample applications illustrated the interest of this method, which should be largely applicable to flavonoids belonging to the same structural families as the

ones employed to develop the method (flavanones, flavanols and flavonols).

Further applications will be developed on complex samples of natural products, which would include a wider range of phenolic compounds (like anthocyanins, oligomeric phenolics). While LC-MS is a very powerful technique to analyse flavonoids, it was demonstrated in several instances (not only with natural products) that SFC-MS is most complementary to LC-MS and that the combined use of the two methods is beneficial [42, 43]. This complementarity is not only due to different chromatographic selectivity but also to different matrix effects. Thus, the number of features that can be detected is the highest when the two methods are employed together, instead of only one. In addition, features that are observed with both methods are also useful in confirming analyte identification. Performance comparison between our UC-MS method and LC-MS methods, particularly in terms of sensitivity, will also be necessary.

Finally, when purified compounds are necessary for bioactivity testing, for precise structure identification with NMR spectroscopy or to serve as synthons in hemisynthesis procedures, it would be good to transfer this method to a preparative scale, where SFC is known to be a very effective, time-saving and ecological method.

Funding information Caroline West is grateful for the support received by the Institut Universitaire de France (IUF), of which she is a Junior Member. Waters Corporation is acknowledged for the support received through the Centers of Innovation program.

Compliance with ethical standards

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

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Supplementary information to:

Analysis of flavonoids with unified chromatography-electrospray ionization mass spectrometry – Method development and application to compounds of pharmaceutical and cosmetic interest

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Figure S1. Comparison of apparent efficiency (EP plate count) measured on the DEA and Diol columns with the gradient method in Table 2a and mobile phase co-solvent containing 0.1% MSA.

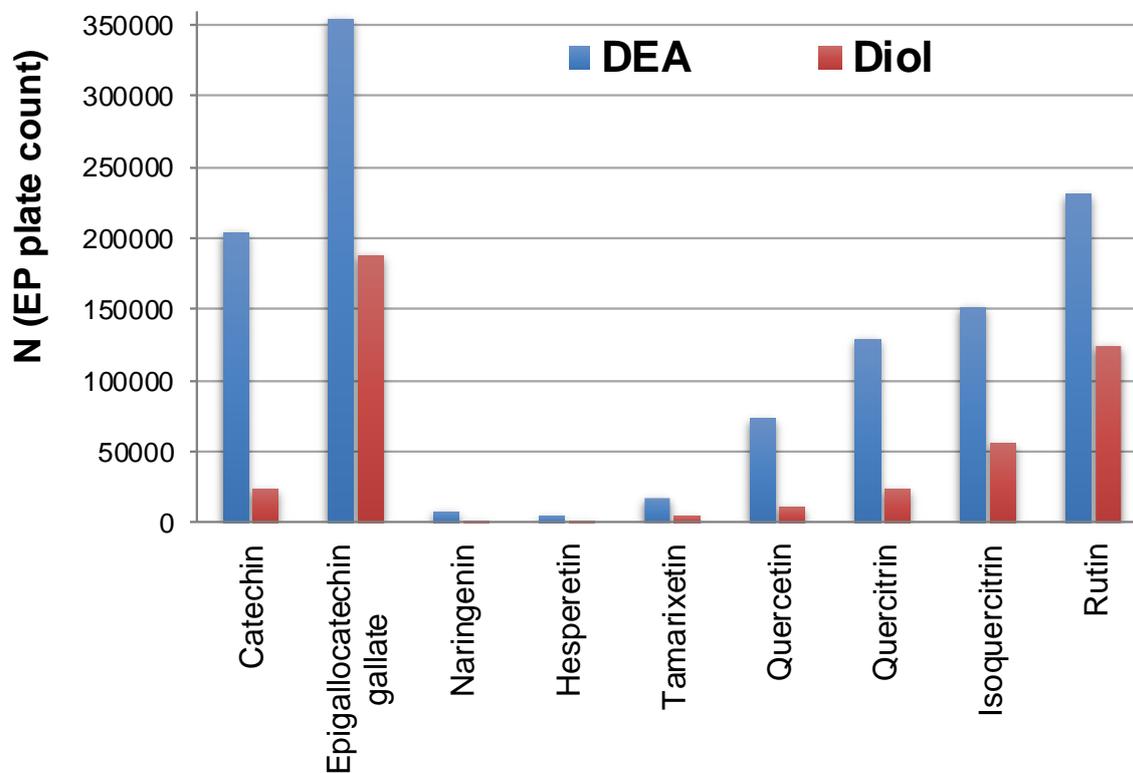
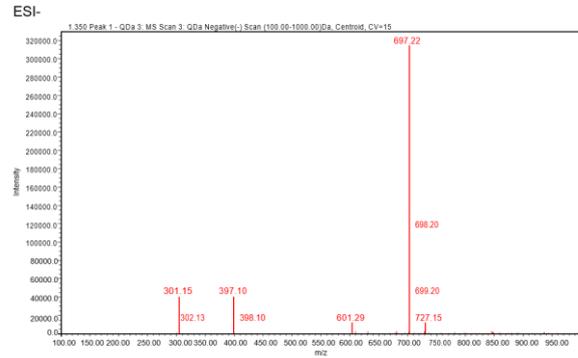
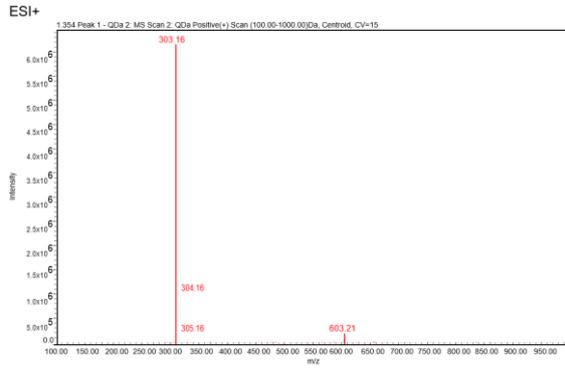
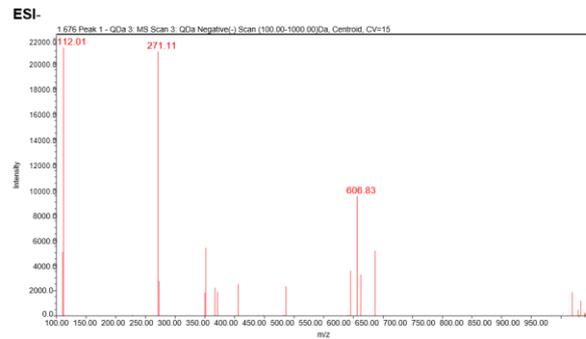
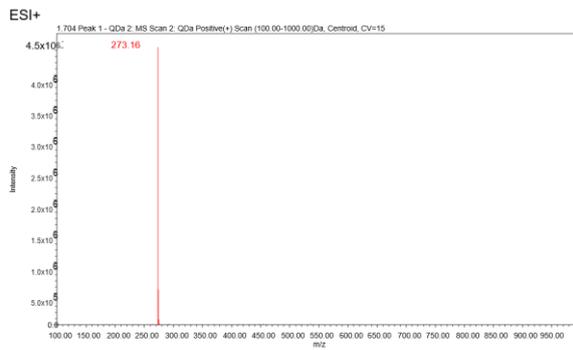


Figure S2. Mass spectra observed for the standard flavonoids (presented accordance to elution order in Figure 5).

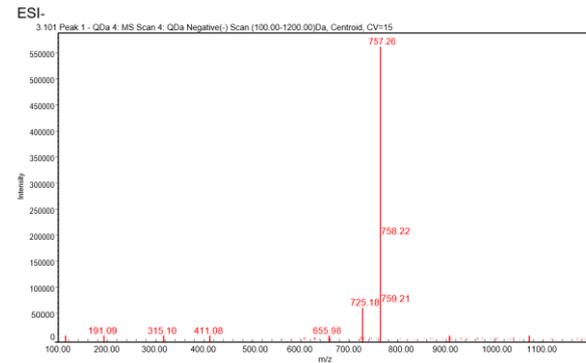
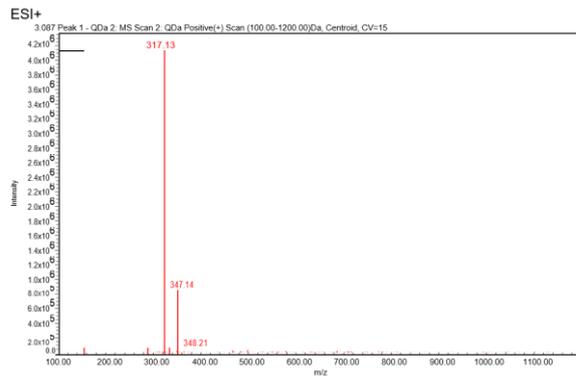
Hesperetin



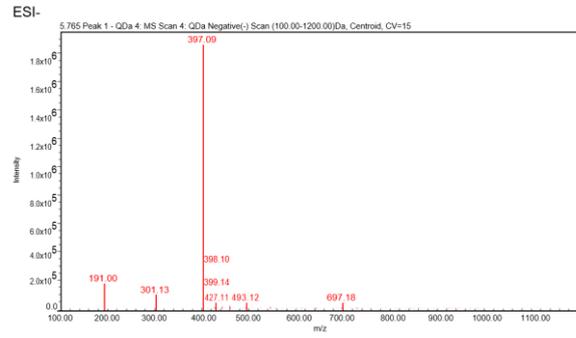
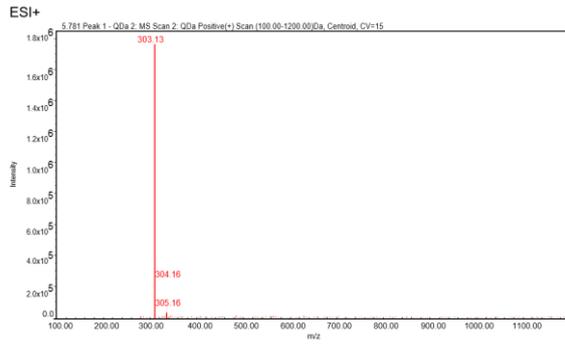
Naringenin



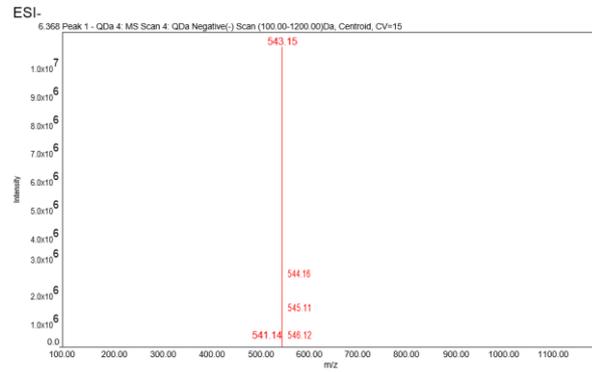
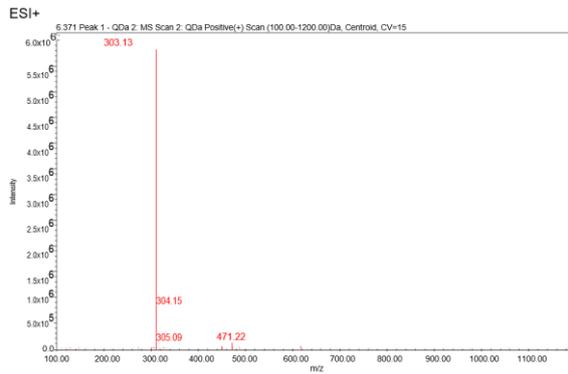
Tamarixetin



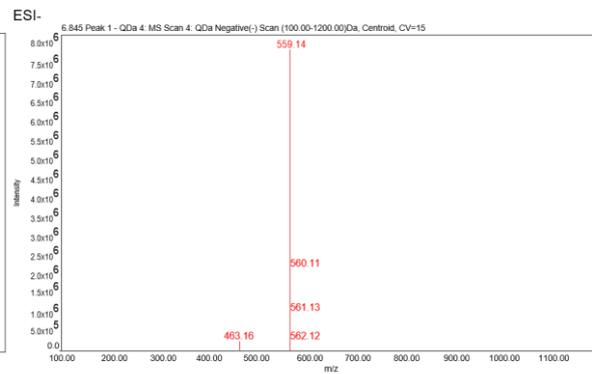
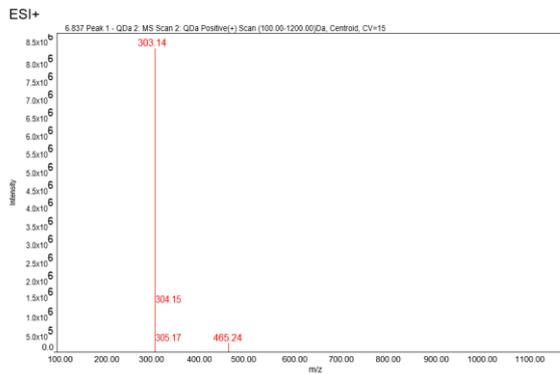
Quercetin



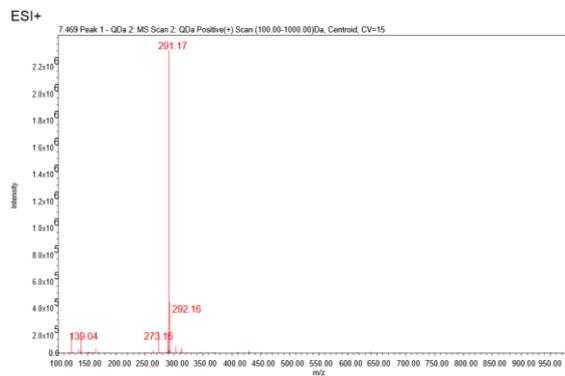
Quercitrin



Isoquercitrin



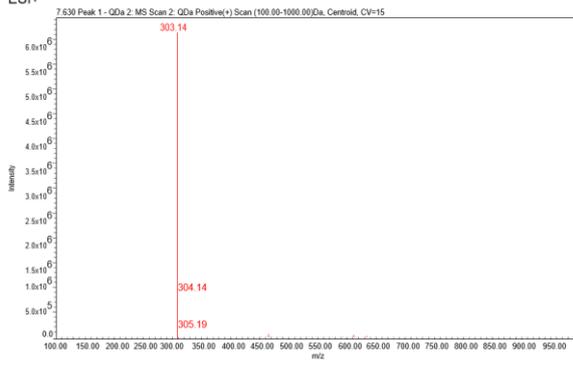
Catechin



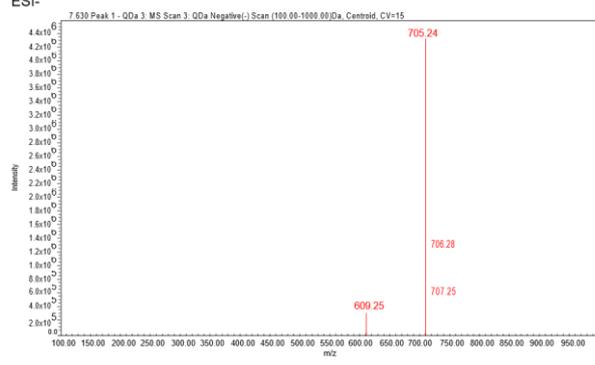
ESI- : nothing observed

Rutin

ESI+

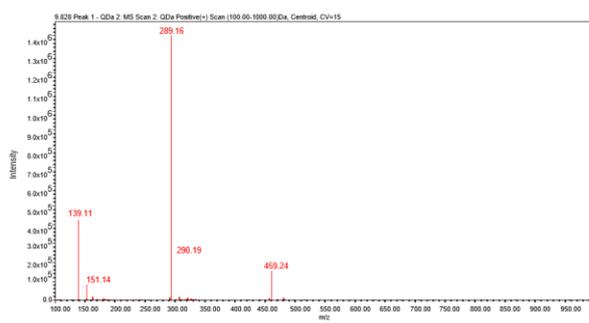


ESI-



Epigallocatechin gallate

ESI+



ESI-

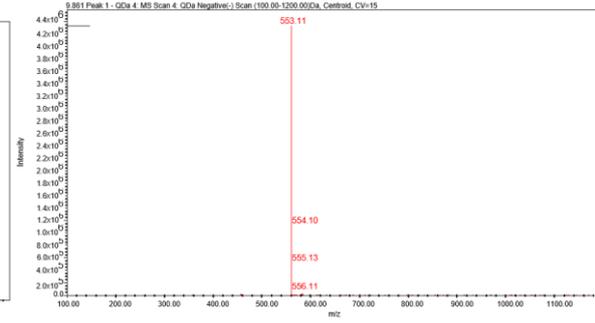
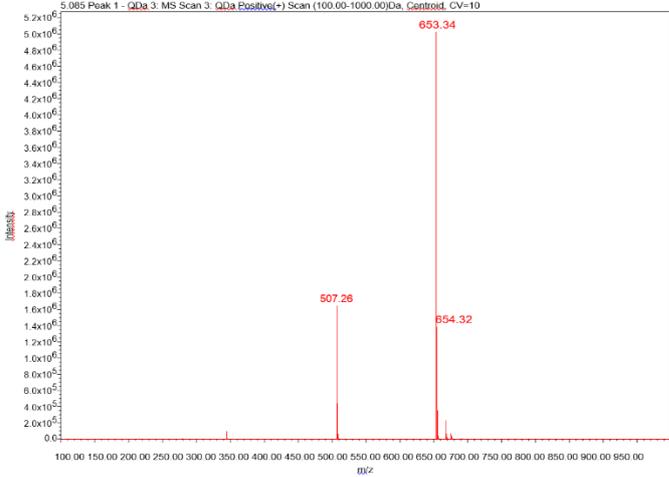


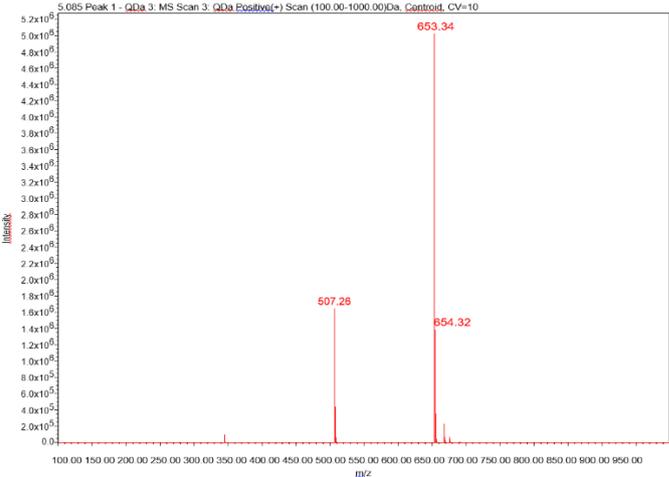
Figure S3. Mass spectra observed for the major peaks in the two sample chromatograms (Figure 7).

Hidrosmín sample, ESI+

Major peak at 4.771 min



Major peak at 5.085 min



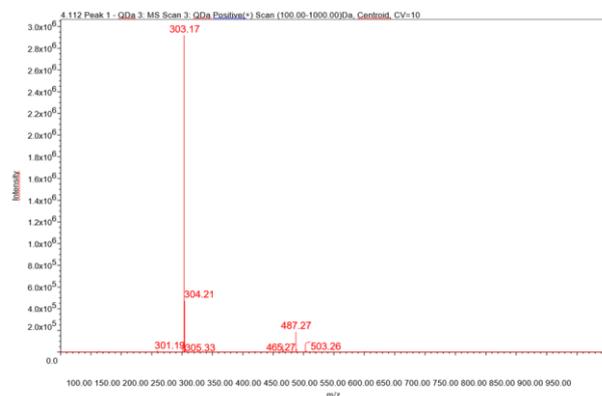
Minor peak at 5.341 min



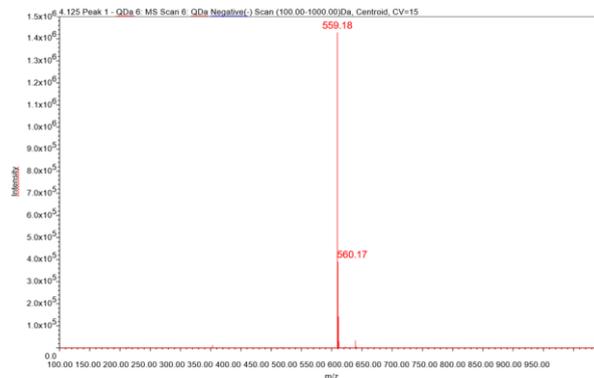
α -Glucosyl-Hesperidin sample:

Minor peak at 4.112 min

ESI+

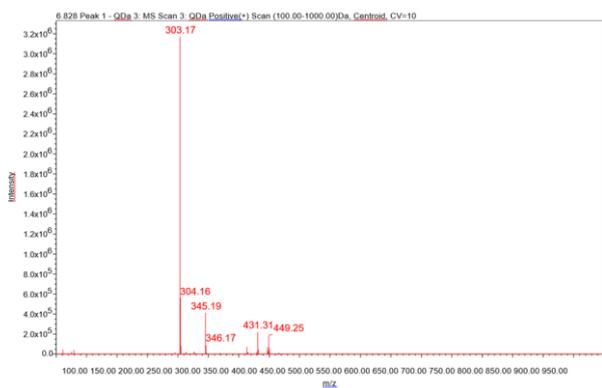


ESI-



Major peak at 6.828 min

ESI+



ESI-



Fig. S4 Reversed-phase LC purification of the Hidrosmin sample (see main text for details). Fractions 2, 3 and 5 were collected and re-analyzed with the UC-MS method

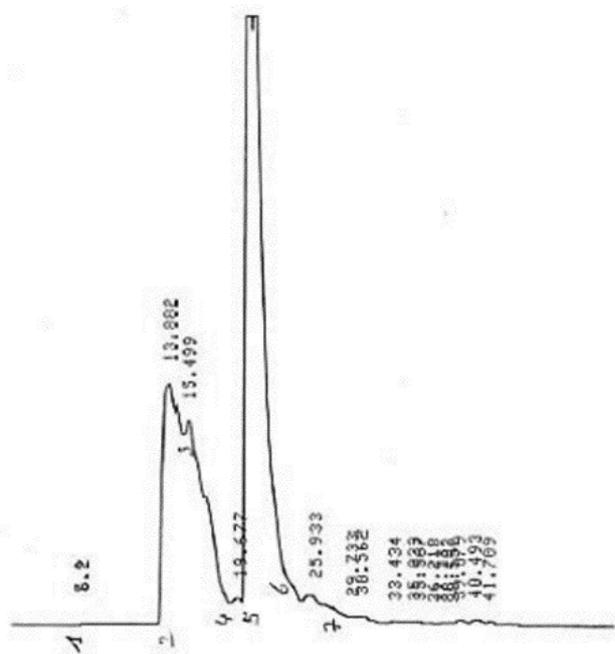
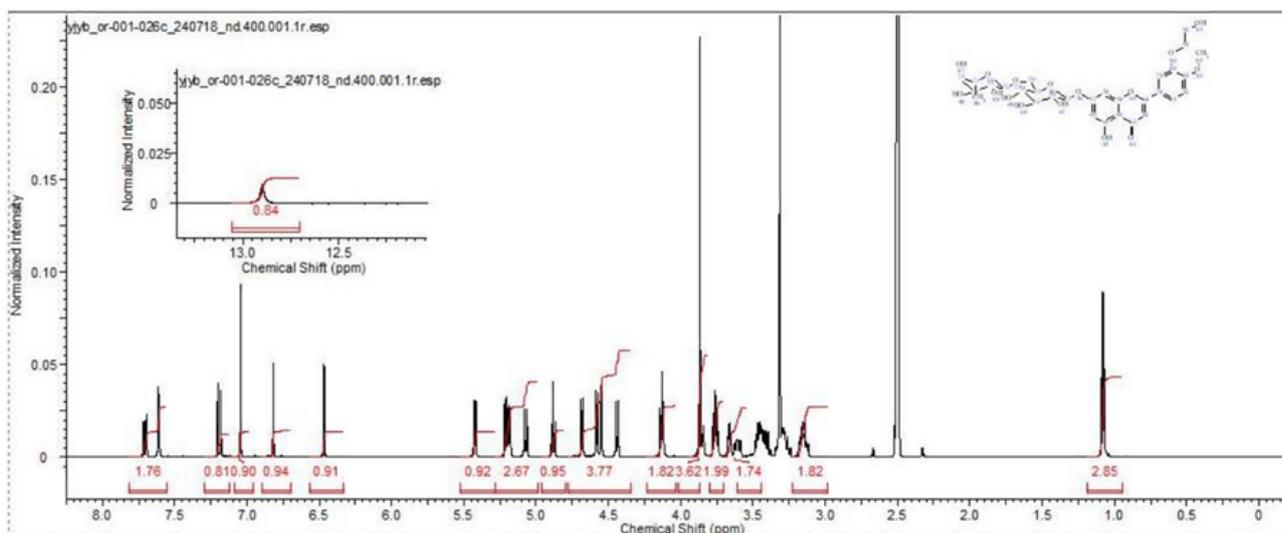


Fig. S5 ¹H NMR analysis of the Hidrosmin sample

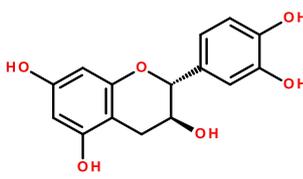
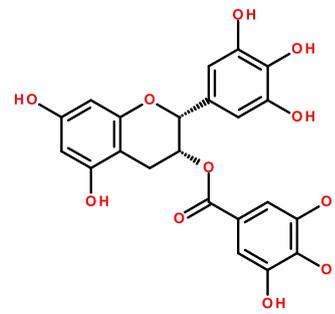
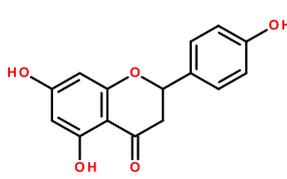
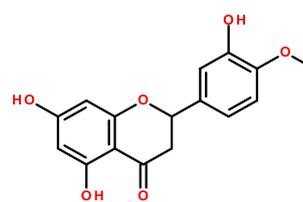


Assignment	Chemical shift δ (ppm)	Type & J-coupling (Hz)	Number of H
85 (OH)	12.9	s	1
83	7.71	dd (8.5-2.2)	1
79	7.61	d (2.2)	1
82	7.19	d (8.5)	1
62	7.04	s	1
58	6.82	d (2.2)	1
66	6.47	d (2.2)	1
OH sugar	5.42	d (4.9)	1
OH sugar	5.21	d (5.4)	1
OH sugar	5.19	d (4.8)	1
51	5.07	d (7.4)	1
93 (OH)	4.88	t (5.4)	1
OH sugar	4.68	d (5.6)	1
OH sugar	4.58	d (4.3)	1
72	4.55	d (1.2)	1
OH sugar	4.44	d (6.1)	1
91	4.13	t (5.0)	2
95	3.87	s	3
70	3.86 + 3.44	m + m	1 + 1
92	3.76	q (5.0)	2
77	3.66	m	1
55	3.61	m	1
74	3.49-3.37	m	1
76	3.49-3.37	m	1
52	3.35-3.25	m	1
53	3.35-3.25	m	1
54	3.18-3.11	m	1
75	3.18-3.11	m	1
86	1.08	d (6.2)	3

Table S1. Previous supercritical fluid chromatographic methods to analyze flavonoids and their full characteristics

Reference	Year	Flavonoid type	Glycosides	Sample	Analysis time (min)	Stationary phase	Column dimensions	Mobile phase co-solvent	Temperature (°C)	Back-pressure (Mpa)	Flow rate (mL/min)	UV or DAD detection	MS detection	Make-up solvent for MS
[11]	1991	Polymethoxylated flavones	Aglycones	Citrus essential oils	8	Silica	250 x 4.6 mm, 5 µm	MeOH; 10% isocratic	40	20.3	1.3-3	UV 313 nm		
[12]	1996	Polymethoxylated flavones	Aglycones	Citrus essential oils	25	Silica	250 x 4.6 mm, 5 µm	MeOH; 10-30% gradient	40	10.1	2.5	UV		
[23]	1999	Flavonols	Aglycones	Standards	na	Phenyl	200 x 4.6 mm, 5 µm	EtOH +0.1% phosphoric acid; 10% isocratic	50	25	1.05	UV 254 nm		
[24]	2002	Flavanols	Aglycones	Grape seed extracts	25	Propanediol-bonded silica	500 x 4.6 mm, 5 µm	MeOH +0.25% citric acid; 7-45% gradient	40	12.7	2	UV 280 nm		
[13]	2014	Flavones	Aglycones & glycosides	Chamomile extracts	15	2-Ethylpyridine	150 x 3.0 mm, 1.7 µm	MeOH:IP:O:H 1:1 +0.5% formic acid; 5-20% gradient	50	10.3	1.7	DAD 190-400 nm	ESI(+)-MS	MeOH + 0.1% formic acid
[6]	2015	Isoflavones	Aglycones & glycosides	Dietary supplements	10	Hybrid silica	100 x 3.0 mm, 1.7 µm	MeOH +0.05% phosphoric acid; 2-25% gradient	50	15	2	UV 254 nm		
[14]	2016	Unidentified		<i>Ganoderma lucidum</i> mushroom extracts	12	Propanediol-bonded silica	250 x 4.6 mm, 5 µm	MeOH +20 mM ammonium acetate; 5-50% gradient	60	13.8	2	DAD	ESI(+)-MS	na
[9]	2016	Flavones, Flavonones, Flavonols & Flavonols	Aglycones & glycosides	Standards and different plant extracts	15	Propanediol-bonded silica, octadecylsiloxane-bonded silica and 2-ethylpyridine-bonded silica	100 x 3.0 mm, 1.7 µm	MeOH +2% H ₂ O and 10 mM ammonium formate; 2-40% gradient	40	15	1.6	DAD	ESI(+)-MS/MS	EtOH, 0.8-0.3 mL/min reversed gradient
[25]	2016	Flavones & Isoflavones	Aglycones	Standards	na	Cyanopropyl-bonded superficially porous silica	100 X3.0 mm, 2.7 µm	MeOH + 20 mM ionic liquid	45	11	2	UV 260 nm		
[8]	2017	Flavones & Flavonols	Aglycones & glycosides	<i>Chrysanthemum morifolium</i> extracts	18	Silica	150 x 4.6 mm, 5 µm	MeOH +0.1% phosphoric acid; 10-20% gradient	40	20	3	UV 313 nm		
[15]	2017	Isoflavones	Aglycones & glycosides	<i>Radix astragal</i> extracts	17	Pentaffluorophenyl	100 x 3.0 mm, 1.7 µm	MeOH; 5-40% gradient	40	11	0.5	DAD		
[21]	2017	Isoflavones	Aglycones & glycosides	Human serum	13	Diethylamino-propyl-bonded silica	100 x 3.0 mm, 1.7 µm	MeOH +0.5% formic acid; 15-40% gradient	35	12.4	1.5	DAD 254 nm		
[20]	2018	Flavonones, Flavones & Flavonols	Aglycones & glycosides	Garlic extracts	8	Propanediol-bonded silica	150 x 4.6 mm, 3 µm	MeOH +0.1 mM oxalic acid + 1 mM ammonium formate; 0-50% gradient elution	40	10	2		ESI(-)-MS/MS	na
[26]	2018	Flavonones, Flavonols & Flavonols	Aglycones	Bee pollen	24	Polybutylene-terephthalate	250 x 4.6 mm, 5 µm	MeOH +0.1% TFA; 5-50% gradient	35	15	2	UV 220, 270, 320 and 370 nm		
[22]	2018	Isoflavones	Aglycones	Rat plasma	3	2-Ethylpyridine	100 x 3.0 mm, 1.7 µm	MeOH +0.1% formic acid; 10-40% gradient	40	13.8	1		ESI(+)-MS/MS	MeOH, 0.2 mL/min
[16]	2019	Flavonone	Aglycones & glycosides	Licorice roots extracts	11	2-Picolylamine	100 x 2.1 mm, 1.7 µm	MeOH +0.2% formic acid; 15-30% gradient	40	13.8	0.8	DAD 190-400 nm		
[17]	2019	Isoflavones	Aglycones & glycosides	<i>Radix puerariae</i> extracts	8	Propanediol-bonded silica	100 x 3.0 mm, 1.7 µm	MeOH +1 mM oxalic acid; 20-40% gradient	50	15.2	1.4	DAD	ESI(+)-MS/MS	MeOH + formic acid; 0.2 mL/min
[17]	2019	Polymethoxylated flavones & Flavonones	Aglycones & glycosides	<i>Chiri reticulatae</i> pericarpium	11	Silica	150 x 2.1 mm, 5 µm	MeOH; 8-36% gradient	45	9.5	0.8	UV 283 nm		
[18]	2019	Flavones & Flavonols	Aglycones	Sweet potato leaves extracts	9	2-Ethylpyridine	100 x 3.0 mm, 1.7 µm	MeOH +0.05% formic acid; 5-40% gradient	50	14	1		ESI(-)-MS/MS	na
[19]	2019	Prenyl-flavonoids	Aglycones & glycosides	<i>Herba epimedii</i> extracts	18	Propanediol-bonded silica	100 x 3.0 mm, 1.7 µm	MeOH:AcN (1:1) +5 mM oxalic acid and 3% H ₂ O; 10-50% gradient	30	11	1.2	DAD 330 nm	ESI(+)-MS/MS	MeOH, 0.3 mL/min

Table S2. m/z values examined with single-ion-recording (SIR) or observed on the mass spectra for each standard flavonoid with tentative identification

Compound name	Structure	SIR	m/z
Catechin (C)		$[M+H]^+$	291
		$[M+Na]^+$	313
		$[M+NH_4]^+$	308
		$[M-H]^-$	289
		$[M+MSA-H]^-$	385
Epigallocatechin gallate (EGCG)		$[M+H]^+$	459
		$[M+Na]^+$	481
		$[M+NH_4]^+$	476
		$[Genin+H]^+$	307
		$[Gallic\ acid+H]^+$	171
		$[M-H]^-$	457
		$[M+MSA-H]^-$	553
Naringenin (N)		$[M+H]^+$	273
		$[M+Na]^+$	295
		$[M+NH_4]^+$	290
		$[M-H]^-$	271
		$[M+MSA-H]^-$	367
Hesperetin (Ht)		$[M+H]^+$	303
		$[M+Na]^+$	325
		$[M+NH_4]^+$	320
		$[M-H]^-$	301
		$[M+MSA-H]^-$	397
		$[2M-H_2-H]^-$	601
		$[2M-H_2+MSA-H]^-$	697

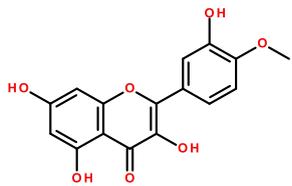
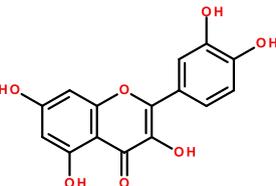
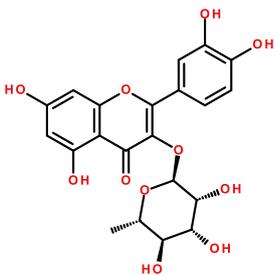
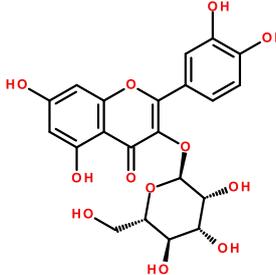
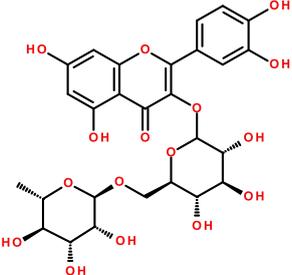
Tamarixetin (T)		$[M+H]^+$	317
		$[M+Na]^+$	339
		$[M+NH_4]^+$	334
		$[M-H]^-$	315
		$[M+MSA-H]^-$	411
		$[2M-H_2+MSA-H]^-$	725
Quercetin (Q)		$[M+H]^+$	303
		$[M+Na]^+$	325
		$[M+NH_4]^+$	320
		$[M-H]^-$	301
		$[M+MSA-H]^-$	397
		$[2M-H_2+MSA-H]^-$	697
Quercitrin (Qi)		$[M+H]^+$	449
		$[M+Na]^+$	471
		$[M+NH_4]^+$	466
		$[Genin+H]^+$	303
		$[M-H]^-$	447
		$[M+MSA-H]^-$	543
Isoquercitrin (IQi)		$[M+H]^+$	465
		$[M+Na]^+$	487
		$[M+NH_4]^+$	482
		$[Genin+H]^+$	303
		$[M-H]^-$	463
		$[M+MSA-H]^-$	559
Rutin (R)		$[M+H]^+$	611
		$[M+Na]^+$	633
		$[M+NH_4]^+$	628
		$[Genin+H]^+$	303
		$[M-H]^-$	609
		$[M+MSA-H]^-$	705

Table S3. Design of experiments to optimize MS parameters

Experiment number	Configuration	Probe temperature (°C)	Cone Voltage (V)	Capillary voltage (ESI+) (kV)
1	231	400	20	0,3
2	233	400	20	1,5
3	222	400	15	0,8
4	123	200	15	1,5
5	133	200	20	1,5
6	212	400	10	0,8
7	331	600	20	0,3
8	223	400	15	1,5
9	221	400	15	0,3
10	323	600	15	1,5
11	213	400	10	1,5
12	132	200	20	0,8
13	112	200	10	0,8
14	333	600	20	1,5
15	311	600	10	0,3
16	121	200	15	0,3
17	312	600	10	0,8
18	131	200	20	0,3
19	000	400	15	0,9
20	313	600	10	1,5
21	332	600	20	0,8
22	211	400	10	0,3
23	232	400	20	0,8
24	113	200	10	1,5
25	111	200	10	0,3
26	322	600	15	0,8
27	321	600	15	0,3
28	000	400	15	0,9
29	122	200	15	0,8

Table S4. m/z values examined with single-ion-recording (SIR) for each sample molecules

Compound name	Structure	SIR	m/z
Hidrosmin (H) and isomer (i)		[M+H] ⁺	653
		[M+Na] ⁺	675
		[Genin+H] ⁺	345
		[M-H] ⁻	651
Disubstituted congener (D)		[M+H] ⁺	697
		[M+Na] ⁺	719
		[Genin+H] ⁺	389
		[M-H] ⁻	695
α-Glucosyl-Hesperidin (αGH)		[M+H] ⁺	773
		[M+Na] ⁺	795
		[Genin+H] ⁺	303
		[M-H] ⁻	771
		[M+MSA-H] ⁻	867
Hesperetin-7-O-glucoside (Ht-Glu)		[M+H] ⁺	465
		[M+Na] ⁺	487
		[Genin+H] ⁺	303
		[M-H] ⁻	463
		[M+MSA-H] ⁻	559

III- Conclusion

Depuis nos travaux appliqués aux flavonoïdes par CO₂ SC, deux articles en lien avec cette thématique ont investigué la famille des flavonoïdes par SFC [173], [174]. Ces deux études mettent en avant des séparations de flavonoïdes hydroxylés. Les premiers travaux s'appliquent sur un extrait de plante fourragère obtenu par SFE [173] alors que pour les seconds, la pharmacocinétique de la Naringénine et de sa prodrogue (Naringénine sur laquelle est additionnée un groupement carbamate) est suivie dans du plasma de rat [174]. Ces deux exemples sont également focalisés sur des flavonoïdes de poids moléculaires réduits et des polarités peu élevées car les flavonoïdes sont hydroxylés mais non glycosylés.

Ce chapitre nous a permis de mettre en avant les étapes de développement analytique pour mettre en place une méthode UC-MS sur un ensemble de standards de flavonoïdes. La méthode optimale fonctionne avec un gradient de co-solvant large allant de 20% jusqu'à 100% de co-solvant, c'est pour cette raison que nous ne parlons plus de SFC mais d'UC. Dans le tableau 3, les flavonoïdes analysés sont classés par ordre d'élution. On observe que certains flavonoïdes éluent à des pourcentages de co-solvant de la gamme SFC comme l'Hespéretine ou la Naringénine tandis que la Quercitrine ou la Catéchine éluent avec un pourcentage de co-solvant de la gamme EFLC. Enfin pour l'élution du gallate d'épigallocatechine (EGCG), l'élution est réalisée à 100% de phase liquide. Les lignes jaunes correspondent aux flavonoïdes sous formes aglycones, les lignes orange aux hétérosides et la ligne bleue de l'EGCG à un flavonoïde estérifié.

Tableau 3 : Paramètres physico-chimiques et élution des flavonoïdes analysés par UC-MS

Flavonoïdes	MW (Da)	clog P	TPSA	Tr (min)	Ce (%)
<i>Hespéretine</i>	302,1	2,5	96,2	1,4	29
<i>Naringénine</i>	272,1	2,5	87,0	1,7	32
<i>Tamarixetine</i>	316,1	2,3	120,4	3,1	46
<i>Hidrosmine</i>	652,2	-1,4	247,4	5,3	69
<i>Quercétine</i>	302,0	2,0	131,4	5,8	73
<i>Quercitrine</i>	448,1	0,5	190,3	6,4	79
<i>α-glucosyle hespéridine</i>	772,2	-3,3	313,4	6,8	84
<i>Isoquercitrine</i>	464,1	-0,5	210,5	6,8	84
<i>Catéchine</i>	290,1	1,5	110,4	7,5	90
<i>Rutine</i>	610,2	-1,7	269,4	7,6	92
<i>EGCG</i>	458,1	2,2	197,4	9,8	100

Nous n'avons pas trouvé de corrélation entre ces paramètres physico-chimiques et la rétention de ces standards de flavonoïdes. Cela peut s'expliquer par le fait que les standards de flavonoïdes proviennent de familles différentes, ce qui affecte notamment leur structure tridimensionnelle, non prise en compte par ces descripteurs moléculaires. L'étude des additifs associés au méthanol comme co-solvant a mis en évidence l'efficacité de l'acide méthanesulfonique sur la forme des pics chromatographiques. Cependant, il ne faut pas oublier que sa compatibilité avec notre détecteur MS nécessitait une adaptation de la vanne d'échappement du QDa. Sur ce même module, nous avons optimisé les quelques paramètres modulables en MS par un plan d'expérience. Les tensions de capillaire et tensions de cône optimisées nécessaires pour maximiser la réponse en masse (mesure du s/n) furent réduites au minimum des points du plan d'expérience. Ce travail a permis la détection en MS de l'EGCG, non visible dans nos conditions initiales. L'optimisation de la nature du solvant de make-up a mis en évidence une amélioration de la réponse MS pour un ensemble d'ions comme les pseudo-moléculaires $[M+H]^+$ et $[M-H]^-$, l'adduit $[M+Na]^+$, ou encore l'ion fragment $[G+H]^+$ (avec G symbolisant la génine), via l'utilisation d'un mélange de méthanol et d'eau (98/2 ; v/v) avec 20 mM d'hydroxyde d'ammonium. Ce travail a permis de remplacer le solvant de make-up usuellement utilisé au sein de l'équipe et le nouveau make-up a été utilisé pour d'autres types d'applications en SFC et en UC.

Tout ce développement de méthode a facilité le travail d'étude des produits commercialisés. L'analyse de l'Hidrosmine a révélé la présence majoritaire de l'isomère de position de la molécule attendue et de la forme disubstituée tandis que l' α -glucosyle hespéridine s'est révélée plus « propre » avec cependant la présence d'une impureté pour laquelle nous avons proposé une structure.

Depuis la fin de ces travaux, cette méthode analytique a été mise en place au sein des laboratoires Servier. De plus, ces travaux ont été poursuivis au sein de l'équipe des fluides supercritiques pour adapter la méthode à la SFC prep en remplaçant l'AMS du-co-solvant par un additif plus adapté. Un complément alimentaire a été analysé par UC-MS. Après une étape de macération, la génine ainsi que plusieurs hétérosides de cette même génine ont été séparés.

Pour compléter ce travail, il serait pertinent d'analyser des flavonoïdes provenant d'autres catégories (chalcones, néoflavonoïdes, ...) que celles de nos standards pour connaître les limites de cette méthode UC-MS. L'utilisation de la modélisation

moléculaire serait également intéressante à étudier pour comprendre les ordres de rétention de ces flavonoïdes glycosylés ou non qui ne suit pas de corrélation évidente entre polarité et temps de rétention.

Chapitre 3 : Les peptides pharmaceutiques par UC-MS

I- Introduction

Le chapitre précédent s'est focalisé sur les flavonoïdes. Il a permis de discuter de la mise en place d'une méthode UC-MS sur des composés à polarité variée de poids moléculaire inférieur à 1 000 Da. Nous souhaitons à présent étudier les peptides à visée pharmaceutique par chromatographie unifiée. Cette famille de composés nous a donné l'occasion d'explorer dans un premier temps des peptides de petites tailles (tripeptides et pentapeptides). Ceux-ci présentent des poids moléculaires du même ordre de grandeur que les flavonoïdes mais une gamme de polarité plus étendue. De plus, les standards de flavonoïdes sélectionnés étaient des composés non ioniques au contraire de ces peptides pouvant être mono ou polychargés. Dans un second temps, nous avons poussé l'étude par UC-MS sur des peptides multichargés de masses moléculaires plus importantes entre 1000 et 5000 Da.

Préalablement à toutes mises en place analytiques, nous avons débuté par un retraitement des analyses de peptides auparavant réalisées à l'IdRS. Cette compréhension du travail analytique au sein de Servier, essentiellement réalisé par RPLC-MS, permet de connaître la méthode analytique employée au quotidien et ses performances. Ce travail a mis en évidence les peptides non résolus chromatographiquement ou non-élus par RPLC-MS après l'application d'un protocole analytique interne à l'IdRS.

L'accès à une grande quantité de « petits » peptides cycliques et linéaires (de masse inférieure à 800 Da), nous a servi à développer une méthode par UC-MS. La méthode devait avant tout être développée à l'échelle analytique pour réaliser le profilage d'impuretés, mais devait éventuellement pouvoir être transposable à l'échelle semi-préparative pour purifier le peptide. Les petits peptides procurés par Servier étant nombreux (76 peptides) mais en faibles quantités (100 µl de peptides en solution à 10 mM dans le DMSO), nous avons développé notre méthode UC-MS à partir de 10 peptides (5 linéaires et 5 cycliques). La sélection de ces 10 peptides nécessitait de recouvrir le mieux possible l'ensemble des 76 peptides disponibles, comme décrit sur la figure 14.A. Cette sélection s'est effectuée à partir de différents critères, (i) couvrir le plus possible la gamme de polarité, (ii) couvrir la gamme de masse moléculaire, (iii) travailler sur des peptides avec/sans présence de sels de TFA et (iv) observer la résolution d'isomères. Le développement de la méthode s'est concentré en premier lieu

sur la PS et la PM (4 colonnes et 3 compositions de PM). Les fonctions de Derringer ont facilité le choix en établissant le meilleur compromis PM/PS à partir de critères analytiques. Puis, la méthode a été optimisée sur différents éléments que sont la température du four colonne, le gradient de co-solvant, la contre-pression, ... Nous avons terminé par l'analyse UC-MS (voir figure 14.B) du lot complet de 76 peptides avec la méthode optimisée et observé s'il existe une corrélation entre la rétention et une des mesures de polarité décrite dans le chapitre 1.II.d.

Nous avons ensuite comparé les résultats obtenus par UC-MS et les performances de la méthode RPLC-MS de l'IdRS. Cette évaluation fut établie sur un set réduit (cf. figure 14.C). Dans un premier temps, nous avons focalisé cette comparaison sur le peptide principal (pic majoritaire) en portant un regard sur l'orthogonalité des deux méthodes. Nous avons par la suite défini et mesuré des critères de réussite. La complémentarité des deux méthodes analytiques est ressortie de cette confrontation avec un taux de succès plus élevé en UC-MS.

Dans un second temps, la comparaison s'est concentrée sur les impuretés d'un lot réduit de peptides correctement élués avec les deux méthodes (cf. figure 14.D) pour en estimer leur pureté. Le nombre d'impuretés ainsi que leurs profils ont été investigués, ce qui a de nouveau pointé la complémentarité des deux méthodes. Nous avons retrouvé des impuretés communes aux deux techniques ainsi que des impuretés observées seulement sur l'une ou l'autre des deux techniques.

Pour finir, la robustesse de la méthode UC-MS a été étudiée par un plan de Doehlert et nous avons comparé les coûts opérationnels des deux méthodes pour un échantillon.

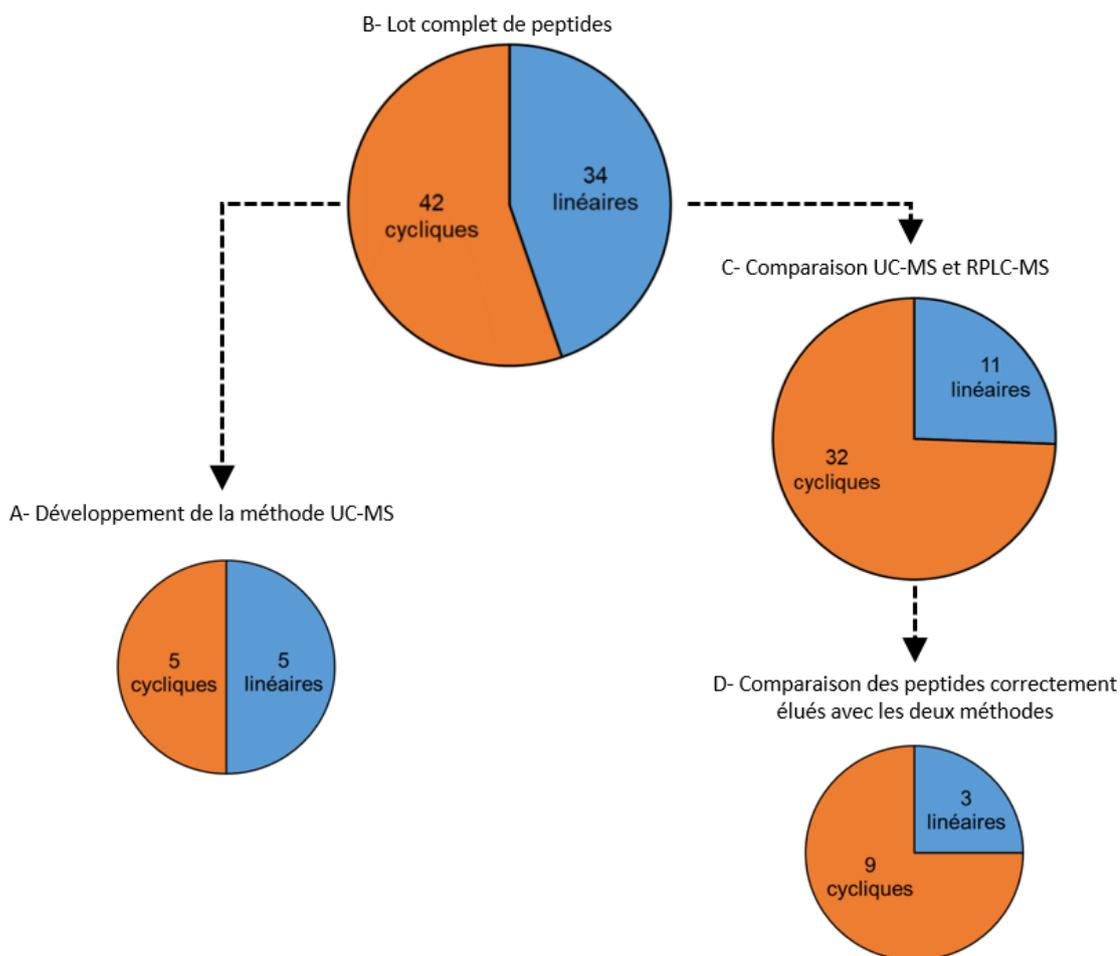


Figure 14 : Composition du set de « petits » peptides selon l'étape d'avancement du travail

La dernière partie de ces travaux sur les peptides a cheminé vers des peptides de plus hauts poids moléculaires, compris entre 1 000 et 5 000 Da. La taille et la polarité de ces « gros » peptides, nous a conduit au développement d'une nouvelle méthode UC-MS. Après de multiples essais de colonnes, de composition de phase mobiles, d'optimisation de la détection, etc... une méthode UC-MS reprise des recherches de Losacco et al. a permis l'élution de ces « gros » peptides [175]. L'adaptation de la méthode publiée à nos peptides de travail a laissé place à l'étude d'autres aspects liés à ces « gros » peptides comme le solvant de mise en solution et le contre-ion associé. Dans notre cas, les contre-ions étudiés étaient des sels de TFA ou des sels d'acétate.

II- Analyse des « petits » peptides (< 1000 Da) par UC-MS

a. Etat des lieux des analyses par RPLC-MS

Avant de débiter tout développement de méthode par UC-MS, nous avons repris les données et métadonnées des analyses réalisées à l'IdRS. L'objectif de ce prélude au développement était de connaître et comprendre les paramètres analytiques appliqués au sein de Servier (PS, PM, concentration de l'échantillon, ...). L'ensemble des analyses de peptides revues correspondent à une tranche de vingt années, c'est-à-dire environ 2000 fichiers. Cet ensemble de 2000 fichiers d'analyse correspond lui-même à 214 peptides investigués au sein de l'IdRS. La quasi-totalité de ces données résulte des analyses par RPLC-MS à l'échelle analytique. Le reste résulte soit d'analyse par RPLC à l'échelle semi-préparative ou d'analyse par électrophorèse capillaire.

Depuis 2016, un protocole analytique pour l'analyse de peptides a été mis en place. Au sein des données retraitées cela concerne 76 des 214 peptides. Ces 76 peptides sont différents de ceux étudiés dans le chapitre 3.II.b. Ce protocole consiste à analyser par RPLC-MS les peptides à l'aide de trois gradients successifs. Ces trois gradients emploient tous une colonne ACQUITY CSH C18, une phase mobile dite A composée d'un mélange eau/acétonitrile/TFA (100/1/0,1 ; v/v/v) et une phase mobile dite B composée d'un mélange acétonitrile/eau/TFA (100/1/0,1 ; v/v/v). Le premier des trois gradients dit « gradient générique » permet de déterminer la composition à l'élution du pic du peptide, c'est-à-dire la proportion d'acétonitrile permettant d'éluer le peptide. Le second gradient appelé « gradient focus court » se focalise sur la zone d'élution autour du peptide et sert principalement à dégager les impuretés co-élues avec le pic majoritaire. Enfin, le troisième gradient nommé « gradient focus long » conserve la focalisation autour de l'élution du peptide avec un temps de gradient deux fois plus long ce qui permet d'en améliorer la résolution. En cas de non-élution ou d'une résolution insatisfaisante, un deuxième protocole par électrophorèse capillaire est appliqué.

L'ensemble de ce retraitement a mis en évidence plusieurs éléments. Les poids moléculaires des peptides analysés à l'IdRS sont compris entre 500 Da et 7 400 Da. Sur cet ensemble, 84% d'entre eux présentent une masse moléculaire strictement supérieure à 1 000 Da, ce qui correspond à la seconde « gamme » de peptides développés dans la partie III de ce chapitre. Nous avons mesuré le taux de succès du

protocole RPLC-MS de l'IdRS sur les 76 peptides concernés par le protocole. Celui-ci est estimé à 86% de réussite sur cet ensemble de sélection. Nous n'avons pas estimé un taux de succès sur l'ensemble des 214 peptides puisque le protocole n'était pas mis en place avant 2016. Pour ce qui est des insatisfactions analytiques par RPLC-MS, la majorité des chromatogrammes retraités sont dus à des co-élutions entre le peptide d'intérêt et les impuretés détectées.

L'ensemble de ces informations cumulées nous amène à vouloir mettre en œuvre une méthode analytique, celle-ci sera capable de couvrir une large gamme de polarité avec un regard particulier sur les peptides les plus polaires. Pour cette raison, l'UC-MS semble être un choix intéressant à investiguer.

b. Développement de l'analyse des « petits » peptides par UC-MS

Cette première partie se focalise sur un ensemble de peptides à courtes chaînes (tripeptides et pentapeptides) pour lesquels nous avons développé une méthode UC-MS. Ces « petits » peptides sont majoritaires parmi les peptides commercialisés à visée pharmaceutiques [176]. Même si la part du nombre de peptides de plus hauts poids moléculaires augmente, les peptides à très courtes chaînes restent majoritaires. Le dernier peptide mis sur le marché en 2021 et recensé par D'Aloisio et al., est lui encore un « petit » peptide linéaire (680 Da) nommé Difelikefalin [177].

Pour développer notre méthode UC-MS, l'utilisation des fonctions de Derringer a été essentielle pour sélectionner en même temps la colonne et la composition de co-solvant la plus adaptée. Cette problématique est liée à la faible quantité de peptides disponibles. Ce développement fut poursuivi par des optimisations multiples de la méthode UC-MS toujours focalisé sur les 10 peptides de départ. La conclusion de ce travail était d'analyser l'ensemble des 76 peptides mis à disposition par l'IdRS par UC-MS.



Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part I. Method development

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ARTICLE INFO

Article history:

Received 15 August 2021

Revised 12 October 2021

Accepted 14 October 2021

Available online 15 October 2021

Keywords:

Derringer desirability functions

Peptides

Supercritical fluid chromatography

Unified chromatography

ABSTRACT

A method to analyse short-chain bioactive peptides (MW < 800 Da) and their impurities was developed with a unified chromatography (UC) analysis, including a wide mobile phase gradient ranging from supercritical fluid to near-liquid conditions, with UV and electrospray ionization mass spectrometry detection (ESI-MS). Four stationary phases and three mobile phase compositions were examined. Ten model peptides were first selected to identify the best operating conditions, including five linear tripeptides and five cyclic pentapeptides, with log P values ranging from -5.9 to 3.6, and including isomeric species. Derringer desirability functions were designed to identify optimal operating conditions based on 7 criteria, namely the number of peaks detected (including all impurities resolved), the proportion of the chromatogram occupied by target peaks, the least favourable resolution observed between the main peptide and impurities, peak shape features (asymmetry and peak width at half height), and finally the signal-to-noise ratio observed both with UV (210 nm) and ESI-MS in positive ionization mode.

The optimum conditions were obtained on Ascentis Express OH5 stationary phase, with a mobile phase composed of carbon dioxide and methanol, comprising 2% water and 20 mM ammonium hydroxide. The final gradient program ranged from 5 to 80% co-solvent in CO₂, with a reversed flow rate gradient ranging from 3.0 to 1.5 mL/min. Back-pressure was set at 120 bar and the column oven temperature at 60°C. Optimal conditions were applied to a large set of 76 peptides (34 linear tripeptides and 42 cyclic pentapeptides) and provided adequate scattering of the peaks in the retention space, together with some separation of isomeric species, particularly for the cyclic peptides.

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1. Introduction

Peptides are oligomers and polymers of amino acids and, as such, can be classified as biomolecules. They may be linear or cyclic, produced synthetically or naturally by microbial fermentation or enzymatic production [1]. They are also the topic of increasing interest in analytical sciences, mostly related to food or pharmaceutical applications [2]. For instance, Shivanna and Nataraj [3] reviewed many therapeutic effects of milk-derived bioactive peptides such as antihypertensive, antidiabetic, anticancer, or others. Although their applicability as drugs may be limited by a short half-life, a poor ability to cross membrane barriers and complex mechanisms of action [4], peptides are however regarded as bioactive compounds with strong medicinal potential and high

efficiency. Indeed, they present good selectivity, high specificity, low toxicity compared to small "chemical" molecules, and they sparsely accumulate in tissues [5,6]. For instance, they can replace inefficient classical antibiotics [7–9]. Cyclic peptides often appear to have superior biological activities to the flexible linear peptides, possibly because they have more conformational rigidity [2,10].

Supercritical fluid chromatography (SFC) is a separation technique employing a mobile phase composed of CO₂ in the sub- or supercritical state, usually combined with a liquid co-solvent (most often a short-chain alcohol like methanol) [11–13]. Thereby, SFC is usually associated to reduced consumption of organic solvent and energy compared to liquid-phase chromatography, especially at the preparative scale. In addition, CO₂ is a low-cost, non-toxic fluid, odorless and non-flammable, which are all desirable properties for a chromatographic solvent. Supercritical fluids are also related to high analyte diffusivity and to low viscosity,

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generating low pressure drops. As a result, SFC usually provides high efficiency at high flow rates. These benefits are visible whatever the proportion of CO₂ in the mobile phase composition, from the large proportions of CO₂ usually employed in SFC (typically above 50%) to the low proportions of CO₂ employed in enhanced-fluidity liquid chromatography (EFLC, typically below 50%) [14]. It was recently demonstrated that SFC, EFLC and HPLC may be joint in a single experiment with wide mobile phase gradients, where the CO₂-solvent composition possibly varies from 100:0 to 0:100 (v/v). This experiment can be called unified chromatography (UC). The concept of unified chromatography was firstly discussed by J. Calvin Giddings in 1965 [15] and may also include other forms of chromatography like GC. Historical developments, fundamentals, instrumentation and applications of unified chromatography have been reviewed by Silva *et al.* [16]. A first application of UC with SFC-EFLC-HPLC combination was proposed in 2014 by Bamba and co-workers, who focused on vitamins covering a large range of polarity [17]. Since then, a few works have presented UC analyses of food samples [18,19], metabolomics samples [20,21], or cosmetic and pharmaceutical samples [22], including peptides [23]. Clearly, the interest of the wide mobile phase gradients employed in such UC experiments is to achieve the elution of polar and non-polar compounds in a single chromatographic experiment. UC is thus an interesting option for biomolecules, where polarity range may vary to a great extent [24]. However, difficulties are expected with some biomolecules like proteins in UC as they may change of conformation. This aspect of structural alterations before and after analysis has been studied by Regalado and co-workers [25] with EFLC gradients. High polarity combined to high molecular weight together complicates the analyses but the addition of a small portion of water in the co-solvent generally improves efficiency [26]. The impact of water and other additives (acids, bases or salts) introduced in the SFC mobile phase were previously examined [27–29]. The choice of additives is significant in SFC and UC, not only to achieve good chromatographic quality but also to ensure sensitive detection, especially when the chromatography is hyphenated to mass spectrometry (MS) [22,30,31].

SFC, EFLC and UC can all employ a wide variety of stationary phases as all stationary phases employed in HPLC are useful, from the polar phases used in normal-phase and hydrophilic interaction modes, to the non-polar phases used in reversed-phase mode. Furthermore, SFC-specific stationary phases have also been designed by some manufacturers, offering additional opportunities for selectivity optimization [13,32]. Regarding the analysis of peptides in SFC and EFLC, a variety of stationary phases have been employed and no general guideline could be drawn for this purpose [24].

This paper presents method development in UC on a set of small linear and cyclic peptides (with molecular weight below 800 Da) starting from a short selection of them to select the most adapted column and co-solvent. Previous studies on this topic rarely examined peptides with molecular weights below 1000 Da. In addition, all of the model peptides in the present included impurities, as the objective was to develop a method that could serve to carry out the impurity profiling of other peptides that would be comparable in size. In previous works, impurities were rarely considered and the sole object was usually to achieve the elution of the major peptide with adequate peak shapes. Derivatives' desirability functions were used to select the best combination of stationary phase (among 4 columns tested) and mobile phase composition (among 3 tested) to achieve adequate chromatographic quality together with UV and MS sensitivity. After this selection, column temperature, back-pressure and flow-rate were also examined. Finally, UC-MS results for the full set of 76 peptides are discussed with correlation between chromatographic retention and physical properties. A particular attention is paid to isomeric species.

Table 1

Small set of peptides for the selection of stationary and mobile phases, with significant features: calculated log P, calculated polar surface area (TPSA), molecular weight and the presence of trifluoroacetic acid (TFA) salt.

Peptide ID	Linear/Cyclic	Salts	MW (g/mol)	log P	TPSA
LP 9905	Linear	-	433.2	-2.01	193.7
LP 10400	Linear	-	433.2	-2.01	193.7
LP 10306	Linear	TFA	456.2	-0.96	179.3
LP 10376	Linear	TFA	458.2	-3.48	272.7
LP 04693	Linear	-	495.3	3.59	137.4
CP 17730	Cyclic	-	539.2	-5.91	255.0
CP 19972	Cyclic	-	539.2	-5.91	255.0
CP 19583	Cyclic	-	566.3	-0.01	190.2
CP 19595	Cyclic	-	566.3	-0.01	190.2
CP 20375	Cyclic	TFA	714.4	1.67	226.1

2. Material and methods

2.1. Chemicals and solvents

Seventy-six peptides were selected from an in-house compound bank at Servier Research Institute. These compounds have confidential structures not exposed in this paper. They are however described by their significant features in Table S1. The values of log P and topological polar surface area (TPSA) were determined with Vortex v2019.04.82972.15-s (Dotmatics Limited). Further description is provided in Section 3.1. A subset of 10 peptides was selected for method development (Table 1). The peptides were provided in dimethylsulfoxide (DMSO) solutions at 10 mM. Some of them having basic residues were present as trifluoroacetic acid salts. The DMSO solutions were further diluted with methanol at 1 mM for analysis.

Gradient-grade methanol (MeOH) was purchased from VWR (Fontenay-sous-bois, France) and CO₂ with a purity of 99.995% was provided by Air Liquide (Bagneux, France). Water was purified by a Purelab Flex instrument from Veolia (Wissous, France). The additives, methanesulfonic acid (MSA), trifluoroacetic acid (TFA) and ammonium hydroxide solution were all purchased from Sigma-Aldrich (Merck, France).

2.2. Instrument

The SFC instrument was an ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) from Waters Corporation (Guyancourt, France). 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, an automated back-pressure regulator, a 2-position column oven compatible with 150 mm-length columns and two detectors: a photodiode-array (ACQUITY PDA®) detector and a single-quadrupole mass spectrometer with electrospray ionization source (ACQUITY QDa®). An isocratic solvent manager was used as a make-up pump and was positioned before the MS. The main flow stream was then split by the on-board flow-splitter assembly. With this system, a portion of the column flow goes to the back-pressure regulator while an unknown portion goes to the MS. Empower® 3 was used for system control, data acquisition and integration of peaks for column efficiency measurements.

2.3. Chromatographic methods

Initially, based on the selection of 10 representative peptides, four columns were compared, comprising different stationary phase chemistries. Their characteristics are presented in Table 2.

Table 2
Four stationary phases examined in this study.

Column name	Manufacturer	Support	Ligand	Dimensions (mm)	Particle size (μm)
Chiralpak ZWIX(-)	Chiral Technologies	Fully porous silica	Zwitterionic quinidine-based ligand	150 × 3.0	3.0
Nucleoshell HILIC	Macherey-Nagel	Superficially porous silica	sulfobetaine ligand	150 × 3.0	2.7
Accucore HILIC	Thermo	Superficially porous silica	-	150 × 4.6	2.6
Ascentis Express OH5	Supelco	Superficially porous silica	Pentahydroxyl ligand	150 × 4.6	2.7

Table 3
Optimized gradient program on Ascentis Express OH5 (150 × 4.6 mm, 2.7 μm) with co-solvent containing ammonium hydroxide and with constant column temperature (60°C) and constant back-pressure (120 bar).

Gradient time (min)	Co-solvent proportion (%)	Flow rate (mL/min)
0.0	5	3.0
0.5	5	3.0
10.5	80	1.5
13.5	80	1.5
13.6	5	1.5
14.0	5	1.5
14.5	5	3.0

Different co-solvent compositions were tested (i) methanol and 20 mM NH_4OH in water 98/2 (v/v), (ii) methanol added with 0.1% v/v methanesulfonic acid (MSA), (iii) methanol added with 0.1% v/v trifluoroacetic acid (TFA).

The column screening was done with a gradient elution program with co-solvent proportion ranging from 5 to 100% (see detailed conditions in Table S2). Because it soon appeared that Chiralpak ZWIX(-) was more retentive than the other phases, a longer step of high proportion of co-solvent was maintained for this column. According to our previous applications of UC elution gradients [18,22], a reversed back-pressure gradient from 150 bar to 110 bar was applied, together with a reversed flow rate gradient from 2.5 mL/min to 1 mL/min. Indeed, pressure drop increases significantly when the co-solvent proportion increases to 100%, thus reducing back-pressure and flow-rates ensures that the inlet pressure remains within the limits of the pumping system. Besides, the reversed flow rate gradient should favor high efficiency at all stages of the elution gradient [20]. The oven temperature was set at 40°C and the sample manager temperature was set at 10°C to limit sample degradation. The injection volume was 5 μL . PDA wavelength was set at 210 nm and spectra were recorded from 190 to 800 nm. MS parameters were set as follows: needle temperature 600°C, capillary voltage 0.3 kV and cone voltage 10 V. The make-up fluid was composed of methanol and 20 mM NH_4OH in water 98/2 (v/v); flow rate was 0.4 ml/min.

The final method was developed on Ascentis Express OH5 (150 × 4.6 mm; 2.7 μm) with a co-solvent composed of methanol comprising 2% water (v/v) and 20 mM NH_4OH . The co-solvent proportion was initially set at 5%, maintained for 0.5 min then increased from 5% to 80% in 10 minutes, where it was maintained for 3 more minutes. A reversed flow-rate gradient from 3.0 to 1.5 ml/min was applied at the same time (see details in Table 3). Back-pressure was maintained constant at 120 bar. The oven temperature was set at 60°C and the sample manager temperature at 10°C. UV detection was done at 210 nm with resolution 1.2 nm. The ESI-MS parameters were as follows: total ion chromatograms (TIC) were registered in positive ionization mode from 100 to 1200 m/z values, while single-ion recordings (SIR) were registered both in positive and negative modes. The needle temperature was set at 600°C and cone voltage at 10 V. Capillary voltage was set at 0.3 kV (ESI+) and -0.8 kV (ESI-). The make-up flow-rate applied a reversed gradient from 0.4 to 0 ml/min in 3 minutes and the solvent composition was identical to the mobile phase cosolvent (methanol comprising

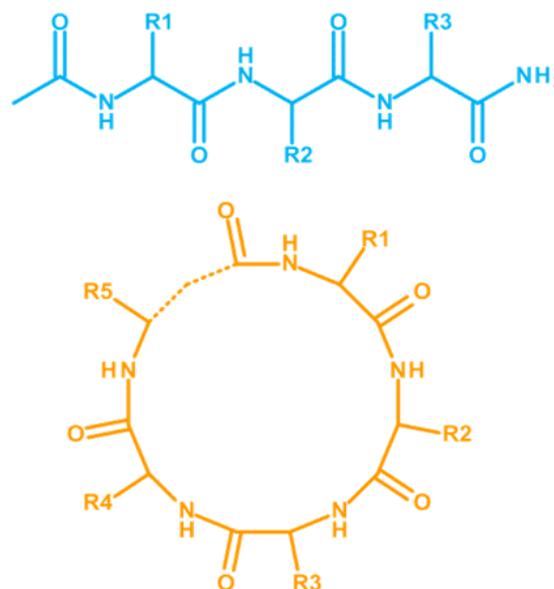


Fig. 1. General structures of the linear tripeptides and cyclic pentapeptides examined in this study.

2% water (v/v) and 20 mM NH_4OH). The injected volume was 5 μL for every blank and sample analysis.

3. Results & discussion

3.1. Description of the peptide set

Model peptides were selected from a library of drug candidates at Servier Research Laboratories. This set included 34 linear tripeptides and 42 cyclic pentapeptides. As represented in Fig. 1, the linear peptides were all acetylated at the N-terminal end, while the C-terminal function had been modified with amidation. For 15 of the cyclic peptides, the ring had been closed through click chemistry with a triazole ring. The residues included hydrophobic and hydrophilic groups, neutral, acidic or basic functions. When basic functions were present, the peptide was solubilized as a TFA salt. Furthermore, the set included several isomeric species: for instance, two or three amino acids could have reversed positions in the peptide chain (positional isomers), or hydrocarbon side-chains could vary (chain isomers). Structural isomers were of particular interest because they are generally difficult to differentiate with reversed-phase liquid chromatography.

Significant features of these peptides are presented in Table S1 and represented in Fig. 2, where molecular weights (MW) are plotted against calculated log P values, and bubble size is related to the topological polar surface area (TPSA). As appears in this figure, in relation to the different number of amino acids in their structure, the linear peptides were generally lighter than the cyclic peptides: the molecular weight of the former ranged from 317 to 526 g/mol, while it ranged from 492 to 736 g/mol for the latter. Calculated log P values were well distributed over a wide range: from -4.1 to 3.6

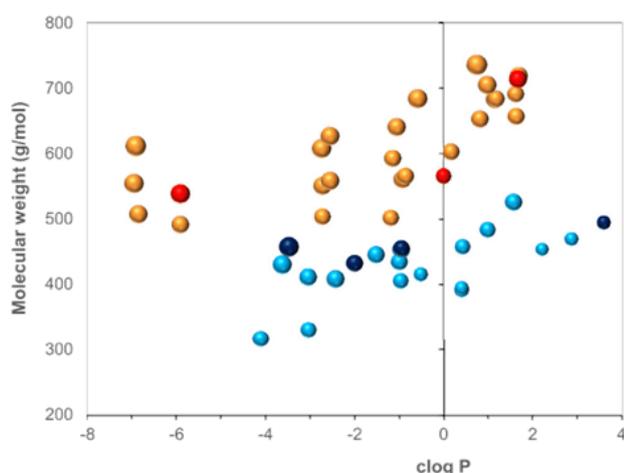


Fig. 2. Significant features of the 76 peptides examined. Molecular weight (g/mol) is plotted against calculated log P values. The bubble size is related to topological polar surface area (TPSA). Blue bubbles are linear tripeptides; orange and red bubbles are cyclic pentapeptides. The dark blue and red bubbles indicate the 10 peptides selected for method development (only 7 bubbles are visible because isomers were selected). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for the linear peptides, and from -7.0 to 1.7 for the cyclic peptides. However, log P may not be the best parameter to reflect analyte retention when polar compounds are concerned. Indeed, log P results from the sum of the positive contributions of hydrophobic fragments and the negative contribution of polar fragments. Two molecules may have the same log P value if they have the same proportions of hydrophobic and hydrophilic fragments. On another hand, TPSA sums the individual contributions of all polar groups in a molecule and is not counterbalanced by hydrophobic groups. In this selection of peptides, TPSA ranged from 121 to 273 for linear peptides, and from 190 to 293 for cyclic peptides.

To determine an adequate chromatographic system to analyze these peptides, ten of them, including five linear and five cyclic peptides, were selected for screening experiments. Their features are presented in Table 1. The selection retained diversity in molecular weight, log P and TPSA values, nature of the amino acids, and included isomers. The method was first developed based on these ten peptides and the optimized method was applied to the larger set.

3.2. Method development

3.2.1. Selection of the stationary and mobile phases

After some initial attempts on different column formats, four stationary phases were retained for further examination (Table 2). First, based on our previous success with amino acids [18,33], Chiralpak ZWIX(-), which possesses a chiral quinine-derived zwitterionic ligand, was selected. The zwitterionic ligand was expected to provide favorable interactions to retain peptides (composed of amino acids), as they may have acidic or basic functions, which could be in their ionic form in the CO_2 -alcohol mobile phase [28]. Following this idea, we selected another zwitterionic achiral stationary phase, Nucleoshell HILIC, which is bonded with a sulfobetaine ligand. Note that enantioresolution was not a purpose in this study thus a non-enantioselective ligand was sufficient. We had previously obtained good results with this column on a large set of chemical drug candidates [34]. In addition, this stationary phase is based on superficially porous particles, which was soon perceived as an advantage to limit the system overpressure issues that could result from the use of wide elution gradients (ending

with large proportions of co-solvent). Then two polar stationary phases also based on superficially porous particles were selected: Accucore HILIC, which is a bare silica gel, and Ascentis Express OH5, which is bonded with a pentahydroxyl ligand.

Three mobile phase additives, which had previously delivered interesting results for biomolecules, were initially selected. Ammonium hydroxide, in conjunction with a small proportion of water, has proved an excellent additive to improve the chromatography of many types of analytes including basic drugs [30], peptides [5] or proteins [25]. TFA was often cited in LC and SFC analysis of peptides and proteins [35–38], although it is sometimes described as an undesirable additive for MS detection, as it would cause ion suppression. Methanesulfonic acid (MSA) was previously employed successfully to analyze polar compounds as flavonoids [22], but also for amino acids [18] and peptides [23,39], all in SFC conditions. Ammonium formate and ammonium acetate have also been frequently cited in recent publications in SFC [40], however they were considered undesirable in the present case, because UV detection would be necessary for the purpose of impurity profiling, and both of these additives would cause extensive baseline drift over the wide mobile phase gradient.

When starting the first experiments, it soon appeared that TFA was unsuccessful as some of the selected peptides were simply not eluted from the columns with this additive, while others had deteriorated peak shapes, as appears in Figure S1 (supplementary information). Therefore, TFA was not tested on more than two stationary phases (Accucore HILIC and Ascentis Express OH5). Similarly, one column (Accucore HILIC) proved less satisfactory with the first compositions of mobile phase tested, typically on peak efficiency, and was therefore not further tested with other mobile phase compositions, especially as the amount of sample available was limited. As a result, nine combinations of stationary phases and mobile phase compositions were fully examined with the ten selected peptides, to identify the best combination for our purpose.

3.2.2. Selection of the best chromatographic system based on Derringer's functions

Due to the small quantity of sample available for each peptide, it was necessary to find an optimal process for method development, to achieve satisfying results in a limited number of experiments. In addition, some means to compare stationary phases and mobile phases on a rational basis were necessary. For this purpose, Derringer's desirability functions [41] are ideal as they provide an overview of results and great flexibility to define the desirability criteria. We had previously employed a similar strategy to develop SFC methods for chemical drug candidates [30,34].

Seven features were selected as being critical attributes to optimize the method and served to define the seven desirability functions (d_1 to d_7). Their features and equations are presented in Fig. 3 and detailed below.

The first Derringer desirability function (d_1) is related to the compounds resolved on each chromatogram. Indeed, each of the ten reference peptides was injected individually, but each of them comprised an unknown number of impurities. Cyclic peptides comprised less impurities than linear peptides, which might be due to better compound stability in cyclic structures. In the purpose of developing an impurity profiling method, the chromatographic system resulting in the largest number of peaks observed is considered the most resolutive, thus the most desirable. To compute d_1 , we counted all peaks with peak area $> 0.1\%$ (in UV detection at 210 nm), including the major peak of the target peptide. The total number of peaks counted for the five linear peptides on the one hand (varying from 29 to 90), and for the five cyclic peptides on the other hand (varying from 10 to 44), served to define d_1 (linear peptides) and d_1' (cyclic peptides) functions varying from

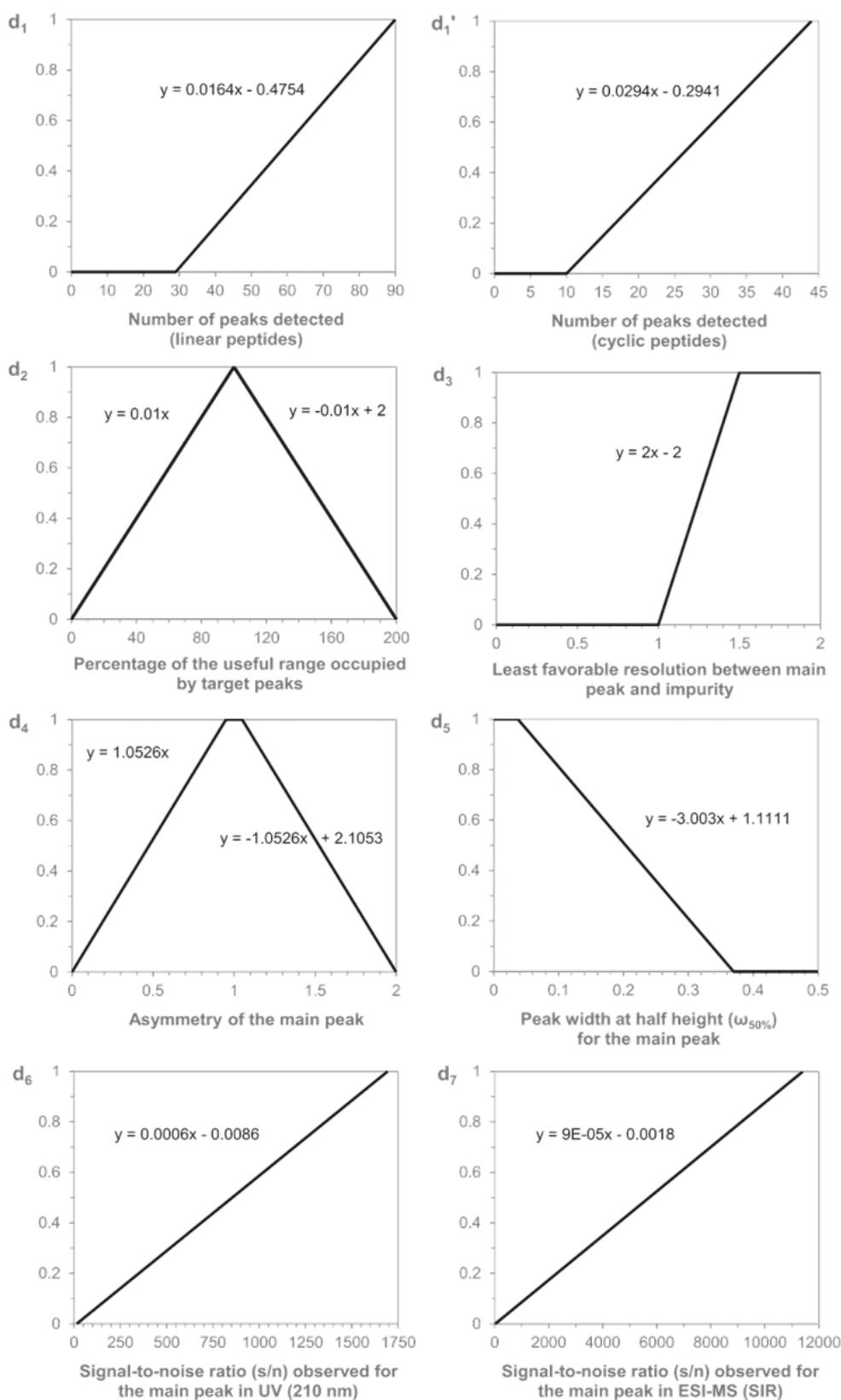


Fig. 3. Derringer's desirability functions (d_i) used to rank the chromatographic systems based on the analysis of the ten selected peptides in Table 1, each of them containing impurities.

0 (smallest number of peaks observed) to 1 (highest number of peaks observed).

The second desirability function d_2 is related to the occupation of the chromatogram by useful information, namely peaks of the major peptide and its impurities. First, we considered the useful window in each chromatogram. Indeed, depending on the stationary phase or mobile phase composition, some disturbances of the UV baseline (at 210 nm) could be observed, which could be related to the sample dilution solvent (comprising DMSO) or to the breakthrough of additives in gradient elution [29]. To be useful for impurity profiling, the baseline should be as devoid of disturbances as possible. Depending on the stationary phase and mobile phase composition, this useful time range varied between 7.5 and 16 min. This point is clearly visible on the chromatograms shown in Figure S1, where the baseline appears to vary greatly depending on stationary and mobile phase combination. Secondly, we considered the time range really occupied by the sample peaks as the useful range defined was not necessarily fully utilized. For instance, the compounds may all elute in a small portion of the chromatogram, e.g. at the end of the gradient if the column is too retentive. This was typically the case with Chiralpak ZWIX(-) with MSA in the mobile phase (Figure S1h). Another undesirable situation occurs when the target peaks elute in an area of the chromatogram where some baseline disturbances would limit the accuracy of peak area measurement. This was the case for Chiralpak ZWIX(-) used with ammonium hydroxide, where the baseline showed several disturbances (Figure S1i). A most desirable situation occurs when both the useful time range (flat baseline) and occupied time range are identical. Therefore, we defined d_2 as the percentage of the useful time range being occupied. This function would increase from 0 (worst case, when none of the useful range is occupied by target peaks) to 1 (when 100% of the useful range is occupied), and then decrease again to 0 (when 200% of the useful range is occupied, meaning that sample peaks eluted in areas of irregular baseline).

The third desirability function d_3 focused on the resolution observed between the main peptide peak and the nearest impurity peak. Ideally, the closest impurities should have the largest resolution, so d_3 function varied from 0 for the cases where the nearest impurity detected had a low resolution to the main peak (resolution < 1), to 1 for the cases where the nearest impurity had a baseline resolution (resolution equal to 1.5 and above).

The fourth desirability function d_4 was based on the peak symmetry of the main peptide. Ideally, peak symmetry should be comprised between 0.95 and 1.05, yielding to a d_4 value equal to 1. Anything below 0.95 or above 1.05 would progressively decrease the d_4 value down to 0.

The fifth desirability function d_5 was based on peak width at half height for the main peptide. Higher resolution should naturally result from thinner peaks so a maximum d_5 value (1) was associated to the thinnest peaks observed (0.04 min) and the minimum d_5 value (0) was associated to the largest peaks observed (0.37 min).

The sixth and seventh desirability functions d_6 and d_7 were related to UV and MS signal-to-noise ratio (s/n) observed for the main peptide peak. For UV detection, the measurement was done on the chromatogram registered at 210 nm, while for ESI-MS detection, the SIR chromatograms were used. Initially, $[M+H]^+$ and $[M-H]^-$ were both considered. When using ammonium hydroxide, negative ionization was favorable to a more sensitive detection, with the $[M-H]^-$ ions being much more visible than the $[M+H]^+$ ions. This can be observed in Figure S2 (supplementary information). However, when using MSA and TFA, the $[M-H]^-$ ions were mostly invisible. Instead, adducts could be observed as $[M+nMSA-H]^-$ or $[M+nTFA-H]^-$, with variable n values, depending on the number of basic residues in the peptide structure. Considering the complexity of the response observed in negative ionization mode,

it was decided to use only the $[M+H]^+$ signal for the d_7 calculation. A 0 value of d_6 and d_7 function would then be obtained for no signal observed (when the peptide is not eluted or when it is seen only with one of the two detectors) and a maximum value (1) would be obtained for the highest s/n obtained for each peptide individually.

For each of the ten peptides, the Derringer's function D_{pi} applied for each of the nine chromatographic systems was defined as the arithmetic mean of the seven d_i individual functions. In cases when the peptide did not elute, the D_{pi} value was automatically set to zero. For instance, when Accucore HILIC and Ascentis Express OH5 were employed with TFA, three peptides did not elute from the column during the gradient program thus D_{pi} values are obtained only for 7 peptides (Fig. 4). The final D value for each chromatographic system, which served to rank the chromatographic systems, was taken as the average of the ten D_{pi} functions. The individual D_{pi} values and the average D values obtained for the 9 chromatographic systems compared can be seen in Fig. 4.

It appears that those chromatographic systems which performed the best overall showed equally good results on all ten peptides, as appears with the low standard deviation in D_{pi} values (error bars on the D values). One stationary phase appeared as being significantly superior to the others, Ascentis Express OH5, when it was employed with ammonium hydroxide or with methanesulfonic acid. This column was then selected. As the results obtained with ammonium hydroxide were slightly superior to the ones obtained with MSA, the former was retained for further optimization.

3.2.3. Method optimization

In the above, the column oven temperature was maintained at 40°C. Previous works have shown that temperature variation could offer significant improvement in the SFC analysis of peptides [23,25]. Besides, it was recently shown that high temperatures employed with large proportions of co-solvent yielded more favorable kinetic properties, with the optimum flow rate being displaced to higher values of mobile phase velocity, and smaller C-terms in the van Deemter curves [42]. In addition, as higher temperatures reduce the fluid density, it may be favorable to faster analysis in reducing the pressure drop, thereby favoring the use of higher flow rate. We then attempted to increase the column temperature to 60°C, which was the maximum temperature recommended by the column manufacturer.

One significant advantage observed when increasing temperature was that the breakthrough of additives causing baseline disturbance appeared much earlier in the chromatogram, thereby promoting a wider useful range of the chromatogram. This can be observed in Fig. 5, where the linear peptide 4693 was analyzed at 40 and 60°C on Ascentis Express OH5 with the optimized gradient and mobile phase containing ammonium hydroxide. At 40°C, the main peak eluted at the same time as the additive breakthrough, making its quantitation uncertain. At 60°C, the main peak eluted much later than the additive breakthrough so its quantitation was easily done. In addition, all peaks of main peptide and impurities were sharpened at the highest temperature, improving resolution and detectability. Retention times of all peaks were only moderately affected by this change of temperature, with most peaks being slightly less retained at 60°C, and some peaks being slightly more retained.

As the increased temperature caused less constraints on the pressure limit, the back-pressure and flow-rate could be further modified. As a constant pressure should ensure a more robust and easier-to-transpose method, we finally privileged a constant back-pressure set at 120 bar. As the maximum inlet pressure was not reached in these conditions, the flow rate could be increased so that the optimized reversed flow rate gradient was 3.0 to 1.5

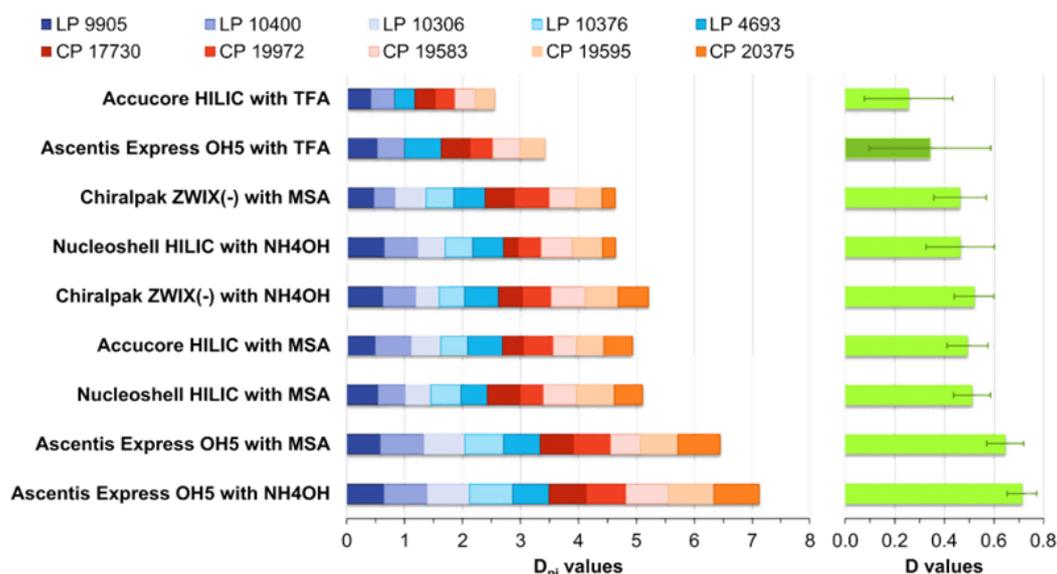


Fig. 4. Derringer desirability functions obtained for the ten individual peptides (D_{pi}) in each chromatographic system assessed, and the average D values with standard deviation. Linear peptides are indicated in blue colors, cyclic peptides in orange-red colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

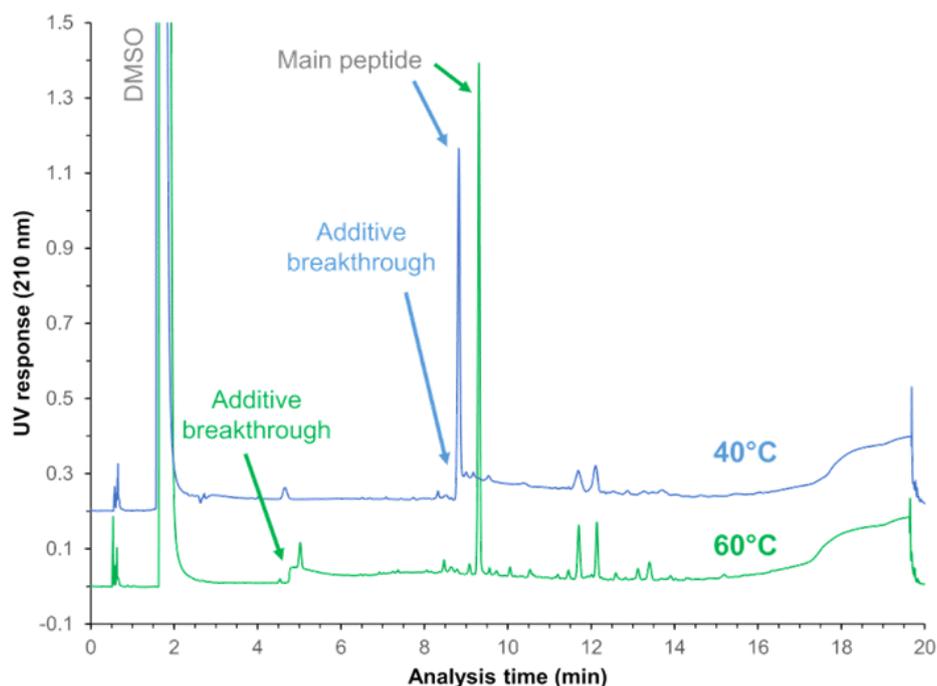


Fig. 5. Chromatograms of a selected degraded linear peptide (4693) with the generic gradient in Table S2 on Ascentis Express OH5 and mobile phase containing ammonium hydroxide at different column temperatures: 40°C or 60°C.

mL/min (against 2.5 to 1.0 mL/min in the original conditions). Constant flow rate had also been examined but yielded worse results than the variable flow rate. Again, this must be related to the different optimum flow rate along the mobile phase gradient: for the early-eluting peaks (SFC conditions), a larger flow rate should be favorable, while a lower flow rate is desirable for the late-eluting peaks (EFLC or LC conditions).

Finally, it was observed that in all cases, all the target peaks (main peptide and impurities) were eluted with mobile phases comprising a maximum amount of 80% co-solvent. In addition,

the increase of co-solvent from 80 to 100% caused a strong baseline drift (Fig. 5), and significantly increased pressure drop at the end of the gradient. Reducing the overall analysis time was also desirable, so the initial gradient of 5 to 100% co-solvent in 15 min was finally modified to be 5 to 80% co-solvent in 10 min. Fig. 6 shows the changes between the screening gradient conditions and final optimized conditions. Judging from the proportion of co-solvent in most of this gradient program, make-up flow was not found necessary during the whole analysis. After several experiments to determine what would be really useful, it was ob-

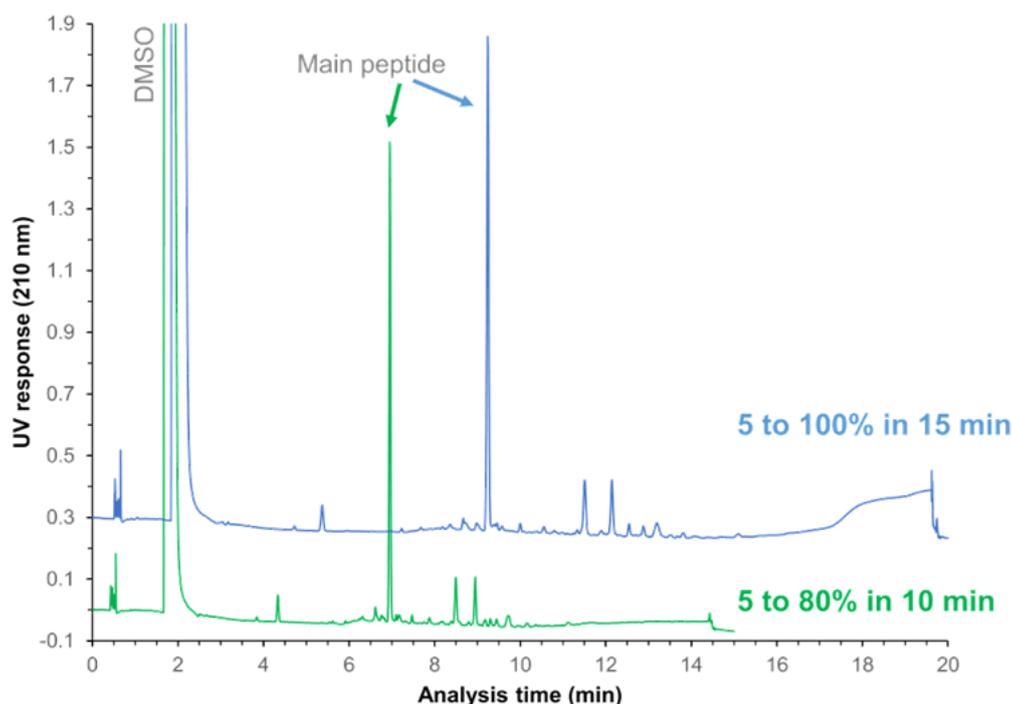


Fig. 6. Screening gradient (conditions in Table S2) and optimized gradient (conditions in Table 3) on Ascentis Express OH5 and mobile phase containing ammonium hydroxide on a selected degraded linear peptide (4693).

served that a low flow rate of make-up fluid was only useful in the first 3 minutes of the analysis. Afterwards, the make-up fluid flow rate was set to zero. The final optimized method is presented in Table 3.

3.3. Application of the optimized method to the larger set of peptides

The large set of 76 peptides (including the 10 peptides that served for method development) was finally analyzed in the optimized conditions. The retention times obtained can be seen in Table S1. For the 34 linear peptides, retention times ranged from 5.6 to 12.7 min. For the 42 cyclic peptides, retention times ranged from 7.5 to 13.8 minutes, thus were generally more retained. In addition, the peptides were adequately scattered along the useful chromatographic range, as is visible in the vertical scattering of points in Fig. 7.

Attempts to correlate retention time with molecular weight yielded no observable trends (Figure S3a). However, retention times could be much better related to polarity parameters: correlation to $\log P$ showed some negative tendency (Figure S3b), particularly for linear peptides, indicating that increasing polarity (lower $\log P$ values) is related to stronger interactions on the poly-hydroxyl stationary phase. This was naturally not a surprise. Positive correlation to TPSA, meaning again that molecules possessing more polar functions are more retained, was even more convincing (Figure S3c) both for linear and cyclic peptides. Again, this did not come as a surprise, especially as polar surface area descriptors were previously demonstrated to be well correlated to peptide retention in SFC on polar stationary phases [43]. Furthermore, the correlation to TPSA was improved when the TPSA values included the TFA counter-ion, for those peptides that were present as TFA salts. This relation can be observed in Fig. 7. There is no reason why the trend should be linear, but attempts at fitting retention time to TPSA with linear or polynomial curves were all improved when the TFA salt was taken into account (Figure S3d). Only one linear peptide was significantly un-

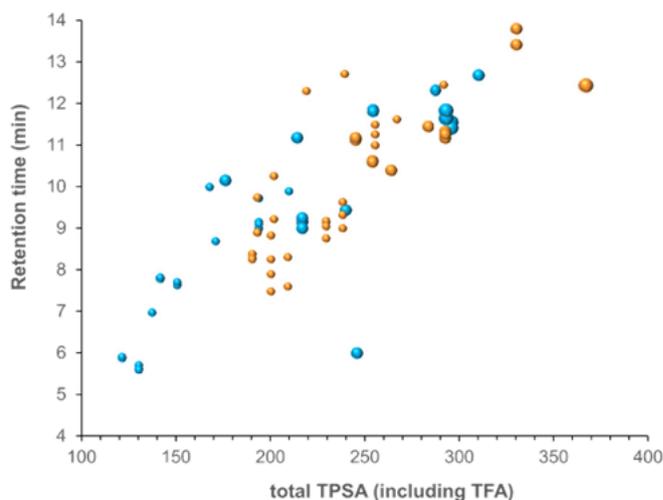


Fig. 7. Relation between retention time in the optimized method (Ascentis Express OH5 and mobile phase containing ammonium hydroxide, gradient conditions in Table 3) and topological polar surface area (TPSA) for the 76 peptides examined. Blue bubbles are linear tripeptides; orange bubbles are cyclic pentapeptides. Bubble size is related to trifluoroacetic acid (TFA) salts: smallest bubbles have no counter-ion, middle-size bubbles have one TFA counter-ion and the largest bubbles have two TFA counter-ions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fit by the general tendency. Furthermore, the trend lines of linear and cyclic peptides were much closer to each other when the polar surface area of the TFA counter-ion was taken into account. This might suggest that the peptides carrying basic chains were eluted as ion pairs and not in their dissociated forms. This point requires further investigation and will be examined in future works.

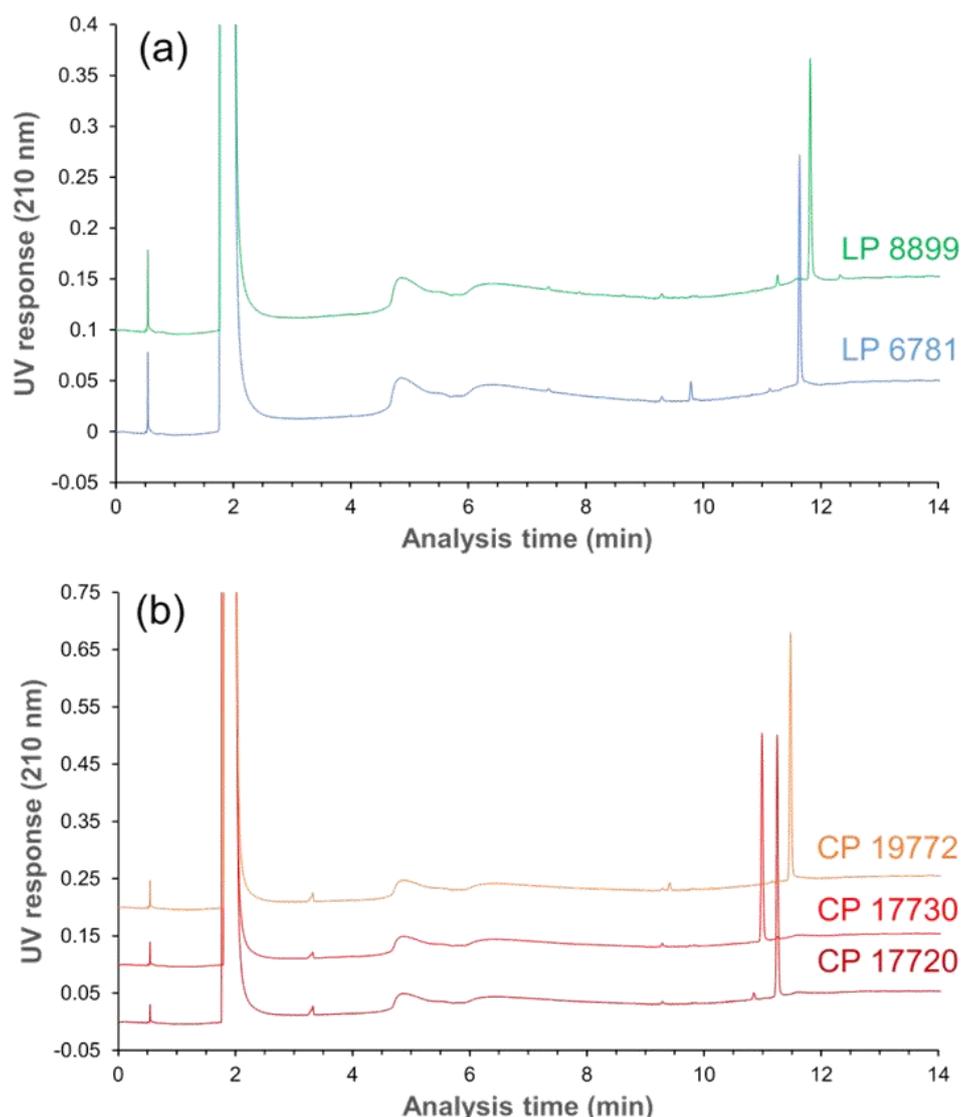


Fig. 8. Optimized conditions on Ascentis Express OH5 and mobile phase containing ammonium hydroxide (gradient conditions in Table 3) for two groups of isomers: (a) linear tripeptide positional isomers (b) cyclic pentapeptide positional and chain isomers.

As mentioned above, isomeric species were of particular interest in this method development because they are usually difficult to resolve with reversed-phase HPLC methods. Previous works in SFC had demonstrated excellent capability for isomeric separation [37]. In the present work, many isomeric pairs can be identified in Table S1. Note that isobaric species in this table may be constitutional isomers that are not necessarily setting any problems. Not all isomeric pairs were resolved with the proposed optimized method but some examples were rather promising, with some isomers being separated by half a minute. Cyclic isomers were generally better resolved than linear isomers, possibly due to the constrained structures of cyclic peptides. Chain isomers were also often better resolved than positional isomers.

For instance, the linear peptides 6781 and 8899 are positional isomers where the first was $\text{Ac-R}_1\text{-R}_2\text{-R}_3\text{-NH}_2$ and the second was $\text{Ac-R}_3\text{-R}_1\text{-R}_2\text{-NH}_2$ (Fig. 8a). For the cyclic peptides, 17720 and 17730 were positional isomers ($\text{R}_1\text{-R}_2\text{-R}_3\text{-R}_4\text{-R}_5$ and $\text{R}_1\text{-R}_4\text{-R}_3\text{-R}_2\text{-R}_5$) while 19972 was a chain isomer to the second ($\text{R}_1\text{-R}_4'\text{-R}_3'\text{-R}_2\text{-R}_5$) (Fig. 8b). In both cases, the isomeric species are baseline resolved with the proposed method.

4. Conclusions and perspectives

In the present paper, we have developed a UC-MS method, covering the usual operating domains of SFC and EFLC, to analyze the purity of short-chain peptides. In previous works utilizing CO_2 -based mobile phases to analyze peptides, short-chain peptides were rarely examined, with only a few studies relating the elution of peptides with molecular weights under 1000 Da [5,23,39], and hardly ever relating the elution of peptides with molecular weights under 500 Da. However, as will be observed in future papers, the adequate conditions to elute and resolve short-chain peptides are clearly different from the adequate conditions to elute and resolve long-chain peptides. The method was carefully optimized, after a rational comparison of four stationary phases and three mobile phase compositions, with the help of Derringer's desirability functions. With this optimized method obtained on a polar stationary phase of poly-hydroxyl ligands, retention was positively correlated to the topological polar surface area of the peptide salts, as demonstrated with a large set of 76 short-chain peptides comprising both linear and cyclic peptides. Some sepa-

ration of isomeric species was possible, especially for the cyclic peptides.

Future works will compare the UC-MS method developed here to a more classical reversed-phase LC-MS analysis, particularly in terms of performance for impurity profiling, which was usually not the purpose of previously published works, where the elution of the major peptide was the principal criterion considered.

Funding

This work was funded by Servier Research Institute.

Compliance with ethical standards

The authors declare they have no conflict of interest.

CRediT author statement

Jérémy Molineau: Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization, **Yasmine Hamel:** Investigation, **Maria Hideux:** Resources, Writing – Review & Editing, Supervision, **Philippe Hennig:** Conceptualization, Resources, Funding acquisition, **Sophie Bertin:** Conceptualization, **Fabien Mauge:** Conceptualization, **Eric Lesellier:** Conceptualization, Resources, Writing – Review & Editing, **Caroline West:** Conceptualization, Methodology, Validation, Resources, Writing – Original Draft, Visualization, Supervision, Project administration

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.

Acknowledgements

ICOA is supported by the University of Orleans, the National Centre for Scientific Research (CNRS), the Labex programs SynOrg (ANR-11-LABX-0029) and IRON (ANR-11-LABX-0018-01), the FEDER programs CHemBio (FEDER-FSE 2014-2020-EX003677) and Techsab (FEDER-FSE-2014-2020-EX011313) and the RTR Motivhealth (2019-00131403).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462631.

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Supplementary information to:

Small peptides analysis by Unified Chromatography-Mass spectrometry

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Figure S1. Superimposed chromatograms for the ten selected peptides in Table 1 in the 9 chromatographic systems compared with Derringer functions in Figure 4. UV detection (210 nm).

Figure S2. Comparison of the MS signal-to-noise values observed for the 10 representative peptides in the different chromatographic systems considered in positive and negative ionization modes, considering the possible adducts formed with MSA and TFA.

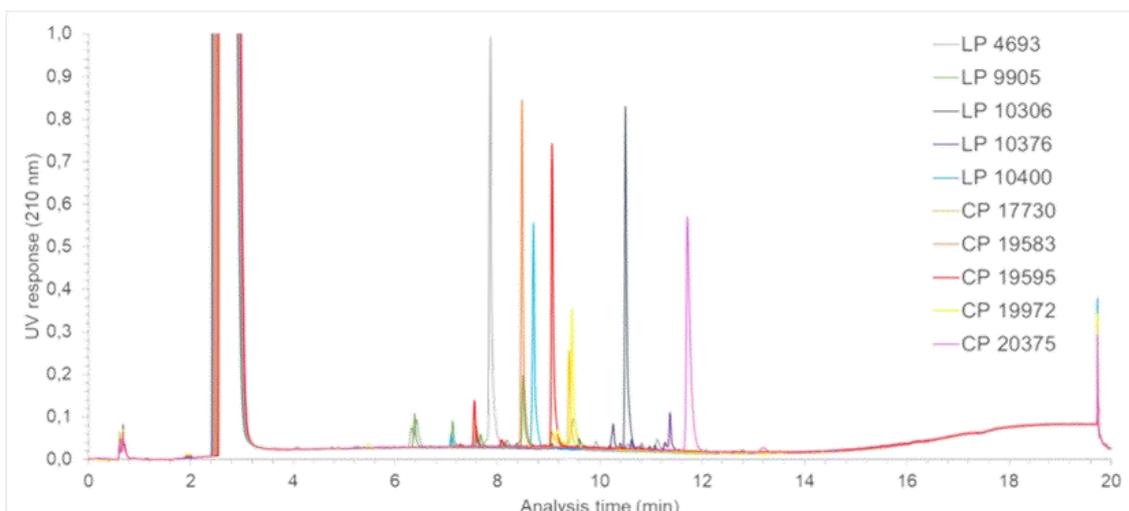
Figure S3. Relation between the chromatographic retention time in optimized conditions (Ascentis Express OH5, mobile phase containing ammonium hydroxide and gradient in Table 3) and different physico-chemical parameters: (a) molecular weight, (b) calculated log P, (c) topological polar surface area, (d), total topological surface area, including the TFA counter-ions. Blue points are linear peptides, orange points are cyclic peptides.

Table S1. 76 peptides examined in this study with significant features: molecular weight (MW), calculated octanol-water partition coefficient (clog P), topological polar surface area (TPSA), presence of trifluoroacetate (TFA) counter-ion and retention time in the optimized conditions (t_R).

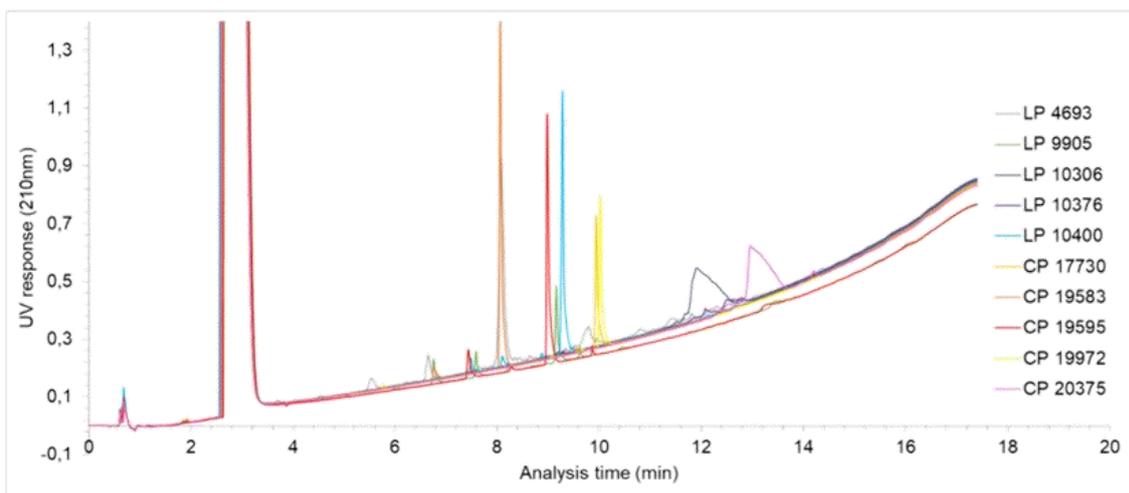
Table S2. Gradient programs for the screening of chromatographic systems with 10 selected peptides.

Figure S1. Superimposed chromatograms for the ten selected peptides in Table 1 in the 9 chromatographic systems compared with Derringer functions in Figure 4. UV detection (210 nm).

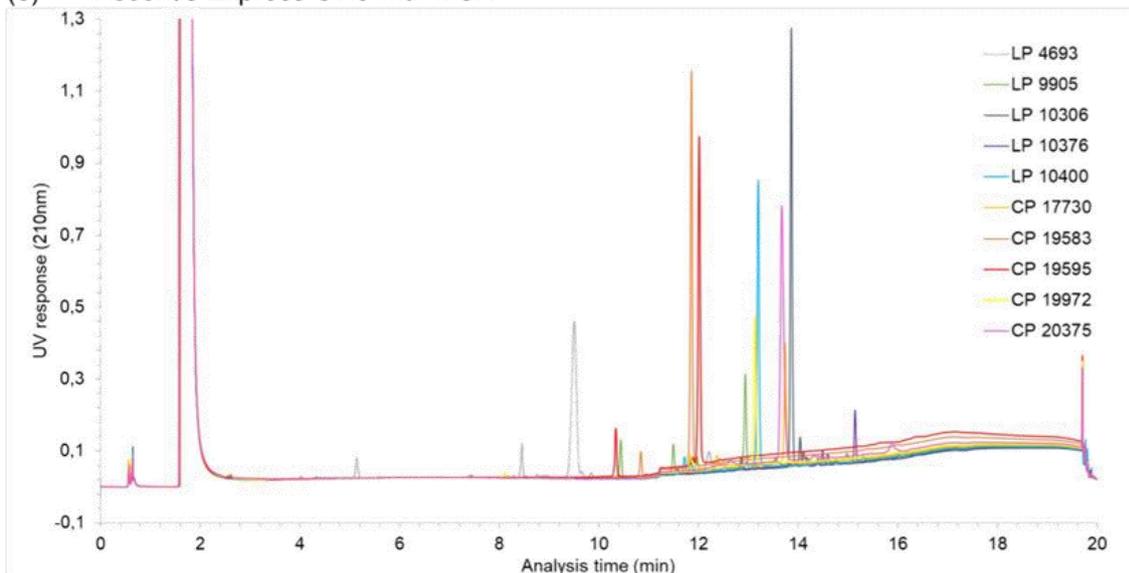
(a) Accucore HILIC with MSA



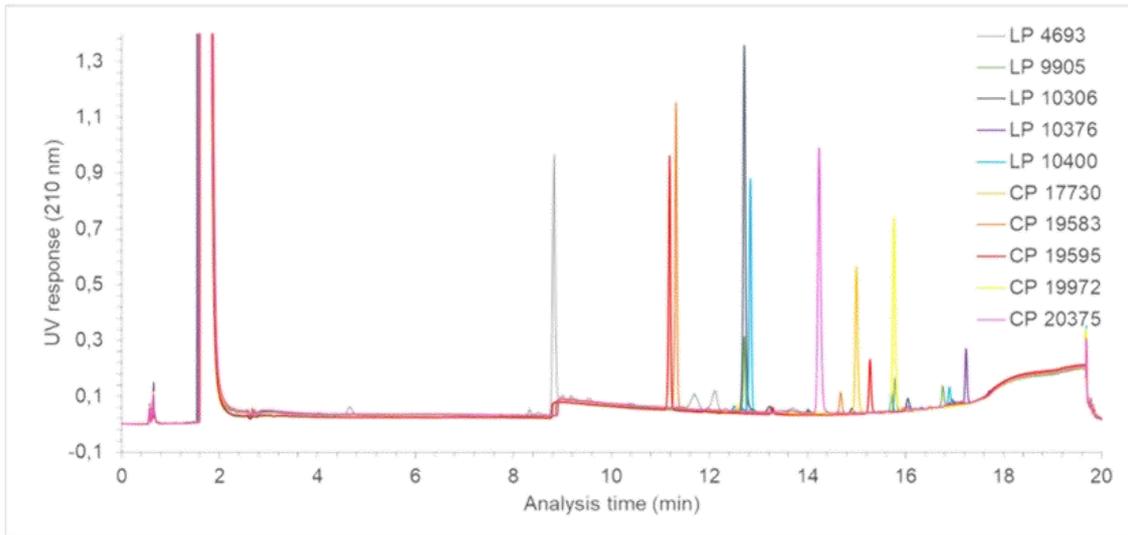
(b) Accucore HILIC with TFA



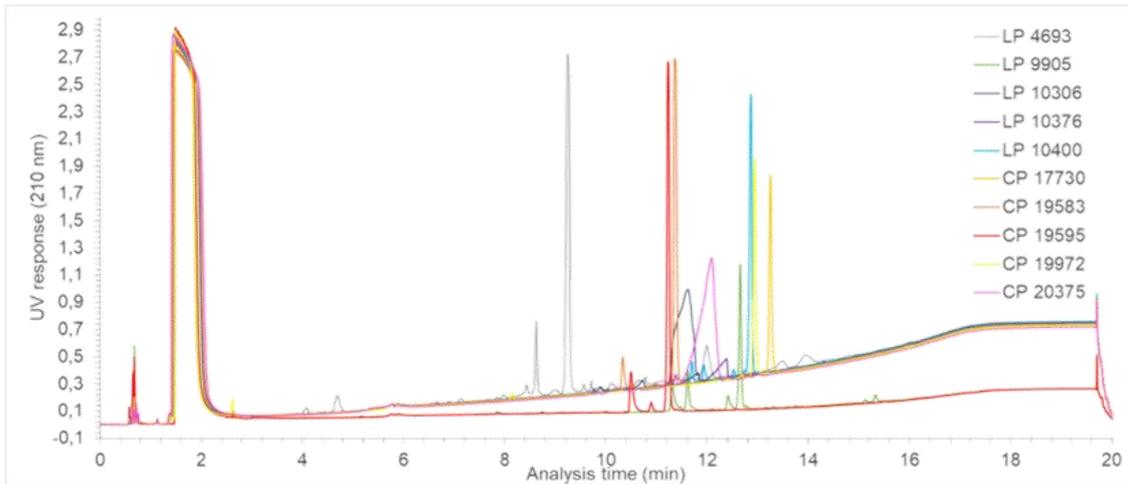
(c) Ascentis Express OH5 with MSA



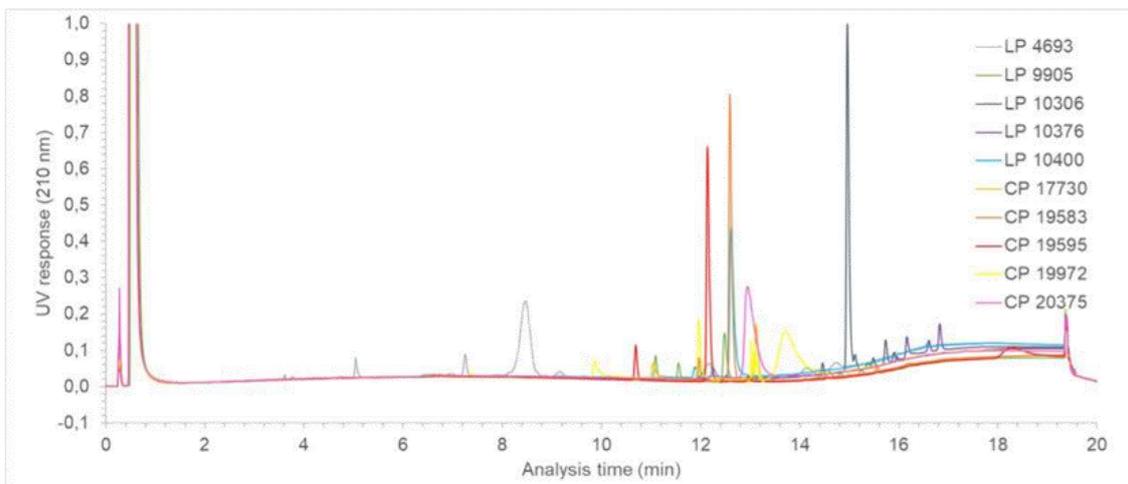
(d) Ascentis Express OH5 with NH₄OH



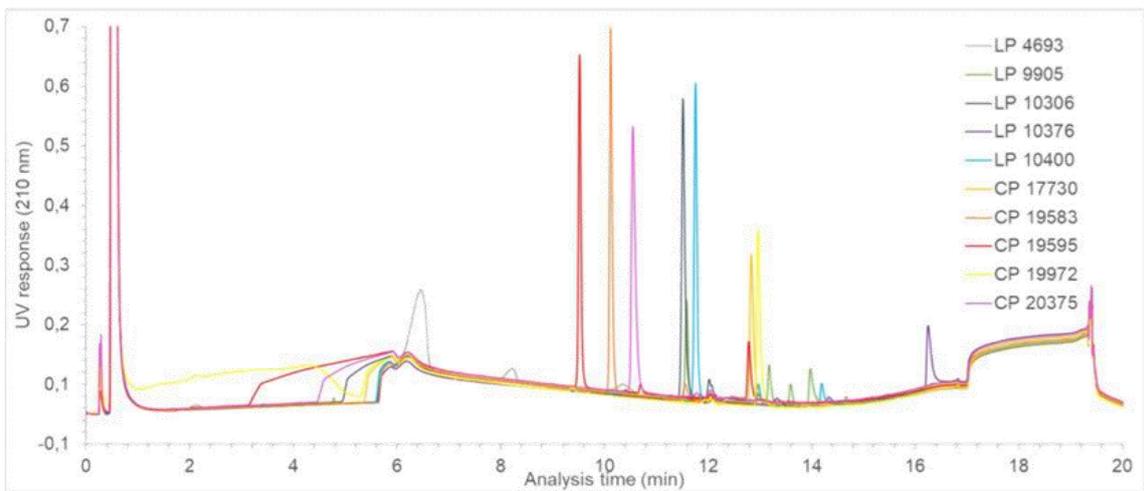
(e) Ascentis Express OH5 with TFA



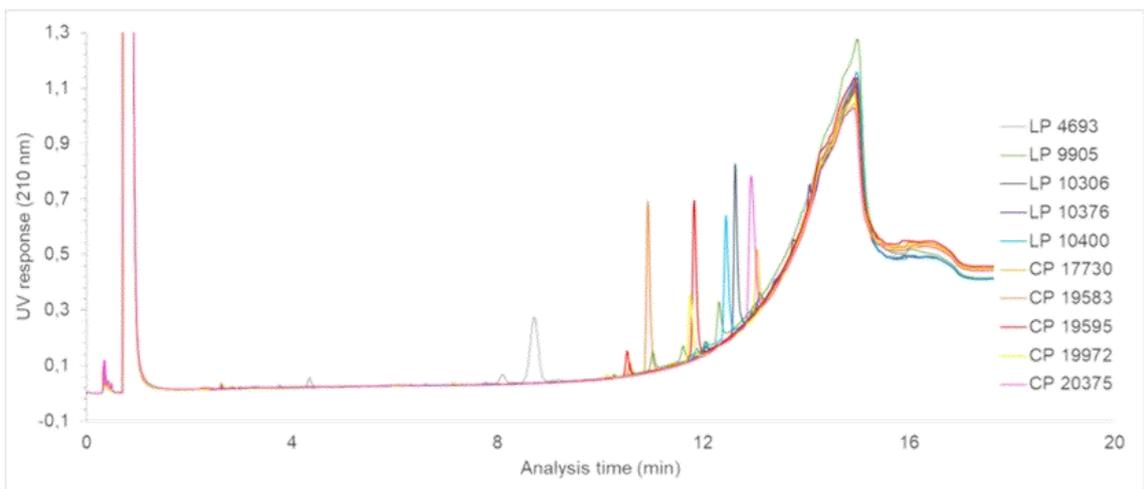
(f) Nucleoshell HILIC with MSA



(g) Nucleoshell HILIC with NH₄OH



(h) Chiralpak ZWIX(-) with MSA



(i) Chiralpak ZWIX(-) with NH₄OH

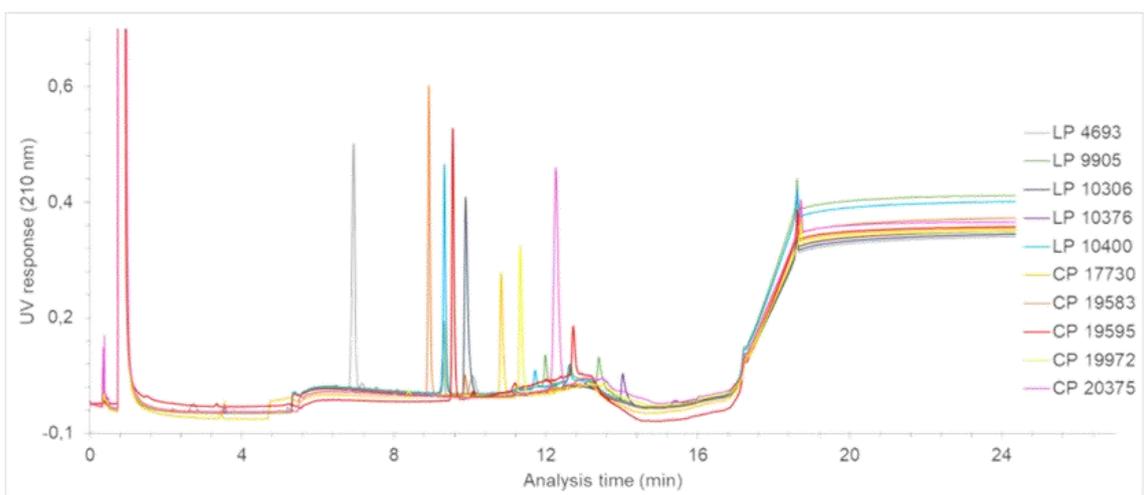


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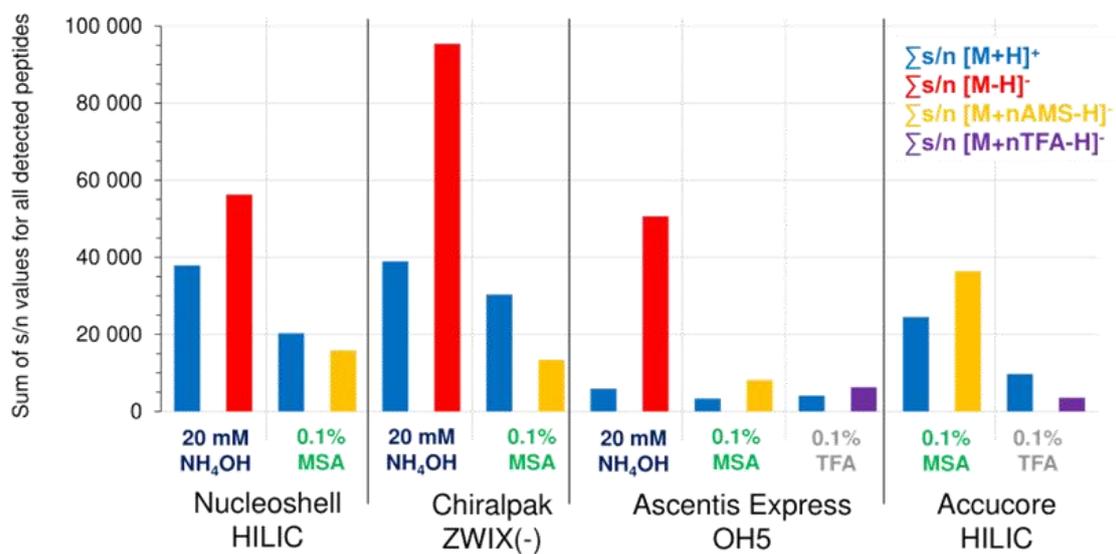
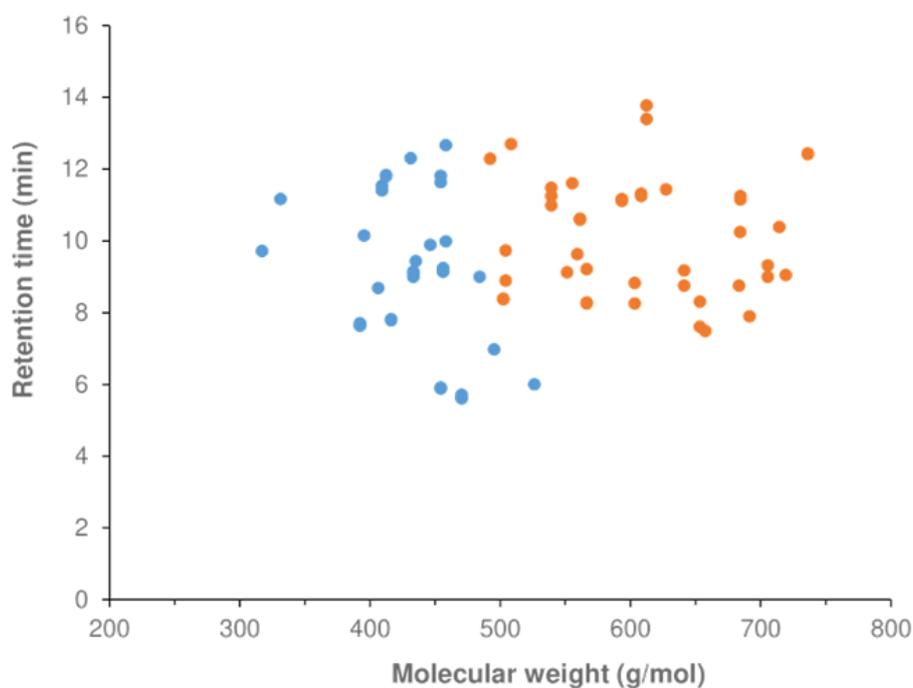
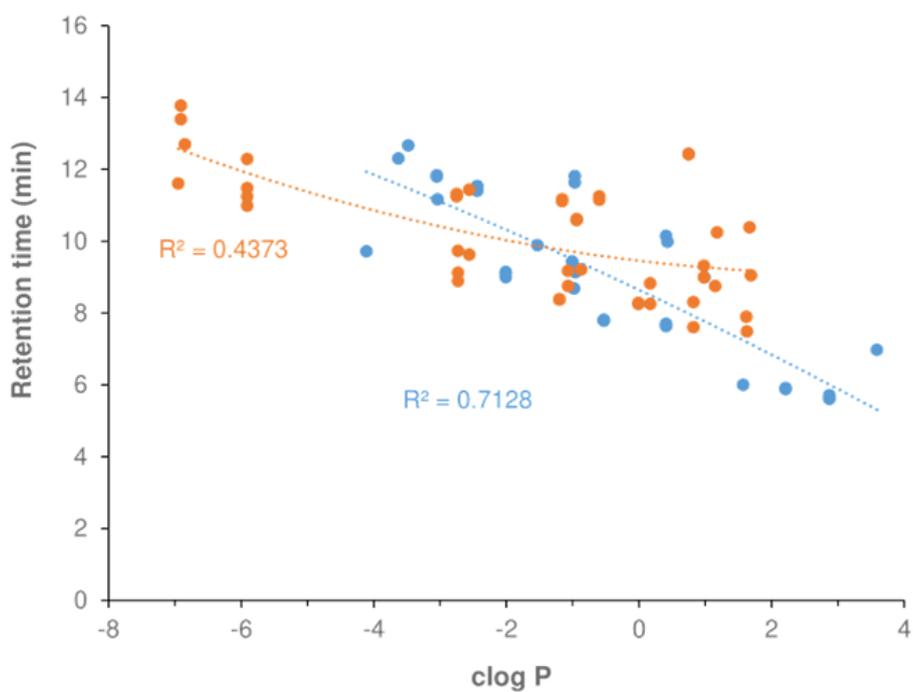


Figure S3. Relation between the chromatographic retention time in optimized conditions and different physico-chemical parameters: (a) molecular weight, (b) calculated log P, (c) topological polar surface area, (d), total topological surface area, including the TFA counter-ions. Blue points are linear peptides, orange points are cyclic peptides.

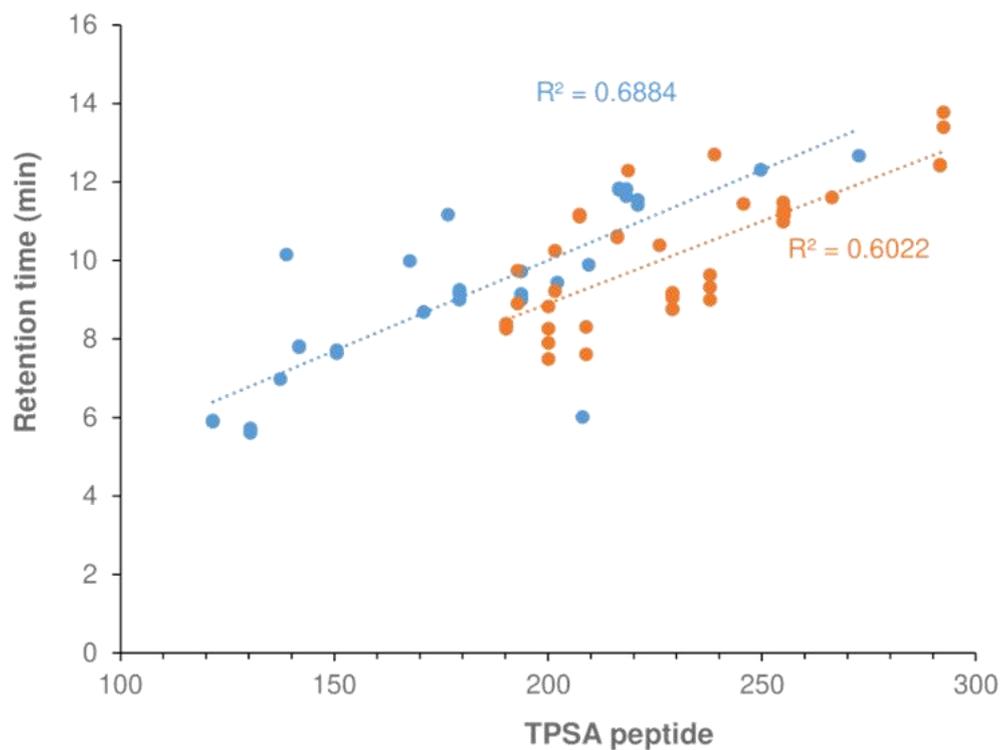
(a)



(b)



(c)



(d)

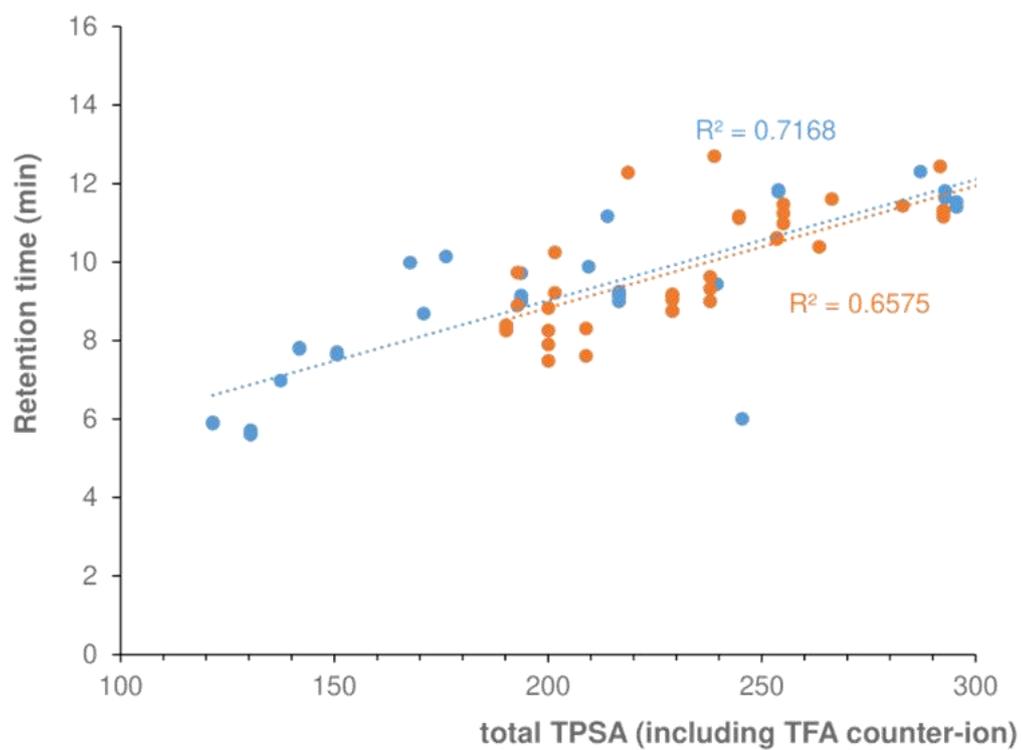


Table S1.

76 peptides examined in this study with significant features: molecular weight (MW), calculated octanol-water partition coefficient (clog P), topological polar surface area (TPSA), presence of trifluoroacetate (TFA) counter-ion and retention time in the optimized conditions (t_R).

Linear peptides	Salt	MW peptide (g/mol)	clog P	TPSA	t_R
LP 9803	-	317.1	-4.11	193.7	9.72
LP 9474	TFA	331.2	-3.04	176.6	11.17
LP 10371	-	392.2	0.41	150.6	7.65
LP 6734	-	392.2	0.41	150.6	7.64
LP 6838	-	392.2	0.41	150.6	7.71
LP 4641	TFA	395.3	0.41	138.8	10.15
LP 9917	-	406.2	-0.98	170.9	8.69
LP 3361	2 TFA	409.2	-2.44	221.0	11.54
LP 3768	2 TFA	409.2	-2.44	221.0	11.41
LP 10333	TFA	412.2	-3.05	216.6	11.84
LP 8777	TFA	412.2	-3.05	216.6	11.80
LP 6401	-	416.2	-0.53	141.8	7.79
LP 7055	-	416.2	-0.53	141.8	7.82
LP 9258	TFA	431.2	-3.63	249.8	12.31
LP 10400	-	433.2	-2.01	193.7	9.15
LP 9887	-	433.2	-2.01	193.7	9.00
LP 9905	-	433.2	-2.01	193.7	9.10
LP 8587	TFA	435.2	-1.01	202.2	9.44
LP 9993	-	446.2	-1.53	209.5	9.89
LP 5811	-	454.3	2.21	121.6	5.89
LP 6189	-	454.3	2.21	121.6	5.92
LP 6781	2 TFA	454.3	-0.97	218.3	11.64
LP 8899	2 TFA	454.3	-0.97	218.3	11.82
LP 10306	TFA	456.2	-0.96	179.3	9.17
LP 6819	TFA	456.2	-0.96	179.3	9.14
LP 9162	TFA	456.2	-0.96	179.3	9.25
LP 10376	TFA	458.2	-3.48	272.7	12.67
LP 7854	-	458.2	0.43	167.7	9.99
LP 5099	-	470.3	2.87	130.4	5.72
LP 6681	-	470.3	2.87	130.4	5.64
LP 7066	-	470.3	2.87	130.4	5.61
LP 7890	TFA	484.2	0.99	179.3	9.00
LP 4693	-	495.3	3.59	137.4	6.98
LP 4303	TFA	526.3	1.57	208.1	6.01

Cyclic peptides	Salt	MW (g/mol)	clog P	TPSA	tR
CP 19088	-	492.2	-5.91	218.7	12.29
CP 19538	-	502.3	-1.20	190.2	8.38
CP 19551	-	502.3	-1.20	190.2	8.39
CP 19212	-	504.2	-2.73	192.9	8.90
CP 19219	-	504.2	-2.73	192.9	9.74
CP 18118	-	508.2	-6.85	238.9	12.70
CP 19972	-	539.2	-5.91	255.0	11.48
CP 17720	-	539.2	-5.91	255.0	11.25
CP 17730	-	539.2	-5.91	255.0	10.99
CP 20081	-	551.3	-2.73	229.1	9.13
CP 17814	-	555.2	-6.95	266.4	11.61
CP 17801	-	559.2	-2.56	237.9	9.63
CP 19711	TFA	561.3	-0.94	216.2	10.62
CP 19742	TFA	561.3	-0.94	216.2	10.59
CP 18604	-	566.3	-0.87	201.6	9.22
CP 19583	-	566.3	-0.01	190.2	8.29
CP 19595	-	566.3	-0.01	190.2	8.26
CP 19846	TFA	593.3	-1.16	207.4	11.12
CP 19850	TFA	593.3	-1.16	207.4	11.17
CP 19370	-	603.3	0.17	200.1	8.83
CP 20297	-	603.3	0.17	200.1	8.26
CP 20365	TFA	608.3	-2.75	255.2	11.32
CP 20048	TFA	608.3	-2.75	255.2	11.25
CP 19325	TFA	612.3	-6.91	292.5	13.40
CP 19308	TFA	612.3	-6.91	292.5	13.78
CP 19196	TFA	627.3	-2.56	245.7	11.44
CP 17992	-	641.3	-1.07	229.1	8.76
CP 18008	-	641.3	-1.07	229.1	9.18
CP 17989	-	653.4	0.82	208.9	7.61
CP 18007	-	653.4	0.82	208.9	8.31
CP 20336	-	657.4	1.63	200.1	7.49
CP 19391	-	683.4	1.15	229.1	8.76
CP 18657	-	684.3	1.18	201.6	10.25
CP 20281	TFA	684.4	-0.60	255.2	11.16
CP 19346	TFA	684.4	-0.60	255.2	11.25
CP 20396	-	691.4	1.62	200.1	7.90
CP 18787	-	705.4	0.98	237.9	9.32
CP 18791	-	705.4	0.98	237.9	9.00
CP 20375	TFA	714.4	1.67	226.1	10.39
CP 19407	-	719.4	1.69	229.1	9.05
CP 21013	-	736.3	0.75	291.7	12.44
CP 20997	2 TFA	736.3	0.75	291.7	12.42

Table S2.

Gradient programs for the screening of chromatographic systems with 10 selected peptides

Gradient time (min)		Co-solvent proportion (%)	Back-pressure (bar)	Flow rate (mL/min)
Chiralpak ZWIX(-)	Other columns			
0	0	5	150	2.5
0.5	0.5	5	150	2.5
15.5	15.5	100	110	1.0
25.5	18.5	100	110	1.0
26	19	5	/	/
27	20	5	150	2.5

c. Comparaison de la méthode UC avec la méthode RPLC de l'IdRS

La partie précédente concernait le développement de la méthode UC-MS et l'analyse de l'ensemble des peptides mis à disposition. Ceci nous a conduit à comparer les performances de cette nouvelle méthode avec celles obtenues par RPLC-MS au sein de l'IdRS. Ce travail de comparaison a débuté sur un lot de 43 peptides analysés via les deux techniques analytiques. Les résultats autour du peptide principal ont d'abord focalisé notre regard pour mesurer les taux de succès des deux méthodes. Cette évaluation s'est appuyée sur trois critères analytiques développés dans l'article.

Dans un second temps, les impuretés détectées et leurs profils, selon la technique, ont été croisés, le but étant de déterminer les impuretés communes à la RPLC et l'UC mais aussi celles détectées uniquement par une seule des deux techniques.

Enfin, nous avons mesuré la robustesse de la méthode UC à l'aide d'un plan de Doehlert. Celui-ci, s'appuie sur trois paramètres analytiques à trois niveaux.



Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part II. Comparison to reversed-phase ultra-high performance liquid chromatography

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ARTICLE INFO

Article history:

Received 10 November 2021

Revised 18 December 2021

Accepted 20 December 2021

Available online 23 December 2021

Keywords:

Peptides

Reversed-phase liquid chromatography

Supercritical fluid chromatography

Unified Chromatography

ABSTRACT

In the first part of this study, a unified chromatography (UC) analysis method, which is similar to supercritical fluid chromatography (SFC) but with wide mobile phase gradients of pressurized CO₂ and solvent, was developed to analyse short-chain peptides, with UV and mass spectrometry (MS) detection. In this second part, the method is compared to a reference reversed-phase ultra-high-performance liquid chromatography (RP-UHPLC) method, based on the analysis of 43 peptides, including 10 linear peptides and 33 cyclic ones.

First, the orthogonality between the two methods was examined, based on the retention patterns. As the UC method was developed on a polar stationary phase (Ascentis Express OH5), the elution orders and selectivities were expected to be significantly different from RPLC on a non-polar stationary phase (ACQUITY CSH C18). Secondly, the success rate of the methods was examined, based on successful retention / elution of the peptides and the absence of observed co-elutions between the main peak and impurities. A successful analysis was obtained for 81% of the peptides in UC and 67% in RPLC. Thirdly, the performance of the methods for the intended application of impurity profiling of peptide drug candidates was assessed, based on the comparison of peak purities, the number of impurities detected and the thorough examination of impurity profiles. Excellent complementarity of the two methods for the specific task of impurity profiling, and for the separation of isomeric species was observed, with only one isomeric pair in this set remaining unresolved. The method sensitivity was however better with RPLC than UC. Finally, the operational costs in terms of solvent cost per analysis were the same between the two methods.

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1. Introduction

The use of biomolecules as active pharmaceutical ingredients (API) has strongly intensified since the beginning of this century. Among them, peptides are amino-acid oligomers and polymers, with a wide diversity of molecular weights and polarities. Peptides have been used as APIs since the introduction of insulin for diabetes treatment in the 1920s' [1]. Compared to the small synthetic chemical molecules mostly used in occidental medicine, the low toxicity usually associated with peptide drugs is attractive to favor patient's safety [2].

Historical perspectives on therapeutic peptides were clearly exposed in previous reviews [3,4]. In addition, pharmaceutical peptides of biological or synthetic origin under 5 000 Da and commercialized in northern countries (Europe, Japan and North America) for therapeutic and diagnostic applications are all freely listed in the PepTherDia database (<http://peptherdia.herokuapp.com/>) [5]. This database currently comprises around 100 compounds information such as physicochemical and pharmacokinetic properties. Examining this database, it appears that short-chain peptides (between 300 Da and 2000 Da) stand for 75% of commercialized pharmaceutical peptides. Among them, about a third (a quarter of the total) has a molecular weight below 1000 Da. Until the 1990s, most of the therapeutic peptides entering in clinical study were short-chain peptides [4]. Then, as the synthesis and manufacturing technologies improved [6,7], peptides with higher molecular weights

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were more often described. Short-chain peptides however continued to be developed in a steady fashion.

Reversed-phase liquid chromatography (RPLC) is the most employed method for peptide analysis [8–11] because of its robustness, high-resolution power, short analysis times and compatibility to mass spectrometry (MS). Different authors described RPLC as a flexible and versatile technique, which could also explain its attractiveness for peptides investigations [11,12]. However, RPLC-UV-MS is not always enough to assess peptide structure and purity as co-elutions may occur, especially with isobaric compounds are isomeric species are frequent in such mixtures. Complementary techniques are always desirable to obtain a more complete picture of a sample [13]. Besides, solubilization of the peptides in adequate dilution solvent and carryover effects have been reported as significant issues in RPLC [14,15]. In addition, retention in RPLC is mostly governed by hydrophobic interactions between the analyte and aqueous mobile phase, and by dispersive interactions between hydrophobic residues and hydrophobic stationary phase ligands. As a result, the more polar peptides (typically encountered in short-chain peptides) are not always adequately retained in RPLC chromatographic systems. Polar peptides should be best analyzed with hydrophilic interaction liquid chromatography (HILIC), which is frequently used as an orthogonal method for peptide purification and identification [16–18]. The solvent-rich mobile phases employed in HILIC are particularly advantageous for sensitive electrospray ionization (ESI) MS detection [16,19]. However, in our experience, HILIC methods often lack the sufficient robustness to be suitable for a generic method in the R&D laboratory. Capillary electrophoresis is also a favorite method to analyze therapeutic biomolecules [20,21]. However, when purification is desirable, transfer to preparative scale is impossible.

Looking for complementary methods that could cover a wide polarity range for short-chain and long-chain peptides, and could possibly be transferred to preparative scale when needed, Unified Chromatography (UC) appears as an interesting option. Indeed, UC mobile phase gradients may start with a non-polar composition comprising large proportions of pressurized carbon dioxide (usual working range of supercritical fluid chromatography – SFC), and end with a rather polar composition containing large proportions of liquid solvents (usual working range of enhanced fluidity liquid chromatography – EFLC), possibly ending in pure-liquid solvent conditions (LC). This promises the possible analysis of non-polar and polar analytes in a single run [22], or makes it possible to propose a single and generic method to analyze both non-polar and polar peptides. Although carbon-dioxide mobile phases are usually perceived as being incapable to elute polar analytes, SFC, EFLC and UC have all been demonstrated to be useful for this purpose [23–27]. Some examples of peptides and proteins separations by SFC, EFLC or UC appeared in literature recently [28–30]. However, most of the peptides appearing in previous works had molecular weights above 1000 Da, which set different issues in terms of solubility and multiple conformations than short-chain peptides [31]. Further information on the topic of biomolecules analysis with SFC, EFLC and UC can be found in a recent review [32].

In the first part of this series of papers [33], a UC method with UV and ESI-MS detection was developed for the purpose of determining the purity of peptide drug candidates, with molecular weights below 1000 Da. The method was developed on a modern instrument included reduced dead volumes, and with a stationary phase based on superficially porous particles offering improved efficiencies, thus the method could be called “ultra-high-performance UC”. In this second part, a thorough comparison between this UC-MS method and a reference, previously optimized, reversed-phase ultra-high-performance liquid chromatography – mass spectrometry (RP-UHPLC-MS) method is proposed. Previously, we had conducted a similar comparison between ultra-

high-performance SFC-MS and RP-UHPLC-MS for small chemical drugs [34] that demonstrated a high level of orthogonality between the two methods. It was therefore of interest to examine the complementarity and compare method performance in more details on these short-chain peptides. For this purpose, 43 peptides comprising 10 linear tripeptides and 33 cyclic pentapeptides were analyzed with both methods. The comparison was focused on performance for the intended application to purity analysis, thereby examined the impurities detection and profile, overall purity comparison, and quality attributes like the symmetry of the main peak and observed peak capacity. Some attention was paid to the separation of isomeric species, which are often encountered in synthetic mixtures of peptides.

2. Material and methods

2.1. Chemicals and solvents

From the 76 peptides used to develop the UC-MS method in the first part of this series [33], 43 peptides were available in sufficient quantity to ensure reliable method comparison. They comprised 10 linear tripeptides and 33 cyclic pentapeptides. These compounds have confidential structures not exposed in this paper. They are however described by their significant features in Table S1. It can be observed in Figure S1 (supplementary information) that adequate diversity was retained with this shorter selection, as the molecular weights ranged from 392 to 736 g/mol, calculated log P values ranged from -6.9 to 2.9 and topological polar surface area (TPSA) ranged from 130 to 292. These features were determined with Vortex v2019.04.82972.15-s (Dotmatics Limited). The scattering of points according to these significant features can be compared to that of the larger set of peptides used to develop the UC-MS method.

The peptides were provided in dimethylsulfoxide (DMSO) solutions at 10 mM. Some of them having basic residues were present as trifluoroacetic acid salts, as indicated in Table S1. The DMSO solutions were further diluted at 1 mM with (i) methanol (MeOH) for UC analysis and (ii) water/acetonitrile/trifluoroacetic acid 98/2/0.1 (v/v/v) for LC analysis.

For UC analysis, gradient-grade MeOH was purchased from VWR (Fontenay-sous-bois, France) and CO₂ with a purity of 99.995% was provided by Air Liquide (Bagneux, France). Water was provided by Purelab flex from Veolia (Wissous, France). Ammonium hydroxide solution was obtained from Sigma-Aldrich (Merck, France). For RPLC analysis, HPLC-grade acetonitrile (ACN) was purchased from Merck (VWR international SAS, France), water was obtained from a Milli-Q Purification System from Millipore (Millipore SAS, France) and trifluoroacetic acid (TFA) for spectroscopy was obtained from Merck (VWR International SAS, France).

2.2. UC-MS instrument

The ultra-high performance supercritical fluid chromatography system was a Waters Corporation (Guyancourt, France) ACQUITY Ultra Performance Convergence Chromatography™ (UPC2®) instrument, equipped with a photodiode-array (ACQUITY PDA) detector and an ACQUITY QDa® single-quadrupole mass detector with electro-spray ionization source. Instrument details were provided in the first part of this work [33]. Empower® 3 was used for system control, data acquisition and integration of peaks for symmetry and peak capacity measurements.

2.3. UC-MS method

The UC-MS method development was previously described [33]. The stationary phase was Ascentis Express OH5 (150 × 4.6

Table 1
Four gradient methods employed in this study.

(a) UC Generic gradient		
Gradient time (min)	Cosolvent proportion (%)	Flow rate (ml/min)
0	5	3
0.5	5	3
10.5	80	1.5
13.5	80	1.5
14	5	1.5
15	5	3
(b) UC Focus gradient		
Gradient time (min)	Cosolvent proportions (%)	Flow rate (ml/min)
0	5	1.5
0.5	5	1.5
1.5	C _e - 5%	1.5
11.5	C _e + 5%	1.5
11.6	100	1.5
14.6	100	1.5
14.7	5	1.5
15	5	1.5
(c) RPLC Generic gradient		
Gradient time (min)	A% (H ₂ O/ACN/TFA)	B% (ACN/H ₂ O/TFA)
0	100	0
0.6	100	0
11.5	40	60
11.6	100	0
14.3	100	0
(d) RPLC Focus gradient		
Gradient time (min)	A% (H ₂ O/ACN/TFA)	B% (ACN/H ₂ O/TFA)
0	100-(C _e -3%)	C _e -3%
0.6	100-C _e	C _e
11.4	100-(C _e +3%)	C _e +3%
11.5	100-(C _e -3%)	C _e -3%
14.3	100-(C _e -3%)	C _e -3%

mm; 2.7 μ m) from Sigma Aldrich. The mobile phase was a CO₂-cosolvent gradient, with a cosolvent composed of MeOH containing 2% H₂O (v/v) and 20 mM NH₄OH. Two different methods were used. The first one, referred to as "generic gradient", is the gradient optimized in the first part of this series. The co-solvent gradient starts at 5% and reaches 80% in 10 min, together with a reverse flow-rate gradient from 3.0 to 1.5 ml/min (see details in Table 1a). Total run duration is 15 min. A second gradient, referred to as "focus gradient", was developed in the course of this study, to enhance the resolution of impurities eluting close to the target peptide. The co-solvent gradient is adjusted individually for each target peptide based on its elution composition C_e, corresponding to the proportion of co-solvent necessary to elute the analyte with the generic gradient. A slow gradient is then conducted with a co-solvent proportion varying from C_e - 5% to C_e + 5% in 10 min. Before and after this slow gradient, a fast gradient is applied, so the total analysis duration is 15 min. Because the C_e values were all rather large, a constant flow-rate of 1.5 ml/min was used in this case to avoid reaching the pressure limit of the pumping system (414 bar). Details are provided in Table 1b.

For both gradient methods, the back pressure was set at 120 bar, the oven temperature was set at 60°C and the sample compartment temperature at 10°C. UV detection wavelength was set at 210 nm with resolution 1.2 nm. ESI-MS parameters were set as follows: both positive and negative ionization single-ion-recordings (SIR) were observed, together with positive ionization for total ion chromatograms (TIC). The needle temperature was set at 600°C and the cone voltage at 10 V. For positive TIC and SIR acquisition, the capillary voltage was set at 0.3 kV, while it was set at -0.8 kV for negative SIR. Injection volume was 5 μ l for every blank and peptide analysis.

When the proportion of co-solvent is low (at the beginning of UC gradient), it is useful to introduce an additional flow of liquid prior to entering the MS. It is however unnecessary when the proportion of co-solvent reaches high values (at the end of UC gradient). Consequently, the MS make-up flow-rate for "large gradient" also applied a reverse gradient from 0.4 to 0 ml/min in 3 min, as previously optimized. However, no make-up fluid was necessary in the case of focus gradients, because all peptides analyzed with a focus gradient were eluted with a large proportion of co-solvent. The composition of make-up fluid was identical to that of the chromatographic co-solvent (MeOH comprising 2% H₂O and 20 mM NH₄OH).

2.4. UHPLC-MS Instrument

The ultra-high-performance liquid chromatography system was an ACQUITY UPLC® I-Class from Waters Corporation. It was equipped with a binary solvent delivery pump compatible with the conditions of the present method (mobile phase flow rate of 0.7 ml/min and pressures up to 528 bar), an autosampler with flow-through-needle injection system, two 2-position column ovens compatible with 150 mm length columns and a photodiode-array (ACQUITY PDA) detector. The main flow stream was splitted by a CORONA VEO RS splitter (Thermo Scientific) placed before the MS entrance. With this system, the split ratio is about 1/10: only 1/10 of the column flow goes to the MS. ACQUITY TQMQ XEVO® triple-quadrupole mass detector (Waters Corporation) with electrospray ionization source was used for mass spectrometry identification. MassLynx® software (V4.1) was used for system control and data acquisition and Empower®3 for integration of peaks for column efficiency measurements. Waters Data Converter (V2.1) was used to convert data from MassLynx to Empower.

2.5. RP-UHPLC-MS method

The RP-UHPLC-MS method serving here as reference has been routinely used at Servier Research Laboratories since 2016 as a generic method to analyze peptides of varied sizes and polarities. The stationary phase was ACQUITY CSH C18 (100 \times 2.1 mm, 1.7 μ m) from Waters. Mobile phase A was H₂O / ACN / TFA 100/1/0.1 (v/v/v) and mobile phase B was ACN / H₂O / TFA 100/1/0.1 (v/v/v). Similarly to the UC methods, two different gradient programs were used: a large gradient referred to as "RPLC generic gradient" and a slower gradient focused on the target peptide, individually adapted to each peptide, referred to as "RPLC focus gradient". In the generic gradient, the B solvent proportion was increased from 0 to 60% in 11 min (see details in Table 1c). The total analysis time was 14.3 min. In focus gradient, the B solvent proportion was slowly increased from C_e -3% to C_e +3. Details are provided in Table 1d.

Flow-rate was set at 0.7 ml/min in both methods with an oven temperature settled at 70°C and sample temperature at 20°C. Injection volume was held at 1 μ l for every blank and peptide analysis. The lower injected volume was necessary to maintain comparable ratios of injected volume to column volume between the UC and RPLC methods (0.3 vs. 0.4%).

UV detection wavelength was set at 210 nm with resolution 1.2 nm. For MS detection, mass to charge ratio between 100 and 1000 Da were recorded with a scan time of 0.2 s. For positive TIC and SIR, acquisition, the MS parameters were set as follows: capillary voltage at 3.8 kV, cone voltage at 30 V, extractor 3 V, source temperature 150°C, desolvation temperature 600°C, cone gas flow 0 L/h, desolvation gas flow 1000 L/h, collision gas flow 0.14 ml/min.

2.6. Design of experiment for UC robustness assessment

To assess the method robustness, a Design of Experiments (DoE) with Doehlert matrix was carried out. The DoE was prepared and evaluated with Ellistat software version 6.5 2021/01 (Ellistat, Chavanod, France). Three parameters were considered: back-pressure (ranging from 111.3 to 128.7 bar), column oven temperature (ranging from 51.8 to 68.2°C) and the final co-solvent concentration in gradient elution (ranging from 70 to 90%). The full matrix is presented in supplementary information (Figure S3).

3. Results & discussion

3.1. Orthogonality of the two methods

Before comparing the performance of the methods, it is useful to examine their orthogonality. Because the UC method was developed on a polar stationary phase (polyhydroxylated ligand), it was expected that elution order would be most different from the elution order obtained in RPLC on an octadecylsiloxane stationary phase. For this purpose, we followed the method proposed by D'Attoma *et al.* [35] and plotted the peptides eluted with both methods in terms of the mobile phase composition (percentage of organic solvent) at the moment of elution. Considering the regression line and the 95% confidence limits, a polygon is drawn to provide a view of the distribution of target peaks in the retention space. The elution composition was computed according to the method proposed by Fekete *et al.* [36] using Eq. (1):

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

Where C_e corresponds to the percentage of ACN (for RPLC analysis) and MeOH (for UC analysis) required to elute the peptide; C_i and C_f are the initial and final compositions of the gradient, respectively; t_g is the gradient time; t_R is the retention time of the peptide and t_D is the system dwell time. The UC dwell time was 0.46 min and the RPLC dwell time was 0.51 min.

The plot obtained is presented in Fig. 1. Note that less than 43 points are visible on this figure because some analytes eluted with the same elution composition with both techniques (typically, the un-retained analytes in RPLC that were strongly retained in UC).

Firstly, it appears that these small peptides elute very early in the RPLC method, with proportions of organic solvent never exceeding 40%. It must be noted that this method was not specifically purposed for such small peptides but is a generic method used for a variety of peptides with all sizes and polarities. However, small polar molecules are unlikely to be well retained and resolved in RPLC mode, even with an optimized mobile phase. On the contrary, the UC method that was specifically designed for these small peptides shows adequate scattering of the target peaks, with elution compositions varying between 40 and 100% organic solvent. It is also worth noting that the scales of the figure were adjusted to have a better view of the scattering of points. The figure with identical scales is visible in supplementary information Figure S2, where it clearly appears that the points are mostly grouped at the bottom of the figure (low proportion of solvent in RPLC mode). The scattering of linear and cyclic peptides in this figure was also interesting to illustrate that this smaller analyte set (compared to the larger set used in the first part of this study) was however providing sufficient diversity.

Secondly, there appears to be little correlation between the two methods. As could be expected, a negative trend is observed, as those peptides that are less retained in the RPLC method were most retained in UC and vice-versa. The polygon drawn with interrupted lines in Fig. 1 is showing the retention space covered by

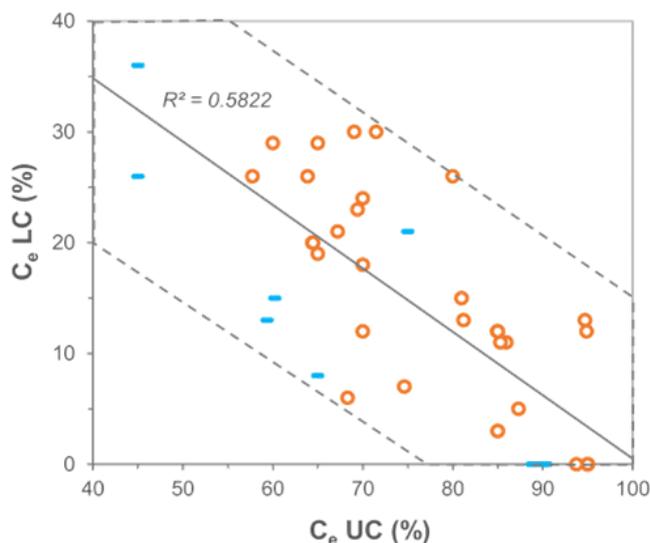


Fig. 1. Comparison of the values of elution composition obtained with the LC method and the UC method, based on the set of 43 peptides and according to Eq. (1). Blue dashes are linear peptides; orange circles are cyclic peptides. The central grey line is the correlation line; the grey interrupted lines are the 95% confidence limits (indicating that 95% of the points in a random set of analytes should be comprised within these lines) and delimit the retention space covered by the two techniques.

the two techniques, based on 95% confidence limit. Truly orthogonal methods would have a square figure for this polygon, while the present polygon leaves significant areas of white. However, reasonable orthogonality was observed, with a determination coefficient equal to 0.58. This suggests that the two methods will provide adequate complementarity also for the purpose of separating impurities from the main peak.

3.2. General chromatographic achievement

The main objective of this study was to compare the performance of the UC and RPLC methods for impurity profiling of short-chain peptide drug candidates. To better understand the purpose of this method, the general context must be explained. In the laboratory where this analysis must be conducted, drug candidates are received from the chemists at an early stage of drug development. At this stage, the possible impurities are unknown and a quick answer is desirable as to the overall purity of the main peak, to decide on the necessity to further purify it. Any information about the number, proportion and identity of impurities is useful and retained for further development. For this purpose, we first considered the success or failure of each analysis method for each of the 43 peptides examined. This achievement was measured based on 3 decision criteria:

- (i) First, the peptide needed to be retained in the chromatographic system and eluted within the gradient time frame. In other words, a measurable peak should be observed for the target peptide. In addition, because impurities are expected to be eluted both before and after the main peak, the fact that the main peak elutes with sufficient but not excessive retention should be favorable to the observation of impurities. An elution of the main peptide occurring 1 min after the dead time and initial baseline disturbances was considered as "sufficient" retention. Indeed, the major disturbance caused by DMSO elution occurred at about 2 min in UC, and some baseline disturbances were observed before 2 min in RPLC, thus "sufficient retention" was considered at

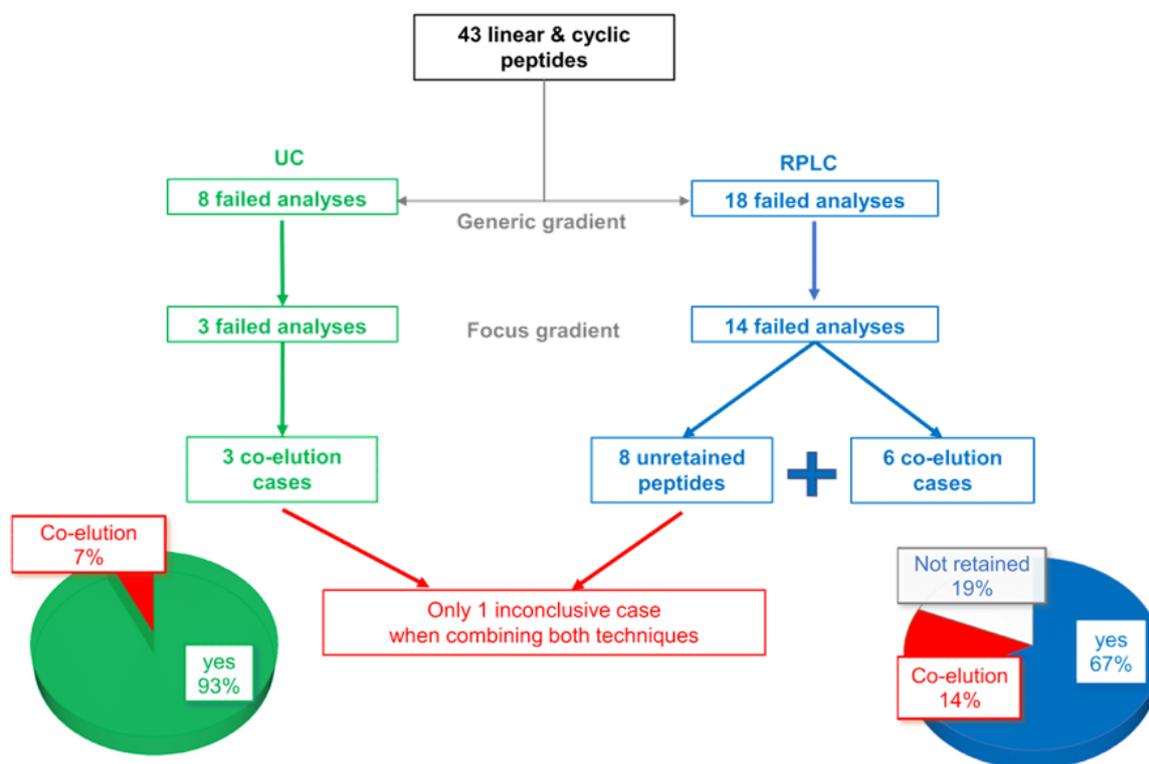


Fig. 2. Cases of failures observed among the 43 peptides analyzed with both gradient methods (generic and focus) in UC and RPLC

2 min in RPLC and 3 min in UC. As no peptide was totally retained on the column, no excessive retention was defined with this set of analytes.

- (ii) Secondly, the target peptide should show no co-elution with impurities, based on the examination of UV and MS spectra.
- (iii) Finally, the target peak should not be eluted in a chromatographic area where some baseline disturbances occur. Indeed, the presence of additives in the mobile phase, adsorbing on the stationary phase and causing breakthrough waves when the stationary phase is saturated, is responsible for baseline disturbances. In the first part of this study [33], the UC generic gradient had been particularly optimized to minimize the impact of such baseline disturbances on impurity profiling.

The overall achievement considering these three criteria was controlled a first time based on the generic gradient analyses. In cases of failure, a “focus gradient”, individually tailored to each peptide, was applied and the achievement was assessed a second time based on this second, supposedly more resolutive analysis.

The results are presented in Fig. 2.

In UC with the generic gradient, all target peptides were eluted and were sufficiently retained to ensure adequate measurement of peak purity. A “successful analysis” was obtained for 81% of the peptides (35 out of 43). The 8 failed cases all resulted from co-elution of impurities. Consequently, they were re-analyzed with tailored focus gradients. After this second analysis, only 3 peptides (7% of the cases) remained as “unsuccessful”, as co-eluted impurities could still be detected. In real-life operation, the gradient program would be further optimized to achieve complete resolution of impurities [37], but naturally this would require time and efforts, while the objective here was to compare the methods based on minimal effort.

In RPLC with generic gradient, only 42% of the analyses were successful (18 out of 43). Two types of failure were observed. The first case of failure was due to the absence of retention for 8 target peptides, which eluted in the dead volume, or too early to ensure accurate purity measurement (19% of cases). Considering the large proportion of water already present at the beginning of the generic gradient, changes in the mobile phase gradient are unlikely to solve this issue and a change of stationary phase would be required to improve their retention. The second case of failure was due to co-elution of impurities with the target peptide (39% cases). Again, in this case tailored focus gradients were carried out for each peptide with observed co-elution. After this second set of experiments, the overall success rate increased to 67% (29 peptides), as 14% of cases (6 peptides) still showed co-elution of impurities.

Clearly, the overall success rate of the UC method on this set of peptides was superior to the success rate of RPLC method. However, an interesting point is that the two methods appeared to be complementary. Indeed, after the whole process of generic and focus gradients, only 1 peptide remained as a “failed case”, as it was unretained in RPLC but showed co-elution of impurities in UC. However, this peptide was strongly retained in UC method, thus optimization would still be possible in changing the mobile phase composition or other operating parameters. In conclusion, all peptides in this set except one could be successfully analyzed with either one or the other method. This process is summarized in Fig. 2.

A final comment is that the UC method also seems more advantageous as it is less labor-intensive to achieve satisfactory results. Indeed, the total number of experiments carried out in UC was equal to 51 (43 generic gradients plus 8 focus gradients), while the total number of experiments carried out in RPLC was 61 (43 generic gradients plus 18 focus gradients). An overall performance criterium, defined as the successful cases divided by the total number of experiments: would yield the following numbers:

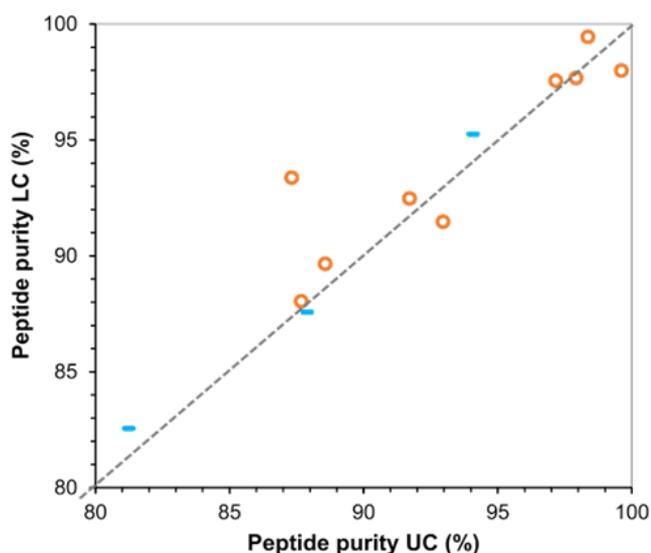


Fig. 3. Comparison of peptide purity measured with both generic gradient methods, for 12 peptides that were successfully analyzed with both methods. Blue dashes are linear peptides; orange circles are cyclic peptides. The interrupted grey line is the first bisector, indicating identical values measured with both methods.

40/51 = 0.78 in UC, and 29/61 = 0.48 in RPLC, which is largely in favor of the UC method.

3.3. Performance for impurity profiling

After this first general view on the data, it was necessary to go into more details and assess the methods specifically for the purpose of impurity profiling. For this purpose, 12 peptides (3 linear and 9 cyclic ones) were retained, which were all eluted in a reasonable time (not too early nor too late) with the generic gradients in both methods. More precisely, the peptides retained for this detailed examination were eluted between 5.7 and 12.4 min in UC, and between 2.1 and 8.1 min in RPLC. Thus the performance of the two methods would be evaluated solely on successful cases.

3.3.1. Purity assessment

A first information that is expected from the method is the overall purity measured for the target peptide, expressed as a percentage of all peak areas integrated, measured on the UV chromatograms. Each chromatogram was compared to blank injection acquired in identical conditions (UC or RPLC, generic or focus gradient) to assign the minor peaks as impurities. All peaks with peak area superior to 0.1% of the major peptide were considered. Supposing that all impurities elute within the chromatographic time frame but are not in dead volume nor are totally retained in the column, the lowest value obtained for the overall purity should indicate the best method, because a lower value means that more impurities were resolved from the main peak. In Fig. 3, the values of peak purity are compared for the 12 selected peptides. In most cases (with the one exception being far from the first bisector), they appear to be very similar. As they are distributed both sides of the first bisector, none of the method is consistently better than the other to provide the lowest purity value. The nature of the peptide (linear or cyclic) seems to have no impact on this measurement.

3.3.2. Detection of impurities

The second information expected is the number and concentration of impurities present in the synthesized peptide. First, we

Table 2

Determination of peak capacity with the two generic methods based on 12 peptides well eluted with both methods

Method	UC	RPLC
Gradient time (min)	10	10.9
Average peak width at 50% height	0.032	0.021
Peak capacity	189	314
RSD%	30.4%	29.9%

focused on the number of impurities detected with each method, again considering only those peaks with UV peak area superior to 0.1% of the main peptide. The best method should be the one showing the largest number of impurities. In Fig. 4a, it appears that, when the number of impurities was low, the two methods were comparable, with a slight advantage to UC. On the contrary, when the number of impurities was large, the best method was always RPLC. No relation to the nature of peptide (linear or cyclic) was observed in this small set. In the larger set of 43 peptides, more impurities were generally observed in linear peptides than in cyclic ones, possibly because the cyclic peptides are more stable. The largest difference observed was of 10 impurities detected in RPLC versus 1 impurity detected with UC. This most significant difference was observed for the peptide that was the most retained in UC (12.4 min), so it can be supposed that the missing impurities were too strongly retained to be eluted within the gradient time. Perhaps, this is an indication that above 12 min retention in UC, the peptide could be considered as being too much retained to provide meaningful information on purity assessment, but a single case is not enough to be conclusive.

Moreover, to better assess the differences between the two methods, all impurities observed were further categorized depending on their relative peak area with UV detection: abundant impurities (area < 1%) and minor impurities (0.1% < area ≤ 1%). Impurities below 0.1% were not considered. The results are shown in Fig. 4b. It appears that the advantage of the RPLC method seems mostly related to minor impurities. This is consistent with the fact that overall purity measurements did not show much difference between the two methods (Fig. 3).

There may be two reasons for the better performance of RPLC method. A first reason is that the resolving power in RPLC would be superior, thus showing more peaks resolved. In UC, these impurities would be either co-eluted with the major peak of the target peptide, or co-eluted with other impurities (thereby reducing the overall number of peaks observed). The impurities might also be eluted in areas where they cannot be measured, very early or very late in the chromatograms, or where a baseline disturbance is complicating accurate measurement. Such cases would be related to the cases in Fig. 3 where the measured purity of the target peak was larger in UC than in RPLC (points below the first bisector).

To examine the hypothesis of a higher resolving power in RPLC, peak capacity was measured. The evaluation of peak capacity was carried out on the 12 peptides. For each of them, the peak width at 50% height was measured. It is interesting to note that peak width in UC showed no relation to retention time and was rather constant (similar to LC), confirming that the strategy of progressively reducing the mobile phase flow rate to better approach the optimum flow rate [24,33] that is decreasing along the gradient [38,39] was not detrimental to peak width. In Table 2, the average maximal peak capacity was calculated for both generic gradient methods. A large difference appears, as UC peak capacity ($P_c = 189$) is significantly lower than RPLC ($P_c = 314$). Gradient times were similar (10 vs. 10.9 min) so this significant difference principally issues from the larger peaks observed in UC (about 60% larger, on average). To a certain extent, this may be related to the different

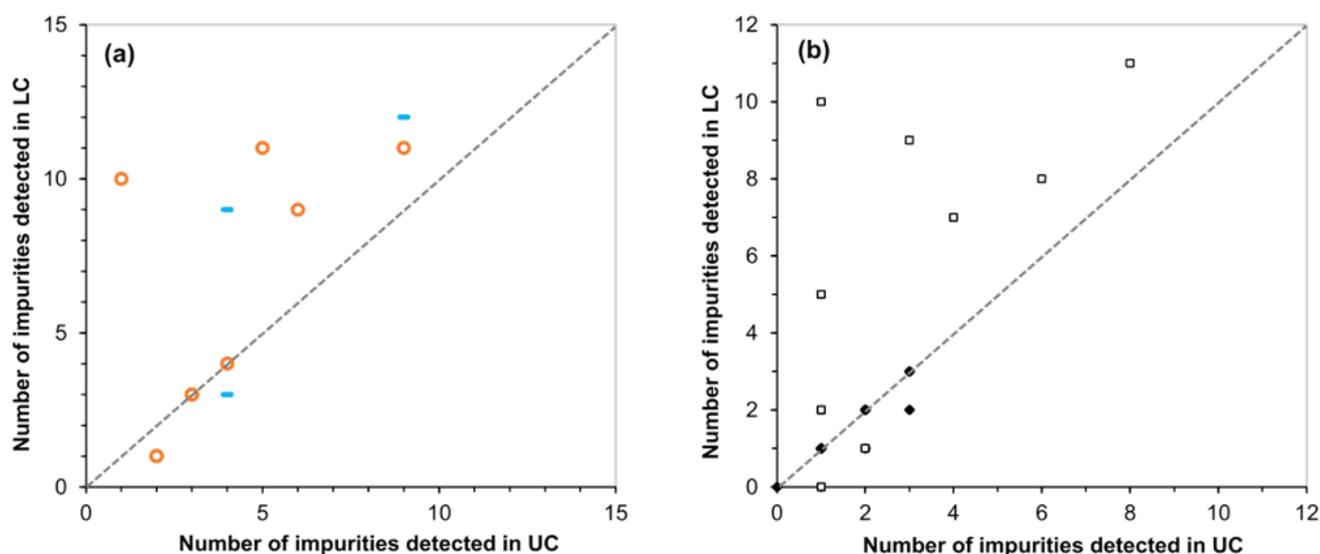


Fig. 4. Comparison of the number of impurities observed with both generic gradient methods, for 12 peptides that were successfully analyzed with both methods. (a) Total number of impurities observed for linear peptides (blue dashes) and cyclic peptides (orange circles). (b) Impurities with large concentration (>1%, black diamonds) and low concentration (<1%, open squares). The interrupted grey line is the first bisector, indicating identical values measured with both methods.

column and particle formats employed in this study as the RPLC method was done with sub-2 μm fully porous particles, while the UC method was done with sub-3 μm superficially porous particles. This observation is also in line with previous studies comparing RPLC to SFC analysis of small chemical drugs on sub-2 μm fully porous particles [34], where the measured efficiency was higher in RPLC than SFC. It is also possible that the slow interaction kinetics due to polar interactions in UC would cause larger band broadening than the hydrophobic interactions in RPLC. It is also worth mentioning that peak asymmetry was not responsible for the larger peaks observed in UC, as the average peak symmetry in this set was $A_{SEP} = 1.10$, slightly better than the average 1.23 measured in LC. In general, it was observed that the peak symmetry on the main peak was better in UC chromatograms than in LC chromatograms, where peak asymmetry frequently exceeded 1.2. In UC chromatograms, asymmetry was slightly worse for late-eluting peaks than for early-eluting peaks.

A second reason that could explain the superior performance of the RPLC method is that the sensitivity would be higher, which should principally show in the number of minor impurities detected. Indeed, as appears in Fig. 4b, the increased number of impurities observed in RPLC was essentially due to minor impurities ($0.1\% < \text{area} \leq 1\%$). For the set of 12 well-eluted peptides, the UV signal-to-noise ratio was higher in RPLC than in UC. Independently of the UV detector performance, this is consistent with larger peaks observed in UC. This sensitivity issue is also well known by SFC-UV chromatographers. Indeed, although modern instruments have largely improved sensitivity compared to older instruments, it is still not as good as comparable instruments in liquid conditions. This is partly inherent to the way the system is working: pressure regulation causes a constant variation of fluid density, which is causing a constant variation of refractive index, thus causing baseline noise in UV [40]. Baseline compensation is somewhat improving this problem but not enough to reach the sensitivity of LC systems.

3.3.3. Impurity profiling

The 12 peptides were investigated in great details on their impurity profiles, trying to match the information from both methods based on peak area and MS spectra. Two representative exam-

ples are shown in Fig. 5 with a linear peptide (Fig. 5a) and a cyclic peptide (Fig. 5b). In these figures, the main peptide is identified with a red square, and was of course detected with both methods. The impurities that were observed with both methods and could be matched are identified with yellow circles. The impurities that were observed with only one method are identified with green triangles. On this figure, they are placed on the right and top of the figure to indicate that they were seen with UC (abscissa) or with RPLC (ordinates) only.

For the linear peptide, 4 impurities were observed with both methods (yellow circles). In addition, 1 impurity was observed with UC only (green triangle at the bottom), while 4 impurities were observed with RPLC only (green triangles on the left). It also appears that the impurities were eluted over a wider time frame in UC than in RPLC. This is consistent with the above observation that retention times of the target peptides in UC were scattered in a wider time frame than in RPLC (Fig. 1).

For the cyclic peptide, 6 impurities were observed with both methods (yellow circles). In addition, 3 impurities were observed with UC only (green triangles at the bottom), while 5 impurities were observed with RPLC only (green triangles on the left). For this sample, some impurities appeared at the end of the UC gradient that were difficult to identify. As the proportion of co-solvent is high at this point, MS sensitivity is somewhat less than at the beginning of the gradient. Further improvement of MS parameters might improve identification of late-eluting impurities.

Among the 12 cases examined in detail, the complementarity of the two techniques was evident. While the RPLC method often showed more minor impurities than the UC method, the two methods mostly provided different information.

3.4. Resolution of isomers

The separation of isomeric species is of interest as isomeric impurities are often present in synthesized peptides. In the set of 43 peptides that could be analyzed with both UC and RPLC methods, no isomeric pairs were present for the linear peptides, but 10 isomeric pairs or trios were present for cyclic peptides. When enough retention could be obtained in RPLC, the resolution of isomeric pairs was generally good with RPLC method. In UC, where

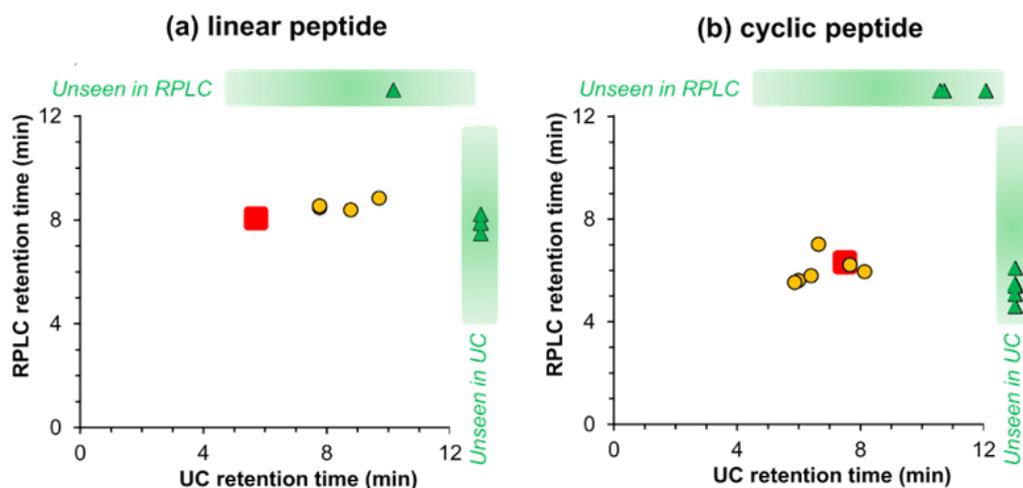


Fig. 5. Comparison of retention times obtained for the API (red square) and impurities. Yellow circles are impurities observed with both RPLC and UC methods, green triangles are impurities observed with only one method: green triangles at the top of the figure were seen with UC only, green triangles at the right of the figure were seen with RPLC only.

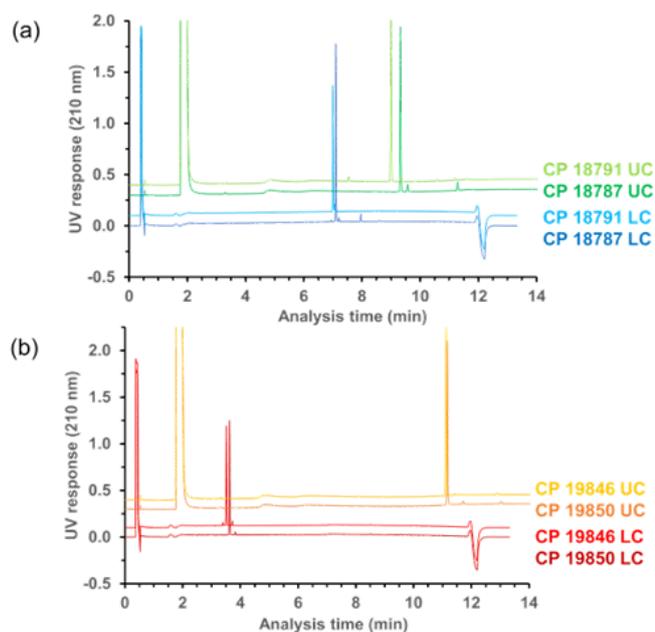


Fig. 6. Chromatograms for two pairs of isomers in UC and RPLC under generic gradient conditions (a) cyclic pentapeptides better resolved with UC (b) cyclic pentapeptides better resolved with RPLC.

all peptides were eluted in an adequate retention time, some isomeric pairs were well resolved while others remained co-eluted. Because the set of compounds was very small for this comparison, it is impossible to draw any conclusion as to the reason for success or failure of isomers separation in one or the other method. Sample chromatograms are presented in Fig. 6, to illustrate the different capabilities for isomer separation. In Fig. 6a, a case where UC offered superior resolution, is observed with positional isomers: CP 18791 was $-R_1-R_2-R_3-R_4-R_5-$ while CP 18787 was $-R_1-R_4-R_3-R_2-R_5-$, i.e. the positions of R_2 and R_4 were exchanged. In Fig. 6b a case where RPLC offered superior resolution is observed with a diastereomeric pair, where CP 19846 was $-R_1-(D-R_2)-R_3-R_4-R_5-$ and CP 19850 was $-R_1-(L-R_2)-R_3-R_4-R_5-$, i.e. one amino acid had opposite absolute configuration in the two peptides (D/L). Fig. 6 also

shows that the two techniques provided comparable chromatographic quality, as previously mentioned when comparing significant features. Finally, only one isomeric pair could not be resolved with any of the two methods, again supporting the high complementarity of the UC and RPLC methods.

3.5. Robustness

The robustness of the well-established LC method was not assessed. The UC method, however, required robustness assessment as it was little explored so far. For this purpose, a Doehlert design of experiment [41] was achieved with three variable parameters: back-pressure, column temperature and final gradient composition. The limit values of the three parameters were voluntarily chosen to be large (20 bars, 20°C and 20 % of co-solvent). Indeed, they are much larger than what would occur in normal operation of the instrument, where the accepted variations of instrument control are about 2 bar, 0.5°C and 2% respectively for back-pressure, temperature and co-solvent proportion. The DoE matrix is detailed in in Figure S3 (supplementary information). Retention factor for one peptide eluting about mid-gradient (with retention time of about 7 min) was examined to assess its variability with these three parameters. The results are presented in Fig. 7 and Table S2. The model was well-fitted, as appears in Figure S3 with $R^2 = 0.982$ and a confidence interval of 0.2. Unsurprisingly, the most influential parameter was the solvent proportion, while back-pressure and temperature were found to be insignificant. Indeed, the large proportion of co-solvent along the gradient and particularly at the end of the gradient make it a low-compressibility fluid, on which pressure and temperature can have only little impact. Whatever the fluid density and compressibility, the effect of changing mobile phase composition is still significant as it will impact the elution strength through changes in polarity, similar to what would be observed in liquid chromatography. However, this impact is largely acceptable as it appears that the normal range of variation that is considered acceptable in instrument control (typically less than 2% variation in the proportion of co-solvent) would yield a variation of retention factor of about 0.1. This corresponds to a variation in retention time of about 0.06 min. As a result and taking account of the instrument reliability, the method looks sufficiently robust to be applied as a generic method in the research and development context.

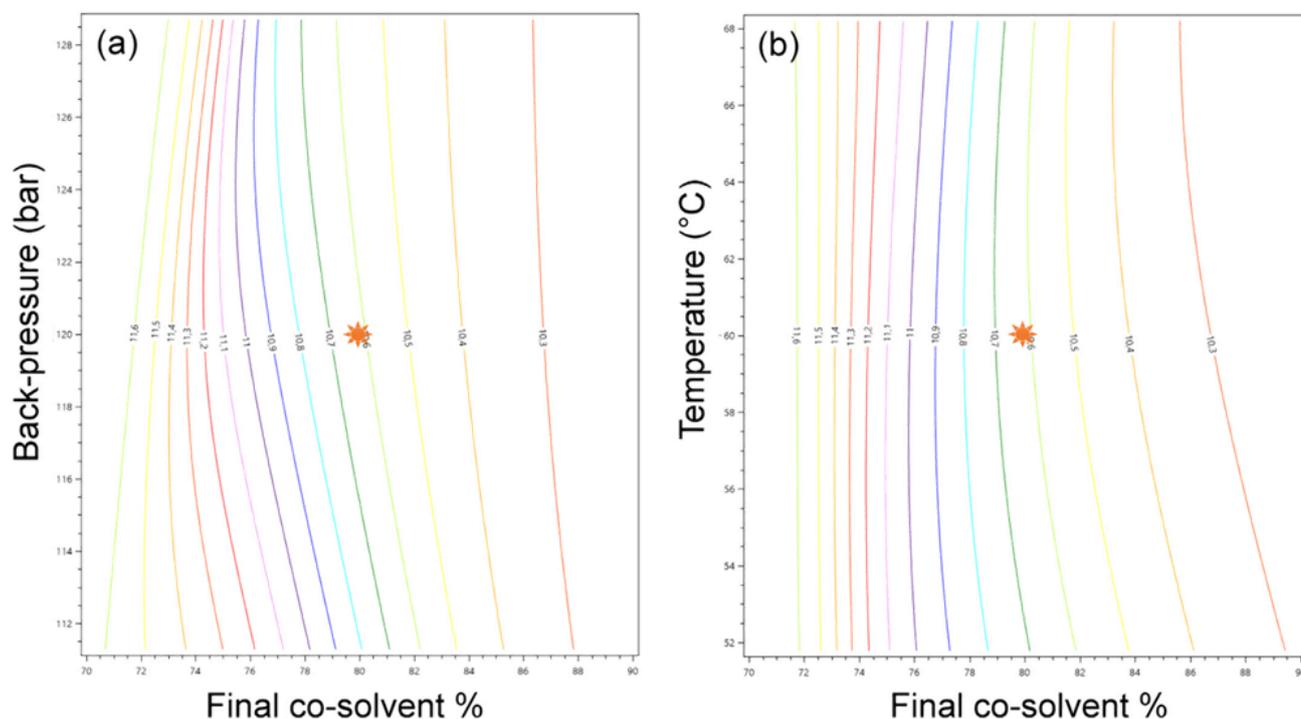


Fig. 7. Design of experiment to assess the variation of retention factor in UC conditions. (a) Effect of back-pressure vs. final gradient composition. (b) Effect of oven temperature vs. final gradient composition. The stars indicate the set conditions in generic UC gradient.

3.6. Operational cost comparison

Finally, comparing the operational cost of the two techniques may be of use. For this purpose, the solvent consumption per analysis was computed for the generic gradient methods. The volume of solvent used (and thus requiring waste treatment after analysis) with the RPLC method was about 10 mL acetonitrile-water, while the volume of solvent used (and requiring waste treatment) with the UC method was 11.9 mL methanol-water. Because acetonitrile is much more expensive than methanol and because carbon dioxide is relatively cheap, the final cost per sample was nearly the same: about 2.3 € per sample. Of course, the UC equipment is somewhat more expensive than the RPLC equipment. However, we must point out that the high success rate of the UC generic method is also providing operational cost savings as it does not require additional expenses to re-analyse the sample with focus gradient or another analytical method.

4. Conclusions and perspectives

Orthogonality between the UC and RPLC methods on this set of peptides is somewhat less significant than was previously observed when small chemical drugs were compared between SFC and RPLC. However, the UC method is particularly interesting for the cases when RPLC retention is too low, which occurred frequently for these small, rather polar molecules. In the present test set, 19% of the samples could not be analyzed with the RPLC method while all of them had adequate retention in UC. When adequate retention could be obtained, less effort was required with UC than with RPLC to obtain satisfying results, as the generic gradient was most successful while the RPLC method often required a second experiment with focus gradient. In terms of capability for impurity profiling, the RPLC method seems superior as it was capable to detect more minor impurities, either due to higher peak capacity or possibly due to higher sensitivity with UV detection. However, the UC

and RPLC methods are most complementary as different impurities could be observed between the two methods. As a conclusion, the joint use of the two methods is advisable to improve the chances of successful analysis with a minimum effort and to obtain a maximum amount of information on a single sample.

Funding

This work was funded by Servier Research Institute.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

CRediT authorship contribution statement

Jérémy Molineau: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Maria Hideux:** Resources, Writing – review & editing, Supervision. **Philippe Hennig:** Conceptualization, Resources, Funding acquisition. **Sophie Bertin:** Conceptualization. **Fabien Mauge:** Conceptualization. **Eric Lesellier:** Conceptualization, Resources, Writing – review & editing. **Caroline West:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Visualization, Supervision, Project administration.

Acknowledgment

ICOA is supported by the University of Orleans, the National Centre for Scientific Research (CNRS), the Labex programs SynOrg (ANR-11-LABX-0029) and IRON (ANR-11-LABX-0018-01), the FEDER programs CHemBio (FEDER-FSE 2014-2020-EX003677) and Techsab (FEDER-FSE-2014-2020-EX011313) and the RTR Motivhealth (2019-00131403).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.chroma.2021.462771](https://doi.org/10.1016/j.chroma.2021.462771).

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Supplementary information to:

Analysis of short-chain bioactive peptides by unified chromatography-electrospray ionization mass spectrometry. Part II. Comparison to reversed-phase ultra-high performance liquid chromatography

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Figure S1. Significant features of the 43 peptides examined. Molecular weight (g/mol) is plotted against calculated log P values. The bubble size is related to topological polar surface area (TPSA). Blue bubbles are linear tripeptides; orange bubbles are cyclic pentapeptides.

Figure S2. Comparison of elution composition obtained with the LC method and the UC method, with identical scales, based on the set of 43 peptides. Blue dashes are linear peptides; orange circles are cyclic peptides. The central grey line is the correlation line; the grey interrupted lines are the 95% confidence limits and delimit the retention space covered by the two techniques.

Figure S3. Robustness design of experiments with Doehlert matrix and resulting model fit.

Table S1. 43 peptides used to compare the two methods, with significant features: molecular weight (excluding counter-ion), calculated log P, topological polar surface area (TPSA) and the presence of trifluoroacetic acid (TFA) salt.

Table S2. Results of the robustness design of experiments for the UC method. (DoE in Figure S3.)

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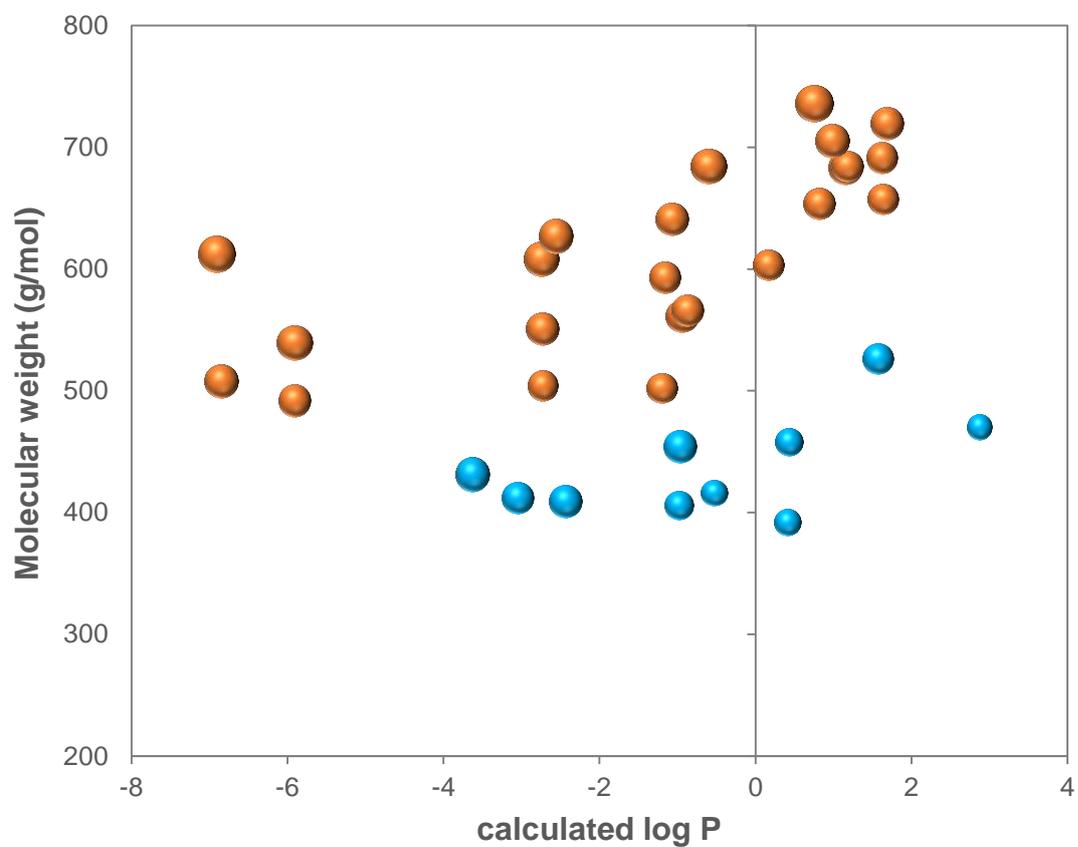


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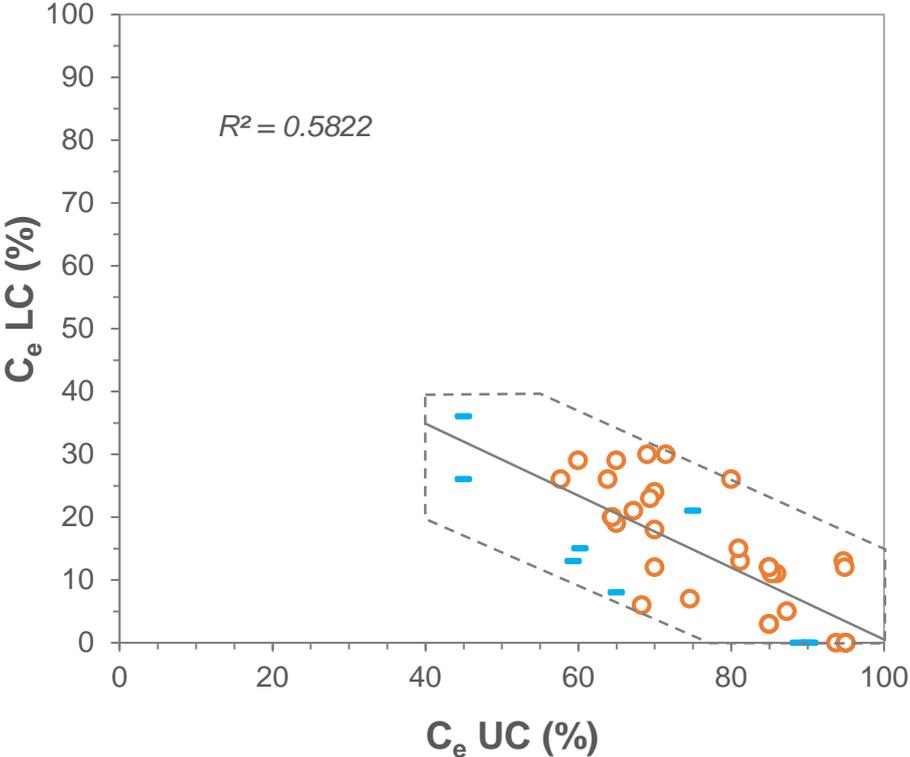
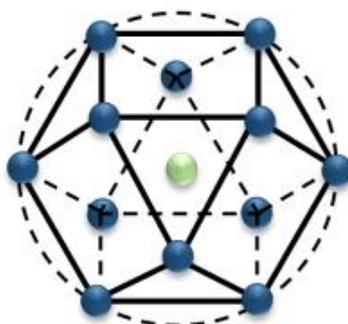


Figure S3. Robustness design of experiments with Doehlert matrix and resulting model fit.



Experiment n°	Final co-solvent proportions (%)	Back-pressure (bar)	Oven temperature (°C)	Retention factor k
1	80	120	60	10,7
2	90	120	60	10,1
3	70	120	60	11,7
4	85	128,7	60	10,3
5	75	111,3	60	11,3
6	85	111,3	60	10,4
7	75	128,7	60	11,2
8	80	120	60	10,7
9	85	122,9	68,2	10,3
10	75	117,1	51,8	11,1
11	85	117,1	51,8	10,5
12	80	125,8	51,8	10,7
13	75	122,9	68,2	11,1
14	80	114,2	68,2	10,8
15	80	120	60	10,5

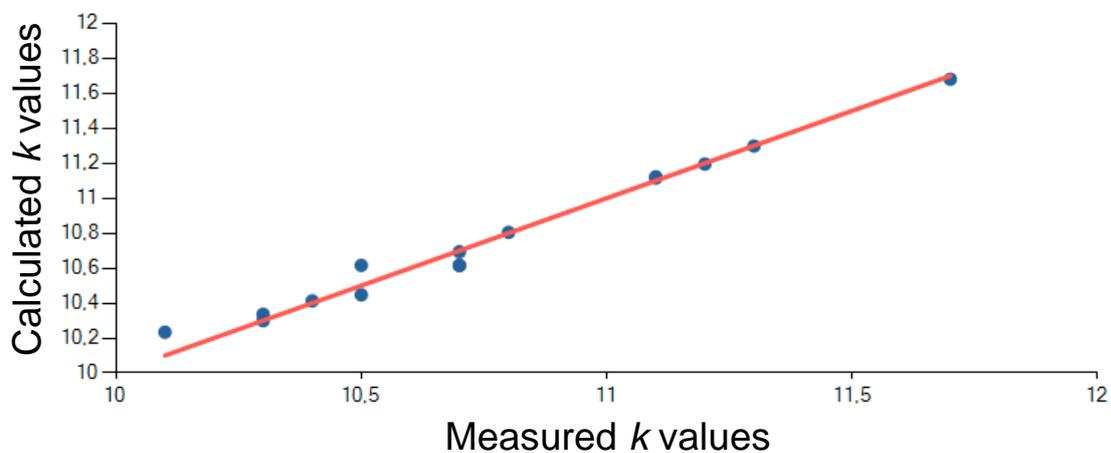


Table S1. 43 peptides used to compare the two methods, with significant features: molecular weight (excluding counter-ion), calculated log P, topological polar surface area (TPSA) and the presence of trifluoroacetic acid (TFA) salt.

Peptide	Salt	MW peptide (g/mol)	clog P	TPSA	tR UC	tR LC
LP 6838	-	392.2	0.41	150.6	7.71	3.98
LP 9917	-	406.2	-0.98	170.9	8.69	3.09
LP 3768	2 TFA	409.2	-2.44	221.0	11.41	0.38
LP 10333	TFA	412.2	-3.05	216.6	11.84	0.41
LP 7055	-	416.2	-0.53	141.8	7.82	4.35
LP 9258	TFA	431.2	-3.63	249.8	12.31	0.41
LP 6781	2 TFA	454.3	-0.97	218.3	11.64	1.31
LP 7854	-	458.2	0.43	167.7	9.99	5.35
LP 5099	-	470.3	2.87	130.4	5.72	8.06
LP 4303	TFA	526.3	1.57	208.1	6.01	6.36
CP 19088	-	492.2	-5.91	218.7	12.29	0.87
CP 19538	-	502.3	-1.20	190.2	8.38	5.16
CP 19551	-	502.3	-1.20	190.2	8.39	5.20
CP 19212	-	504.2	-2.73	192.9	8.90	2.62
CP 19219	-	504.2	-2.73	192.9	9.74	2.90
CP 18118	-	508.2	-6.85	238.9	12.70	0.75
CP 17720	-	539.2	-5.91	255.0	11.25	2.14
CP 20081	-	551.3	-2.73	229.1	9.13	3.82
CP 19711	TFA	561.3	-0.94	216.2	10.62	3.90
CP 19742	TFA	561.3	-0.94	216.2	10.59	4.39
CP 18604	-	566.3	-0.87	201.6	9.22	4.76
CP 19846	TFA	593.3	-1.16	207.4	11.12	3.76
CP 19850	TFA	593.3	-1.16	207.4	11.17	3.64
CP 19370	-	603.3	0.17	200.1	8.83	5.97
CP 20297	-	603.3	0.17	200.1	8.26	6.74
CP 20365	TFA	608.3	-2.75	255.2	11.32	2.20
CP 20048	TFA	608.3	-2.75	255.2	11.25	2.62
CP 19325	TFA	612.3	-6.91	292.5	13.40	0.41
CP 19308	TFA	612.3	-6.91	292.5	13.78	0.41
CP 19196	TFA	627.3	-2.56	245.7	11.44	2.56
CP 17992	-	641.3	-1.07	229.1	8.76	5.01
CP 18007	-	653.4	0.82	208.9	8.31	6.42
CP 20336	-	657.4	1.63	200.1	7.49	6.32
CP 19391	-	683.4	1.15	229.1	8.76	5.42
CP 18657	-	684.3	1.18	201.6	10.25	6.42
CP 20281	TFA	684.4	-0.60	255.2	11.16	3.82
CP 19346	TFA	684.4	-0.60	255.2	11.25	3.64
CP 20396	-	691.4	1.62	200.1	7.90	6.84
CP 18787	-	705.4	0.98	237.9	9.32	7.00
CP 18791	-	705.4	0.98	237.9	9.00	7.11
CP 19407	-	719.4	1.69	229.1	9.05	5.78
CP 21013	-	736.3	0.75	291.7	12.44	3.78
CP 20997	2 TFA	736.3	0.75	291.7	12.42	3.96

Table S2. Results of the robustness design of experiments for the UC method. (DoE in Figure S3.)

Parameter	Coefficient	Standard deviation	t	p-value	Significance
Constant	18.144	0.920	19.721	0.000	Yes
Final B%	-0.080	0.006	-13.544	0.000	Yes
Back-pressure	-0.007	0.006	-1.222	0.247	No
Oven temperature	-0.002	0.006	-0.346	0.736	No

III- Analyse des « gros » peptides par UC-MS

Les peptides analysés précédemment par UC-MS étaient des composés de bas poids moléculaires (< 800 Da). La suite de ce chapitre, va se consacrer aux recherches par UC-MS sur de nouveaux gros peptides (entre 1 000 et 5 000 Da). Le poids moléculaire de ces nouveaux peptides est semblable à celui d'une majorité des composés retraités dans le chapitre 3.II.a. Ces peptides, mis à disposition par l'IdRS, sont associés pour la plupart soit à des contre-ions TFA, soit à des contre-ions acétates. Pour étudier ces composés, il a été nécessaire de redévelopper une méthode UC-MS pour les éluer. La publication présentée ci-dessous discute d'investigations en cours au sujet de ces gros peptides. Elle relate des observations sur l'élution des peptides associés aux contre-ions et s'étendra par la suite au choix du solvant de solubilisation ainsi que du volume d'injection.

Effect of dilution solvent and injection volume on the analysis of basic polypeptide salts with enhanced-fluidity liquid chromatography

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Article in process, to be submitted in 2022

Abstract

The chromatographic analysis of long-chain therapeutic peptides, with molecular weight mostly in the 3500-4500 Da range (31-34 amino acids), is explored with pressurized CO₂ in the mobile phase. The optimal method was obtained on a Torus 2-PIC column, with a gradient elution of 50 to 90% co-solvent in CO₂, which is relevant of enhanced-fluidity liquid chromatography (EFLC). Both UV (210 nm) and mass spectrometric detection modes were employed to assess the purity of the major peak and its resolution from impurities. Most of the peptides in this set were basic, thus they were analyzed as acetate or trifluoroacetate salts. As significant peak distortion was observed in some cases, thorough examination of dilution solvent and injection volume was conducted to improve peak shape and resolution from impurities. Finally, the best injection volume was 1 μL, as any other volume (smaller or larger) yielded distorted peaks, and the best dilution solvent composition was the same as the mobile phase co-solvent (methanol comprising 5% water and 0.1 % methanesulfonic acid). However, not all peptide salts were fully soluble in this solvent thus further investigations will be required.

Keywords: counter-ion, enhanced-fluidity liquid chromatography, peptides, supercritical fluid chromatography, unified chromatography

1. Introduction

The interest of peptides as therapeutic drugs has been known for several decades. The effects of varied peptides on human health have been described in literature for treatment of cardiovascular disease [1], cancer [2,3] or SARS-CoV pathology [4,5]. While both are polymers of amino acids linked by peptide bonds, the exact difference between peptides and proteins is however not very clear. One criterion is the molecular size as proteins are bigger than peptides, with 50-100 amino acids being a possible cut-off between the two. Lau and Dunn pointed the evolution of chain length in therapeutic peptides observed between the 1980s' and 2018 [6]. Short-chain peptides containing less than 10 amino acids (molecular weight below 1000 Da) were still major in clinical development and standing for 75% of commercialized peptides in northern countries until 2021 [7]. However, following the developments in peptide synthesis and manufacturing improvement to achieve molecular weights up to 5000 Da [8,9], larger peptides appeared and are now more commonly observed in newly developed drugs. Those bigger peptides could present multi-cyclization due to disulfide bridges but also C-C bonds and/or C-O-C bonds such as in Oritavancin. Another significant criterion to distinguish between peptides and proteins is the existence of a 3D structure in proteins (like β -sheets and α -helices), while the structure of peptides would be less well defined. For instance, Insulin was considered as the first therapeutic peptide from the 1920s' [10] but some elements of 3D structure due to disulfide bridges and β -sheet may relate it to proteins.

The historical development of therapeutic peptides has been reviewed, highlighting target diversification over time [6,11]. The attractiveness of peptides as active pharmaceutical ingredients compared to small chemical molecules is related to their low toxicity and reduced side-effects compared to chemical drugs, their high target specificity, selectivity and affinity [12,13]. Compared to proteins, their smaller size favors the crossing of membrane barriers [14] and facilitates their chemical synthesis. Indeed, therapeutic peptides can be natural or synthetic, and the amino acid chain may be linear or cyclic. Cyclic peptides are usually more stable as they are less prone to enzymatic degradation.

Supercritical or sub-critical carbon dioxide is employed as a mobile phase component in different separation techniques such as supercritical fluid chromatography (SFC),

enhanced fluidity liquid chromatography (EFLC) or unified chromatography (UC). CO₂ displays many attractive properties because it is a non-toxic and non-flammable fluid, available at a low cost and producing no solvent waste requiring treatment. However, in all three chromatographic methods, CO₂ is usually associated to a liquid co-solvent, which is most often methanol, possibly containing a small concentration of water and/or acidic, basic or salt additives [15]. Whatever the proportion of liquid, the presence of pressurized CO₂ in the mobile phase favors the high diffusivity of analytes and low viscosity of the fluid. Those parameters enable the use of high flow-rate and favor high efficiency. While the concept of unified chromatography is rather old [16], it has notably been used to describe the successive use of SFC, EFLC and LC in the course of a single experiment, through a gradient of mobile phase composition, possibly starting with 100% CO₂ and ending with 100% liquid. In a seminal paper, Taguchi et al. [17] employed UC analysis to elute vitamins with a large range of polarities. The wide polarity range is an interesting feature in the context of peptide analysis, as therapeutic peptides may be lipophilic (like Gramicidin) or hydrophilic (like Oxytocin). The analysis of biomolecules (comprising peptides and proteins) with supercritical CO₂, achieved in SFC, EFLC or UC conditions, was reviewed in a recent paper [18]. Since then, new UC methods were published for short-chain [19,20] and long-chain therapeutic peptides [21].

Depending on their side-chain residues, peptides might be acidic, basic or uncharged. Indeed, the acidic and basic functions in natural amino acids will be charged at physiological pH. For instance, when the number of basic functions (e.g. arginine, lysine and the N-terminal amino group), is superior to the number of acidic functions (e.g. aspartic acid, glutamic acid and the C-terminal acid group), the peptide is basic. Then the peptide should be associated to a counter-ion to obtain the electrical neutrality in the form of a salt. Usual counter-ions for basic peptides are chloride, acetate (AcO⁻) and trifluoroacetate (TFA⁻). The selection of this counter-ion could impact the final formulation as reviewed Sikora et al. [22]. The authors also pointed at structural effects due to counter-ion nature and concentration. For improved biological effects, understanding inserted salts must be investigated. As a result, in the course of therapeutic peptide development, the different salts obtained with one or the other counter-ion would require chromatographic analyses.

In the mobile phases containing pressurized carbon dioxide, the exact pH cannot be known, thus the exact ionization state of the peptide remains unsure. However, the apparent pH in CO₂-methanol was estimated to be around 5 [23]. The presence of basic additives and salts like ammonium acetate may offer some buffering of the mobile phase apparent pH around 5-6, while the introduction of acidic additives would lower the apparent pH. Similarly, the pK values of amino acid acidic and basic functions in such environment cannot be known.

In this paper, we examine the chromatographic analysis of long-chain therapeutic peptides, with molecular weight mostly in the 3500-4500 Da range, with pressurized CO₂ in the mobile phase. The screening gradient was wide, relevant of UC, but the final method was achieved with CO₂ proportion inferior to 50%, which is more relevant of EFLC. Both UV and mass spectrometry (MS) detection modes were examined. Most of the peptides in this set were basic, thus they were analyzed as acetate or trifluoroacetate salts. As significant peak distortion was observed in some cases, thorough examination of dilution solvent and injection volume was conducted to improve peak shape and resolution from impurities.

2. Material and methods

2.1 Chemicals and Solvents

Eleven peptides were selected from an in-house compound bank at Servier Research Institute. These compounds have confidential structures not exposed in this paper. They are however described by their significant features in Table S1: molecular weight, isoelectric point (pI) and GRAVY number. The values of GRAVY number (Grand average of hydropathicity) and pI were determined with ProtParam tool from ExPASy [24]. Further description is provided in section 3.1. Peptides were provided as powder with either AcO⁻ or TFA⁻ salts. Solubilization was studied using different compositions of dilution solvent at 1 mg/ml of peptide.

Gradient-grade methanol (MeOH) and water LC-MS grade were purchased from VWR (Fontenay-sous-Bois, France) and CO₂ with a purity of 99.995% was provided by Air Liquide (Bagneux, France). Methanesulfonic acid (MSA), trifluoroacetic acid (TFA) and ammonium hydroxide solution (NH₄OH) were purchased from Sigma-Aldrich (Merck, France), acetic acid (AA) was purchased from VWR (Fontenay-sous-Bois,

France) and dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (Illkirch, France).

2.2 Instrument

The instrument was an ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) from Waters Corporation (Guyancourt, France). 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, an automated back-pressure regulator, a 2-position column oven compatible with 150 mm-length columns and two detectors: a photodiode-array (ACQUITY PDA®) detector and a single-quadrupole mass spectrometer with electrospray ionization source (ESI-MS) (ACQUITY QDa®). An isocratic solvent manager was used as a make-up pump and was positioned after the PDA and before the MS. The main flow stream was then split by the on-board flow-splitter assembly. With this system, a portion of the column flow goes to the back-pressure regulator while an unknown portion goes to the MS. Empower® 3 was used for system control, data acquisition and integration of peaks.

2.3 Stationary phases

Initially, several stationary phases providing different selectivity [25] were tested. They are presented in Table S2. The optimal results were obtained on ACQUITY Torus 2-PIC (100 x 3.0 mm, 1.7 µm).

2.4 Chromatographic methods

The stationary phases in Table S2 were tested with different mobile phase compositions based on CO₂ and methanol comprising 2% H₂O and (i) 20 mM NH₄OH, or (ii) 0.1% TFA or (iii) 0.1% MSA. The screening method applied a gradient elution from 1 to 100% of co-solvent in 10 min, which is relevant of unified chromatography. Back-pressure was set at 120 bar, the oven temperature was set at 25°C and the sample manager temperature was set at 10°C to limit sample degradation. The flow-rate (isocratic and reverse gradient) was adapted to column dimensions. The injection volume was 5 µL. PDA wavelength was set at 210 nm and spectra were recorded from 190 to 800 nm. ESI-MS parameters were set as follows: needle temperature 600°C, capillary voltage 1.5 kV and cone voltage 20 V. The make-up fluid was composed of

methanol and 20 mM NH₄OH in water 98/2 (v/v), identical to the mobile phase. The make-up flow rate was adapted to the flow-rate of liquid coming from the mobile phase.

After some optimization steps, the best method was obtained on the Torus 2-PIC column with a co-solvent composed of methanol comprising 5% H₂O (v/v) and 0.1% MSA. This is similar to the chromatographic system selected by Losacco et al. [21], but the gradient had to be adapted to obtain adequate retention. In the final gradient program, the co-solvent proportion was initially set at 50%, maintained for 1 min then increased from 50% to 90% in 6.2 min, where it was maintained for 0.3 min. This gradient is perhaps more relevant of EFLC than of SFC or UC. The oven temperature was set at 25°C and the sample manager temperature at 10°C. Back-pressure was maintained constant at 120 bar. Similarly to our previous UC experiments [19,26], a reversed flow-rate gradient was applied from 0.7 ml/min to 0.5 ml/min to avoid reaching the upper pressure limit of the pumping system. UV detection was done at 210 nm with resolution 1.2 nm. The ESI-MS parameters were as follows: single-ion recordings (SIR) and total ion chromatograms (TIC) were registered in positive ionization mode from 100 to 1250 m/z values. The needle temperature was set at 600°C and cone voltage at 20 V. Capillary voltage was set at 1.5 kV (ESI+). The make-up flow-rate applied a reversed gradient from 0.5 to 0.4 ml/min in 6.2 minutes and the solvent composition was identical to the mobile phase cosolvent (methanol comprising 5% H₂O (v/v) and 0.1% MSA). The injected volume was varied between 0.5 and 5 µL.

3. Results & discussion

3.1 Description of the peptide set

11 model peptides were selected from a library of drug candidates at Servier Research Laboratories. This set included 9 peptides available both as AcO⁻ and TFA⁻ salt (P3 to P11), 1 peptide available only as TFA⁻ salt (P1) and one acidic peptide, with sodium counter-ion (P2). All of them were linear, except P1 that was cyclic, comprising 3 disulfide bridges. The N-terminal function could be free or protected with acetylation (P6) or with a biotin group (P7 to P11). The C-terminal function could be free or modified with amidation (P2 to P6). The amino acids included hydrophobic and hydrophilic groups, neutral, acidic and basic functions. All of these peptides were

composed of proteinogenic amino acids, apart from a few D-amino acids in peptides P3 to P6.

The exact structure of the peptides cannot be shared, but their significant features are presented in Table S1 and Figure 1. In Figure 1, GRAVY number, which is an indicator of the peptide hydrophobicity or hydrophilicity [27], was plotted against molecular weight. As appears from the figure, most of the peptides had molecular weights varying in a narrow range, from 3 400 Da to 4 200 Da (31 to 34 amino acids), while P1 was much heavier (5245 Da, 48 amino acids) and P2 was much lighter (1515 Da, 13 amino acids). GRAVY number ranged from -0.2 to -2.1, indicating that the peptides were all more or less hydrophilic. Note that GRAVY number does not take terminal modification into account as it only considers the hydropathy of proteinogenic amino acids in the calculation.

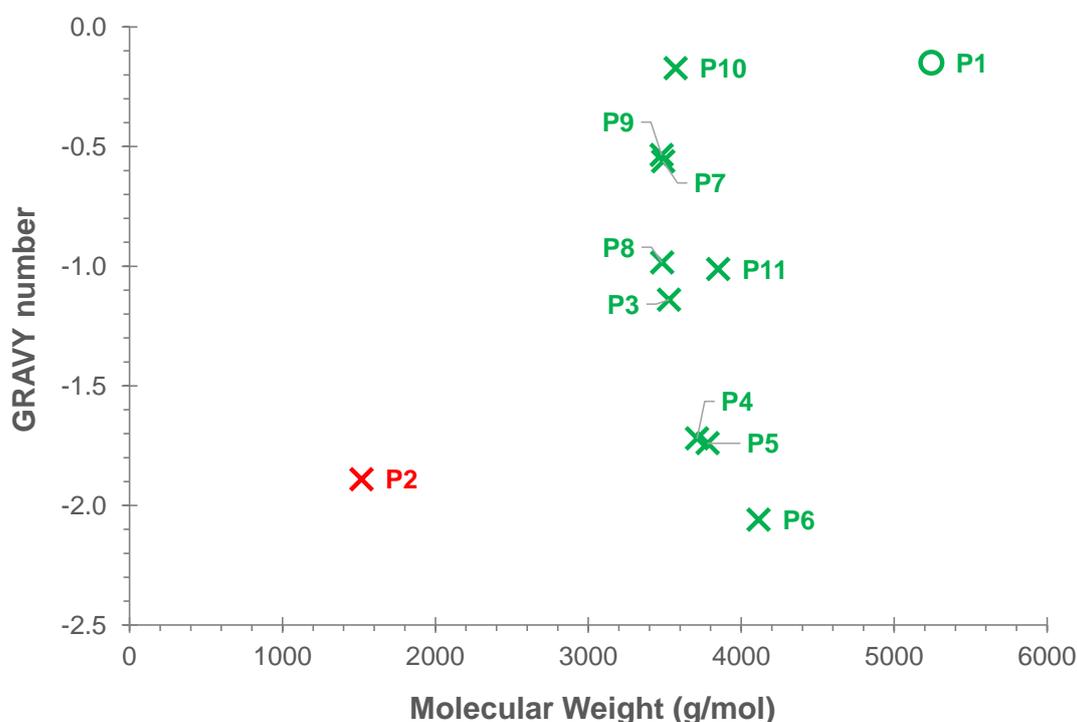


Figure 1. Description of the peptide set by physical properties. Crosses are linear peptides, the circle is a cyclic peptide (disulfide bridges). The red cross is an acidic peptide while all others are basic peptides.

3.2 Development of UC method

Firstly, a previous method that had been developed for short-chain bioactive peptides [19,20] based on a polar stationary phase with pentahydroxyl ligand (Ascentis Express OH5) and a methanol co-solvent comprising 20 mM NH₄OH and 2% H₂O was assessed. However, with this method the bigger peptides examined here were not

eluted from the column. Then several other stationary phases offering a range of retention properties [25] were tested (Table S2), with different mobile phase compositions comprising either NH_4OH , TFA or MSA. While the acidic peptide (P2) could be eluted with good peak shape in different chromatographic systems, the basic peptides set more difficulties. Peptides were only eluted with three columns: Torus DEA, Torus 2-PIC and Viridis BEH 2-EP, which are all polar stationary phases that are appropriate to analyze basic compounds [25]. However, the peak shapes were not always satisfying, but Torus 2-PIC provided the most promising results. After some optimization steps, the best method was an adaptation from Losacco *et al.* [21] using Torus 2-PIC column, with a co-solvent composed of methanol comprising 5% H_2O (v/v) and 0.1% MSA. However, as the elution gradient ranged from 50 to 90% co-solvent, the present method is perhaps more relevant of EFLC, while the method developed by Losacco *et al.* was relevant of UC. Sample chromatograms are presented in Figure 2.

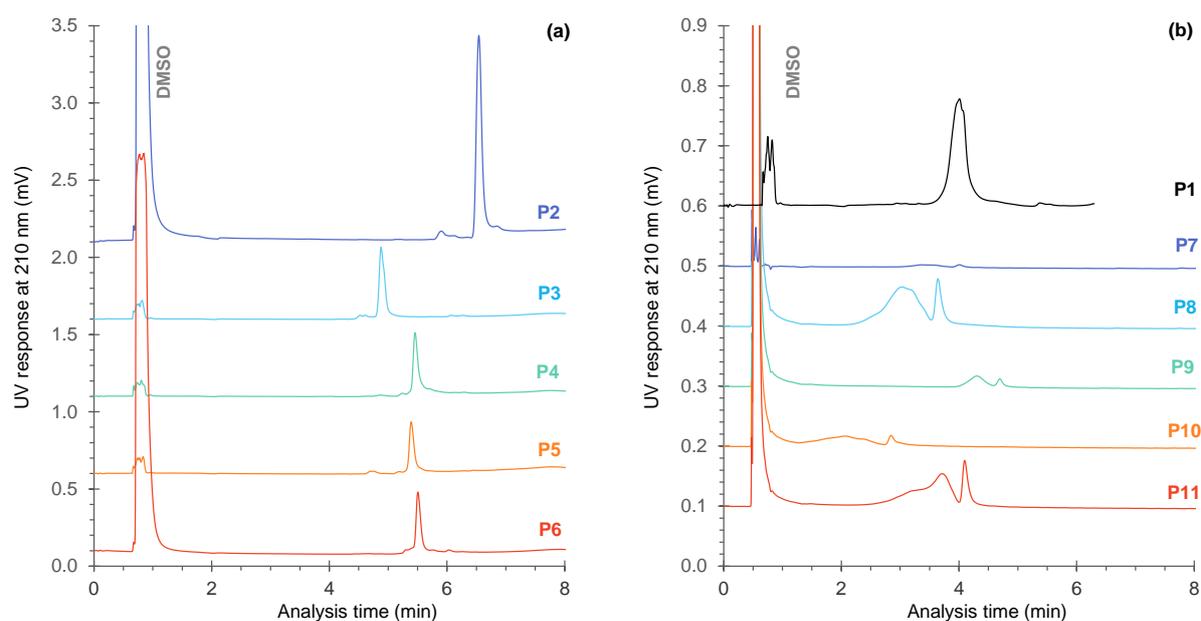


Figure 2. EFLC analysis of acidic (P2) and basic peptides TFA- salts (P1, P3-P11) on Torus 2-PIC with a co-solvent of methanol comprising 5% H_2O (v/v) and 0.1% MSA. Gradient elution from 50% to 90% co-solvent, with reversed flow-rate gradient from 0.7 to 0.5 ml/min. (a) Acceptable chromatographic peak shapes (b) distorted chromatographic peaks. Oven temperature 25°C, back-pressure 120 bar.

However, some problems appeared at this stage as some peptides had satisfying peak shapes, allowing for the resolution of several impurities (P2 to P6, in Figure 2a), while other peptides showed strongly distorted peak shapes (P7 to P11, in Figure 2b).

Note that both UV and MS traces were examined in all cases to ensure that the deformed peaks corresponded to the target peak and were not showing the resolution from a major impurity. In all cases in Figure 2b, the same UV and MS spectra were observed along the double and distorted peaks, indicating that the main peptide was indeed eluted with these strange distortions. It is important to note that in these initial experiments the injection volume was rather large (5 μL). The injection solvent was not always the same, because the solubility of the peptide salts was not always sufficient in MeOH, thus some of them were dissolved with MeOH-DMSO.

At this stage, some hypotheses could be proposed to explain the peak distortions.

First, the large plug of MeOH or MeOH-DMSO introduced in the initial mobile phase composition might have caused a viscosity mismatch, possibly resulting in viscous fingering effect [28–30]. This is especially true to the samples that had been prepared in MeOH-DMSO mixtures, as the viscosity of DMSO is much larger than the viscosity of MeOH (2.24 cP vs. 0.59 cP) [31]. However, distortion was not always observed when DMSO was present, as can be observed with P2 and P6 (Figure 2a).

Secondly, there may also be a solvent strength mismatch [28]. Again, this should be particularly true to the samples that had been prepared in MeOH-DMSO mixtures, as the solvent strength of DMSO should be larger than that of any other solvent in this mobile phase. Partial elution of the analyte with the dilution solvent plug (breakthrough effect), occurring in cases of solvent strength mismatch could contribute to the distorted peak shapes. Chapel and co-workers [32] have described breakthrough effects for basic analytes, among which was a peptide (Leucine enkephalin) [33] in liquid chromatography experiments. They observed that the effect was related to (i) the nature of the analyte, as basic compounds were affected differently from acidic analytes; (ii) the composition of the dilution solvent and (iii) the volume of sample injected, with different volumes injected from 0.5 to 4.5 μL .

Thirdly, the injection of a large volume may cause peak deformation related to the adsorption of the co-solvent and injection solvent molecules on the stationary phase, as previously described by Rajendran [34] and by Fornstedt and co-workers [35–38].

Finally, the peptide salts may partly dissociate during the elution, thus a portion of the peptide would elute as a free cation, while another portion would elute as an ion pair,

resulting in deformed peaks. Assuming, which is probable, that a basic peptide with such high pI values would still be positively charged in the CO₂-solvent mobile phase, the peptide could be eluted as a salt with its counter-ion, or it could be dissociated from the counter-ion and eluted as a free ion, with the counter-ion eluting at a different time. Indeed, SFC elution of chemical drugs as hydrochloride salts was demonstrated by Geiser *et al.*[39], while separate elution of chemical drugs and their counter-ions in SFC was demonstrated by Foulon *et al.* and by Kostenko and Prokovskiy [40]. Depending on the dissociation equilibrium, the effect would be more or less visible. The presence of a strong acid (methanesulfonic acid) in the mobile phase may also impact this dissociation equilibrium.

Further experiments were required to understand the variations of peak shapes observed.

3.3 Comparison of the salt counter-ions

Most peptides in this set (9 out of 11) were available with two different counter-ions, either acetate or trifluoroacetate. The optimal method described above had been developed solely on TFA⁻ salts and was later applied to AcO⁻ salts. Figure 3 shows the analysis of two such pairs of peptides. A clear difference appears between the two salts, as the peptide peak intensity was significantly lower in the case of AcO⁻ salts than in the case of TFA⁻ salts. Our first hypothesis was that, again the dilution solvent was responsible for this difference as the TFA⁻ salts were diluted in MeOH while the AcO⁻ salts were diluted with MeOH-DMSO. Because DMSO has a higher eluting strength than MeOH, we suspected that a portion of the AcO⁻ salts had been eluted in the solvent peak, thus faced some breakthrough effect. In addition, if some dissociation occurred between the basic peptides and their counter-ions, the dissociation equilibrium should differ between the two salts, as AcO⁻ counter-ions should be more loosely attached than TFA⁻ counter-ions, especially as MSA was present in the mobile phase.

Following these observations and the above peak distortions observed for some peptides, further experiments were then conducted to explore the effects of the injection solvent and volume on the peptide chromatograms.

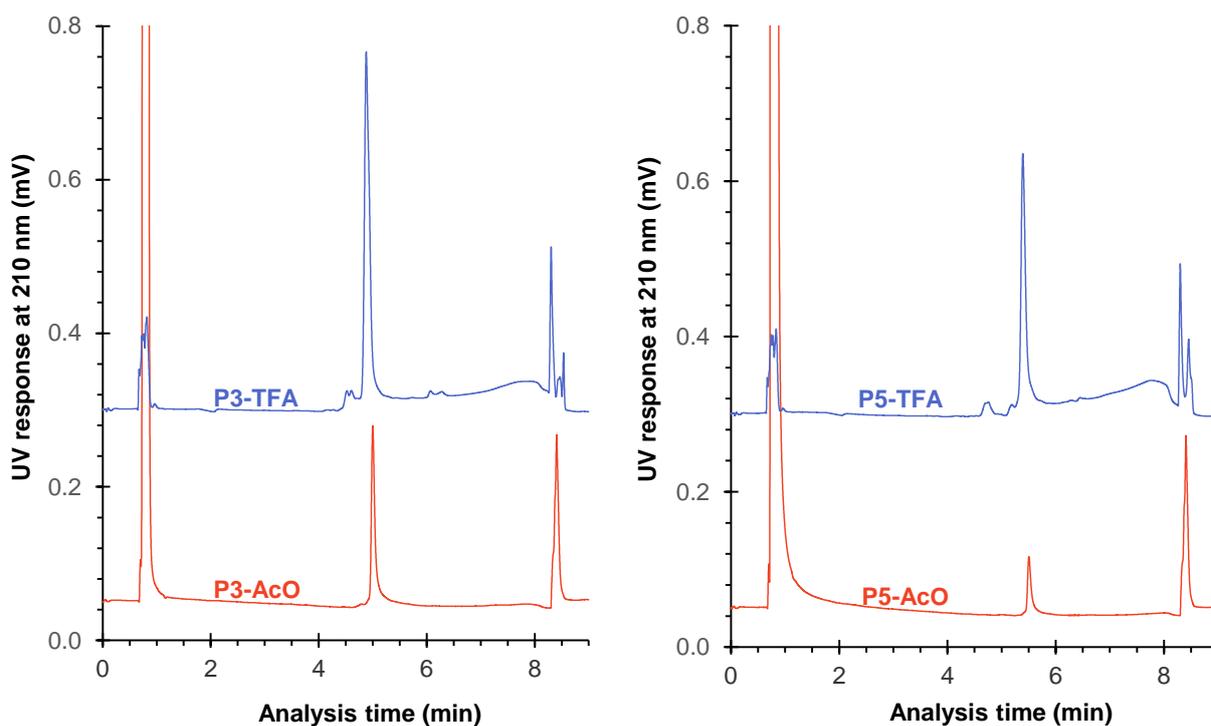


Figure 3. EFLC analysis of the same basic peptides (P3 and P5) with TFA- salts in blue or AcO- salts in red. Analysis on column Torus 2-PIC with a co-solvent of methanol comprising 5% H₂O (v/v) and 0.1% MSA. Gradient elution from 50% to 90% co-solvent, with reversed flow-rate gradient from 0.7 to 0.5 ml/min. Oven temperature 25°C, back-pressure 120 bar.

3.4 Solubilization solvent and injection volume

Due to low quantities of sample available, the effect of solubilization and injection volume was investigated on two peptides, one with TFA (P11-TFA) and one with acetate (P8-AcO). The two peptides had very close properties, both being linear basic peptides bearing the same biotin protection, with identical GRAVY numbers and close values of pI and molecular weight. As observed in Figure 2b, both peptides presented peak distortions under the optimal chromatographic conditions. Seven different dilution solvent compositions were tested, always using methanol as the main solvent. Our intention was to verify whether another solvent (DMSO, H₂O) or different acid additives introduced in the dilution solvent were responsible for different band profiles. Each preparation was injected 6 times with increasing injection volumes, varying from 0.5 to 5 μ L, to observe the potential variation of peak shapes. Each analysis was compared to a blank analysis injecting the same solvent composition and volume.

First, Figure 4 shows the variation of peak shapes when the injection volume increased. UV and MS detection chromatograms are both shown for the purpose of clarity. Only one example is shown for each of the two peptides, with the dilution

solvent made of the same mixture as the mobile phase co-solvent (methanol containing 5% H₂O and 0.1% MSA), but similar observations were made on all 7 solvent compositions for both peptides. A surprising effect was observed, as only the 1 μ L injection showed satisfying peak shapes. Below and above, the peaks were strongly distorted, the target molecules being eluted in very broad peaks for about 1 min. While viscous fingering may be suspected in the case of large volumes injected, the distortion observed with smaller injected volumes may be related to breakthrough or plug effect [29]. However, why the 1 μ L volume should (fortunately) show no deformation was quite mysterious.

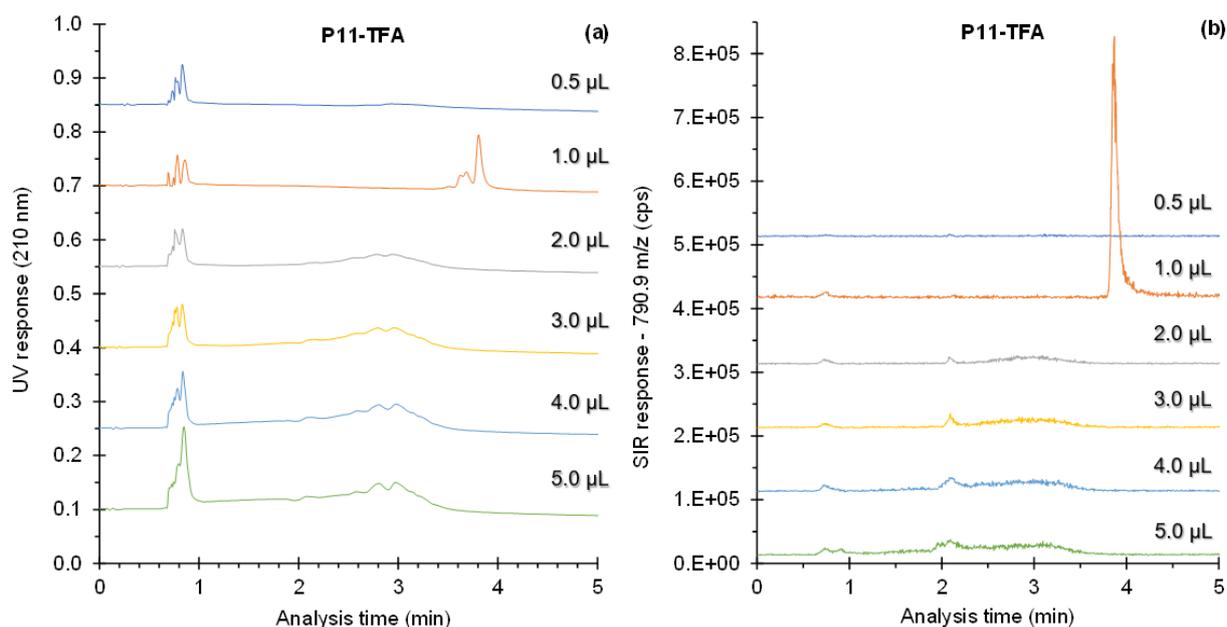


Figure 4. Impact of the injection volume (from 0.5 to 5 μ L) on UV (a, c) and SIR (b, d) chromatograms in EFLC analysis on the peptides (a, b) P11-TFA and (c, d) P8-AcO. Each peptide was solubilized with the same solvent as the mobile phase co-solvent (methanol comprising 5% water (v/v) and 0.1% MSA). Other conditions as Figure 2.

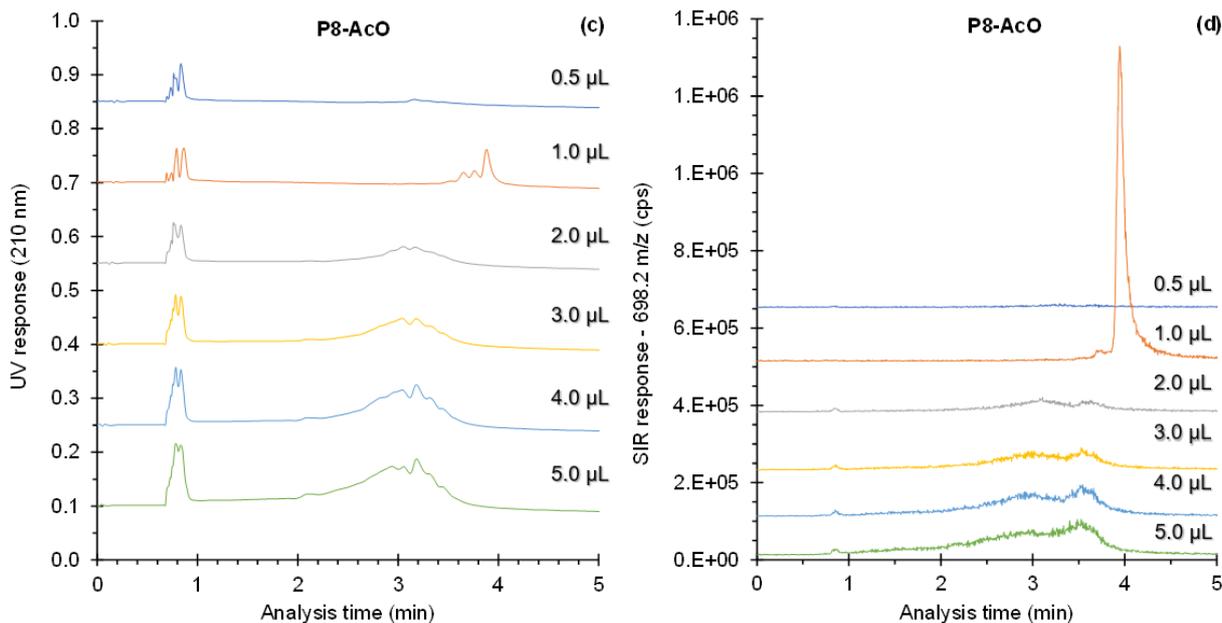


Figure 4. (continued). Impact of the injection volume (from 0.5 to 5 μL) on UV (a, c) and SIR (b, d) chromatograms in EFLC analysis on the peptides (a, b) P11-TFA and (c, d) P8-AcO. Each peptide was solubilized with the same solvent as the mobile phase co-solvent (methanol comprising 5% water (v/v) and 0.1% MSA). Other conditions as Figure 2.

We then resolved to keep a 1 μL injection volume and compared the different solvent compositions. The comparison is presented in Figure 5. As expected, for both peptides, the nature of the injection solvent is seen to impact peak shape, which is resulting in different resolution of the impurities, but is also impacting analysis time of the main peptide.

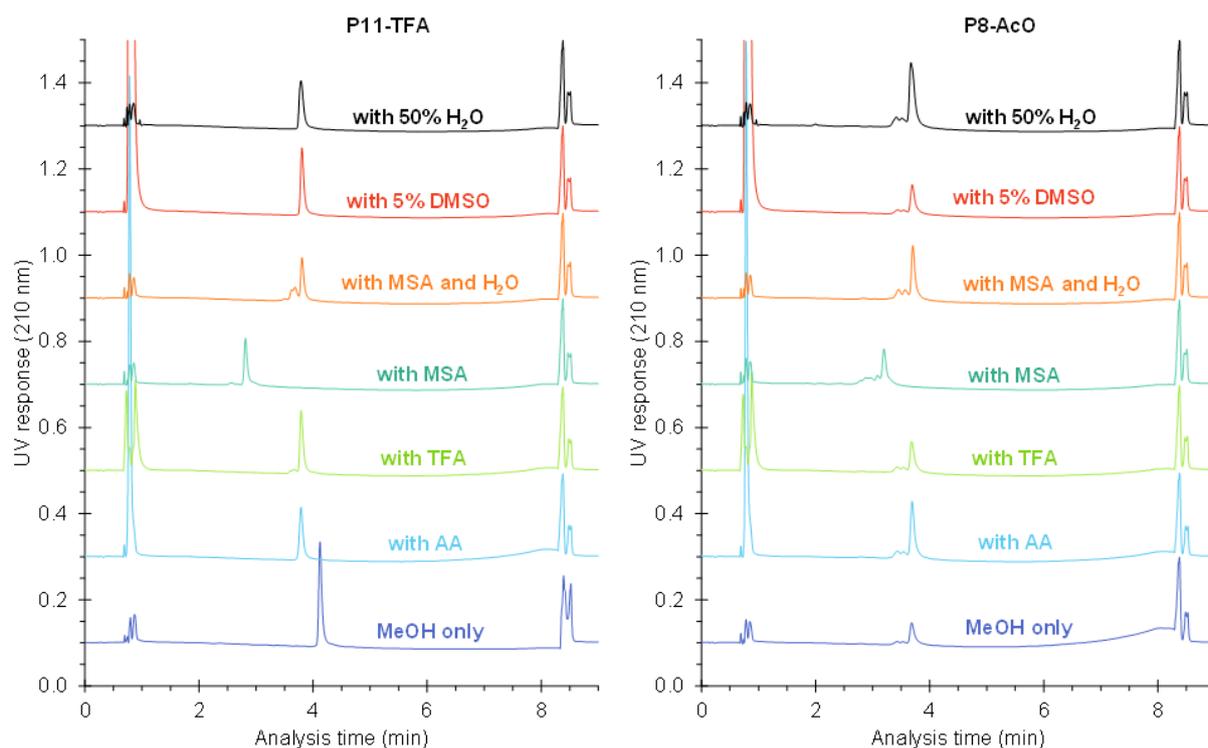


Figure 5. Impact of the dilution solvent composition on UV chromatograms in EFLC analysis on the basic peptide salts P11-TFA and P8-AcO. 1 μ L injected. Other conditions as Figure 2

The most significant effect on peptide retention is observed when comparing the composition containing MeOH, H₂O and MSA (orange trace, third chromatogram from the top in Figure 5) (with identical proportions to the mobile phase co-solvent) to the composition containing only MeOH and MSA (green trace, fourth chromatogram from the top in Figure 5): a significant decrease in retention time (about half a minute) was observed when water was not present in the dilution solvent. We may suppose that water is competing with the solute to adsorb on the stationary phase, acting as an additive rather than a modifier.

Regarding peak shape, some solvent compositions provided rather sharp peaks but failed to resolve the impurities from the main peak, e.g. pure MeOH for P11-TFA. Indeed, the acidic compositions (MeOH with MSA, MeOH with MSA and H₂O, MeOH with TFA) were the only ones where some impurities could be observed in peptide P11-TFA. In peptide P8-AcO, some impurities could be observed in all conditions.

Introducing an additive in the dilution solvent to obtain symmetrical peak shapes of basic compounds in SFC conditions was proposed by Cazenave-Gassiot [41], but it was later demonstrated that, when the dilution solvent contained different components from the mobile phase composition, repeatability of retention times and peak shapes

was not good [42], and it was necessary to inject about 25 samples before adequate repeatability could be obtained. This is due to the progressive adsorption of the components of the injection solvent onto the stationary phase. Thus, equilibration can only be reached when the stationary phase is fully covered with these components. From these observations, it can be concluded that it would be preferable to maintain identical chemicals between the mobile phase co-solvent and the dilution solvent. In the present case, MeOH containing H₂O and MSA would be the chosen composition. In addition, as mentioned above, good peak shapes and some resolution of impurities were observed on the two peptides tested. However, it appeared that some of the acetate salts were not soluble with this solvent. To date, a satisfying solution that could yield both full dissolution and good chromatograms (with good peak shapes and resolution of impurities) has not been found for these acetate salts.

4. Conclusions

The complexity of large basic peptides, considering their size, polarity, free charges or their existence as salts when associated to counter-ions is clearly setting much difficulties in understanding their retention mechanisms, particularly in pressurized CO₂-based mobile phases that cannot be so simply described as liquids. In the above, a reasonably good chromatographic method in EFLC mode was designed, although the appropriate dilution solvent that would yield both full dissolution and satisfying chromatographic quality for all peptide salts was not identified. The only acidic peptide examined caused no difficulty, only basic peptides were prone to peak deformation. Different solutions might finally be selected for TFA⁻ and AcO⁻ salts. This point requires further investigation.

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Supplementary information to:

Effect of dilution solvent and injection volume on the analysis of basic polypeptide salts with unified chromatography

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Table S1. Significant features of the peptides examined in this study.

Table S2. Stationary phases examined at the screening stage.

Table S1. Significant features of the peptides examined in this study.

Peptide name	Number of amino acids	MW (g/mol)	pI	GRAVY number	Free N-terminal	N-terminal acetylation	N-terminal biotin	Free C-terminal	C-terminal amidation	3D structure
P1	48	5245.2	8.9	-0.2	X			X		cyclic
P2	13	1515.5	3.5	-1.9	X				X	linear
P3	32	3527.9	12.0	-1.1	X				X	linear
P4	32	3711.0	11.2	-1.7	X				X	linear
P5	32	3779.1	11.2	-1.7	X				X	linear
P6	34	4113.4	9.7	-2.1		X			X	linear
P7	31	3488.8	9.5	-0.6			X	X		linear
P8	31	3481.8	9.8	-1.0			X	X		linear
P9	31	3478.8	9.5	-0.5			X	X		linear
P10	32	3570.9	9.5	-0.2			X	X		linear
P11	33	3849.0	9.3	-1.0			X	X		linear

Table S2. Stationary phases examined at the screening stage.

Column name	Manufacturer	Type of particles	Ligand	Dimensions (mm)	Particle size (µm)
Accucore HILIC	Thermo	Superficially porous silica	-	150 x 4.6	2.6
Accucore PFP	Thermo	Superficially porous silica	Pentafluorophenyl	150 x 4.6	2.6
Ascentis Express OH5	Supelco	Superficially porous silica	Pentahydroxyl	150 x 4.6	2.7
Kinetex XB-C18	Phenomenex	Superficially porous silica	Octadecyl	150 x 4.6	2.6
Cosmosil 3HP	Nacalai Tesque	Fully porous silica	3-Hydroxyphenyl	150 x 4.6	3.0
Torus 2-PIC	Waters	Fully porous silica	2-Picolylamine	100 x 3.0	1.7
Viridis BEH 2-EP	Waters	Fully porous silica	2-Ethylpyridine	100 x 3.0	1.7
HSS C18 SB	Waters	Fully porous silica	Octadecyl	100 x 3.0	1.8
CSH Fluoro-Phenyl	Waters	Fully porous silica	Pentafluorophenyl	100 x 3.0	1.7
Torus DEA	Waters	Fully porous silica	Diethylamine	100 x 3.0	1.7
Hypersil Gold Cyano	Thermo	Fully porous silica	Cyanopropyl	100 x 3.0	1.9

IV- Conclusion

L'analyse de peptides pharmaceutiques par UC-MS est un sujet considérable à étudier. Nous avons divisé ce chapitre en deux parties correspondant au lot des « petits » peptides et au lot des « gros » peptides.

Dans la première partie, nous avons revisité les analyses RPLC-MS de l'IdRS pour faire un état des lieux de leur méthode. Cette méthode, qui s'applique à des peptides de diverses tailles, présente un taux de satisfaction de 85%. Les peptides qui n'ont pas apporté satisfaction, après analyse selon le protocole mis en place, sont le résultat de co-élutions avec leurs impuretés.

Par la suite, une première approche par UC-MS s'est focalisée sur des peptides à courtes chaînes. Un ensemble de 10 « petits » peptides linéaires ou cycliques fut utilisé pour développer notre méthode UC-MS. L'application de fonctions de Derringer pour sélectionner l'association PS/PM a réduit le nombre d'expériences à mener. La colonne Ascentis Express OH5 associée à un co-solvant composé de méthanol dans lequel est ajoutée une solution d'hydroxyde d'ammonium à 20 mM compose notre système PS/PM. La méthode a été optimisée sur d'autres paramètres analytiques pour permettre l'analyse rapide des peptides en dégageant le maximum d'impuretés tout en obtenant une résolution du peptide principal la plus satisfaisante possible ($R_s > 1,5$ par rapport aux impuretés). C'est à partir de cette méthode UC-MS optimisée que le lot complet des 76 peptides fut analysé. Un regard particulier a été posé sur la séparation d'isomères qu'ils soient linéaires ou cycliques. Enfin, nous avons établi une corrélation entre la polarité des peptides et leur temps de rétention. Cependant, pour que cette corrélation soit viable, il était nécessaire d'indiquer la présence ou non de sel de TFA accompagnant le peptide, suggérant que les peptides basiques étaient élués sous forme de sel.

Pour établir les performances de la méthode UC-MS, nous avons défini des critères de mesure de la « satisfaction » analytique. Afin de comparer les performances de la méthode UC-MS avec celles par RPLC-MS, 43 « petits » peptides ont été analysés en RPLC-MS selon le protocole interne. L'orthogonalité des deux méthodes a été étudiée par la mesure des compositions à l'élution de chacun des peptides. Sur la question de la pureté, 12 peptides dont l'analyse était « satisfaisante » ont permis la comparaison entre les deux techniques. Cette même sélection de 12 peptides nous a servi pour

confronter le nombre d'impuretés détecté après analyse UC ou RPLC. Comme lors d'une précédente étude menée sur des petites molécules synthétiques, plus d'impuretés sont détectées en RPLC car la technique est plus sensible [178]. Enfin, sur ces « petits » peptides, deux exemples de séparations d'isomères présentent dans un cas, une meilleure résolution en UC et dans l'autre, une meilleure résolution en RPLC.

Dans la seconde partie, les recherches UC-MS se sont concentrées sur un nouveau lot de gros peptides. Une nouvelle méthode, relevant plutôt de la chromatographie liquide à fluidité améliorée (EFLC) a dû être mise au point car la méthode préalablement développée pour les petits peptides ne permettait pas de les éluer. Cette méthode a néanmoins révélé des difficultés : formes de pics dégradées ou diminution de l'intensité du signal de façon difficilement compréhensible. L'étude de la nature du solvant de mise en solution ainsi que du volume injecté ont montré qu'un volume d'injection de 1 μ L devait être privilégié. Cependant, les essais menés sur la nature du solvant de solubilisation n'ont pas résolu le problème dans le cas des peptides basiques associés à des contre-ions acétate. Cette question devra encore être approfondie pour finaliser cette méthode et proposer une solution générique fonctionnant pour tous les gros peptides, qu'ils soient acides ou basiques et quel que soit le contre-ion associé.

Conclusion générale et perspectives

I. Conclusion

Au sein de ce manuscrit, nous souhaitons discuter de la mise en place de nouvelles méthodes chromatographiques s'appuyant sur le CO₂ pressurisé pour analyser de petites biomolécules. La chromatographie unifiée (UC) nous a permis de travailler sur deux familles moléculaires à polarité très diverses que sont les flavonoïdes et les peptides. Au fil des chapitres expérimentaux, le poids moléculaire et la gamme de polarité des composés étudiés par UC-MS se sont étendus, nous amenant à des adaptations.

Dans le premier chapitre, nous avons revisité les techniques chromatographiques s'appuyant sur le CO₂ sc dans la phase mobile. La SFC, l'EFLC et l'UC sont des techniques aux applications larges et pas encore totalement exploitées. L'UC est la technique la plus récemment employée grâce aux évolutions technologiques et c'est avec cette chromatographie que nous avons débuté nos recherches. Deux familles de biomolécules ont été décrites, les flavonoïdes et les peptides, avec l'objectif de les étudier par UC-MS.

Dans le second chapitre, nous avons décrit le développement d'une méthode UC-MS pour l'analyse de standards de flavonoïdes. Cette sélection de 9 flavonoïdes, des aglycones et des hétérosides, représente trois catégories de flavonoïdes, les flavanols, les flavonols et les flavanones. Ceux-ci sont considérés non ionisés dans les conditions de pH estimées. Après des essais sur plusieurs colonnes, les colonnes Torus DEA et Torus Diol sont celles qui ont apporté de la rétention, de la sélectivité et des formes de pics les plus acceptables. Plusieurs additifs acides ont été également testés tels que l'acide phosphorique, l'acide citrique, l'acide trifluoroacétique ou l'acide méthanesulfonique. Les deux premiers acides étant non compatibles avec une détection MS tandis que l'AMS nécessite une adaptation instrumentale pour l'utiliser en MS sur un appareil UPC² de Waters™. L'acide méthanesulfonique additionné au méthanol s'est avéré être l'additif le plus adapté pour les flavonoïdes sélectionnés en termes d'efficacité et de forme de pic. Cette composition de co-solvant fut testée sur les deux colonnes retenues. Finalement, la méthode UC-MS emploie une colonne Torus DEA associée à un co-solvant comprenant du méthanol et de l'acide méthanesulfonique à 0.1%. Pour éluer cet ensemble de standards à polarité (log P) variant de -1,7 à 2,5, un gradient de co-solvant allant de 20% à 100% fut nécessaire. La détection par MS fut optimisée par plan d'expérience focalisé sur la température de l'aiguille, la tension de

cône et la tension de capillaire. De plus, nous avons perfectionné la composition du solvant make-up. Le méthanol mélangé à une solution d'hydroxyde d'ammonium présentait les mesures de signal-sur bruit les plus importantes sur un ensemble d'ions. Cette méthode analytique a permis l'investigation de deux produits commercialisés. L'Hidrosmine qui est un actif pharmaceutique s'est avéré très impur après analyse UC-MS, avec des pics majoritaires correspondant à l'isomère de position et à une forme disubstituée. De son côté, l' α -glucosyle hespéridine qui est un actif cosmétique, s'est avéré plus propre avec la détection d'un seul pic minoritaire potentiellement identifié.

Dans le troisième chapitre, dédié aux peptides par UC-MS, nous avons tout d'abord effectué un état des lieux des analyses de peptides réalisées par RPLC-MS à l'IdRS. Ce travail, en amont de tout développement analytique, a permis d'établir une estimation du taux de succès analytique du protocole RPLC-MS appliqué à l'IdRS. Les insatisfactions analytiques ont été décortiquées et sont majoritairement dues à une résolution insuffisante entre les peptides et leurs impuretés.

A partir de 10 « petits » peptides (5 cycliques et 5 linéaires) couvrant le mieux possible l'ensemble des 76 peptides mis à disposition par l'IdRS, nous avons commencé à développer une méthode par UC-MS. Pour cela, 4 phases stationnaires et 3 compositions de phases mobiles ont été investiguées ensemble en raison des faibles quantités de peptides disponibles (100 μ l en solution à 10 mM dans le DMSO pour chaque peptide). Afin de sélectionner le meilleur compromis de ce système PS/PM, nous avons employé des fonctions de Derringer basées sur 7 critères analytiques (résolution, asymétrie, signal-sur-bruit, ...). La meilleure association, c'est-à-dire le meilleur score de Derringer, s'est avérée être la colonne Ascentis Express OH5 associée à un co-solvant composé de méthanol dans lequel est ajoutée une solution d'hydroxyde d'ammonium. Par la suite d'autres optimisations, telles que la température du four de la colonne, la contre-pression, le débit de PM et le gradient de co-solvant ont été étudiées. Un exemple, l'optimisation de la température de travail a donné des différences significatives lorsque l'on a augmenté celle-ci à 60°C. On a observé une réduction du temps avant l'apparition du phénomène de relargage de l'additif ainsi qu'une amélioration de la résolution des pics des peptides d'intérêts. La méthode UC-MS développée permet ensuite l'analyse du lot complet de 76 peptides. La corrélation entre le temps de rétention et la mesure de polarité dite TPSA semble appropriée lorsque l'on tient compte de la présence de sels de TFA accompagnant les peptides.

Cette méthode UC-MS fut comparée aux performances de la méthode RPLC-MS en place au sein de l'IdRS depuis 2016. Ce travail s'est effectué sur un set plus réduit de 43 « petits » peptides. Tout d'abord, l'orthogonalité des deux méthodes s'est avérée bien différente du fait de la nature des phases stationnaires (groupements hydroxyles polaires en UC et chaînes C18 apolaires en RPLC). Ensuite, le taux de « réussite » des deux méthodes a été comparé après une double analyse par gradient dit « générique » puis par gradient dit « focus ». On observe 93% de succès par UC-MS et 67% par RPLC-MS. En termes de pureté des peptides, la comparaison s'est focalisée sur 12 peptides chromatographiquement retenus et dans une zone de temps d'élution raisonnable au vu des méthodes. On retrouve sur cet ensemble de peptides des puretés tout à fait comparables selon les deux méthodes. Pour ce qui est des impuretés détectées, leur nombre s'avère plus important en RPLC qu'en UC pour celles de faibles concentrations. Une mesure de la robustesse de la méthode UC fut également établie par plan de Doehlert sur la contre-pression, la température du four et la proportion finale de co-solvant. Le facteur de rétention k était le paramètre suivi comme réponse du plan. Les variations de k acceptables correspondent à des amplitudes de 120 ± 2 bars de contre-pression, de 600 ± 5 °C dans le four colonne et 80 ± 2 % de proportion finale de co-solvant en fin de gradient.

Les fonctions de Derringer étant adaptables à la problématique, il sera possible de mettre en place une nouvelle fonction pour l'échelle préparative. Celle-ci prendra en compte la consommation de solvants pour éluer le peptide, elle prêter attention à la résolution entre le peptide et l'impureté, et encore d'autres paramètres à définir. La possibilité d'analyser deux peptides composés de la même séquence d'acide aminés mais différenciés par leur conformation, l'un sous forme linéaire et l'autre sous forme cyclique serait un indicateur de performance de la méthode intéressant à étudier. Nous nous sommes concentrés sur des tripeptides linéaires et des pentapeptides cycliques mais nous n'avons pas pu tester notre méthode UC-MS sur d'autres « petits » peptides avec un nombre d'acide aminé différents.

Finalement, nous avons poursuivi les investigations avec des peptides de plus hauts poids moléculaires en développant une nouvelle méthode EFLC-MS. La solubilisation de ces « gros » peptides n'étant pas complète pour l'ensemble de notre lot, nous devons poursuivre les investigations pour définir un ou des solvants de mise en solution adapté(s). Cette composition dépendra peut-être de la nature du contre-ion associé au

peptide. Une fois cette problématique résolue, nous pourrions analyser les composés avec la méthode EFLC-MS optimale pour comparer les performances avec la méthode RPLC-MS.

Pour conclure, l'analyse des deux familles de biomolécules par chromatographie unifiée a pointé le besoin d'utiliser des phases stationnaires polaires pour obtenir une rétention adéquate des analytes. Ces phases stationnaires combinées à un co-solvant polaire et en proportion importante ont permis l'élution des molécules les plus polaires. L'intérêt de la chromatographie unifiée, dans notre cadre de travail, se trouve dans sa capacité à analyser des familles de poids moléculaires et de polarité diverses avec une méthode unique.

II. Perspectives

D'autres projets ont été partiellement avancés au cours de ces travaux. Notre volonté de nous intéresser à d'autres familles de biomolécules, comme celles à bases nucléiques, nous a amené à les explorer également par UC-MS. Nous avons aussi travaillé sur la construction de modèles de prédiction de la rétention chromatographique focalisée sur des petites molécules synthétiques. Ces deux sujets sont présentés ci-dessous.

a. Composés à bases nucléiques

Les composés à bases nucléiques sont essentiels pour tous les organismes vivants. Les nucléobases sont les unités de construction des nucléosides avec l'ajout d'un ribose. Les nucléotides sont les briques élémentaires de l'ADN et de l'ARN. Nous nous sommes focalisés sur les bases canoniques que sont l'adénine (base purique) ainsi que la cytosine et l'uracile (bases pyrimidiques). Pour ce qui est des nucléosides, nous avons eu accès aux plus courants. Nous parlons des bases canoniques liées à un ribose mais aussi des désoxyribonucléosides pour lesquels la base est liée à un pentose. Nous avons travaillé sur un ensemble de standards dont les caractéristiques sont représentées sur la Figure 15, les structures sont disponibles en Annexe 2. Cet ensemble de composés présente une variation de polarité restreinte avec des log P variant de -3 à 0. Cependant, ils sont intéressants à étudier puisque ce sont des biomolécules polaires avec des faibles poids moléculaires (masses molaires inférieures aux « petits » peptides étudiés précédemment).

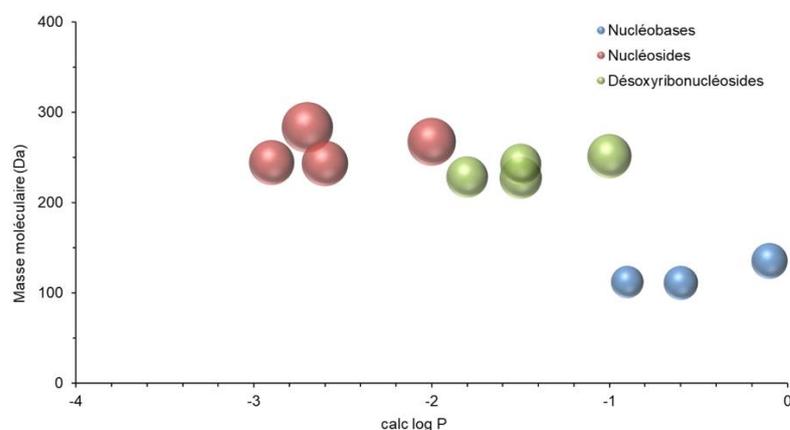


Figure 15 : Répartition des bases nucléiques étudiées selon le poids moléculaire et la polarité. La taille des bulles représente la TPSA ou surface topologique polaire

Nous les avons analysés à partir d'une solution mélange de ces 11 standards solubilisés dans le méthanol. L'échantillon de mélange a été injecté en UC-MS et l'on retrouve le chromatogramme sur la figure 16. On observe une bonne sélectivité entre les familles de bases nucléiques. Cependant, cette méthode n'est que partiellement développée et la détection masse n'a pas été approfondie pour l'instant.

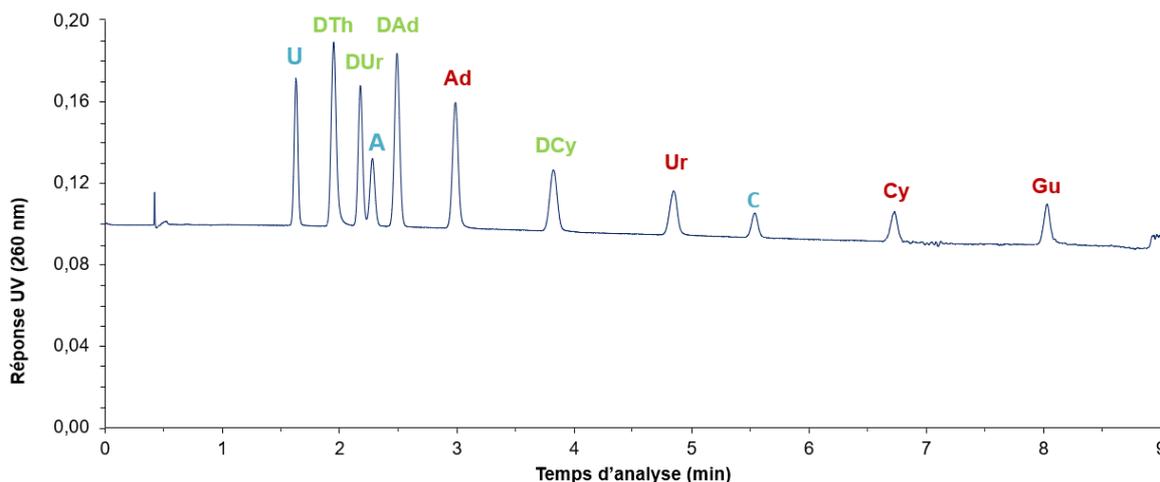


Figure 16 : Chromatogramme UV du mélange de 11 standards de bases nucléiques par UC.

Avec en bleu les nucléobases U pour uracile, A pour adénine et C pour cytosine. En vert les désoxyribonucléosides DTh pour désoxythymidine, DUr pour désoxyuridine, DAd pour désoxyadénosine et DCy pour désoxycytidine. En rouge les nucléosides Ad pour adénosine, Ur pour uridine, Cy pour cytidine et Gu pour Guanosine.

De plus, nous avons examiné deux paires de composés avec cette méthode UC-MS. La première concerne la séparation de l'uridine et la pseudouridine, deux isomères qui se différencient par la position de la liaison entre la base et le ribose. La pseudouridine ou Ψ est le nucléoside modifié, présent dans l'ARN, le plus abondant [179]. C'est un biomarqueur permettant notamment le diagnostic du développement du cancer de la prostate [180].

La seconde s'intéresse à la séparation de l'uracile et de la 5-fluorouracile ou 5-FU. Ces deux composés se différencient par l'insertion d'un fluor en position 5 de l'uracile. La 5-FU est une nucléobase modifiée préconisée dans le traitement des tumeurs ou du cancer colorectal [181]. A cause de certaines de ses propriétés physico-chimiques qui présentent des effets toxiques indésirables, l'utilisation de la 5-FU se fait sous forme de pro-médicament. Cela correspond à l'association de la molécule 5-FU avec un autre composé, par exemple un anticancéreux, pour améliorer l'activité biologique de la 5-FU

[182]. Sa forte activité biologique fait de la 5-FU un template idéal pour la recherche de nouveaux antiviraux par synthèse.

La figure 17 représente les deux chromatogrammes de ces deux paires de composés analysées avec la méthode UC-MS. La paire d'uraciles n'est pas parfaitement résolue ($R_s = 1,27$) au contraire de la paire d'uridines. Les uraciles présentent des log P et des TPSA calculés quasi-identiques expliquant la difficulté à les résoudre, au contraire des uridines qui sont calculés avec des log P similaires mais des TPSA plus dissemblables. Notons cependant que les deux uraciles ont des masses molaires distinctes et sont donc différenciés par la spectrométrie de masse, alors que les uridines sont isomères, ce qui implique la nécessité de leur résolution chromatographique. Les structures de la 5-FU et de la Ψ sont disponibles en Annexe 2.

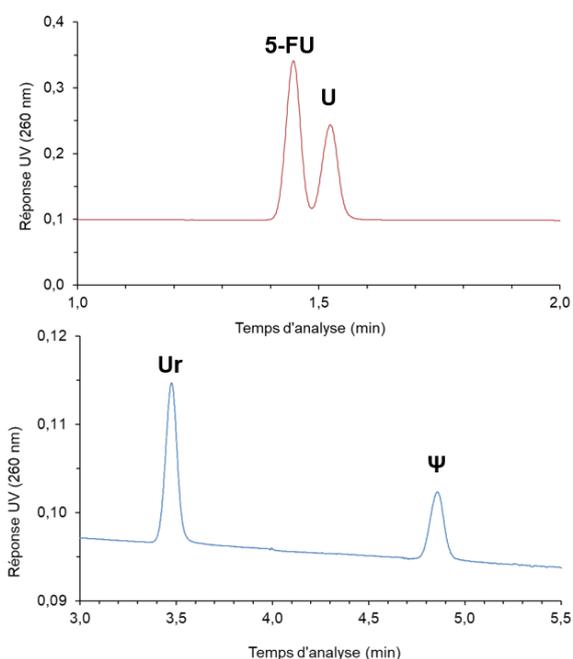


Figure 17 : Analyses par chromatographie unifiée de deux paires de bases nucléiques.

Chromatogrammes UV du mélange uracile (U), 5-fluorouracile (5-FU) en haut et du mélange uridine (Ur), pseudouridine (Ψ) en bas.

En termes de perspectives, une pré-validation de notre méthode nous permettrait de travailler sur des bases nucléiques présentes dans la composition de produits cosmétiques ou pharmaceutiques. Les produits cosmétiques emploient essentiellement l'adénosine à de faibles quantités, en général $< 1\%$, pour sa propriété anti-âge. Concernant les produits pharmaceutiques, les laboratoires Servier produisent notamment une formulation destinée au traitement du cancer (Lonsurf) contenant un

nucléoside (trifluridine) et une nucléobase modifiée (tipiracil), pour lesquels cette méthode pourrait avoir un intérêt.

L'analyse de nucléotides sera aussi un défi en UC si nous poursuivons le travail sur les molécules à bases nucléiques. Les nucléotides sont difficiles à éluer et/ou détecter avec une méthode UC-MS partant d'une portion majeure de CO_{2 sc} [213]. Losacco et al. proposent des hypothèses pour expliquer ces difficultés rencontrées (i) par une adsorption des composés phosphorylés sur les parois métalliques du système chromatographique, (ii) une précipitation des composés, liée à une incompatibilité avec la phase mobile lorsque celle-ci est majoritairement composée de CO_{2 sc} ou (iii) une non-élution dans leurs conditions UC-MS. En 2021, nous n'avons pas trouvé de publications décrivant l'analyse réussie de nucléotides et employant du CO_{2 sc} à l'exception de l'équipe de Mme Olesik en EFLC [183]. Le travail sur les composés à bases nucléiques pourra s'étendre aux oligonucléotides. Ces composés polaires nécessiteront peut-être une adaptation de la méthode analytique liée à la taille des oligonucléotides, comme rencontré dans le cas des peptides.

b. Modèles de prédiction de petites molécules synthétiques

Pour chaque famille discutée dans ce manuscrit, nous avons étudié la rétention chromatographique obtenue avec les quelques paramètres physicochimiques. Cela nous a conduit à travailler sur la mise en place d'un modèle prédictif. Cependant, avant de nous lancer sur des biomolécules qui sont complexes du fait de leurs structures dans l'espace, nous avons débuté par des petites molécules synthétiques. Ces 136 molécules furent préalablement analysées par Mme Lemasson, par SFC ou LC, lors de ses travaux de thèse [184].

Deux objectifs ont été déterminés derrière la construction de ce modèle. Le premier est de diriger l'analyste vers le système chromatographique le plus adapté à partir de la structure de la molécule. Le second objectif est d'apporter un soutien à l'identification d'impuretés lorsque celles-ci sont suggérées par le chimiste organicien (en fonction de la synthèse) et par l'analyste (en fonction des observations MS).

Pour cela, la construction du modèle s'est effectuée en deux étapes. Tout d'abord, nous devons déterminer les descripteurs moléculaires de nos composés de travail. Pour cela, nous avons modélisé chacune des molécules étudiées à l'aide du

logiciel MOE (version 2020) de Chemical Computing Group. A partir des SMILES, chacune des molécules est modélisée puis mise en condition selon le pH estimé de la phase mobile. Cette modélisation des charges s'appuie sur la connaissance du ou des pK_a et pK_b des composés. Une fois ce travail effectué, nous avons calculé la conformation d'énergie minimale de chacune des molécules. Pour cela, le logiciel simule la molécule entourée d'un solvant comme sur la Figure 18 et détermine un ensemble de configurations spatiales possibles. Celles de moindres énergies sont conservées et c'est à partir de cette structure avec cette conformation spatiale que nous avons calculé une multitude de descripteurs physico-chimiques.

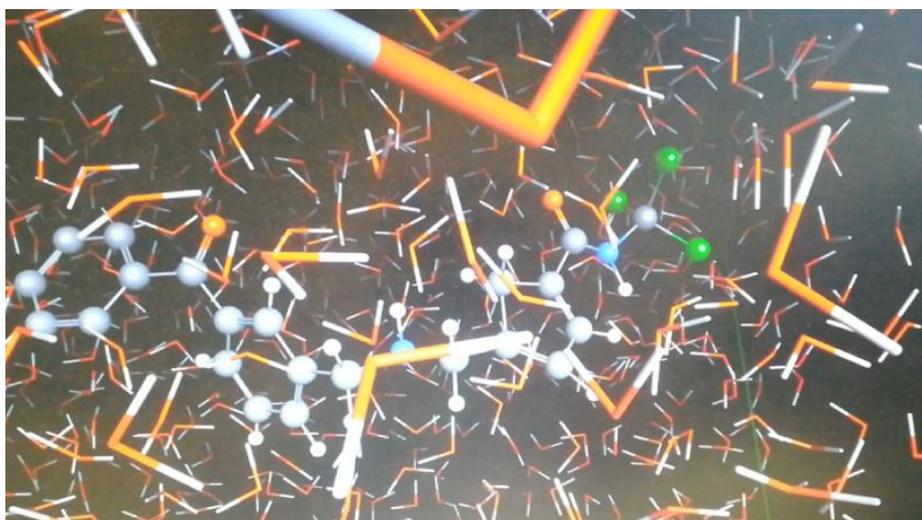


Figure 18 : Représentation d'une molécule synthétique dans un solvant (l'eau) simulé lors du calcul des conformations d'énergie minimale.

A ce moment, la seconde étape d'analyse des données démarre à partir de ces descripteurs calculés. Nous souhaitons obtenir un modèle prédictif de la rétention des molécules à partir de leurs structures calculées, on parlera dorénavant de modèle QSRR (Quantitative Structure-Retention Relationships). Dans le cadre de cet essai, nous avons défini notre mesure de la rétention à prédire par la composition à l'élution de la molécule (pourcentage de solvant organique dans la phase mobile au moment où la molécule est éluée). C'est cette composition à l'élution que nous tenterons de prédire le plus précisément possible.

L'outil Jupyter Notebook, une application web de programmation, a réalisé les étapes suivantes à l'aide du langage de programmation Python. Nous avons appelé le logiciel à réaliser un échantillonnage aléatoire des molécules étudiées, dans les proportions suivantes : 80% de lot d'apprentissage du modèle et 20% de lot de test du modèle. C'est

à partir de cette division des données, que la régression des moindres carrés partiels ou PLS-R a été appliquée. L'avantage du Jupyter Notebook est sa rapidité d'exécution puisque pour relancer une PLS-R en changeant le nombre de composantes principales, quelques secondes sont suffisantes. Les composantes principales sont les représentatives réduites et indépendantes des descripteurs. Sur la figure 19, les bâtons verts (clairs et foncés) représentent le nombre de molécules prédites avec succès, pour le lot de molécules étudié, dans une condition chromatographique. La satisfaction d'une prédiction correspond à une différence entre la valeur prédite et la valeur mesurée inférieure à 10 points d'écart. Pour cet exemple lorsque l'on applique 14 composantes principales, nous observons le meilleur taux de prédictions. Ici, 127 des 134 molécules présentent une prédiction satisfaisante.

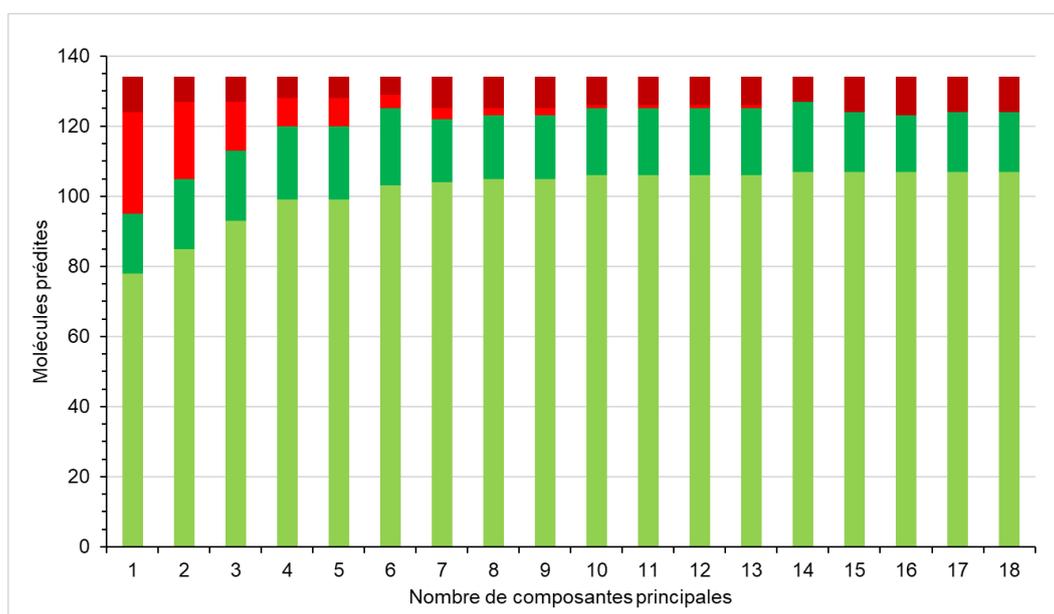


Figure 19 Qualité de la prédiction des molécules en fonction du nombre de composantes après calcul par PLS-R

En vert clair, les prédictions satisfaisantes du set d'apprentissage, en vert foncé, les prédictions satisfaisantes du set de test. En rouge, les prédictions non-satisfaisantes du set d'apprentissage, en rouge foncé, les prédictions non-satisfaisantes du set de test.

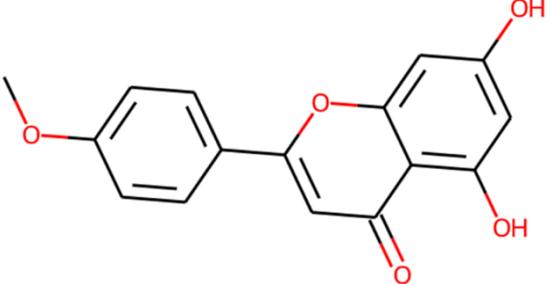
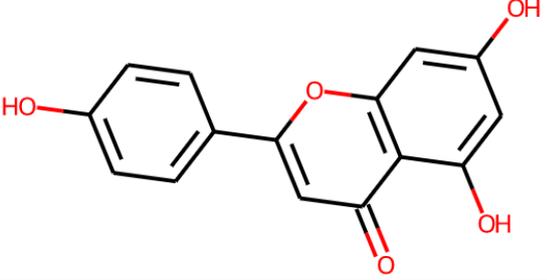
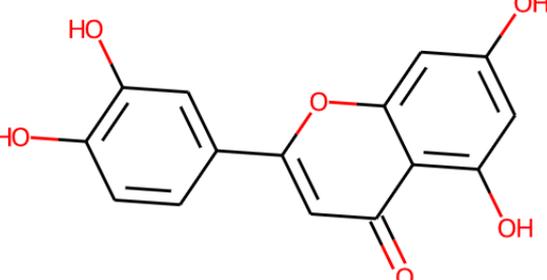
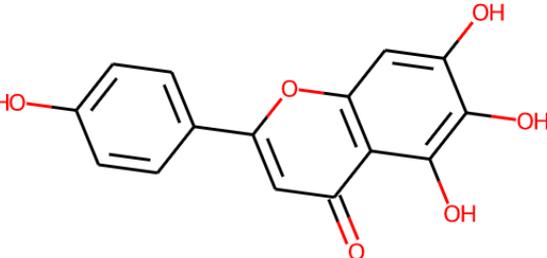
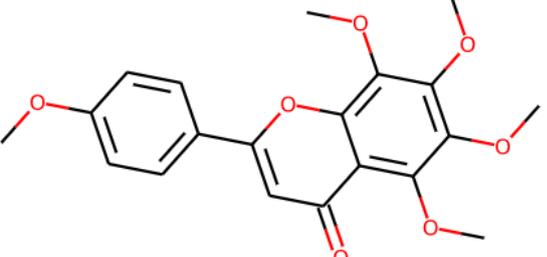
A travers ce court descriptif de notre modèle prédictif, nous souhaitons mettre en avant notre méthodologie. La capacité de calcul des postes informatiques de l'ICOA combinés aux connaissances des Jupyter Notebook permettent d'envisager la création d'un outil permettant de diriger dans un futur plus ou moins proche, un analyste vers la technique chromatographique la plus appropriée à partir de la structure de sa molécule. Un travail équivalent a été réalisé lors des travaux de thèses de Canault pour la mise en place d'une plateforme de prédiction in silico des propriétés ADME-Tox [185].

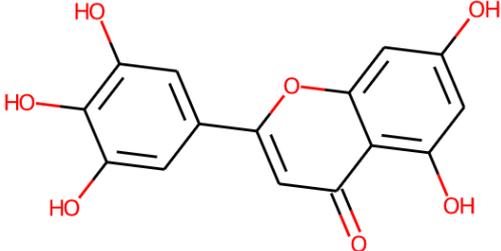
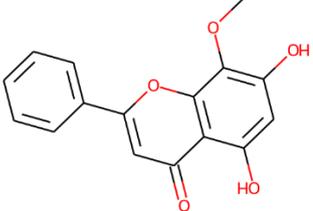
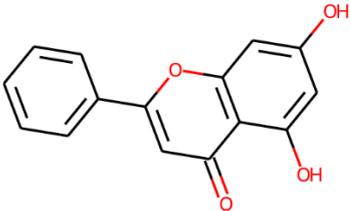
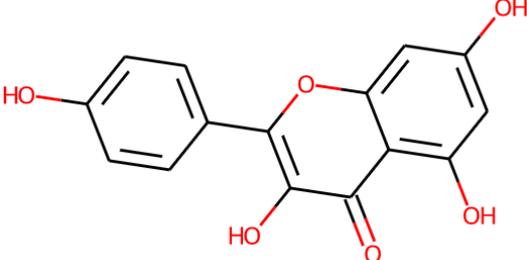
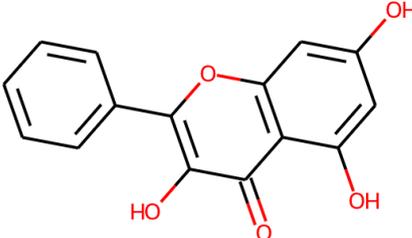
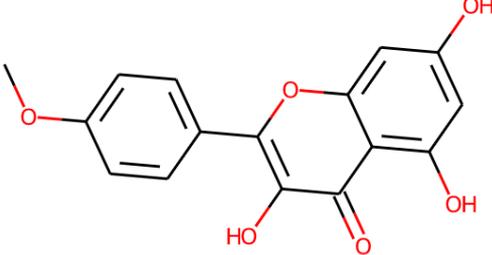
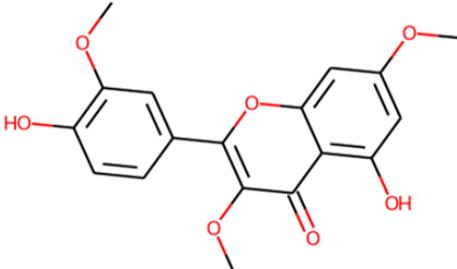
Ce travail devra être poursuivi, sur ce lot de molécules, dans d'autres conditions chromatographiques. Pour le cas d'analyse SFC, la simulation du solvant devra être testée avec du CO₂ pour déterminer la conformation d'énergie minimale des molécules. L'ensemble des modèles récoltés, nous définirons des critères de choix dans la sélection d'une méthode pour, qu'à partir d'une molécule, le logiciel prédise sa composition dans chacune des conditions et propose la ou les plus adaptées. Nous espérons poursuivre ces travaux prometteurs à la suite de ce projet de thèse.

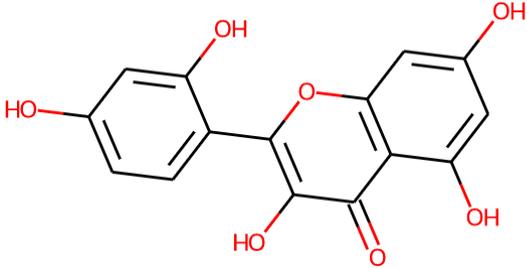
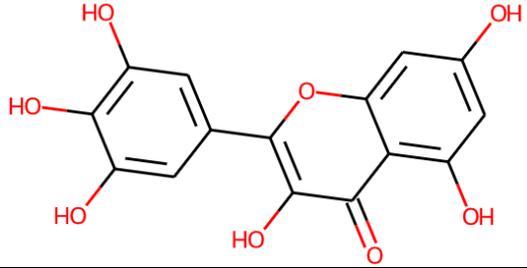
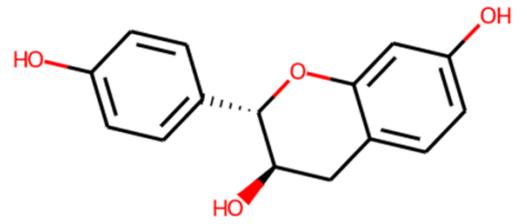
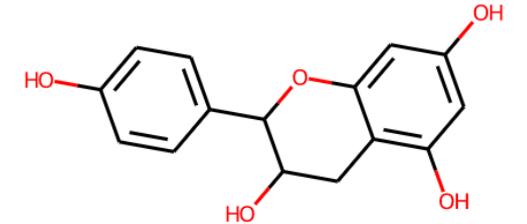
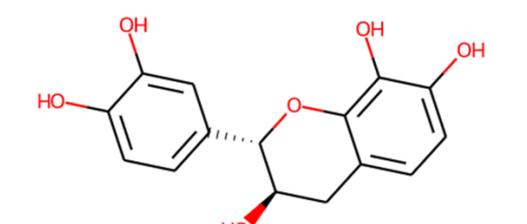
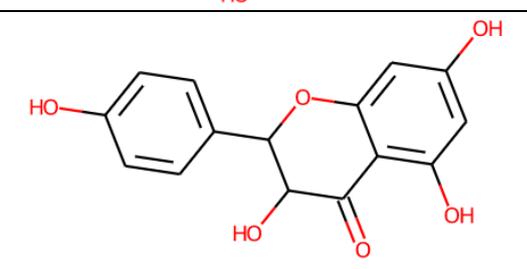
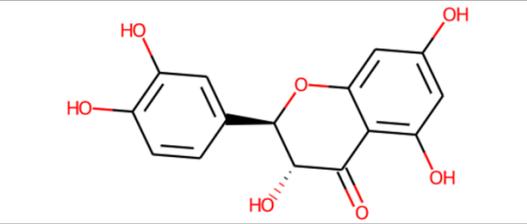
Annexes

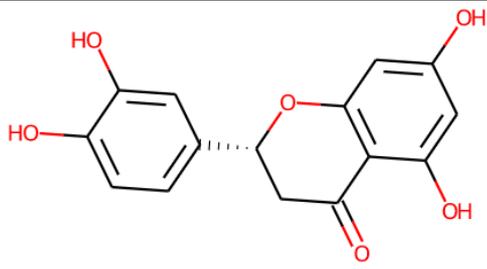
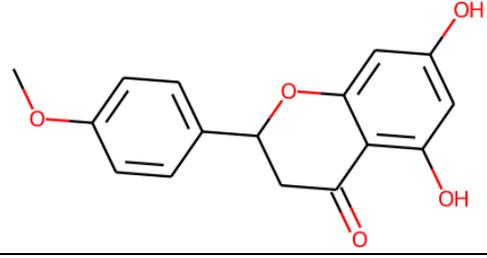
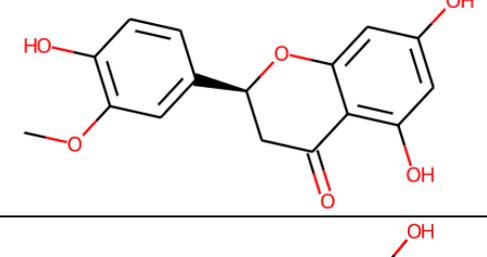
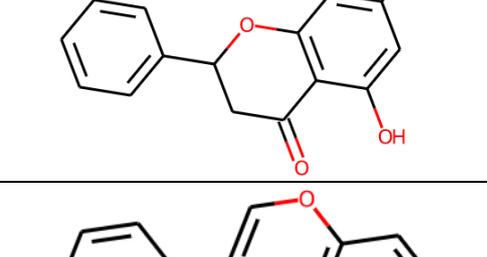
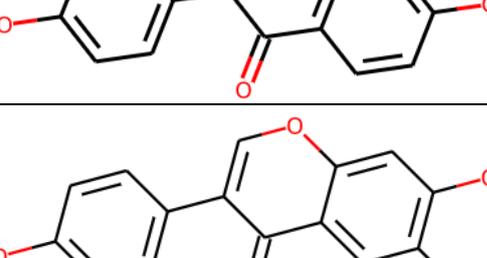
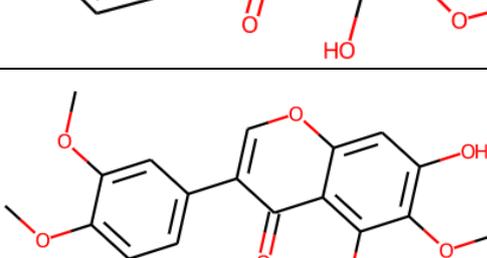
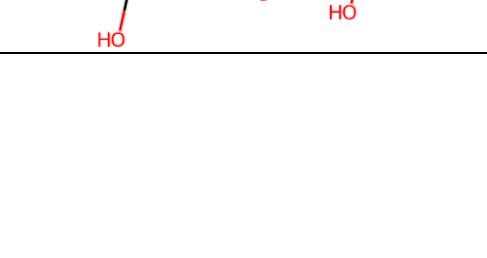
Annexe 1 : Structures des flavonoïdes (Chapitre 2)

Tableau 4 : Structure des flavonoïdes non glycosylés

Nom	Structure développée	Famille de flavonoïdes
Acacétine		Flavone
Apigénine		Flavone
Lutéoline		Flavone
Scutellaréine		Flavone
Tangéritine		Flavone

<i>Tricetin</i>		Flavone
<i>Wogonine</i>		Flavone
<i>Chrysin</i>		Flavone
<i>Galangine</i>		Flavonol
<i>Kaempférol</i>		Flavonol
<i>Kaempféride</i>		Flavonol
<i>Pachypodol</i>		Flavonol

<i>Morine</i>		Flavonol
<i>Myricétine</i>		Flavonol
<i>Guibourtinidol</i>		Flavanol
<i>Afzéléchine</i>		Flavanol
<i>Mesquitol</i>		Flavanol
<i>Aromadédrine</i>		Flavanonol
<i>Taxifoline</i>		Flavanonol

<i>Eriodictyol</i>		Flavanone
<i>Isosakuranétine</i>		Flavanone
<i>Homoériodictyol</i>		Flavanone
<i>Pinocembrine</i>		Flavanone
<i>Daidzein</i>		Isoflavone
<i>Tectorigénine</i>		Isoflavone
<i>Irigénine</i>		Isoflavone

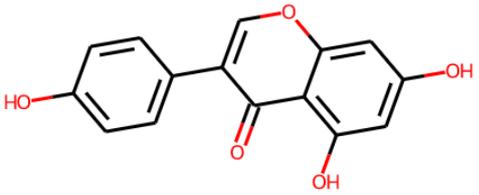
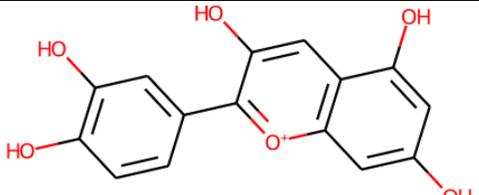
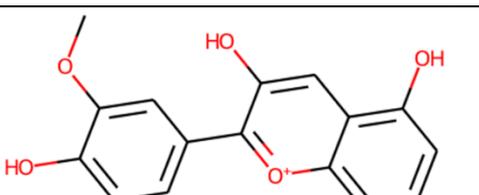
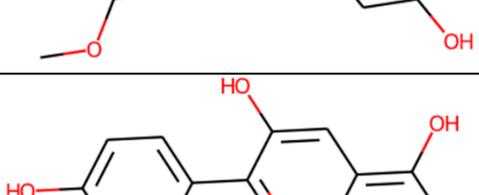
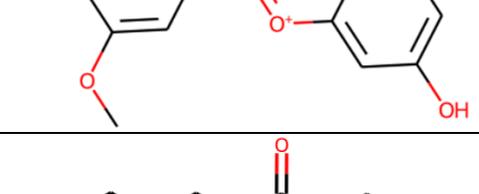
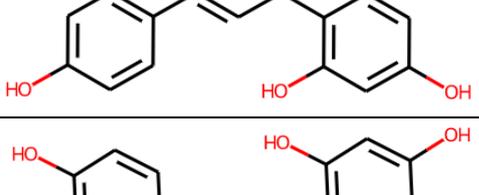
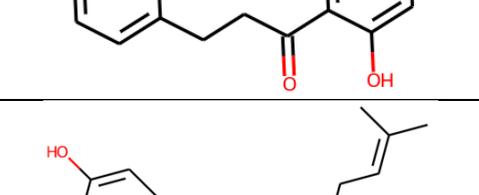
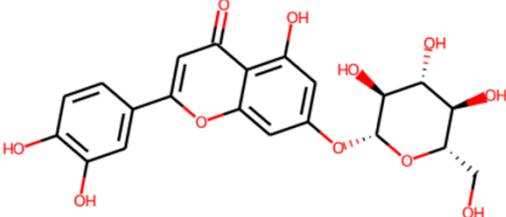
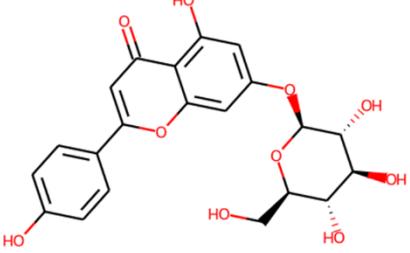
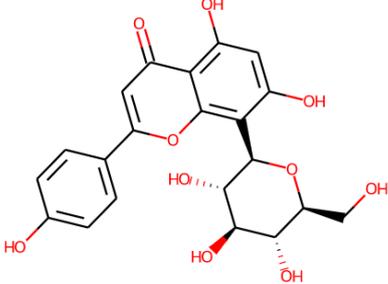
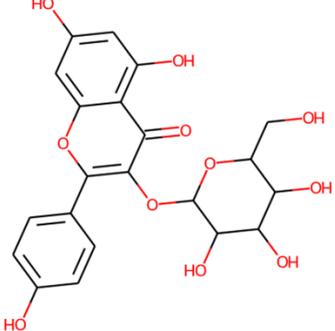
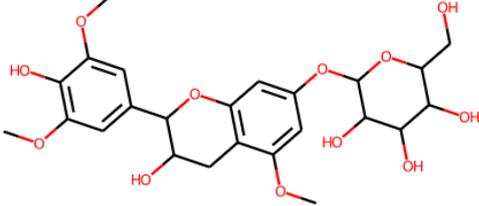
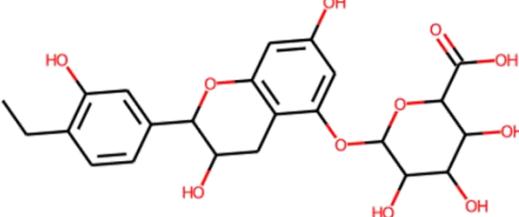
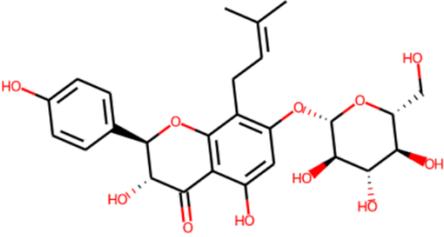
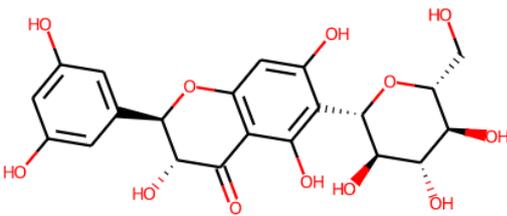
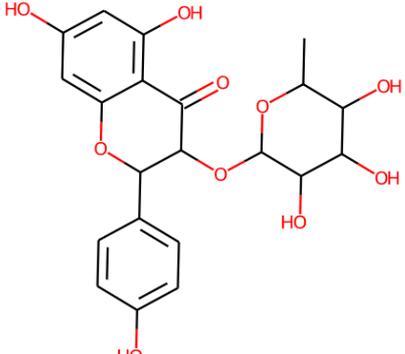
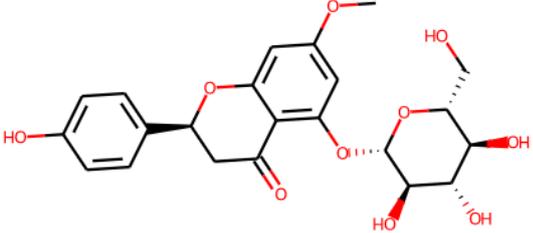
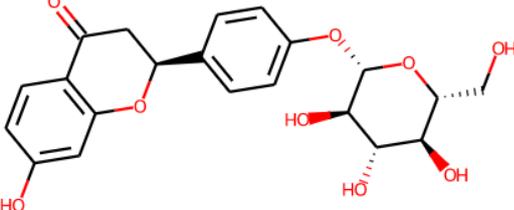
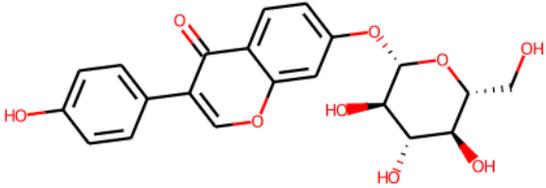
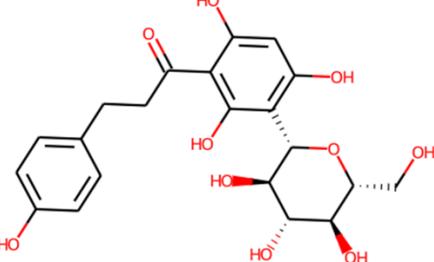
<i>Génistéine</i>		Isoflavone
<i>Cyanidine</i>		Anthocyanidol
<i>Malvidine</i>		Anthocyanidol
<i>Pétunidine</i>		Anthocyanidol
<i>Isoliquiritigénine</i>		Chalcone
<i>Phlorétine</i>		Chalcone
<i>Xanthohumol</i>		Chalcone

Tableau 5 : Structure des flavonoïdes monoglycosylés d'un monosaccharide

Nom	Structure développée	Famille de flavonoïdes
<i>Cynaroside</i>		Flavone
<i>Apigétrine</i>		Flavone
<i>Vitexine</i>		Flavone
<i>Astragaline</i>		Flavonol
<i>Glochiflavanoside B</i>		Flavanol
<i>4'-O-methyl-epicatechin-5-O-glucuronide</i>		Flavanol

<p><i>Phellamurin</i></p>		<p>Flavanonol</p>
<p><i>Xeractinol</i></p>		<p>Flavanonol</p>
<p><i>Engeletin</i></p>		<p>Flavanonol</p>
<p><i>Sakuranine</i></p>		<p>Flavanone</p>
<p><i>Liquiritoside</i></p>		<p>Flavanone</p>
<p><i>Daidzin</i></p>		<p>Isoflavone</p>
<p><i>Nothofagin</i></p>		<p>Chalcone</p>

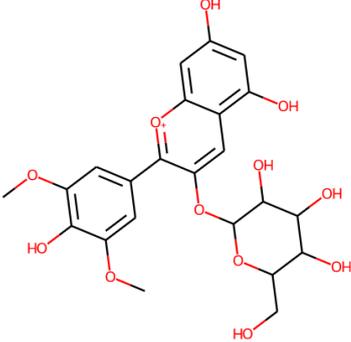
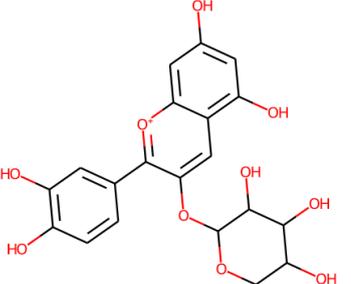
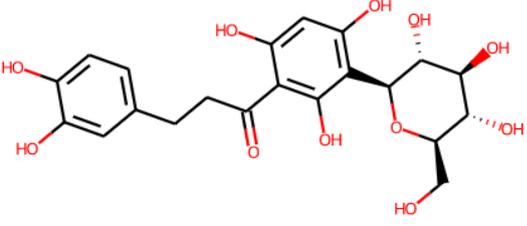
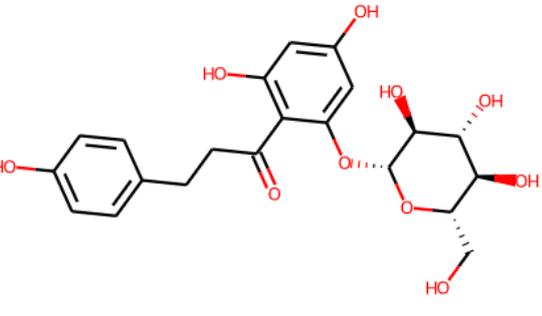
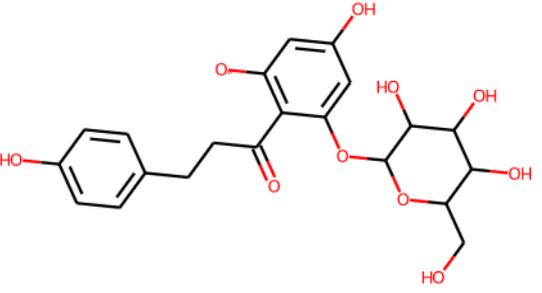
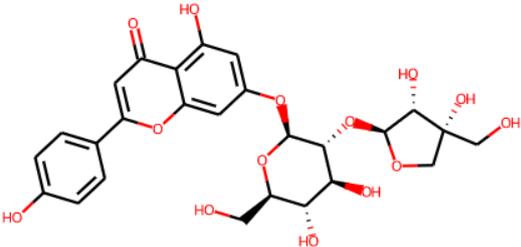
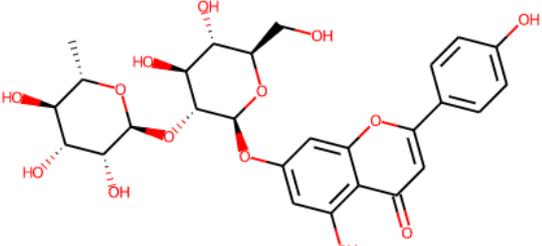
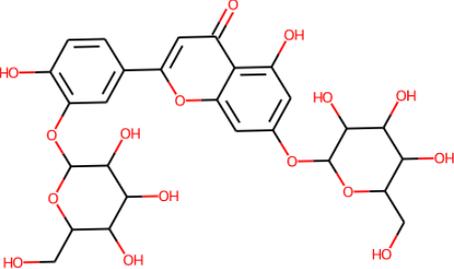
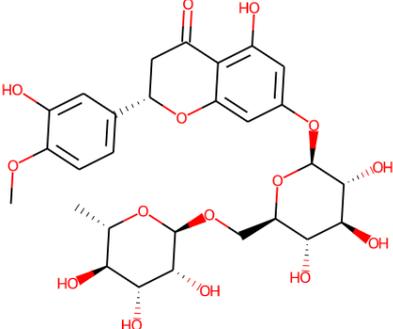
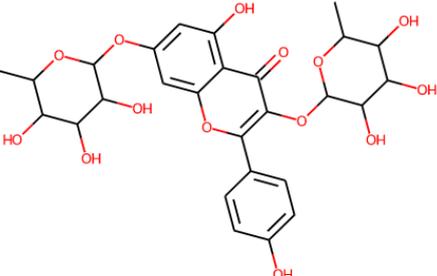
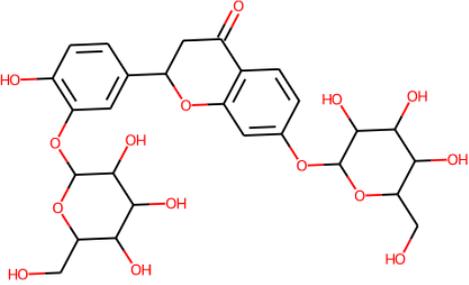
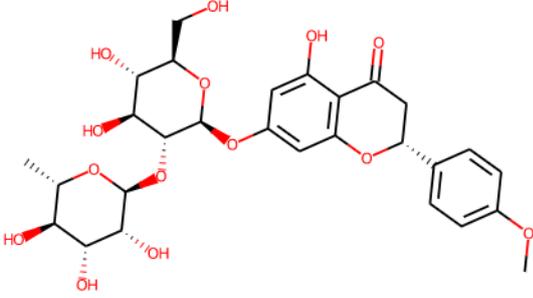
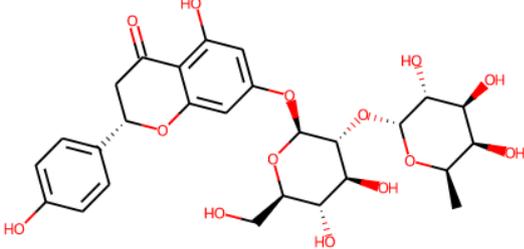
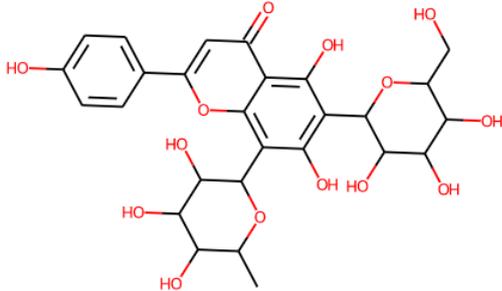
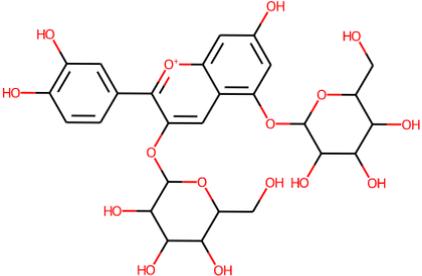
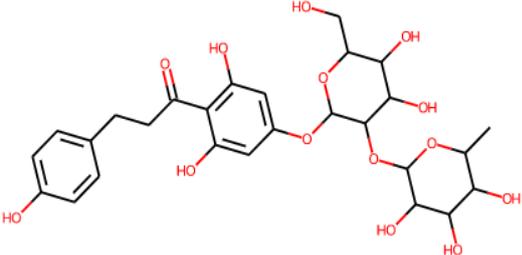
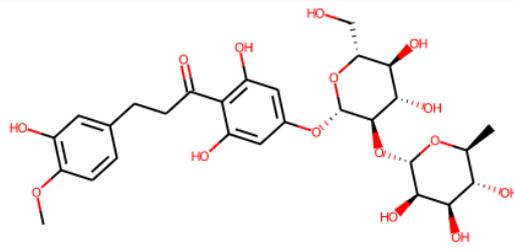
<p><i>Malvidine 3-glucoside</i></p>	 <p>The structure shows a malvidine aglycone (3,5,7-trimethoxyflavylium) linked at the C3 position to a glucose molecule. The glucose is in its cyclic pyranose form with hydroxyl groups at C2, C3, C4, and C6, and a hydroxymethyl group at C5.</p>	<p>Anthocyanidol</p>
<p><i>Cyanidine-3-arabinoside</i></p>	 <p>The structure shows a cyanidine aglycone (3,5-dihydroxyflavylium) linked at the C3 position to an arabinose molecule. The arabinose is in its cyclic furanose form with hydroxyl groups at C2, C3, and C4, and a hydroxymethyl group at C5.</p>	<p>Anthocyanidol</p>
<p><i>Aspalathine</i></p>	 <p>The structure shows a chalcone aglycone (3,5,7-trihydroxychalcone) linked at the C7 position to a glucose molecule. The glucose is in its cyclic pyranose form with hydroxyl groups at C2, C3, C4, and C6, and a hydroxymethyl group at C5.</p>	<p>Chalcone</p>
<p><i>Phloridzine</i></p>	 <p>The structure shows a chalcone aglycone (3,5,7-trihydroxychalcone) linked at the C7 position to a glucose molecule. The glucose is in its cyclic pyranose form with hydroxyl groups at C2, C3, C4, and C6, and a hydroxymethyl group at C5.</p>	<p>Chalcone</p>
<p><i>Phlorétine 2'-glucoside</i></p>	 <p>The structure shows a chalcone aglycone (3,5,7-trihydroxychalcone) linked at the C2' position to a glucose molecule. The glucose is in its cyclic pyranose form with hydroxyl groups at C2, C3, C4, and C6, and a hydroxymethyl group at C5.</p>	<p>Chalcone</p>

Tableau 6 : Structure des flavonoïdes monoglycosylés d'un disaccharide ou des disubstitués d'un monosaccharide

Nom	Structure développée	Famille de flavonoïdes
<i>Apiine</i>	 <p>The structure shows a flavone core (apigenin) with a hydroxyl group at the 7-position. This hydroxyl group is glycosylated with a beta-D-glucopyranose unit. The glucose unit is further substituted with a disaccharide chain consisting of a galactose unit and a glucose unit, both in their pyranose forms.</p>	Flavone
<i>Rhoifoline</i>	 <p>The structure shows a flavone core (rhegenin) with hydroxyl groups at the 5 and 7 positions. The 7-hydroxyl group is glycosylated with a beta-D-glucopyranose unit. The glucose unit is further substituted with a disaccharide chain consisting of a galactose unit and a glucose unit, both in their pyranose forms.</p>	Flavone
<i>Lutéoline-7,3'-diglucoside</i>	 <p>The structure shows a flavone core (luteolin) with hydroxyl groups at the 5, 7, and 3' positions. The 7-hydroxyl group is glycosylated with a beta-D-glucopyranose unit, and the 3'-hydroxyl group is also glycosylated with a beta-D-glucopyranose unit.</p>	Flavone
<i>Violanthin</i>	 <p>The structure shows a flavone core (violanthin) with hydroxyl groups at the 5, 7, and 3' positions. The 7-hydroxyl group is glycosylated with a beta-D-glucopyranose unit. The glucose unit is further substituted with a disaccharide chain consisting of a galactose unit and a glucose unit, both in their pyranose forms.</p>	Flavone
<i>Kaempféritrine</i>	 <p>The structure shows a flavone core (kaempferol) with hydroxyl groups at the 5, 7, and 3' positions. The 7-hydroxyl group is glycosylated with a beta-D-glucopyranose unit, and the 3'-hydroxyl group is also glycosylated with a beta-D-glucopyranose unit.</p>	Flavonol

<p><i>Butrine</i></p>	 <p>The structure of Butrine consists of a central flavanone core. It features a 4-hydroxyphenyl group at the 7-position of the A-ring and a 4-hydroxyphenyl group at the 2-position of the C-ring. Both phenyl rings are glycosylated with glucose units at their respective 4-positions.</p>	<p>Flavanone</p>
<p><i>Poncirine</i></p>	 <p>The structure of Poncirine features a flavanone core with a 4-methoxyphenyl group at the 7-position of the A-ring and a 4-hydroxyphenyl group at the 2-position of the C-ring. The 4-hydroxyphenyl group is glycosylated with a glucose unit at its 4-position, and the glucose unit is further substituted with a galactose unit at its 6-position.</p>	<p>Flavanone</p>
<p><i>Hesperidine</i></p>	 <p>The structure of Hesperidine consists of a flavanone core with a 4-hydroxyphenyl group at the 7-position of the A-ring and a 4-hydroxyphenyl group at the 2-position of the C-ring. The 4-hydroxyphenyl group at the 2-position is glycosylated with a glucose unit at its 4-position, which is further substituted with a galactose unit at its 6-position.</p>	<p>Flavanone</p>
<p><i>Naringine</i></p>	 <p>The structure of Naringine features a flavanone core with a 4-hydroxyphenyl group at the 7-position of the A-ring and a 4-hydroxyphenyl group at the 2-position of the C-ring. The 4-hydroxyphenyl group at the 2-position is glycosylated with a glucose unit at its 4-position, which is further substituted with a galactose unit at its 6-position.</p>	<p>Flavanone</p>
<p><i>Cyanidine 3,5-diglucoside</i></p>	 <p>The structure of Cyanidine 3,5-diglucoside consists of a cyanidin core with hydroxyl groups at the 3 and 5 positions of the A-ring. It is glycosylated with two glucose units at the 3 and 5 positions of the A-ring.</p>	<p>Anthocyanidine</p>
<p><i>Naringine dihydrochalcone</i></p>	 <p>The structure of Naringine dihydrochalcone features a dihydrochalcone core with a 4-hydroxyphenyl group at the 7-position of the A-ring and a 4-hydroxyphenyl group at the 2-position of the C-ring. The 4-hydroxyphenyl group at the 2-position is glycosylated with a glucose unit at its 4-position, which is further substituted with a galactose unit at its 6-position.</p>	<p>Chalcone</p>

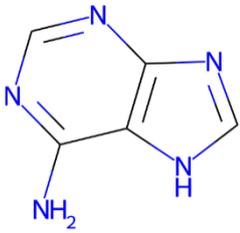
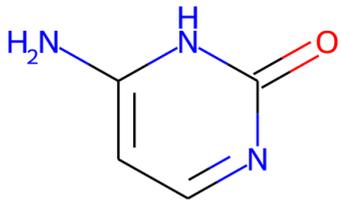
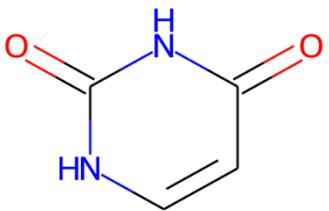
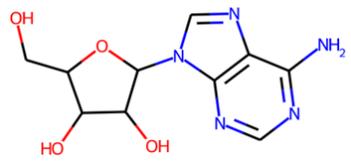
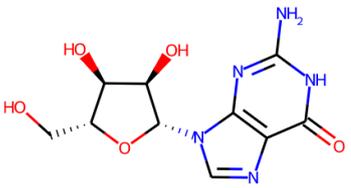
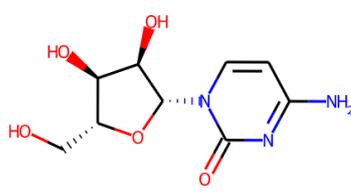
Néohespéridine
dihydrochalcone



Chalcone

Annexe 2 : Structures des bases nucléiques (Perspectives)

Tableau 7 : Structure des bases nucléiques analysées

Familles à bases nucléiques	Nom	Abréviation	Structure
Nucléobases	<i>Adénine</i>	A	
	<i>Cytosine</i>	C	
	<i>Uracile</i>	U	
Nucléosides	<i>Adénosine</i>	Ad	
	<i>Guanosine</i>	Gu	
	<i>Cytidine</i>	Cy	

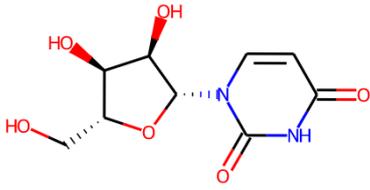
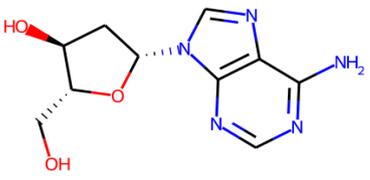
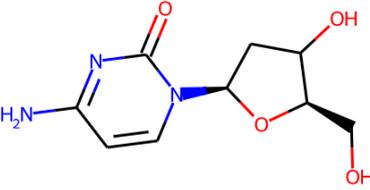
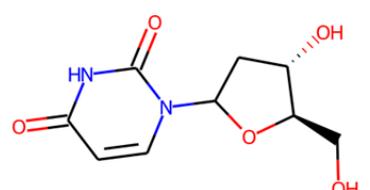
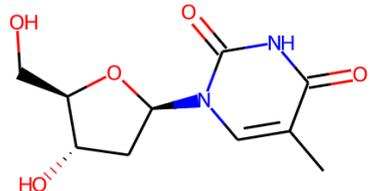
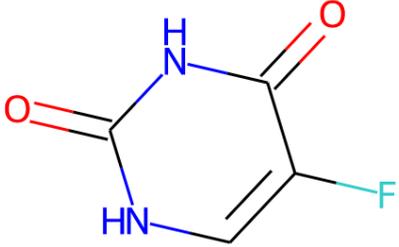
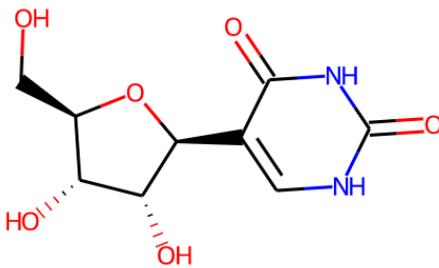
	<i>Uridine</i>	Ur	
Déoxyribonucléosides	<i>Déoxyadénosine</i>	DAd	
	<i>Déoxycytidine</i>	DCy	
	<i>Déoxyuridine</i>	DUr	
	<i>Déoxythymidine</i>	DTh	

Tableau 8 : Structure des bases nucléiques modifiées

Nom	Abréviation	Structure
<i>5-Fluorouracile</i>	5-FU	
<i>Pseudouridine</i>	ψ	

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Développement de nouvelles méthodologies chromatographiques pour l'analyse de petites (bio)molécules d'intérêt pharmaceutique

Résumé :

Le profilage d'impuretés d'actifs pharmaceutiques (identification et quantification de toutes les impuretés) est une nécessité à tous les stades de développement du médicament. Pour ce faire, les méthodes chromatographiques, en phase liquide (LC) ou supercritique (SFC), sont largement employées. Une autre méthode en récent essor, nommée chromatographie unifiée (UC), permet l'analyse de familles moléculaires s'étalant sur une large gamme de polarités. En effet, une telle analyse UC débute avec une majorité de CO₂ pressurisé (mode SFC) puis se termine avec une majorité de phase liquide, voire 100% de phase liquide (mode LC).

Dans le cadre de cette thèse en lien avec l'Institut de Recherches Servier (IdRS), nous souhaitons explorer l'utilisation de l'UC pour l'analyse des biomolécules d'intérêt pharmaceutique, produites chimiquement ou par des organismes biologiques. En particulier les molécules cibles étaient les flavonoïdes et les peptides de petite taille (inférieure à 5000 Da).

Pour les flavonoïdes, une méthode générique a d'abord été développée à l'aide de standards pour permettre ensuite l'investigation d'échantillons commerciaux d'intérêt cosmétique et pharmaceutique. Dans le cas des peptides, nous nous sommes appuyés sur une diversité de composés issus de l'IdRS afin d'adapter nos méthodes aux molécules propres à l'entreprise. Dans un premier temps, une méthode UC applicable aux peptides linéaires et cycliques de taille inférieure à 1000 Da a été développée et comparée à la méthode LC établie depuis quelques années. Dans un second temps, les peptides de taille supérieure à 1000 Da, posant d'autres défis, ont nécessité un second développement analytique en chromatographie liquide à fluidité améliorée (EFLC). Enfin, la possibilité de prédire le comportement chromatographique des actifs pharmaceutiques chimiques ou biologiques a également été abordée.

Mots clés : Biomolécules, Fluides supercritiques, UC, Développement de méthode

New chromatographic methods development for small (bio)molecules analysis with a pharmaceutical interest

Summary:

Impurity profiling of active pharmaceutical ingredients (identification and quantification of every impurity) is necessary at each step of the development of a drug. To achieve this purpose, chromatographic methods are largely used, mostly in liquid phase (LC) or supercritical fluid phase (SFC). Another method that has recently emerged, named unified chromatography (UC), allows the analysis of molecular families with wide polarities. Indeed, UC analyses start with a majority of pressurized CO₂ (SFC mode) and end with a majority of liquid phase (LC mode).

In the context of this thesis in partnership with Servier Research Institute (IdRS), we wished to explore the use of UC to analyze pharmaceutical biomolecules, which may be produced chemically or by living organisms. The target molecules were flavonoids and short-length peptides (molecular weight inferior to 5000 Da).

Regarding flavonoids, a generally applicable method was first developed based on a selection of standard molecules, to be later applied to commercial samples of cosmetic and pharmaceutical interest. Regarding peptides, we developed our methods based on a diversity of IdRS in-house compounds, to better adapt the UC method to the industry's requirements. Firstly, a UC method applicable to short-chain linear and cyclic peptides (molecular weight inferior to 1000 Da) was developed and compared to the previously-existing LC method. Secondly, longer-chain peptides (molecular weight superior to 1000 Da), setting other challenges, required the development of a second enhanced-fluidity liquid chromatography (EFLC) method.

Finally, the possibility to predict the chromatographic behavior of active pharmaceutical ingredients was also assessed.

Keywords: Biomolecules, Supercritical fluids, UC, Method development

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