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*ÉCOLE DOCTORALE SANTE, SCIENCES BIOLOGIQUES ET CHIMIE DU VIVANT
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Institut De Chimie Organique Et Analytique - ICOA UMR7311

THÈSE présentée par :

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Développement d'outils chromatographiques multidimensionnels en phase supercritique pour l'analyse de produits naturels

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Liste des communications scientifiques et formations suivies

Publications acceptées ajoutées au manuscrit

1. Development of a two-dimensional supercritical fluid chromatography system in multiple heart-cutting mode. Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West
Analytical Chemistry 2024, 96, 29, 11969-11976
2. Chiral and achiral analysis of chiral flavonoids with liquid and supercritical fluid chromatography – A review. Laurine Réset, Clément De Saint Jores, Caroline West
Journal of Chromatography Open 6 (2024) 100183.
3. Examining the conditions to achieve successful fraction transfer in two-dimensional supercritical fluid chromatography. Laurine Réset, Clément De Saint Jores, Caroline West
Journal of Chromatography A 2025, 1762, 466355.
4. Determination of the diastereomeric excesses of chiral flavonoids in organs of Citrus aurantium with two-dimensional supercritical fluid chromatography. Laurine Réset, Bibi Ousseni, Laetitia Fougère, Christophe Hano, Clément De Saint Jores, Caroline West
Food Chemistry 2025, 496, 146882.
5. Stereoisomeric separation of flavonoids by two-dimensional supercritical fluid chromatography: identification of adequate chiral columns and application to honey analysis. Laurine Réset, Bibi Ousseni, Mélodie Degrelle, Clément De Saint Jores, Caroline West
Chirality 2025, 37(11), e70058.

Publication acceptée non incluse dans le manuscrit

1. Cu/Pd Dual Catalyzed Stereoselective Construction of Vicinal Tri-and Tetrasubstituted Stereocenters Connected to Chiral α -Benzylnitriles. Mimoun Liliane, Sanaa Hamdi, Nicolas Cyril, Gerard Hélène, Vanthuyne Nicolas, Réset Laurine, West Caroline, Retailleau Pascal, Heppner Florian, Gillaizeau Isabelle
Advanced Synthesis & Catalysis 2024, 366 (11), 2495-2500.

Communications orales

1. Development of an SFC-SFC configuration for the analysis of chiral flavonoids from bitter orange (*Citrus aurantium* L.)
Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West
Ecole thématique AFSEP – Juin 2023 – Vichy, France
2. Development and challenges of an SFC-SFC configuration in multiple heart-cutting to analyze chiral flavonoids from bitter orange (*Citrus aurantium* L.).
Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West
HTC-18 – Mai 2024 – Louvain, Belgique
3. Flavanone diastereomeric excesses in bitter orange (*Citrus aurantium* L.) measured with SFC-SFC-MS in multiple heart-cutting mode.
Laurine Réset, Clément De Saint Jores, Caroline West
ISC 2024 – Octobre 2024 – Liverpool, Royaume-Uni
4. Development of a two-dimensional supercritical fluid chromatography system in multiple heart-cutting mode.
 Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West
SFC – Octobre 2024 – Princeton, New jersey, USA
5. Development and challenges of an SFC-SFC configuration in multiple heart-cutting to analyze chiral flavonoids from bitter orange (*Citrus aurantium* L.).
Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West
AFSEP club Île de France – Novembre 2024 – Paris, France
6. Development of a multiple heart-cut SFC-SFC setup.
 Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West
16th MDCW – 3-5 février 2025 Liège, Belgique
7. Recent developments in hyphenated supercritical fluid systems including extraction and chromatography.
Caroline West, Clément De Saint Jores, Laurine Réset, Benjamin Caux, Ramy Abou-Naccoul, Shinnosuke Horie, Isabelle François
Pittcon – Mars 2025 – Boston, USA

8. Optimisation du transfert et de l'injection dans un SFC-SFC en ligne.

Laurine Réset, Clément De Saint Jores, Caroline West

SEP25 – Mars 2025 – Paris, France

9. From Science-Fiction chromatography to modern SFC.

Caroline West, Clément De Saint Jores, Laurine Réset, Benjamin Caux, Ramy Abou-Naccoul, Shinnosuke Horie

HPLC – Juin 2025 – Bruges, Belgique

Communication Posters :

1. Development of an SFC-SFC configuration in multiple heart-cutting to analyze chiral flavonoids from bitter orange (*Citrus aurantium* L.).

Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West

Le studium – Octobre 2023 – Orléans, France

2. Conception et optimisation d'un système chromatographique bidimensionnel pour l'analyse de flavonoïdes chiraux de l'orange amère (*Citrus aurantium* L.).

Laurine Réset, Clément De Saint Jores, Isabelle François, Caroline West

Biotechnocentre – Octobre 2024 – Nouan-le-Fuzelier, France

3. Study of the transfer conditions for an online 2D SFC system.

L. Réset, C. De Saint Jores, C. West

HPLC 2025 – 15-19 juin 2025 – Bruges, Belgique

Liste des formations suivies

Formations doctorales

- Pédagogie active : 20 h
- Intégrité scientifique dans les métiers de la recherche : 15 h
- Rédiger et publier un article scientifique : 20 h
- Ecole thématique « du traitement de l'échantillon au couplage à la masse : un voyage analytique » : 15 h
- Le temps, l'énergie, la lumière, l'univers : quelles limites vers l'infiniment grand ? : 21h
- Ma thèse en 180 secondes : préparation au concours : 24 h
- Development of measurement and prediction tool to determine solubility in supercritical fluids : 40 h

Formations scientifiques

- Journée thématique AFSEP : chromatographie multidimensionnelle – *Novembre 2022, Paris, France*
- Congrès SEP-23 – *Mars 2023, Paris, France*
- Congrès SFC Europe 2023 – *Mai 2023, Bâle, France*
- Journée thématique AFSEP : chromatographie à l'échelle préparative – *Mai 2023, Paris, France*
- Ecole thématique « du traitement de l'échantillon au couplage à la masse : un voyage analytique » - *Juin 2023, Vichy, France*
- Congrès du Studium : Plant rediscovery with advanced tools for well-being applications – *Octobre 2023, Orléans, France*
- Journée thématique AFSEP : La dynamique des phases stationnaires – *Novembre 2024, Paris, France*
- Journée thématique AFSEP : Validation, normalisation, réglementation, attention ! – *Mars 2024, Paris, France*
- Formation sur la solubilité : Development of measurement and prediction tool to determine solubility in supercritical fluids – *Avril 2023, Université de Kyushu, Japon*
- Congrès HTC-18 – *Mai 2024, Louvain, Belgique*
- Congrès ISC 2024 + short course (6h) : multi-dimensional separations – *Octobre 2024, Liverpool, Royaume-Uni*
- Congrès Biotechnocentre 36^e colloque – *Octobre 2024, Nouan-le-Fuzelier, France*
- Congrès SEP-25 – *Mars 2025, Paris, France*

Liste des abréviations

1D-LC	Chromatographie liquide unidimensionnelle
2D-GC	Chromatographie en phase gazeuse bidimensionnelle
2D-LC	Chromatographie liquide bidimensionnelle
2D-SFC	Chromatographie en phase supercritique bidimensionnelle
ACN	Acétonitrile
ACOX	Acyl-CoA oxydase
Akt-GSK3 β	Voie de signalisation Akt - glycogène synthase kinase 3 β
AMPK	Protéine kinase activée par l'AMP
ASM	Modulation active de solvant
BBD	Plan de Box-Behnken
BHT	Hydroxytoluène butylé (antioxydant)
BPR	Régulateur de contre pression
C/EBP α	Protéine de liaison à l'élément CCAAT/enhancer α
CAT	Catalase
CCC	Chromatographie à contre-courant
CD	Dichroïsme circulaire
CDK1	Kinase dépendante des cyclines 1
COV	Composés organiques volatils
CSP	Phase stationnaire chirale
d.e.	Excès diastéréoisomérique
DAD	Détecteur à barrette de photodiodes
Dim1	Première dimension
Dim2	Deuxième dimension
DoE	Plan d'expériences
DPPH	2,2-Diphényl-1-picrylhydrazyle (réactif antioxydant)
e.e.	Excès énantiomérique
ESI	Ionisation par électrospray
EtOH	Ethanol
FA	Acide formique

FAS	Acide gras synthase
FID	Détecteur à ionisation de flamme
FLD	Détecteur de fluorescence
GC	Chromatographie en phase gazeuse
GC × GC	Chromatographie bidimensionnelle gazeuse en mode compréhensif
GSH	Glutathion peroxydase
HAP	Hydrocarbures aromatiques polycycliques
HILIC	Chromatographie d'interaction hydrophile
HPLC	Chromatographie liquide à haute performance
IEX	Chromatographie d'échange d'ions
iNOS	Synthase inductible de l'oxyde nitrique
LC	Chromatographie en phase liquide
LC × LC	Chromatographie bidimensionnelle liquide en mode compréhensif
LC-LC	Chromatographie bidimensionnelle liquide en mode heart-cut
LOQ	Limite de quantification
LSER	Relation linéaire d'énergie de solvation
<i>m/z</i>	Rapport masse sur charge
MAPK	Protéine kinase activée par des mitogènes
MeOH	Méthanol
MHC	Multiple Heart-Cut
MS	Spectrométrie de masse
MSA	Acide méthanesulfonique
mSFC-SFC	Système en fluide supercritique bidimensionnel en mode multi-heart-cut
MTX	Méthotrexate
NADES	Solvants eutectiques profonds naturels (Natural Deep Eutectic Solvents)
NF-κB	Facteur nucléaire kappa B
NPLC	Chromatographie liquide en phase normale
PDA	Détecteur à barrette de photodiodes
PeDS	Échantillon sec d'écorce (Peel Dry Sample)
PO	Polarité organique

PPAR γ	Récepteur activé par les proliférateurs de peroxyosomes γ
RPLC	Chromatographie liquide en phase inversée
RSD	Écart-type relatif
SCD-1	Stéaroyl-CoA désaturase 1
SEC	Chromatographie d'exclusion stérique
SFC	Chromatographie en phase supercritique
SFC $\times$ SFC	Chromatographie bidimensionnelle supercritique en mode compréhensif
SFC-SFC	Chromatographie bidimensionnelle supercritique en mode heart-cut
SFE	Extraction par fluide supercritique
SOD	Superoxyde dismutase
SPAM	Modulation assistée par phase stationnaire
SPE	Extraction en phase solide
SPP	Particules superficiellement poreuses
Sys1D	Système de séparation unidimensionnel
Sys2D	Système de séparation bidimensionnel
TCM	Médecine traditionnelle chinoise
TFA	Acide trifluoroacétique
TNF- α , IL-6, IL-1 β	Cytokines pro-inflammatoires (facteur de nécrose tumorale α , interleukines 6 et 1 β)
TOF	Temps de vol
TPSA	Aire de surface polaire topologique (Topological Polar Surface Area)
UAE	Extraction assistée par ultrasons
UC	Chromatographie unifiée
UHPLC	Chromatographie liquide ultra haute performance
UHPSFC	Chromatographie supercritique ultra haute performance
UV	Ultraviolet
VEM	Modulation de l'évaporation sous vide
w50%	Largeur de pic à mi-hauteur (50 %)

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Partie 1 : Excès diastéréoisomérique des flavonoïdes du *Citrus aurantium* L. dans les différentes parties de l'orange

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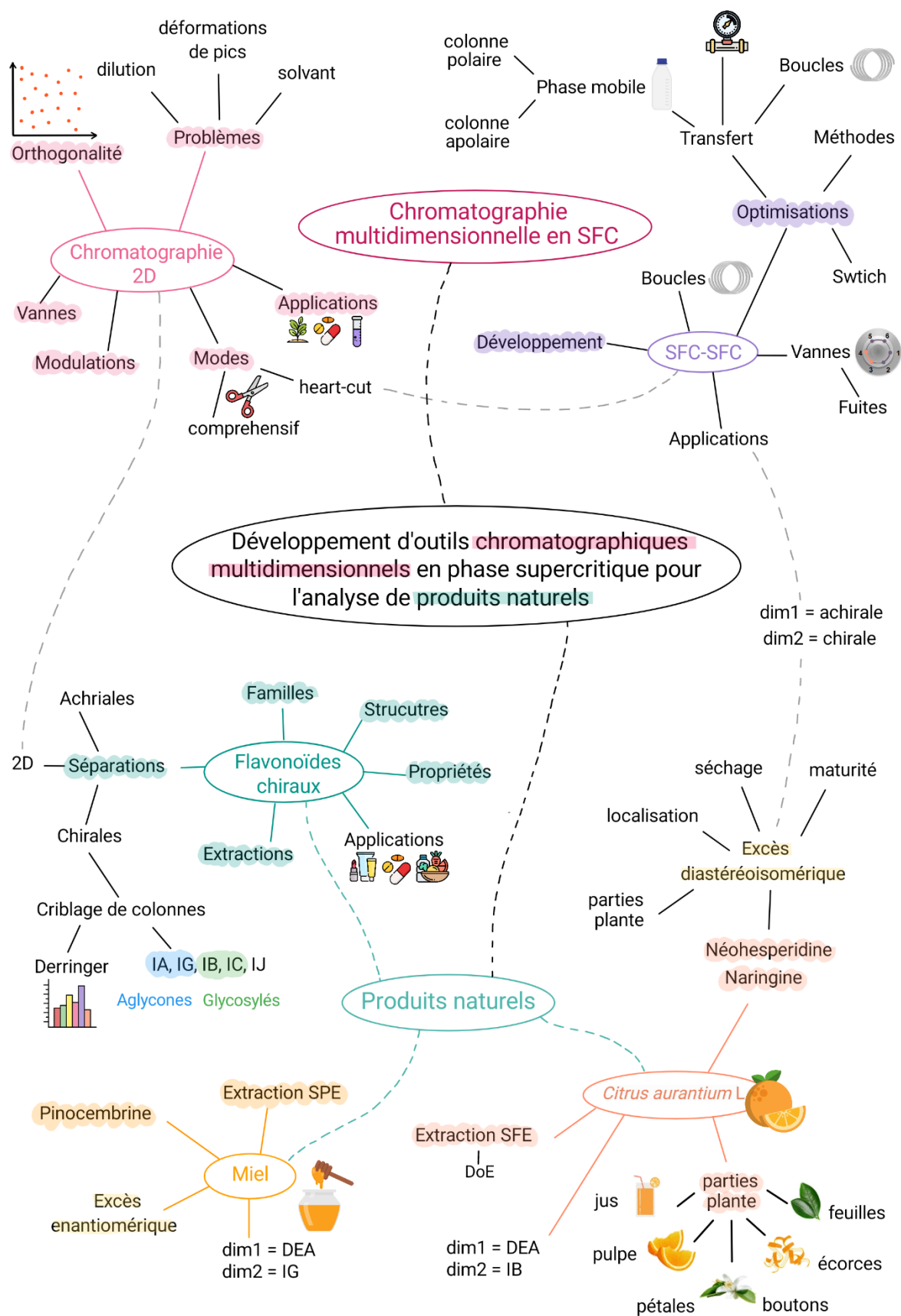
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Introduction générale





L'analyse de mélanges complexes issus de matrices naturelles représente aujourd'hui un enjeu analytique important. Les produits naturels contiennent une grande diversité de métabolites secondaires tels que les flavonoïdes, présentant une grande diversité structurale. Ces composés peuvent présenter des centres chiraux ce qui rend leur identification plus difficile mais nécessaire car leurs propriétés biologiques peuvent dépendre de leur chiralité. Cependant, les méthodes chromatographiques unidimensionnelles montrent rapidement des limites. Pour analyser ces mélanges complexes, il est donc nécessaire d'utiliser des techniques plus performantes capables de séparer un grand nombre de composés.

La chromatographie bidimensionnelle (en phase liquide 2D-LC ou supercritique 2D-SFC) est apparue comme une solution adaptée pour répondre à ces défis. En couplant deux dimensions chromatographiques orthogonales, cette technique permet d'augmenter significativement la résolution globale, tout en conservant des temps d'analyse raisonnables. En particulier, la chromatographie en phase supercritique bidimensionnelle (2D-SFC), encore peu développée par rapport à la 2D-LC, offre des perspectives intéressantes pour l'analyse de tous types de composés y compris les composés chiraux.

C'est dans ce contexte que s'inscrit le travail présenté dans cette thèse, qui a pour objectif de développer un système 2D-SFC, pour l'analyse de composés bioactifs issus de produits naturels comme le *Citrus aurantium* L. Cette plante est utilisée dans divers domaines d'applications et est particulièrement riche en flavonoïdes chiraux. Ils ont des structures variées, avec des formes glycosylées ou aglycones. Leur séparation constitue un défi analytique pertinent pour mettre à l'épreuve un système chromatographique bidimensionnel.

Le premier chapitre de cette thèse est consacré à un état de l'art de la chromatographie bidimensionnelle, en particulier dans sa configuration en phase supercritique. Il permet de donner les bases théoriques et techniques du fonctionnement des systèmes 2D. Les mécanismes de modulation, de transfert de fraction, de sélection de colonnes orthogonales, et de gestion des paramètres critiques seront abordés. Ceci afin de mieux comprendre le bon fonctionnement d'un système 2D.

Le deuxième chapitre est consacré aux flavonoïdes chiraux et à la complexité de leur analyse. Leur chiralité joue un rôle clé dans leurs effets biologiques, rendant l'analyse chirale indispensable. L'évolution des techniques chromatographiques, notamment la chromatographie liquide (LC), la chromatographie en phase supercritique (SFC) ainsi que la chromatographie bidimensionnelle ont

permis d'analyser ce types de composés, grâce aussi au développement de phases stationnaires chirales. Dans ce contexte, le *C. aurantium*, ou orange amère, représente une source végétale d'intérêt contenant des flavonoïdes chiraux dans ses différents organes. Il est utilisé en médecine traditionnelle, en cosmétique ou en agroalimentaire. Ce chapitre propose une revue des méthodes analytiques appliquées aux flavonoïdes chiraux, ainsi qu'une étude particulière de ceux du *C. aurantium*, en lien avec leurs propriétés biologiques et leurs applications.

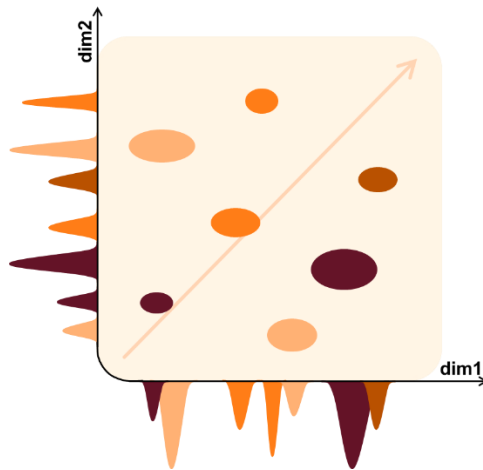
Le troisième chapitre, basé sur nos travaux déjà publiés, décrit le développement d'un système de chromatographie bidimensionnelle en phase supercritique en configuration multiple heart-cut. Ce nouvel instrument basé sur un système unidimensionnel avec un ajout simple de vannes externes et de boucles a permis un transfert précis et efficace des fractions de chromatogramme en limitant la perte d'échantillon. Une deuxième partie de ce chapitre est axée sur les optimisations du transfert et de l'injection en fonction des conditions opératoires comme la taille de boucle, la pression ou encore la composition de la phase mobile. Ces optimisations ont pour but de montrer les limites d'utilisations du système pour pouvoir ensuite l'appliquer à un grand nombre d'applications et envisager l'évolution vers un système compréhensif.

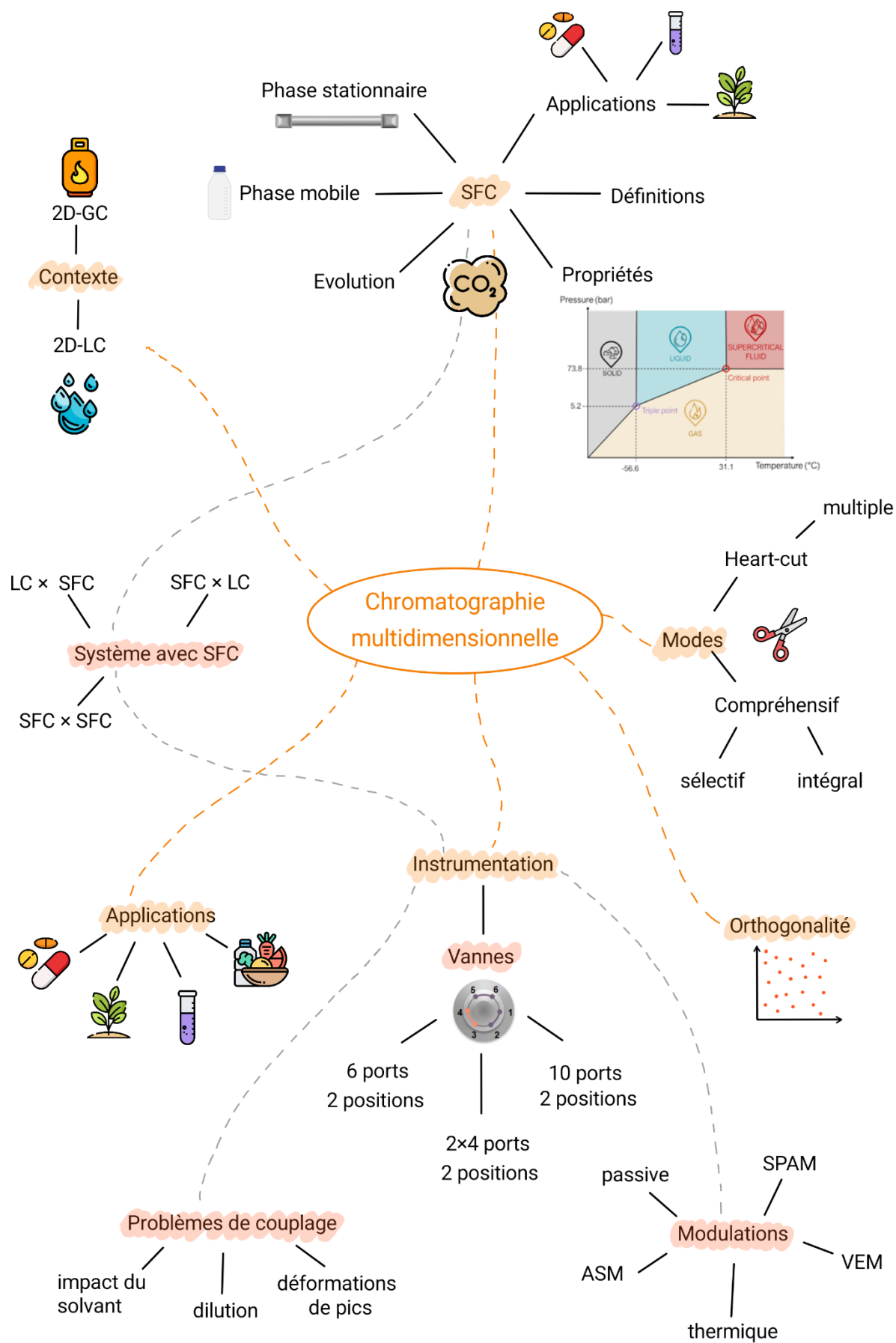
Dans le quatrième chapitre, ce système SFC-SFC est appliqué à la séparation de flavonoïdes chiraux majoritaires du *C. aurantium* : la naringine et la néohespéridine. Leur extraction par fluide supercritique est préalablement optimisée par un plan d'expérience afin d'améliorer le rendement d'extraction des flavonoïdes cibles. Puis leurs excès diastéréoisomériques sont déterminés grâce au système SFC-SFC, en montrant un lien entre ces excès et les différentes parties de la plante, mais également les conditions de récolte et de séchage. Une deuxième partie de ce chapitre est consacrée à l'étude plus large des séparations chirales des flavonoïdes. Un screening de colonnes a été réalisé sur un ensemble de composés pour mettre en avant des liens entre structures de flavonoïdes et nature de la phase stationnaire, notamment les formes glycosylées ou aglycones. En complément, un cas d'application sur des miels de Guyane est présenté pour déterminer l'excès énantiomérique de la pinocembrine dans cet échantillon complexe.

L'ensemble de ce travail vise à démontrer l'intérêt de la 2D-SFC comme outil analytique pour l'étude de composés bioactifs au sein de mélanges complexes, en alliant performance technique et innovation instrumentale. Il contribue ainsi à enrichir les approches analytiques dédiées aux produits naturels. Il ouvre de nouvelles perspectives de méthodes de séparation plus sélectives et rapides dans le domaine de la chimie analytique, s'ouvrant ainsi à un grand nombre d'applications.

Chapitre I

La multi-dimension en chromatographie





Introduction

Dans de nombreux domaines l'identification et la quantification de mélanges complexes représentent un défi analytique majeur. Ces échantillons sont souvent hétérogènes et riches en composés structurellement proches, ce qui complique considérablement leur séparation. Néanmoins, bien que les techniques chromatographiques unidimensionnelles soient très largement utilisées dans de nombreux cas, elles peuvent s'avérer insuffisantes.

Afin de surmonter ces limitations, la chromatographie multidimensionnelle s'est imposée comme une approche analytique particulièrement efficace. Elle repose sur le couplage de systèmes séparatifs ayant des mécanismes de rétention distincts et complémentaires. Ce couplage, basé sur le principe d'orthogonalité, permet d'augmenter significativement la sélectivité et la capacité de séparation. En combinant ainsi plusieurs dimensions de séparation, la chromatographie multidimensionnelle offre une meilleure séparation des composés, y compris lorsqu'ils sont structurellement proches au sein de matrices complexes.

L'intérêt croissant pour la chromatographie multidimensionnelle reflète sa capacité à résoudre des problèmes, traiter des échantillons dont la complexité dépasse les capacités analytiques des méthodes unidimensionnelles. Cependant, sa mise en place repose sur une instrumentation spécifique et l'optimisation de nombreux paramètres opératoires, ce qui en fait une technique à la fois performante et exigeante.

Ce chapitre donne une introduction à la chromatographie multidimensionnelle. Il commence par une mise en contexte des différents types de chromatographie utilisant la multi-dimension : chromatographie en phase gazeuse, en phase liquide, et en phase supercritique. La suite du chapitre explore les fondements de la chromatographie bidimensionnelle, en précisant les différents modes de couplage (notamment le mode heart-cutting et le mode compréhensif) et le rôle central de l'orthogonalité dans l'efficacité des séparations. Enfin, une partie sera consacrée à l'instrumentation, notamment les systèmes de commutation et de modulation, indispensables à la mise en place de ces systèmes pour les chromatographies liquides et supercritiques.

1. Différents types de chromatographie

1.1. Chromatographie en phase gazeuse

La chromatographie en phase gazeuse (GC) a été introduite dans les années 1950 par James et Martin [1]. Elle est très rapidement devenue une technique de référence pour l'analyse des composés volatils et semi-volatils. Cependant, l'augmentation de la complexité des échantillons étudiés a rapidement montré les limites de la GC monodimensionnelle, notamment en raison d'une résolution insuffisante, qui entraîne la co-élution de plusieurs analytes.

C'est dans ce contexte que, quelques années plus tard, en 1990, Phillips et Liu ont introduit la chromatographie bidimensionnelle en phase gazeuse (2D-GC) [2]. Ils ont démontré qu'une meilleure séparation pouvait être obtenue en utilisant deux colonnes de sélectivités différentes couplées à un modulateur thermique ou pneumatique [3,4]. Cette avancée a permis d'améliorer significativement la capacité de séparation des mélanges complexes, rendant possible son application dans de nombreux domaines.

La 2D-GC est utilisée pour la caractérisation de mélanges complexes tels que les carburants, huiles, bio-huiles ou fractions de raffinage [5,6]. Dans le domaine alimentaire, la 2D-GC est souvent utilisée couplée avec la spectrométrie de masse (MS) pour la caractérisation d'arômes ou pour réaliser des discriminations sensorielles d'aliments ou de boissons [7–9]. Par exemple, Stefanuto *et al.* ont optimisé une méthode GC×GC-TOFMS pour l'analyse des composés volatils du profil aromatique de la bière [7]. Elle est également utilisée pour s'assurer de la fraîcheur des aliments et des contaminations [8].

Dans le contexte environnemental, la 2D-GC est un outil incontournable pour la détection des composés organiques volatils (COV) et des hydrocarbures aromatiques polycycliques (HAP) dans l'atmosphère, les sols et l'eau [10–12]. C'est également le cas pour les mycotoxines volatiles [13] et les pesticides. Wang *et al.* ont développé une méthode GC×GC-TOFMS pour détecter simultanément 100 pesticides dans des matrices végétales, démontrant une sensibilité et sélectivité accrues [10]. Un des derniers secteurs est celui de la médecine et du domaine pharmaceutique. En médecine, la 2D-GC joue un rôle clé dans la recherche de biomarqueurs volatils pour le diagnostic précoce de maladies [14–16]. Elle permet aussi la détection précise de résidus de solvants et d'impuretés dans les formulations pharmaceutiques [17].

Ainsi la 2D-GC offre des avantages significatifs par rapport à la chromatographie unidimensionnelle classique [18,19]. Sa meilleure résolution chromatographique permet une meilleure séparation des analytes, même dans les matrices complexes. Elle nécessite souvent

l'utilisation d'un couplage GC×GC-MS afin d'améliorer l'identification des composés et l'interprétation du chromatogramme bidimensionnel, notamment en mettant en évidence l'organisation de familles moléculaires au sein de l'échantillon. [20].

1.2. Chromatographie en phase liquide

Depuis les années 1970, la chromatographie liquide (LC) s'est imposée comme une méthode de référence, en particulier pour l'analyse de composés polaires ou apolaires, thermolabiles ou de masse molaire élevée, domaines dans lesquels la chromatographie en phase gazeuse (GC) présente des limites. Avec l'augmentation constante de la complexité des échantillons issus de matrices environnementales, biologiques, pharmaceutiques ou alimentaires, les performances de la chromatographie liquide unidimensionnelle (1D-LC) ont montré leurs limites, notamment en ce qui concerne la résolution des analytes dans les mélanges complexes.

C'est dans ce contexte que s'est développée la chromatographie liquide bidimensionnelle (2D-LC), initialement théorisée par Giddings dans les années 1980 [21]. Cette approche repose sur le couplage de deux systèmes présentant des mécanismes de séparation orthogonaux, reliées par un modulateur assurant le transfert temporel de fractions de la première vers la seconde dimension. Ce montage permet d'augmenter considérablement la capacité de séparation, rendant possible la résolution de centaines de composés dans des matrices particulièrement complexes [22,23].

Le développement de la 2D-LC a été favorisé par des avancées techniques majeures, notamment l'introduction de modules de commutation rapide, de modulateurs performants, et de systèmes de collecte automatisée, qui ont significativement renforcé la robustesse et la fiabilité des analyses [24,25]. En parallèle, les progrès des logiciels de traitement de données ont permis une meilleure exploitation des chromatogrammes bidimensionnels, rendant la technique plus accessible.

Dans la pratique, la plupart des configurations 2D-LC utilisent deux dimensions en phase inversée (RPLC×RPLC). Cette combinaison est très répandue en raison de la compatibilité totale entre les phases mobiles utilisées [26–29], ce qui facilite la mise en place et réduit les risques de perturbations en deuxième dimension. Toutefois, l'orthogonalité limitée de ce couplage incite à explorer d'autres associations de modes chromatographiques, telles que HILIC×RPLC, RPLC×HILIC ou encore SEC×RPLC, dans le but d'améliorer la caractérisation de l'échantillon.

L'efficacité globale de la 2D-LC repose fortement sur la complémentarité des deux dimensions, mais cette configuration soulève aussi plusieurs défis pratiques. L'un des principaux est la compatibilité entre les phases stationnaires et les phases mobiles : le solvant de la première

dimension, transféré avec la fraction collectée dans la seconde dimension, peut altérer la performance de la seconde dimension, en particulier dans les montages RPLC×HILIC ou HILIC×RPLC [30,31]. D'autres contraintes concernent les effets de dilution lors du transfert entre les dimensions, qui peuvent entraîner une perte de sensibilité. Pour y remédier, des stratégies comme la modulation active (*Active Solvent Modulation*) ont été proposées [32–35].

Les applications de la 2D-LC se sont étendues à de nombreux domaines. En analyse environnementale, elle permet la détection de micropolluants, résidus pharmaceutiques ou perturbateurs endocriniens dans les eaux usées et naturelles [27]. En bioanalyse, cette technique s'avère particulièrement utile pour la caractérisation de biomolécules complexes telles que les protéines et anticorps thérapeutiques, notamment lorsqu'elle est couplée à la spectrométrie de masse haute résolution (HRMS) [28,30,36,37]. Dans le secteur agroalimentaire, la 2D-LC a été utilisée pour étudier les anthocyanes dans le vin [31], évaluer la qualité des huiles ou encore détecter divers contaminants [38,39].

Au-delà de ces champs d'application classiques, la 2D-LC s'avère également pertinente pour des domaines plus spécialisés, comme l'analyse de nanoparticules [40], l'étude de médicaments traditionnels [41] ou la conservation du patrimoine culturel [24], grâce à sa capacité à résoudre des mélanges particulièrement complexes.

Les récents développements méthodologiques ont permis de rendre la 2D-LC plus accessible pour une utilisation en routine. Les efforts se sont concentrés sur l'optimisation des temps d'analyse, la réduction de la dilution entre les dimensions, la gestion automatisée des données [34,42,43] et du développement de méthode [44]. Le couplage systématique de la 2D-LC avec la spectrométrie de masse (LC×LC-MS) renforce les performances analytiques globales, en combinant une séparation de haute résolution avec une capacité de détection et d'identification avancée [22,26,45].

Grâce à l'amélioration continue des équipements, la 2D-LC s'impose comme une technologie adaptée aux exigences analytiques. Elle répond efficacement aux besoins croissants de sélectivité, de sensibilité, et de robustesse.

1.3. Chromatographie en phase supercritique (SFC)

1.3.1. Définition d'un fluide supercritique

La chromatographie en phase supercritique, plus communément appelée SFC, est une technique de séparation qui utilise un fluide supercritique comme phase mobile. Un fluide est supercritique lorsqu'il est soumis à certaines conditions de pression et de température. Ces conditions doivent se trouver au-delà du point critique, c'est-à-dire la limite au-delà de laquelle la distinction entre phase liquide et phase gazeuse disparaît. À cet état, le fluide est dans un état intermédiaire entre les deux phases et adopte des propriétés particulières qui le rendent intéressant pour des applications chromatographiques.

Le diagramme de phase permet de visualiser les différents changements d'état en fonction de la température et de la pression. Celui du dioxyde de carbone (CO_2) est présenté Figure I.1. Il comporte trois zones principales : l'état solide, l'état liquide et l'état gazeux, séparées par des courbes de changement de phase. La courbe de sublimation relie l'état solide à l'état gazeux, tandis que la courbe de vaporisation définit l'équilibre entre l'état liquide et l'état gazeux. Enfin, la courbe de fusion sépare l'état solide de l'état liquide. Sur chacune de ces courbes, le composé se retrouve en équilibre entre deux états de la matière. Au niveau du point triple, il y a coexistence des trois états de la matière. Partout ailleurs ce composé existe sous un seul état de la matière.

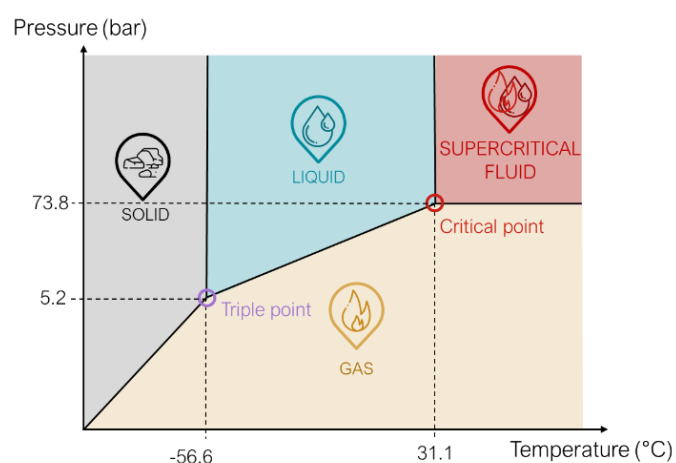


Figure I.1 – Diagramme de phase simplifié du dioxyde de carbone (CO_2)

Le point où la courbe de vaporisation cesse d'exister est appelé le point critique. Pour le CO_2 , ce point se situe à $31,1^{\circ}\text{C}$ et $73,8\text{ bars}$. Au-delà de ces conditions, les phases liquide et gaz du CO_2 se mélangent et forment la phase supercritique.

1.3.2. Evolution de la SFC

L'histoire de la SFC commence en 1822, lorsque le scientifique français Charles Cagniard de Latour observa, au cours d'expériences sur l'éthanol, qu'à une certaine température, la distinction entre les phases liquide et gazeuse disparaissait [46]. Cette découverte posa les bases du concept de fluide supercritique. En 1869, Thomas Andrews confirma ce phénomène en étudiant les transitions d'état de l'acide carbonique, introduisant ainsi la notion de point critique [47].

Cependant ce n'est que durant la seconde moitié du XXème siècle, que les fluides supercritiques furent envisagés pour des applications analytiques. En 1962, Klesper *et al.* réalisèrent la première séparation chromatographique en utilisant un fluide supercritique, le CO₂, pour la séparation de composés aromatiques [48].

Le développement de la technique a cependant été freiné par l'émergence et la popularité croissante de la chromatographie en phase liquide à haute performance (HPLC). De plus, les premiers systèmes SFC souffraient de nombreuses limitations techniques, notamment une faible reproductibilité des analyses, due à une instrumentation encore peu robuste et sensible [49,50].

Malgré cela, la SFC n'a pas été totalement oubliée durant toutes ces années en grande partie grâce à l'intérêt croissant pour les séparations de composés chiraux et la possibilité de les purifier à l'échelle préparative [51]. L'amélioration de l'instrumentation au cours des décennies suivantes a permis à la SFC de s'imposer comme une méthode performante et respectueuse de l'environnement, tant en mode analytique qu'en mode préparatif, notamment dans les industries pharmaceutique et chimique [52,53].

Dans ce contexte d'amélioration continue, plusieurs études comparatives ont été menées pour évaluer les performances actuelles de la SFC face aux techniques plus pratiquées. Parmi elles, l'étude de Grand-Guillaume Perrenoud *et al.* en 2012 a comparé la SFC et l'HPLC pour l'analyse de produits pharmaceutiques [54]. Les auteurs ont apporté des précisions essentielles sur l'évolution des courbes de Van Deemter en fonction de la taille des particules des colonnes chromatographiques (Figure I.2). Les performances des colonnes chromatographiques sont fortement influencées par la taille des particules qui les composent. Plus elles sont petites, plus l'efficacité est élevée. Cependant, leurs utilisations engendrent des pertes de charge plus importantes, ce qui impose l'usage de pompes capables de délivrer des pressions élevées. Ces pompes sont nécessaires pour maintenir un débit suffisant et atteindre ainsi des conditions proches de l'optimum de la courbe de Van Deemter. Cependant le fluide supercritique est beaucoup moins visqueux qu'un liquide, ce qui limite les contraintes de pression des pompes par rapport à celles de l'UHPLC.

L'étude a mis en évidence que l'UHPSFC dépasse l'UHPLC en termes de performance en maintenant une faible hauteur de plateau (H) sur une plus large gamme de vitesses linéaires (u). Or, pour exploiter pleinement les performances des colonnes sub-2 μm à haut débit, des pressions encore plus élevées sont nécessaires. Cette contrainte a conduit à des développements de nouveaux systèmes permettant d'exploiter complètement les capacités théoriques de l'UHPSFC.

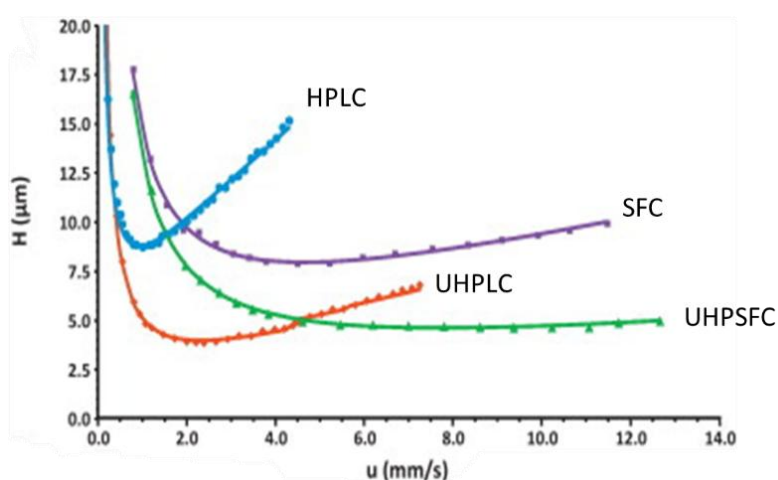


Figure I.2 – Courbes de van Deemter du butylparabène en HPLC (bleu) avec une colonne Xterra RP18 50 mm $\times$ 2.1 mm, 1.7 μm , SFC (violet) avec une colonne Acquity UPC² BEH 2-EP 100 mm $\times$ 3.0 mm, 3.5 μm , UHPLC (rouge) avec une colonne Acquity Shield C18 50 mm $\times$ 2.1 mm, 1.7 μm , UHPSFC (vert) avec une colonne Acquity UPC² BEH 2-EP 100 mm $\times$ 3.0 mm, 1.7 μm . Adapté de [54]

1.3.3. Propriétés physico-chimiques d'un fluide supercritique

Cet état particulier permet au fluide d'adopter des propriétés uniques situées à l'intermédiaire entre celles d'un liquide et celles d'un gaz. Il a une viscosité beaucoup plus faible qu'un liquide et proche de celle d'un gaz. De plus, il a un coefficient de diffusion intermédiaire entre celui des phases gazeuse ou liquide ce qui permet de travailler à des débits élevés tout en maintenant une bonne efficacité de séparation, sans générer de pression excessive dans le système.

Table I.1 – Propriétés physiques des fluides liquides, supercritiques et gazeux [55]

Nature du fluide	Viscosité (cP)	Coefficient de diffusion (m ² .s ⁻¹)	Masse volumique (g.m ⁻³)
Liquides (30 °C, 1 atm)	0,2 - 3	(0,2 - 2) .10 ⁻⁹	0,6 – 1,6
Supercritique (Tc et Pc)	(1 - 3) .10 ⁻²	0,7 .10 ⁻⁷	0,2 – 1,2
Gaz (30 °C, 1 atm)	(1 - 3) .10 ⁻²	(1 - 4) .10 ⁻⁵	(0,6 – 2) .10 ⁻³

Ces propriétés intermédiaires rendent le dioxyde de carbone particulièrement efficace pour la chromatographie, notamment au niveau de la résistance au transfert de masse correspondant à une pente plus faible de la courbe de van Deemter (Figure I.2). En effet, sa faible viscosité permet de travailler à des débits élevés, tout en réduisant la pression dans la colonne. De plus, sa haute diffusivité améliore les interactions entre la phase mobile et la phase stationnaire, de qui conduit à des séparations plus rapides et plus efficaces. Un autre grand avantage est de pouvoir faire varier sa densité en jouant sur la pression et la température, ce qui permet d'adapter la force d'élution en fonction de la nature des analytes à séparer.

1.3.4. Phase mobile

Le CO₂ est le fluide supercritique le plus utilisé en chromatographie. Ce choix s'explique par ses propriétés physico-chimiques intéressantes, sa non-toxicité, son caractère ininflammable, ainsi que par ses conditions critiques modérées (température critique de 31,1 °C et pression critique de 73,8 bar), facilement atteignables.

Cependant d'autres fluides peuvent être utilisés à l'état supercritique (Tableau 2). Le principal facteur limitant reste la difficulté d'atteindre leurs conditions critiques, souvent plus extrêmes. Par exemple, l'eau possède une température critique de 374 °C et une pression critique de 221 bar, rendant son usage plus complexe. Pourtant, l'eau supercritique présente des propriétés uniques : elle devient un solvant non polaire, avec la diminution de sa constante diélectrique. Elle peut donc solubiliser des composés organiques et catalyser des réactions d'oxydation sans ajout de réactifs [56]. Ces caractéristiques sont utilisées dans la gazéification de la biomasse pour produire de l'hydrogène [57,58], dans la liquéfaction hydrothermale ainsi que dans le traitement des boues huileuses et des déchets dangereux [59]. Certains articles utilisent l'eau subcritique (haute pression mais température modérée) pour l'extraction de composés dans des matrices naturelles [60].

Table I.2 – Températures et pressions critiques de différents fluides supercritiques [61]

	T _c (°C)	P _c (bar)
Dioxyde de carbone (CO ₂)	31,1	73,8
Oxyde nitreux (N ₂ O)	36,5	72,5
Ammoniaque (NH ₃)	132,3	113,5
Méthanol (MeOH)	239,5	80,9
Eau (H ₂ O)	373,9	220,6

De la même façon, le méthanol supercritique est un autre fluide prometteur malgré des conditions difficiles à atteindre avec une température de 240°C. Il est largement utilisé pour la transestérification des huiles végétales et animales permettant la production de biodiesel [62–67].

Malgré ses nombreux avantages, le CO₂ supercritique est apolaire, ce qui limite son utilisation pour solubiliser les composés les plus polaires. C'est pourquoi, dans la pratique, le CO₂ supercritique est souvent combiné à un co-solvant polaire, aussi appelé modificateur, comme le méthanol ou l'éthanol. Cet ajout permet d'augmenter la polarité de la phase mobile et d'élargir la gamme de composés analysables. Cette modulation de polarité rend la SFC particulièrement puissante pour l'analyse de composés complexes dans les domaines pharmaceutique, des produits naturels et de l'environnement [68]. En plus de ses performances analytiques, la SFC présente un intérêt écologique majeur. Contrairement à la chromatographie liquide, qui utilise de grandes quantités de solvants organiques, la SFC réduit considérablement leur utilisation. Ainsi la SFC permet de diminuer l'empreinte environnementale des analyses.

En plus du co-solvant, des additifs peuvent être ajoutés à la phase mobile. L'eau, lorsqu'elle est introduite en faible concentration, est considérée comme un additif. Elle est ajoutée à la composition du co-solvant comme solvant tertiaire. Il doit être ajouté en faible proportion, maximum 10% dans le co-solvant, pour éviter une démixtion entre le CO₂ et l'eau due à leur faible miscibilité. Malgré ça, 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 [69,70]. Roy *et al.* ont mis en évidence dans une revue que ces effets peuvent être bénéfiques, tant en chromatographie chirale qu'achirale, avec différence de sélectivité et l'augmentation de l'efficacité obtenue en fonction de la teneur en eau du mélange modificateur. Cependant, ils notent aussi que ces effets ne sont pas systématiquement positifs : un excès d'eau peut réduire la sélectivité ou perturber la stabilité du mélange modificateur. Finalement, ajouter une petite quantité d'eau dans le co-solvant permet généralement d'augmenter l'efficacité [71].

Des additifs acides ou basiques peuvent également être ajoutés. Ces ajouts permettent de réduire les interactions ioniques non spécifiques et d'améliorer la symétrie des pics. Cependant, Edge *et al.* ont montré dans leur étude sur trois colonnes polaires achirales et deux additifs assez similaire que le choix de l'additif avait un impact significatif sur la rétention, mais un impact moins important sur la sélectivité [72]. D'autres études plus étendues ont mis en évidence un impact bien plus marqué du type d'additif. En séparation chirale, l'additif peut complètement modifier la résolution. C'est également le cas pour les séparations achirales, où changer l'additif peut également influencer la sélectivité [73,74].

Dans l'étude de Rimbault *et al.* l'effet des fortes concentrations d'additifs a été exploré, permettant de montrer leurs influences sur les mécanismes de rétention [75]. Speybrouck *et al.* quant à eux ont démontré leur utilité spécifique dans les séparations chirales [76]. West *et al.* ont mis en avant en 2017 le rôle des additifs sur la polarité et l'acidité de la phase mobile [74]. La figure suivante (Figure I.3) montre la fréquence d'utilisation des additifs selon le type de séparation, chirale ou achirale [77]. On observe que les séparations achirales utilisent massivement les additifs, en particulier les sels d'ammonium. A l'inverse, les séparations chirales ont une légère préférence pour des phases mobiles sans additif. Cela s'explique notamment par le fait que de nombreuses méthodes chirales sont développées pour l'échelle préparative, où l'on cherche à éviter les additifs difficiles à éliminer des fractions purifiées. Lorsqu'ils sont utilisés à l'échelle analytique, les additifs servent principalement à symétriser les pics, ce qui permet d'obtenir une bonne résolution avec une moindre sélectivité. Ce choix d'utilisation d'additifs dépend finalement de la nature des composés à séparer.

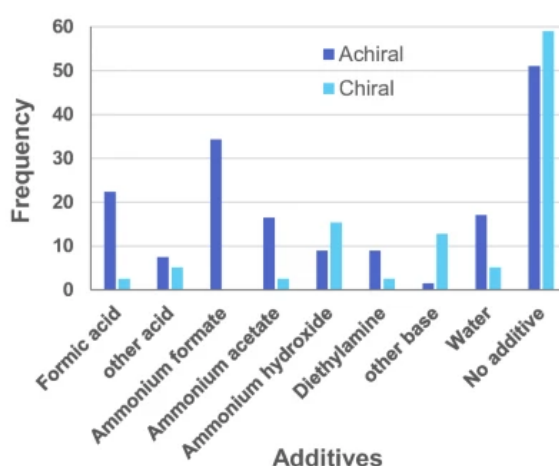


Figure I.3 – Choix des additifs en SFC chirale et achirale sur des applications entre 2016 et 2018 [77]

1.3.5. Phase stationnaire

L'un des éléments clés de la performance en SFC repose sur le choix de la phase stationnaire, qui influence fortement la sélectivité, l'efficacité et la robustesse des séparations, notamment pour les composés basiques et pharmaceutiques.

Contrairement à une idée reçue selon laquelle la SFC serait une extension de la phase normale, aujourd'hui il est reconnu que toutes les chimies de phase stationnaire développées pour la LC peuvent potentiellement être utilisées en SFC. Cela à condition que ces phases résistent aux contraintes du CO₂ supercritique [78]. Ainsi, tous les modes de rétentions sont accessibles selon les choix de phase mobile et de phase stationnaire : phase normale, phase inverse, échange d'ions, mode mixte, exclusion.

De nombreux travaux de West *et al.* se sont penchés sur l'évaluation des phases stationnaires et leurs classifications selon les descripteurs d'Abraham et un modèle LSER (Linear Solvation Energy Relationships) [79–83].

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

Les termes décrits dans cette équation sont les suivants :

- c : constante, correspondant principalement au rapport de phase
- E : capacité de l'analyte à interagir via les électrons π et non liants
- e : interactions π - π et interactions de Van der Waals (liées à E)
- S : présence de dipôles dans l'analyte
- s : interactions dipôle-dipôle (liées à S)
- A : capacité de l'analyte à être donneur de proton (liaison hydrogène)
- a : interactions par liaison hydrogène avec les donneurs de H (liées à A)
- B : capacité de l'analyte à être accepteur de proton (liaison hydrogène)
- b : interactions par liaison hydrogène avec les accepteurs de H (liées à B)
- V : volume caractéristique de McGowan
- v : interactions dispersives (liées à V), et effet de la cavité dans les phases fortement auto-associées (telles que l'eau)
- D^- : capacité de l'analyte à interagir par ses charges anioniques
- d^- : interactions avec les charges négatives (liées à D^-)
- D^+ : capacité de l'analyte à interagir par ses charges cationiques
- d^+ : interactions avec les charges positives (liées à D^+)

Ces approches permettent de générer des diagrammes en toile d'araignée (Figure I.4) utilisés pour comparer les interactions dominantes entre différentes colonnes. Ces outils facilitent la sélection de phases stationnaires similaires ou orthogonales, accélérant le développement de méthodes analytiques.

En parallèle de cette caractérisation, des avancées technologiques ont permis d'améliorer les performances chromatographiques. L'introduction de particules superficiellement poreuses (SPP) et des particules sub-2 μm , a notamment conduit à des gains significatifs en efficacité, avec des hauteurs réduites de plateaux permettant une amélioration notable de la résolution [84,85]. C'est ce qui est illustré dans la Figure I.2. La comparaison des courbes de Van Deemter entre l'HPLC et la SFC montre que de petites particules, combinée à un fluide mobile peu visqueux comme dans le cas de l'UHPSFC, permet d'atteindre des performances optimales à des vitesses linéaires plus élevées, tout en conservant une faible hauteur de plateau (H). Au-delà de la simple efficacité, le concept de sélectivité optimisée prend une place croissante dans les stratégies de développement. Des approches prédictives permettent aujourd'hui d'anticiper les combinaisons de phases stationnaires les plus pertinentes pour maximiser la différenciation entre analytes proches [86].

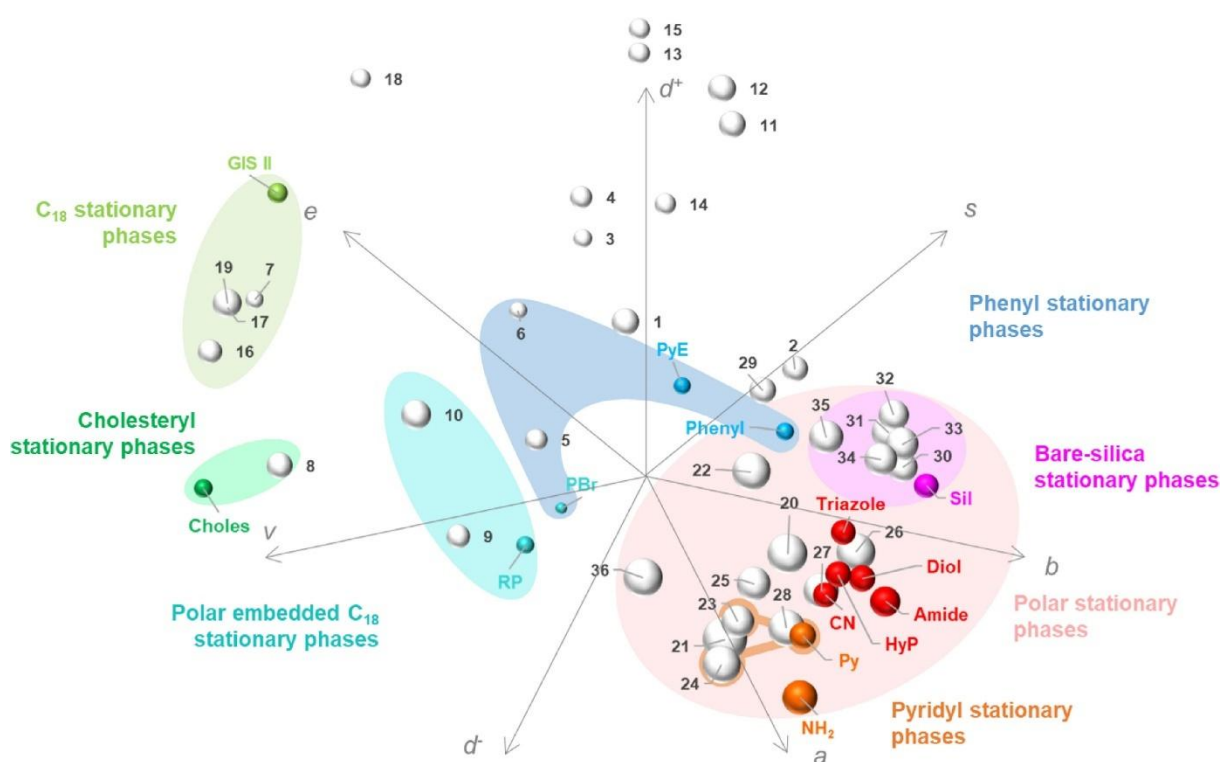


Figure I.4 – Diagramme en toile d'araignée obtenu à partir du modèle LSER calculé en utilisant l'équation (1) pour 160 composés, 14 nouvelles colonnes (couleurs) et 36 anciennes colonnes (blanc) [83]

1.3.6. Applications

La SFC s'est imposée comme une technique de choix dans de nombreux domaines analytiques grâce à ses performances élevées, sa rapidité d'analyse, sa sélectivité, ainsi que sa compatibilité avec des approches respectueuses de l'environnement. West a montré dans une revue de 2018 un récapitulatif des domaines d'applications de la SFC (Figure I.5) [77].

En effet, l'un des principaux champs d'application est l'analyse des produits naturels, représentant environ 27 % des études [87,88]. Ces recherches couvrent un large éventail de composés comme les alcaloïdes [89–92], les flavonoïdes [93], ou encore les extraits de plantes médicinales telles que *Mahonia bealei* [89] ou *Rheum officinale* [94].

Dans le secteur de la bioanalyse, la SFC permet d'effectuer la séparation chirale de biomarqueurs ou de médicaments d'intérêt dans des matrices biologiques plus ou moins complexes [95,96]. Son couplage à la spectrométrie de masse améliore la sensibilité et la sélectivité dans l'analyse de composés endogènes ou exogènes [97].

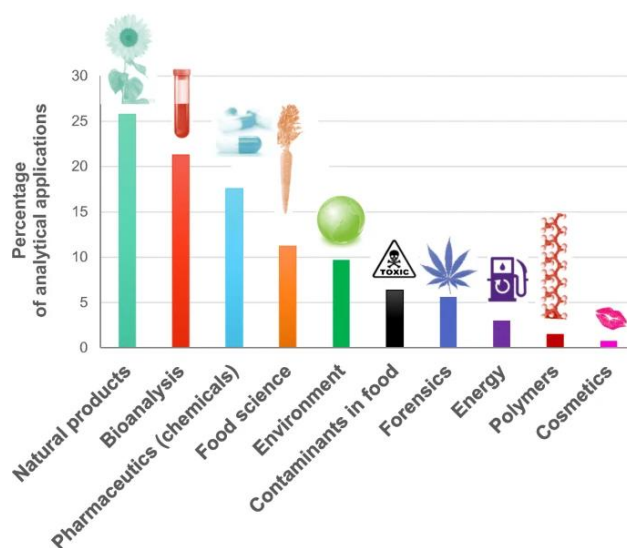


Figure I.5 – Domaines d'application analytiques de la SFC entre 2014 et 2018 [77]

Le domaine pharmaceutique est aussi un domaine majeur d'application avec environ 17% des applications publiées. La SFC est utilisée pour les enantioséparations [98,99], mais également pour l'analyse de formes formulées [100], le contrôle qualité [101], ou encore la détection des impuretés [102,103]. La SFC est utilisée aussi bien en phase de développement analytique qu'en purification [104]. La SFC s'impose également dans la lipidomique pharmaceutique, notamment pour le profilage des lipides complexes [105–107].

Pour le domaine alimentaire, la SFC est utilisée pour l'analyse de composés bioactifs tels que les caroténoïdes, stérols ou stéroïdes [108,109]. Elle permet aussi d'étudier les extraits de plantes utilisées en médecine traditionnelle [110], notamment dans les médecines asiatiques.

Concernant l'environnement, la SFC se montre prometteuse dans l'analyse des contaminants dans l'eau [111] et des pesticides [112], offrant une alternative verte et efficace aux méthodes classiques.

D'autres domaines émergents sont les cosmétiques, les polymères, ou encore l'énergie, mais leur part dans les publications reste encore minoritaire. Cependant, la diversité des matrices étudiées donne une vue d'ensemble de la polyvalence de la SFC dans l'analyse.

2. Principe de la chromatographie bidimensionnelle

2.1. Modes

En chromatographie bidimensionnelle, il existe plusieurs modes d'analyse en fonction du degré de transfert des analytes de la première dimension (dim1) vers la deuxième dimension (dim2). On distingue deux modes : le mode heart-cut, noté avec « - » et le mode compréhensif, noté avec « × » (voir détails plus loin).

Même s'il existe différents modes, certains éléments sont communs à toutes les configurations bidimensionnelles. Chacun de ces modes assurent un transfert partiel ou total de l'échantillon entre les deux dimensions afin d'augmenter la capacité de séparation par rapport à une séparation unidimensionnelle. En revanche, selon le mode utilisé, les exigences instrumentales peuvent varier. Le mode heart-cut ne nécessite pas forcément deux systèmes chromatographiques complets. En effet, il est possible de stocker la ou les fractions en attendant la fin de la première analyse en dim1. De cette façon, il n'est pas nécessaire d'avoir deux pompes et deux détecteurs en parallèle. À l'inverse, le mode compréhensif impose généralement l'utilisation de deux systèmes couplés, capables de fonctionner simultanément.

Cette différence instrumentale a également des conséquences sur la durée des analyses. Théoriquement, le mode compréhensif permet une durée d'analyse équivalente à celle de la dim1, puisque la dim2 fonctionne en parallèle. En pratique, comme ce mode impose généralement de ralentir la dim1 pour laisser le temps à la dim2 d'analyser chaque fraction, le temps d'analyse total peut être allongé. En mode heart-cut, on n'analyse généralement pas en parallèle la ou les fractions, donc son temps d'analyse global peut être également plus long que dans une analyse unidimensionnelle.

2.1.1. Heart-cut

Le mode heart-cut consiste à extraire une portion relativement large d'un chromatogramme dans la première dimension, correspondant à au moins une largeur de pic, et à l'injecter directement dans la deuxième dimension. Lorsque ce processus est répété et que plusieurs fractions sont extraites pour être injectées dans la seconde dimension, on parle alors de mode Multiple Heart-Cut (MHC).

Le principe de base du mode heart-cut repose sur la collecte de fractions spécifiques du "cœur" de pics chromatographiques. En effet, un échantillon est injecté dans la colonne chromatographique, où une première séparation des composés se fait. Le pic d'intérêt va être collecté puis redirigé vers une autre colonne pour une seconde séparation (Figure I.6A). Cela permet de séparer et quantifier des composés qui auraient pu co-éluer en première dimension, ce qui rendrait leurs identifications difficiles. Le MHC fonctionne de la même façon, mais avec plusieurs coupures effectuées au cours de l'analyse (Figure I.6B).

Un des avantages du MHC est la possibilité de se concentrer uniquement sur les zones d'élution problématiques, tout en réduisant la consommation de solvant par rapport au mode compréhensif complet. Ce mode nécessite généralement des modifications instrumentales mineures pour adapter un système 1D en un système capable de réaliser du MHC. En effet, pour sélectionner, stocker et transférer les fractions, il suffit généralement d'ajouter des vannes et des boucles de collecte au système existant.

Finalement, le MHC est une méthode puissante pour optimiser la séparation de mélanges complexes, en particulier pour optimiser la séparation de certaines zones cibles difficiles à séparer avec une seule colonne. Cette technique améliore la résolution chromatographique, la précision de l'analyse et la capacité de quantification, tout en réduisant les problèmes de co-élution.

2.1.2. Compréhensif

Le mode compréhensif permet d'exploiter de manière plus large le potentiel de la chromatographie bidimensionnelle. Deux variantes principales peuvent être distinguées : le mode compréhensif sélectif, qui ne réanalyse qu'une portion ciblée du chromatogramme en plusieurs fractions, et le mode compréhensif intégral, qui transfère l'intégralité du chromatogramme de dim1 en plusieurs fractions dans la dim2. Chacun de ces modes présente des avantages spécifiques en termes de couverture analytique et dépend des objectifs de l'analyse.

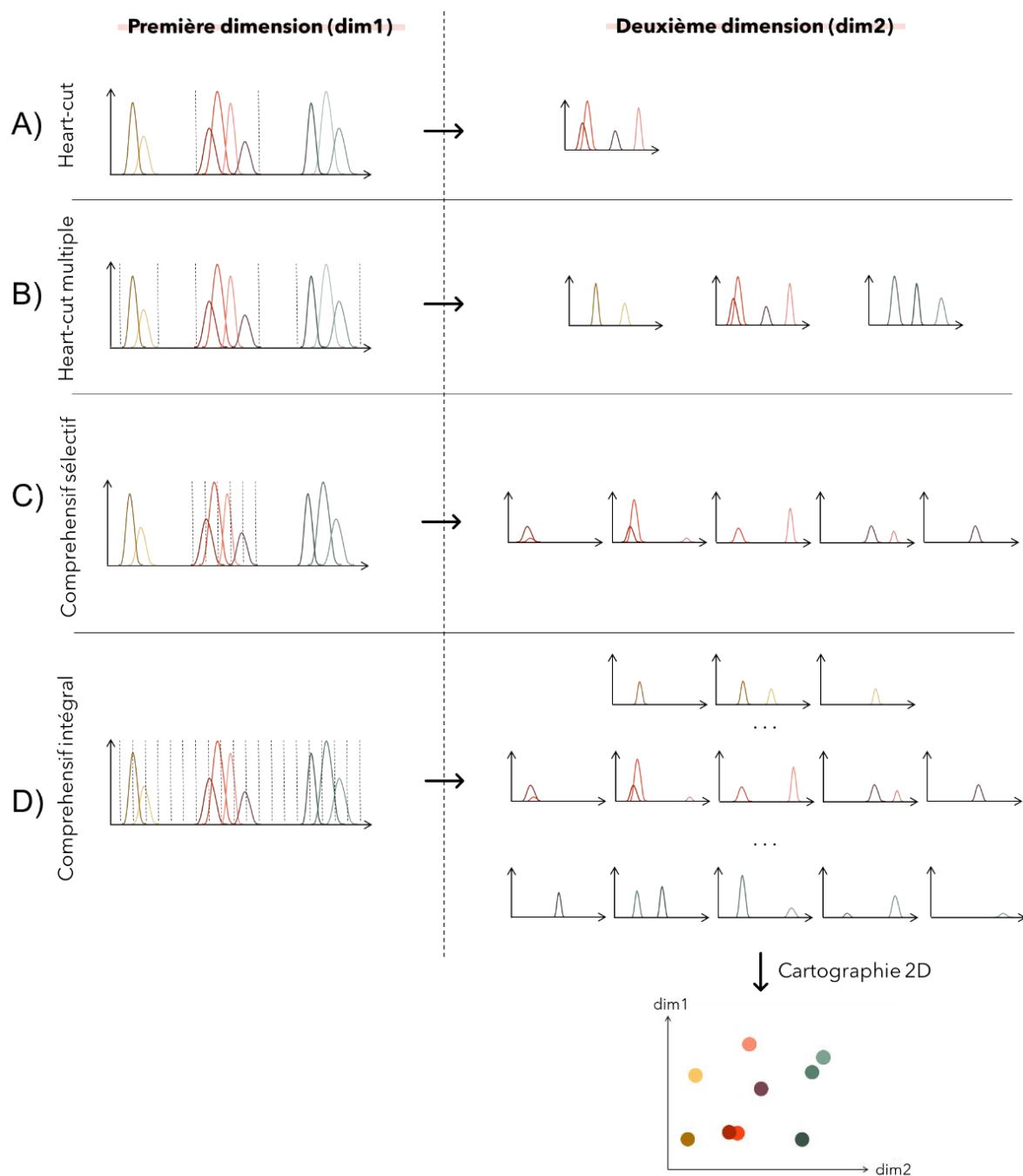


Figure I.6 - Représentation des quatre modes en chromatographie bidimensionnelle. A) heart-cut, B) Heart-cut multiple, C) Comprehensif sélectif et D) Comprehensif intégral. Figure adaptée de [113]

Le mode compréhensif sélectif repose sur un transfert partiel des analytes séparés de la dim1 vers la dim2. Ce mode est à l'intermédiaire entre le MHC et le compréhensif intégral. En effet ce mode permet d'analyser en détail une région du chromatogramme de dim1 (Figure I.6C).

Analyser seulement une partie du chromatogramme permet de réduire la quantité de données et la complexité du traitement, ce qui est un réel avantage. De plus, en limitant le nombre de fractions transférées, ce mode permet d'améliorer la sensibilité et la robustesse de l'analyse en diminuant les risques de superposition et de co-élution en dim2. Cette méthode est particulièrement adaptée aux applications ciblées, où l'objectif est de détecter ou quantifier certaines classes de composés spécifiques plutôt que d'analyser l'ensemble de l'échantillon.

Le mode compréhensif sélectif a aussi des limites. En ne transférant qu'une partie des analytes, il peut conduire à une perte d'information sur la composition globale de l'échantillon, ce qui peut être un inconvénient dans des études exploratoires. De plus, la mise en place de ce mode nécessite une certaine modulation du système pour être capable de détecter les zones d'intérêt et de diriger leur fractionnement, ce qui peut rendre l'instrumentation complexe.

Le mode compréhensif intégral, quant à lui, consiste à transférer l'ensemble des analytes séparés en dim1 vers la dim2, sans aucune sélection préalable. Chaque fraction du chromatogramme de dim1 est collectée et réinjectée en continu dans la seconde colonne. Ainsi l'orthogonalité des deux dimensions chromatographiques est bien exploitée. Cette approche garantit une résolution maximale, puisqu'elle applique une séparation supplémentaire à tous les composés.

L'un des principaux avantages du mode intégral est qu'il fournit une cartographie complète de l'échantillon. C'est un outil idéal pour réaliser des analyses exploratoires et pour caractériser de nouveaux échantillons. Il est particulièrement utilisé dans des secteurs nécessitant un niveau de détail très élevé, comme la pétrochimie ou l'analyse des arômes et parfums.

Ce mode produit une quantité de données très élevée, ce qui requiert des ressources informatiques puissantes pour isoler et identifier efficacement les pics chromatographiques. De plus, la modulation continue des analytes impose une excellente maîtrise des conditions expérimentales, c'est-à-dire une bonne synchronisation entre les deux dimensions.

2.2. Orthogonalité

L'efficacité de la chromatographie bidimensionnelle repose sur le choix judicieux des deux dimensions de séparation. Si les mécanismes de rétention utilisés dans les deux dimensions offrent une sélectivité similaire (mécanisme de rétention comparable), alors les performances globales du système ne seront pas significativement améliorées par rapport à la chromatographie unidimensionnelle. Dans ce cas, les facteurs de rétention dans les deux dimensions sont corrélés, entraînant un regroupement des pics le long d'une diagonale dans l'espace bidimensionnel de séparation (Figure I.7A).

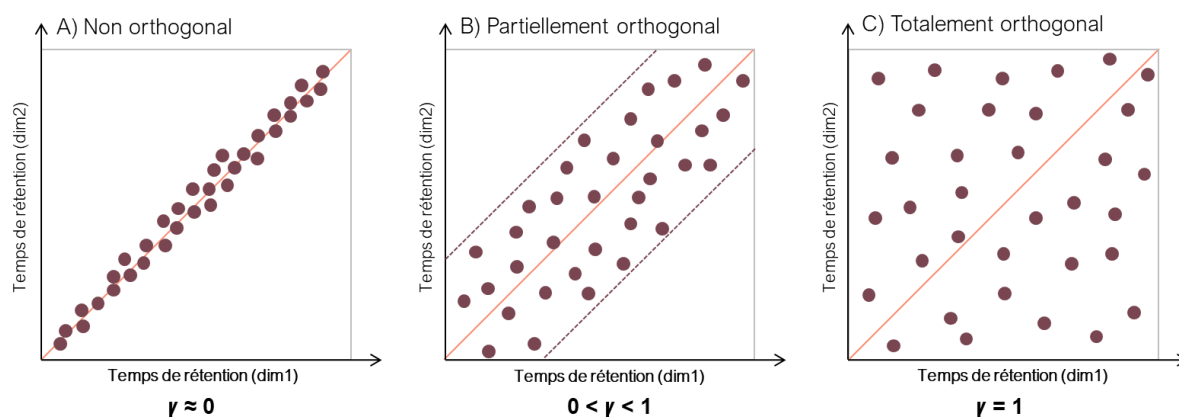


Figure I.7 – Représentation schématique de l'orthogonalité pour des analyses LC×LC. A) système non orthogonal, B) système partiellement orthogonal, C) système totalement orthogonal

A l'inverse, si les deux dimensions présentent une sélectivité complémentaire, les pics seront uniformément répartis dans l'espace de séparation (Figure I.7C). Cette capacité à distribuer les composés de manière homogène est appelée orthogonalité. Cette orthogonalité peut être définie comme le taux d'occupation γ qui représente la proportion de l'espace total de séparation en 2D. Il intervient dans le calcul de la capacité de pics (équation 1) [114].

$$n_{2D, effective} = \alpha \times \beta \times \gamma \times n_1 \times n_2 \quad (1)$$

- $n_{2D, effective}$: capacité de pics effective du système
- n_1, n_2 : capacités théoriques des première et seconde dimensions
- γ : taux d'occupation, mesurant l'efficacité d'utilisation de l'espace 2D
- α : facteur de correction lié à l'échantillonnage insuffisant en première dimension
- β : facteur tenant compte de l'élargissement des bandes en seconde dimension, généralement dû à un transfert non idéal entre les deux dimensions

L'orthogonalité est un descripteur de qualité pour évaluer un système 2D. Elle n'apparaît pas directement dans l'équation précédente, mais elle a un impact sur le taux d'occupation (γ), qui montre la distribution des composés dans l'espace 2D. Une bonne orthogonalité permet de mieux répartir les composés et donc d'avoir une meilleure efficacité.

Pour que le fonctionnement d'une méthode 2D soit optimale, il faut choisir deux dimensions qui soit compatibles. Plusieurs critères doivent être pris en compte lors de cette sélection : l'orthogonalité des mécanismes de séparation, la compatibilité des solvants, les contraintes liées à la détection, ainsi que la faisabilité instrumentale du couplage.

Le tableau ci-dessous (Table I.3) est adapté des travaux récents de van den Hurk *et al.* [115] et de Heinisch *et al.* [113]. Il présente une table de compatibilité entre les quatre grands modes chromatographiques utilisés en 2D-LC/SFC : la chromatographie en phase inverse (RPLC), la chromatographie d'interaction hydrophile (HILIC), la chromatographie en phase normale (NPLC) et la chromatographie par fluide supercritique (SFC). Chaque combinaison est évaluée selon plusieurs critères techniques, représentés par des barres de notation (entre 1 et 5) et du vert (facile à développer) au rouge (complexe à développer).

Table I.3 – Compatibilité des principaux couplages en chromatographie liquide bidimensionnelle selon la complexité de développement et 4 critères : Breakthrough (B), Orthogonalité (O), Compatibilité avec la masse (M), et Compatibilité des phases mobiles (X)

		Deuxième dimension (dim2)			
		RPLC	HILIC	NPLC	SFC
Première dimension (dim1)	RPLC	B O M X	B O M X	B O M X	B O M X
	HILIC	B O M X	B O M X	B O M X	B O M X
	NPLC	B O M X	B O M X	B O M X	B O M X
	SFC	B O M X	B O M X	B O M X	B O M X

+ Complexité de développement -

Les quatre critères considérés sont les suivants :

- Breakthrough (B) : ce critère reflète l'impact de la force d'élution du solvant injecté de la dim1 sur la séparation en dim2. Celui-ci donne généralement des effets indésirables sur la séparation chromatographique, qui peuvent aller d'un léger élargissement à une déformation sévère du pic. Dans le cas le plus extrême, le facteur de rétention de l'analyte en tête de la colonne est si faible au moment de l'injection que l'analyte passe par la colonne avec très peu d'interaction avec la phase stationnaire et sort donc très proche du temps mort dans la colonne. C'est ce qu'il peut se passer lorsque le solvant de la dim1 est trop fort par rapport à la phase mobile de la 2D. Cela compromet fortement la qualité de la séparation.
- Orthogonalité (O) : il s'agit du degré d'indépendance entre les mécanismes de séparation de la dim1 et de la dim2. Une orthogonalité élevée reflète une complémentarité maximale entre les

deux dimensions, ce qui permet d'explorer une gamme de composés plus étendue et d'améliorer la résolution globale.

- Compatibilité avec la MS (M) : Ce critère évalue la capacité de la dim2 à être directement couplée à la MS. Cette compatibilité repose notamment sur la possibilité d'utiliser des solvants et des additifs compatibles avec les techniques d'ionisation les plus couramment utilisées : l'électrospray (ESI) ou l'ionisation chimique à pression atmosphérique (APCI). Cette compatibilité est devenue un paramètre essentiel, car le couplage avec la MS est aujourd'hui une nécessité pour l'analyse de la majorité des échantillons complexes.
- Compatibilité des phases mobiles (X) : la miscibilité entre les phases mobiles des deux dimensions est essentielle pour éviter les précipitations ou les problèmes de pression. Une faible miscibilité peut perturber le transfert de fractions entre les dimensions et altérer la reproductibilité de la méthode.

Une attention particulière est nécessaire pour les combinaisons impliquant la SFC. En effet, selon le choix de phase stationnaire, la SFC peut adopter des comportements très variés, similaires aux modes NP ou RP en phase liquide. Ainsi, toutes les combinaisons sont possibles avec une bonne orthogonalité, et résumer la SFC à un mode unique dans ce tableau est donc une vision un peu réductrice.

L'analyse de ce tableau permet néanmoins de mettre en évidence certaines combinaisons particulièrement intéressantes. Par exemple, les couples RPLC-HILIC et HILIC-RPLC sont largement utilisés [116] car ils offrent une bonne orthogonalité, même si le couplage RPLC-HILIC est moins évident que l'inverse (Table I.3). Ces configurations ont cependant une compatibilité satisfaisante des solvants et une excellente aptitude au couplage avec la détection MS [31]. De même, RPLC-SFC s'avère être un couplage prometteur, notamment grâce à la faible utilisation de solvant en SFC mais aussi dans son efficacité dans la séparation de composés hydrophobes ou peu polaires [117,118]. En revanche, certaines combinaisons comme RPLC-NPLC ou NPLC-RPLC présentent de fortes limitations, dues à une mauvaise miscibilité des solvants et un manque de compatibilité avec la détection MS dans les configuration ou la NPLC se retrouve en dim2 [119].

Finalement, cette approche multicritère fournit une vue d'ensemble et une bonne base pour le développement de méthodes 2D-LC. Elle permet d'anticiper les incompatibilités potentielles et d'optimiser la performance globale de la séparation en choisissant des paires de dimensions qui sont à la fois orthogonales et techniquement compatibles. Dans ce contexte, il est pertinent d'examiner les tendances actuelles en matière de sélection des techniques chromatographiques utilisées en première et seconde dimension.

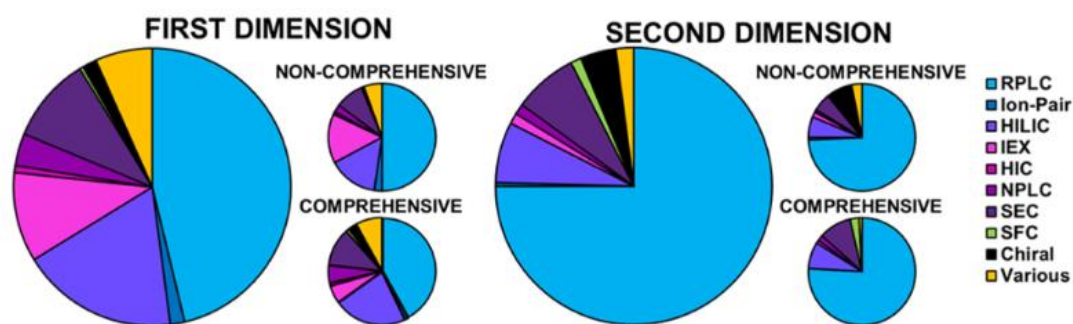


Figure 1.8 – Vue d’ensemble de l’utilisation des différentes techniques de chromatographie en phase liquide entre la première et la deuxième dimension et la répartition entre les modes compréhensif et non-compréhensif [115]

Cette figure illustre l’utilisation des différentes techniques chromatographiques dans la première et la seconde dimension utilisées usuellement dans les différents types de chromatographie liquide bidimensionnelle, en distinguant les approches compréhensive et non-compréhensive.

Dans la première dimension, la chromatographie en phase inverse (RPLC) est largement prédominante, aussi bien pour les approches compréhensive que non-compréhensive [43]. D’autres techniques, telles que l’HILIC (chromatographie à interactions hydrophiles) [30,120] et l’IEX (échange d’ions) [121], sont également employées en première dimension.

Dans la seconde dimension, la RPLC reste la technique dominante, avec une prépondérance encore plus marquée qu’en première dimension [122]. Quelques autres approches, comme l’HILIC et les techniques chirales, sont utilisées mais restent plus faiblement utilisées. La diversité des techniques est plus importante dans les modes compréhensif que non-compréhensif.

Globalement, cette figure met en évidence que la RPLC est la méthode la plus couramment utilisée en 2D-LC, tant en première qu’en seconde dimension. Toutefois, la première dimension présente une plus grande diversité de techniques que la seconde, où la RPLC domine encore davantage. L’approche compréhensive semble également favoriser une utilisation plus variée des différents modes chromatographiques [123].

2.3. Applications

La chromatographie bidimensionnelle s’est imposée ces dernières années. Grâce à sa capacité à augmenter considérablement le pouvoir de séparation par la combinaison orthogonale de deux dimensions chromatographiques, elle est une technique puissante pour l’analyse de mélanges complexes. Elle est aujourd’hui appliquée dans un grand nombre de domaines. Toutefois, le choix

du mode d'analyse (compréhensif ou non-compréhensif) dépend fortement des objectifs analytiques et des contraintes propres à chaque secteur.

Van den Hurk *et al.* ont présenté le nombre d'applications de la chromatographie liquide bidimensionnelle dans divers domaines [115]. Pour chacun, ils ont différencié les approches non-compréhensive et compréhensive. Dans le domaine biopharmaceutique, on observe une grande prédominance du mode non-compréhensif, utilisé environ deux fois plus souvent que les méthodes compréhensives [124,125]. Ce constat montre une préférence pour des analyses plus ciblées ou simplifiées dans ce secteur. Le domaine des produits pharmaceutiques est celui qui compte le plus grand nombre d'applications en 2D-LC, avec également une forte utilisation des approches non-compréhensive [126,127]. En revanche, la médecine traditionnelle chinoise (TCM) montre une tendance opposée, avec une utilisation majoritairement compréhensive [128,129]. Le secteur alimentaire compte aussi beaucoup d'applications mais seulement une légère préférence pour le mode compréhensif [38,130].

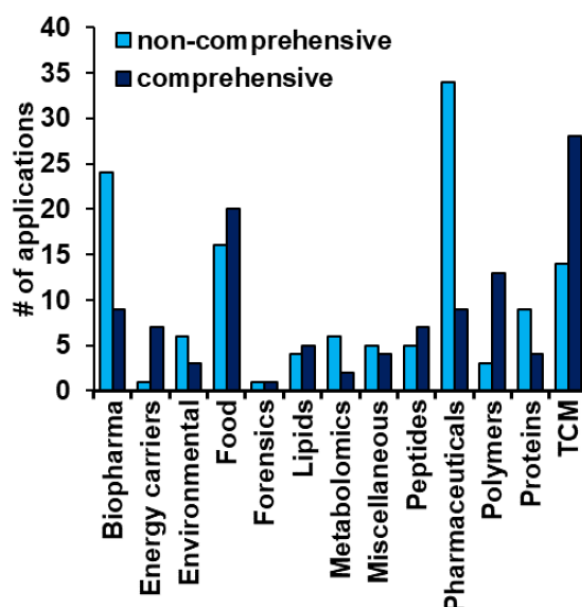


Figure I.9 – Nombre d'applications publiées par domaine pour le mode non-compréhensif (bleu clair) et compréhensif (bleu foncé) [115]

D'autres secteurs comme l'environnement, la métabolomique, les polymères et les protéines présentent une répartition plus homogène entre le compréhensif et le non-compréhensif. Dans d'autres domaines, comme pour les lipides, la criminalistique ou dans le secteur de l'énergie, le nombre d'applications est plus restreint, même si un léger avantage est observé pour le compréhensif. Finalement cette figure montre que le choix d'un mode ou de l'autre varient fortement en fonction des besoins analytiques de chaque secteur.

Que ce soit dans le choix de combinaison de techniques chromatographique ou bien celui du mode compréhensif ou non un contrôle précis du transfert de l'échantillon est nécessaire. Des progrès récents et diversifiés comme ceux sur les vannes de commutation et sur les technologies de modulation ont considérablement amélioré la robustesse et la reproductibilité de ces transferts.

3. Instrumentation

3.1. Vannes

La chromatographie bidimensionnelle nécessite un transfert d'échantillons entre deux dimensions. Ce transfert repose sur des vannes multiports permettant la commutation entre le chargement en dim1 et l'injection en dim2, tout en limitant les volumes morts et en assurant une bonne répétabilité. Ces optimisations sont cruciales dans les configurations impliquant des phases mobiles compressibles ou à faible viscosité, comme en SFC. En effet, la rapidité et la fiabilité de la commutation est directement liée à la qualité du transfert. Elles restent néanmoins tout aussi importantes en LC, où la stabilité des débits et le contrôle des volumes morts sont essentiels pour assurer un transfert efficace, sans dispersion ni distorsion des pics transférés.

3.1.1. Vannes 6 ports – 2 positions

L'utilisation de deux vannes 6 ports à 2 positions est une solution robuste et simple à mettre en place pour les configurations en mode heart-cutting comme en mode compréhensif. En position A, le contenu d'une première boucle (boucle 1) est injecté dans la seconde dimension, tandis que la seconde (boucle 2) est remplie avec l'échantillon et la phase mobile de la dim1 (Figure I.10). Au moment de la rotation, les rôles s'inversent. Cette alternance garantit une bonne symétrie de parcours entre les deux boucles, et permet une modulation efficace avec un minimum de dispersion.

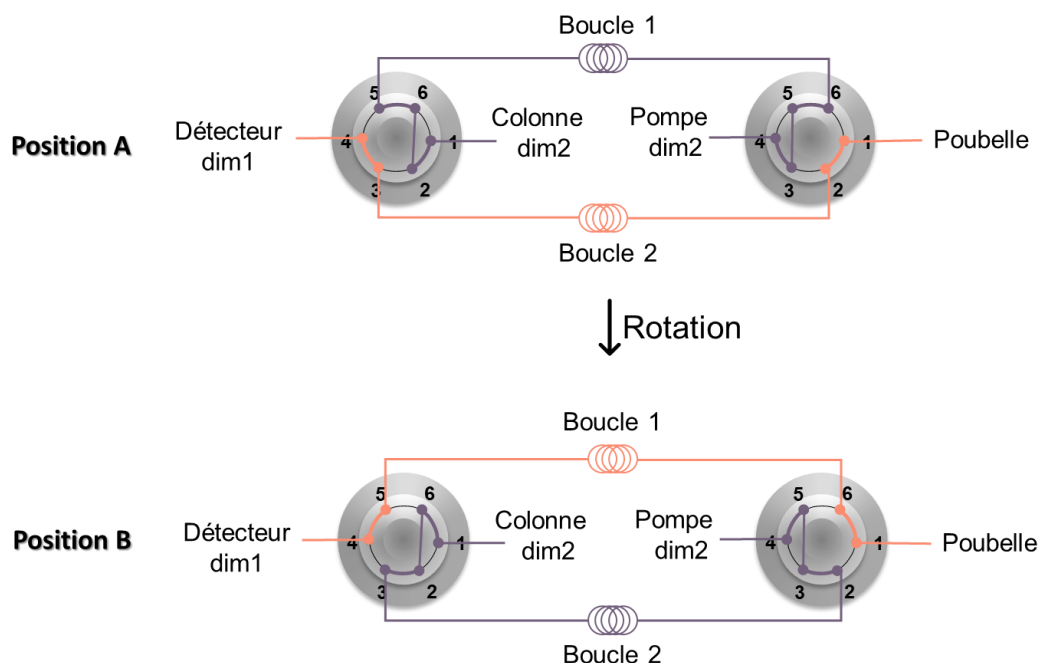


Figure I.10 - Fonctionnement d'une configuration avec deux vannes 6 ports 2 positions

Leur faible coût et leur facilité d'installation en font une bonne solution pour les laboratoires [131]. Dixon *et al.* proposent également une revue des applications biomédicales et pharmaceutiques des systèmes 2D. Ils présentent notamment 2 systèmes avec une configuration en MHC et une en compréhensif confirmant leur efficacité pour le transfert et la modulation [45]. Stevenson *et al.* ont utilisé des vannes à 6 ports et deux positions dans des systèmes 2D-LC pour évaluer les performances de séparation. Ils ont mis en œuvre une méthode automatisée de détection des pics appelé « peak picking », ce qui permet de quantifier objectivement l'efficacité du couplage entre les deux dimensions [23].

3.1.2. Vannes 10 ports – 2 positions

La vanne à 10 ports et 2 positions est largement répandue dans les systèmes de chromatographie bidimensionnelle, notamment en LC×LC, LC×SFC et SFC×SFC. Ce type de vanne intègre les deux boucles directement dans une même vanne. A chaque changement de position, une boucle est injectée tandis que l'autre se remplit. Cette solution est particulièrement efficace en mode compréhensif. C'est ce que montre la revue de Shi *et al.* en recensant les différentes vannes et boucles utilisées dans différentes configurations de 2D-LC [22]. Les volumes reportés vont de 1,57 μL à 500 μL , selon les objectifs analytiques et les contraintes instrumentales. Par exemple, Uliyanchenko *et al.* utilisent deux boucles de 100 μL dans un système LC×LC pour la séparation

de polymères [132], tandis que Mondello *et al.* utilisent des boucles de 11 μL pour la séparation de triglycérides [133].

Ces vannes ont aussi été utilisées dans des configurations contenant la SFC dans l'une des dimensions. François *et al.* ont implémenté un circuit SFC×RPLC avec ce type de vanne et une boucle de make-up pour optimiser le piégeage des analytes [134]. De même Hirata *et al.* ont réalisé un système SFC×SFC en mode pressure-stop démontrant l'efficacité des vannes 10 ports [135]. Sanchez et Devaux ont également utilisé ce type de vannes dans des configuration RPLC×SFC [117,136].

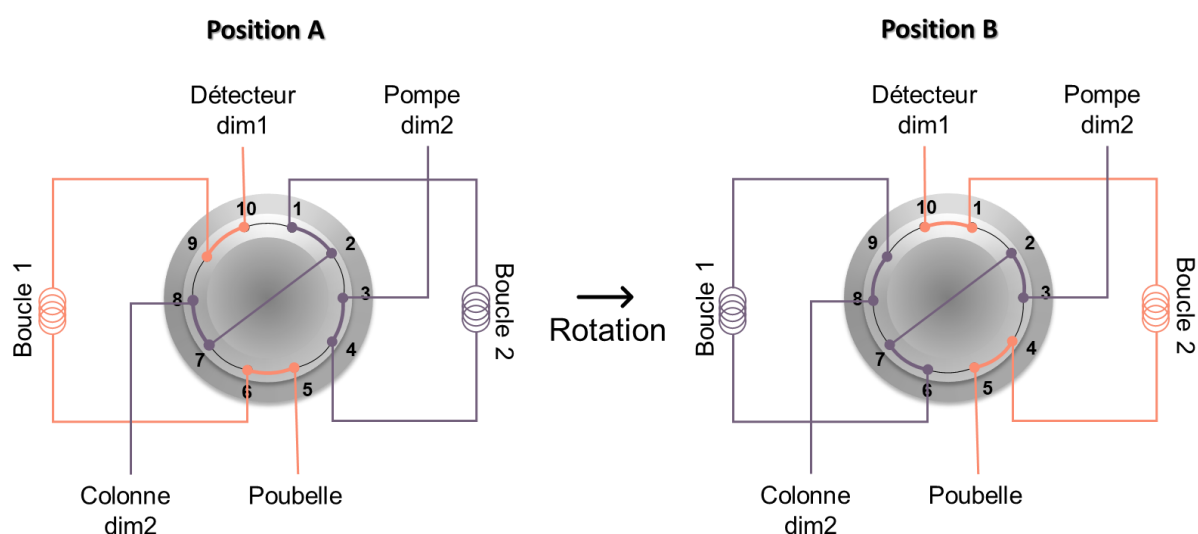


Figure I.11 - Fonctionnement d'une configuration avec une vanne 10 ports 2 positions

Cependant, dans cette configuration l'échantillon ne parcourt pas le même trajet en position A et en position B (Figure I.11). En effet un raccord est présent entre les positions 7 et 2. En position A (en violet), ce raccord se situe après la boucle, ce qui allonge le chemin parcouru par l'échantillon avant son injection en dim2. En revanche, en position B (en orange), le raccord intervient avant la boucle, réduisant ainsi la longueur du parcours. Cette asymétrie engendre un décalage systématique des temps de rétention entre les fractions en dim2. Pour corriger cette différence, il est nécessaire de procéder à un réalignement numérique des pics en dim2, afin de garantir une comparaison fiable et une interprétation correcte des données obtenues.

3.1.3. Vannes 2×4 ports – 2 positions

La vanne double à 4 ports et 2 positions est une alternative à la vanne 10 ports 2 positions. Cette configuration est symétrique et assure alternativement le remplissage et l'injection des boucles. Cela simplifie l'instrumentation tout en conservant une modularité élevée, particulièrement

précieuse en SFC pour gérer les contraintes de pression, de compatibilité des solvants et de dilution [137].

Ce type de vanne est également utilisé dans d'autres travaux comme ceux de Stoll *et al.* sur le couplage RPLC×IEX [138] ou de Caño-Carrillo *et al.* sur la LC-2D avec les modes comprehensif et heart-cutting [39], démontrant sa polyvalence.

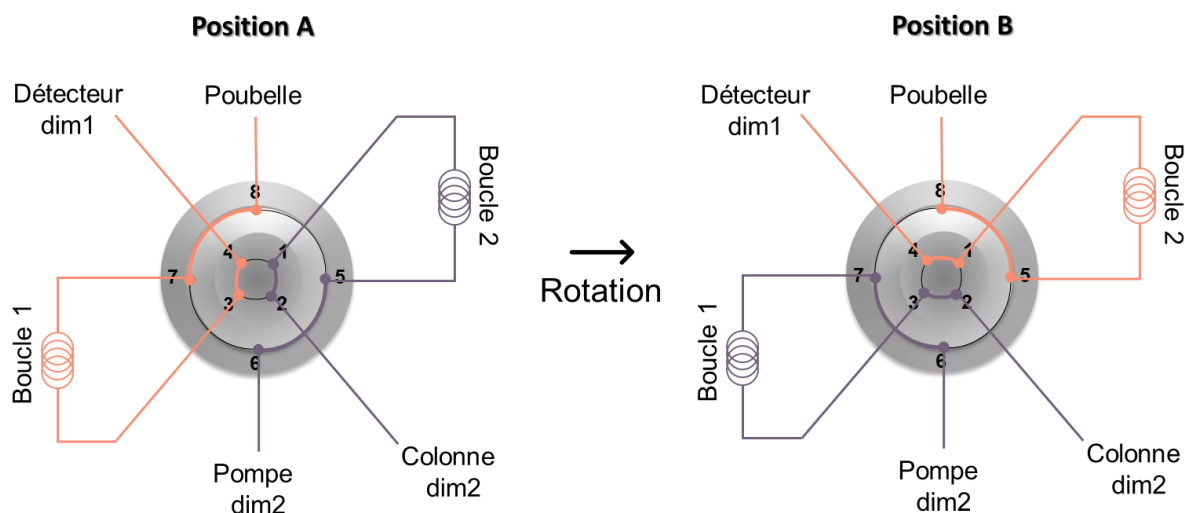


Figure I.12 - Fonctionnement d'une vanne double 4 ports 2 positions

Quel que soit le type de vanne, pour passer d'un mode heart-cut à un mode multiple heart-cut, il suffit de changer les boucles simples par des sélecteurs contenant plusieurs boucles, que l'on appelle aussi des decks.

3.2. Modulation

Avec l'évolution de la chromatographie bidimensionnelle, la modulation est devenue un élément central pour assurer un transfert efficace entre les deux dimensions. Plusieurs types de modulation ont été développés pour répondre à des besoins analytiques spécifiques [115,139]. Le choix dépend du domaine d'application et des contraintes chromatographiques.

3.2.1. Modulation passive

La modulation passive est la forme la plus simple de transfert de fraction entre deux dimensions. Ce mode consiste à utiliser les vannes telles que présentées précédemment. Aucun ajout n'est nécessaire. C'est-à-dire que le solvant d'injection de la dim2 est la phase mobile de la dim1. Cette modulation est simple à mettre en place mais a certaines limitations. En effet, la compatibilité des

phases mobiles entre les dimensions est limitée et elle induit aussi un risque de dilution des analytes, qui peut affecter la sensibilité de la méthode.

Pour atténuer ces effets, des variantes ont été proposées, comme l'utilisation de boucles de modulation remplies (*packed loops*). Ces dernières, en remplissant les boucles de particules inertes, permettent de limiter la diffusion radiale et de réduire l'élargissement des pics. En 2023, Knol *et al.* ont démontré que l'utilisation de ces boucles permet de limiter la dispersion des analytes et d'éviter le sur-remplissage de la boucle [140].

Ce concept, déjà exploré dans les années 1980 par Coq *et al.*, avait montré un potentiel de réduction de l'élargissement des bandes d'injection en chromatographie unidimensionnelle (1D-LC) à grand volume, et reste pertinent dans un contexte 2D moderne [141].

3.2.2. Modulation assistée par phase stationnaire (SPAM)

Dans ce type de modulation, les analytes issus de la dim1 sont piégés sur de petites colonnes de rétention, appelées colonnes de piégeage, qui remplacent les traditionnelles boucles de stockage. Tandis que la phase mobile de dim1 traverse le système sans être retenue, les analytes d'intérêt sont capturés temporairement par la phase stationnaire. Ensuite, une vanne active permet l'élution rapide de ces analytes vers la dim2.

Cette approche présente de nombreux avantages comme la réduction du temps d'analyse, la suppression de la phase mobile incompatible, l'amélioration de la sensibilité et de la reproductibilité. Il est également possible d'adapter les diamètres de colonnes dim1/dim2 sans perte de performance [142].

La SPAM a été appliquée avec succès dans de nombreux contextes analytiques complexes, notamment pour l'étude des antioxydants naturels [143], la caractérisation protéomique de levures [142], ou encore l'analyse chirale de médicaments *in vivo* via un couplage 2D-LC-SFC-MS [144]. Elle est également utilisée pour optimiser les performances dans les combinaisons HILIC×RPLC, où des approches de modulation focalisantes ou non sont comparées pour maximiser la sensibilité [145].

3.2.3. Modulation active de solvant (ASM)

Contrairement à la modulation passive et à la SPAM, l'ASM modifie activement la composition du solvant de l'effluent de la dim1 avant son injection dans la dim2, en le diluant avec un éluant faible spécifique à la dim2. Ce processus permet une focalisation efficace des analytes en tête de

colonne de dim2, ce qui améliore la résolution, réduit les effets de breakthrough et limite la dilution chromatographique [35].

Développée principalement pour des systèmes RPLC×RPLC, cette technique s'est révélée particulièrement utile pour gérer l'incompatibilité de solvant entre des séparations de type HILIC×RPLC [30,146] ou SEC×LC [147], où la forte proportion d'acétonitrile ou de solutés aqueux pose souvent problème.

Gargano *et al.* ont démontré que l'ASM permettait de réduire le volume des fractions collectées, accélérer la dim2, et diviser par deux le temps d'analyse global, tout en réduisant significativement la consommation de solvant [25].

Enfin, l'étude de Sanchez *et al.* a élargi le champ d'application de l'ASM à la chromatographie LC×SFC. Six interfaces ont été comparées, notamment le transfert du contenu de la boucle à l'aide d'un flux de CO₂ ou d'un flux de modificateur, cette dernière étant la plus facile à mettre en œuvre et la plus flexible (Figure I.13) [136]. Ces résultats ouvrent des perspectives prometteuses pour le couplage efficace LC×SFC, jusque-là limité par des incompatibilités de phases mobiles.

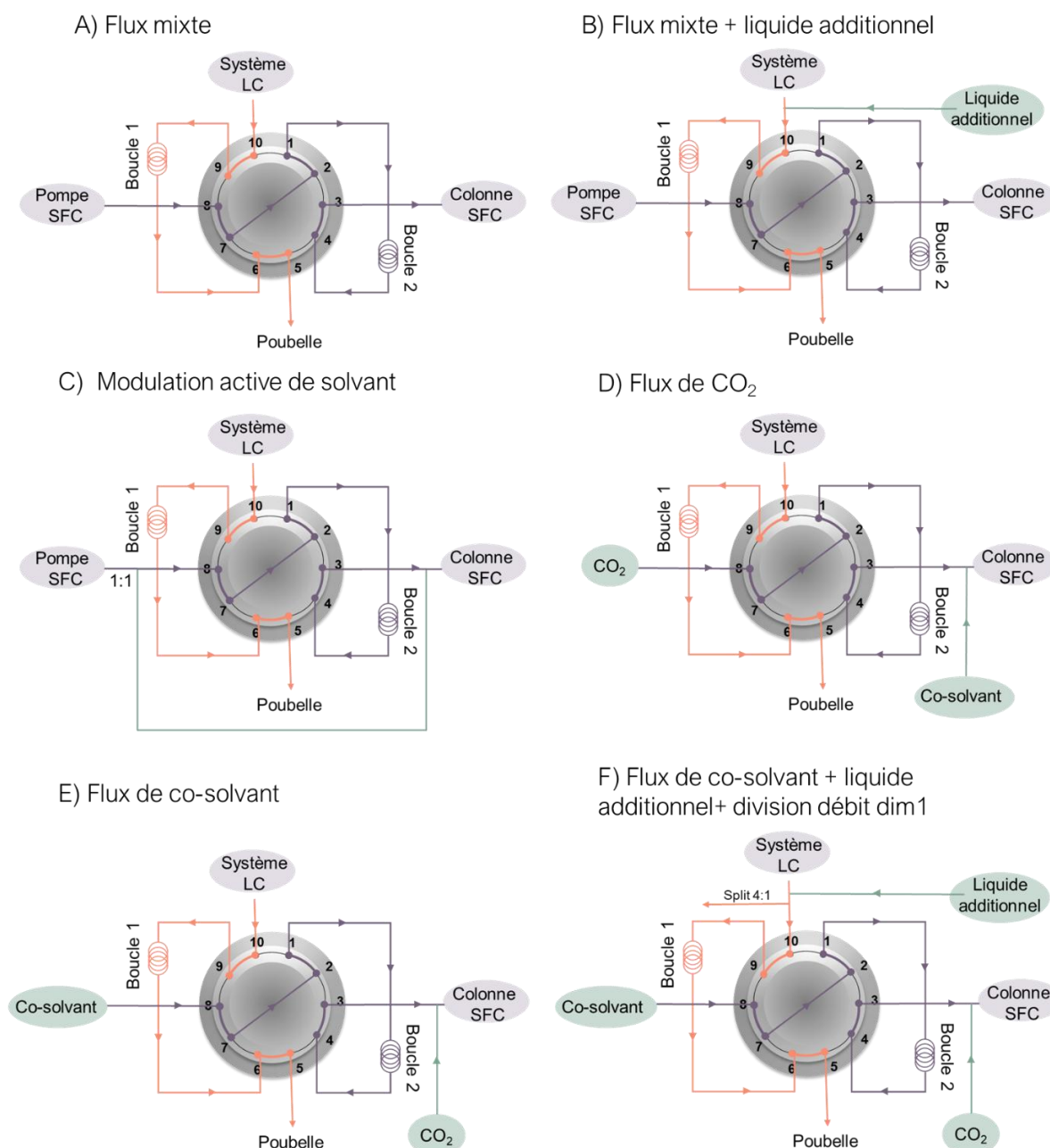


Figure I.13 – Modulations de configurations LC×SFC A) flux mixte, B) flux mixte + liquide additionnel C) modulation active de solvant, D) flux de CO₂, E) flux de co-solvant, and F) flux de co-solvant + liquide additionnel + division débit dim1. Adaptée de [136]

3.2.4. Modulation de l'évaporation sous vide (VEM)

La VEM (*Vacuum Evaporation Modulation*) permet l'évaporation du solvant de la dim1 sous vide, avant l'introduction dans la dim2. Cette approche est principalement utilisée pour les systèmes NPLC×RPLC, où les phases mobiles sont fortement incompatibles.

Des travaux ont démontré que cette technique permet un transfert rapide et efficace entre les deux dimensions, réduisant la dispersion de bande et améliorant la performance globale du

système 2D-LC, sans affecter l'efficacité ou la sélectivité des séparations individuelles [148,149]. Cependant, la perte d'analytes, notamment les composés volatils, reste un problème potentiel. Cette limitation a été explorée dans l'étude de Ding *et al.*, qui a montré que les pertes sont négligeables pour les composés non volatils ou ayant un point d'ébullition supérieur à 340 °C [149]. De plus, la VEM a été appliquée avec succès dans l'analyse de matrices complexes, telles que des extraits de plantes médicinales [150] ou des lipides plasmatiques dans le cadre de recherches biomédicales [151].

Un autre type de module basé sur l'évaporation a été développé, la modulation par membrane d'évaporation (EMM). Cette technique repose sur l'évaporation sélective du solvant à travers une membrane hydrophobe poreuse, permettant de réduire considérablement le volume des fractions transférées à la dim2. En 2018, Fornells *et al.* ont démontré que ce procédé permet d'atténuer les incompatibilités de phase mobile et d'améliorer à la fois la reproductibilité et la résolution des séparations 2D [152]. Le volume transféré à la dim2 étant jusqu'à 10 fois plus faible, cela conduit à une meilleure concentration des analytes et une amélioration significative des performances chromatographiques.

3.2.5. Modulation thermique

La modulation thermique repose sur le principe de la désorption thermique : une impulsion de température rapide est appliquée pour remobiliser les analytes, qui sont ensuite injectés dans la deuxième dimension. Ce processus peut être répété cycliquement pour assurer une modulation continue et précise.

Dans l'étude de Verstraeten *et al.*, un système de chromatographie liquide à faible masse thermique a été utilisé pour concevoir un dispositif de modulation thermique [153]. Cette modulation est capable à la fois de concentrer les analytes et de servir d'interface d'injection pour la deuxième colonne. Ce qui permet de minimiser la dispersion des pics et d'améliorer la sensibilité.

De plus, la modulation thermique longitudinale sur colonne, développée par Creese *et al.*, a montré des avantages par rapport à la modulation par vanne [154]. En effet, une réduction significative des perturbations de la ligne de base et une amélioration du rapport signal/bruit allant de 6 à 14 fois ont été observées, pouvant être dues à la cryo-focalisation.

3.3. Problèmes liés au couplage

Le couplage de deux dimensions soulève des défis majeurs, parmi lesquels la dilution, les incompatibilités de solvants, les problèmes d'injection et les pertes de performances analytiques.

3.3.1. Effets de dilution entre les dimensions

Il existe une dilution des analytes entre les dimensions de la chromatographie bi-dimensionnelle. Ce qui est une contrainte inévitable et qui a une incidence sur la sensibilité. Cependant, elle peut être prédite. Aebischer *et al.* ont modélisé cet effet pour la HILIC×RPLC à partir d'un facteur de dilution, dépendant du volume, de la nature et du pourcentage de solvant dans la phase d'injection. Cette prédiction permet d'identifier des valeurs minimales de dilution nécessaires pour obtenir des pics symétriques [42]. Guilleme *et al.* ont décrit le facteur de dilution comme un des descripteurs de qualité importants variant selon les conditions expérimentales [43]. En effet, une dilution excessive peut réduire la sensibilité, ce qui montre l'importance d'optimiser les conditions pour maintenir un bon équilibre entre capacité de pic et dilution [34,155].

3.3.2. Problèmes d'injection et de compatibilité de solvant

La compatibilité entre les solvants utilisés dans chaque dimension est essentielle pour maintenir une bonne forme de pic et éviter les phénomènes de dégradation chromatographique. L'un des défis les plus importants concerne l'injection dans la dim2, notamment lorsque celle-ci repose sur des conditions chromatographiques très différentes. C'est notamment le cas dans le couplage RPLC×SFC. Bien que ce type de couplage offre une forte orthogonalité, il reste techniquement contraignant, en raison de la mauvaise miscibilité entre l'eau (issue de la dim1) et le CO₂ supercritique de la dim2 [156,157]. Ce couplage a été étudié par Devaux *et al.* [158]. La Figure I.14 issue de leur étude démontre l'impact direct de la composition du solvant d'injection sur la forme et l'intensité des pics en SFC. Les injections de solutions très aqueuses provoquent un élargissement ou une disparition des pics, dues à la mauvaise miscibilité de l'eau avec le CO₂ supercritique.

Sarrut *et al.* montrent également les limites pratiques du couplage RPLC×SFC pour des matrices très hydrophiles, telles que les extraits aqueux de bio-huile [159]. L'injection d'eau dans une colonne SFC peut poser des problèmes de reproductibilité, principalement en raison de l'adsorption de l'eau sur la phase stationnaire. Ce phénomène dépend fortement de la nature de la phase stationnaire utilisée, ainsi que de la composition de la phase mobile. Il est généralement atténué lorsque l'eau est déjà présente, même en faible proportion, dans la phase mobile.

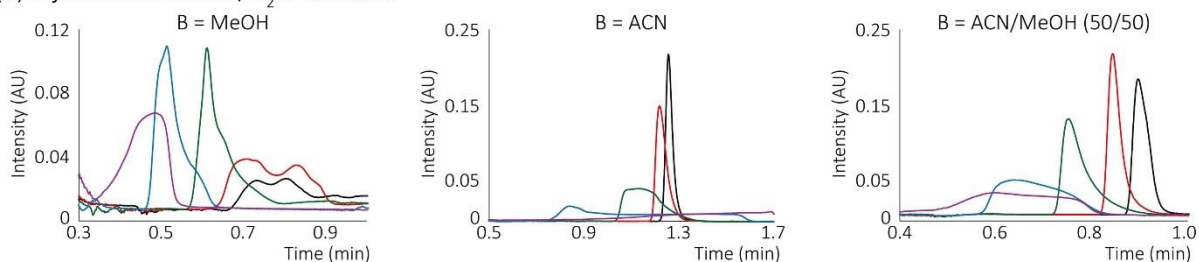
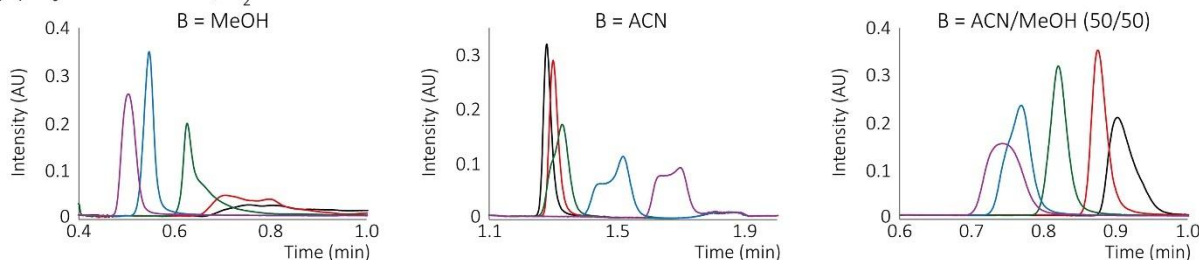
(a) Injection in MeOH/H₂O mixtures(b) Injection in ACN/H₂O mixtures

Figure I.14 – Injection de caféine sur une colonne BEH 2-EP a) MeOH/H₂O et b) ACN/H₂O. Comparaison de différents solvants d'injection : 5% H₂O (violet), 25% H₂O (bleu), 50% H₂O (vert), 75% H₂O (rouge) et 95% H₂O (noir) [158]

De la même façon, Chapel *et al.* ont évalué différentes stratégies pour préserver la symétrie des pics lors du couplage HILIC×RPLC [160]. Malgré une forte différence de polarité entre les deux phases, ils ont montré que l'utilisation de gradients progressifs permet de limiter les distorsions de pics et conserve la symétrie.

Finalement, les performances analytiques d'un système 2D ne dépendent pas uniquement du choix des dimensions de colonnes et de phase stationnaire, mais aussi de la gestion du couplage entre les deux dimensions.

3.4. Systèmes incluant la SFC

D'après la Figure I.8 de la section 2.2., la SFC est très peu utilisée en chromatographie bidimensionnelle. Cependant, la SFC s'est révélée extrêmement prometteuse grâce à sa rapidité et sa sélectivité complémentaire à d'autres modes chromatographiques. L'intégration de la SFC dans des montages bidimensionnels, qu'ils soient purement en SFC ou hybrides avec la RPLC, a permis d'élargir considérablement les capacités de séparation.

Les couplages hybrides entre la RPLC et la SFC sont possibles dans les deux sens. Dans les systèmes RPLC×SFC, la première dimension utilise un solvant majoritairement aqueux, ce qui introduit une complexité technique : le CO₂ supercritique est peu miscible avec l'eau, rendant le transfert entre les dimensions délicat. Pour contourner cette incompatibilité, plusieurs stratégies

ont été proposées : évaporation de la phase mobile, piégeage sur cartouches ou colonnes courtes, dilution avec un solvant miscible avec le CO₂, ou encore couplage par évaporation rapide suivi de reconcentration [157,159]. Ces approches ont permis de développer des dispositifs efficaces pour l'analyse d'extraits naturels [161,162], d'échantillons pharmaceutiques complexes [163], de composés issus de bio-huiles [159] ou encore de biocarburants [117,118].

À l'inverse, certains montages exploitent la configuration SFC×RPLC, où le fluide supercritique constitue la phase mobile de la première dimension. Dans ce cas, le transfert est techniquement plus simple puisque l'élimination du CO₂ est généralement plus rapide. Yang *et al.* ont proposé un dispositif SFC×RPLC-MS en ligne pour le profilage lipidique, démontrant l'intérêt de la complémentarité des mécanismes de rétention [164]. Dans certains cas, comme chez François *et al.*, la RPLC est utilisée pour affiner la séparation de classes de composés déjà partiellement résolus en SFC [134,165].

En parallèle de ces systèmes mixtes, des développements majeurs ont également été réalisés avec des configurations purement basées sur la SFC, parmi lesquelles on distingue les couplages SFC-SFC, SFC×SFC, et le plus complexe SFE-SFC×SFC. Un des points importants dans les configurations incluant la SFC est de réussir à maintenir la pression dans le système pour maintenir le fluide dans son état supercritique. La figure suivant présente ces trois configurations (Figure I.15).

La configuration SFC-SFC en mode heart-cut (Figure I.15A) présentée par Zeng *et al.* a été utilisée pour la purification énantiomérique de principes actifs pharmaceutiques [166]. Cette configuration ne contient qu'une seule boucle de stockage et quatre vannes. Cette configuration permet de n'avoir qu'un seul régulateur de pression (BPR), ce qui simplifie sa mise en place.

La configuration SFC×SFC est plus complexe, nécessitant une modulation complète du flux de la dim1 vers la dim2. Hirata *et al.* se sont penchés sur ce système (Figure I.15B) [135,167]. Pour leur configuration, ils ont utilisé deux vannes, une 6 ports 2 positions et une de 10 ports 2 positions. Du côté du maintien de la pression, ce système se complexifie car il y a deux BPR sur chacune des voies, plus un restricteur de pression. Sur la même base, Okamoto *et al.* ont pu inclure une dimension supplémentaire à la configuration SFC×SFC, celle de l'extraction par fluide supercritique (SFE) [168]. La première vanne 6 ports 2 positions a simplement été échangée par une vanne 10 ports 2 positions.

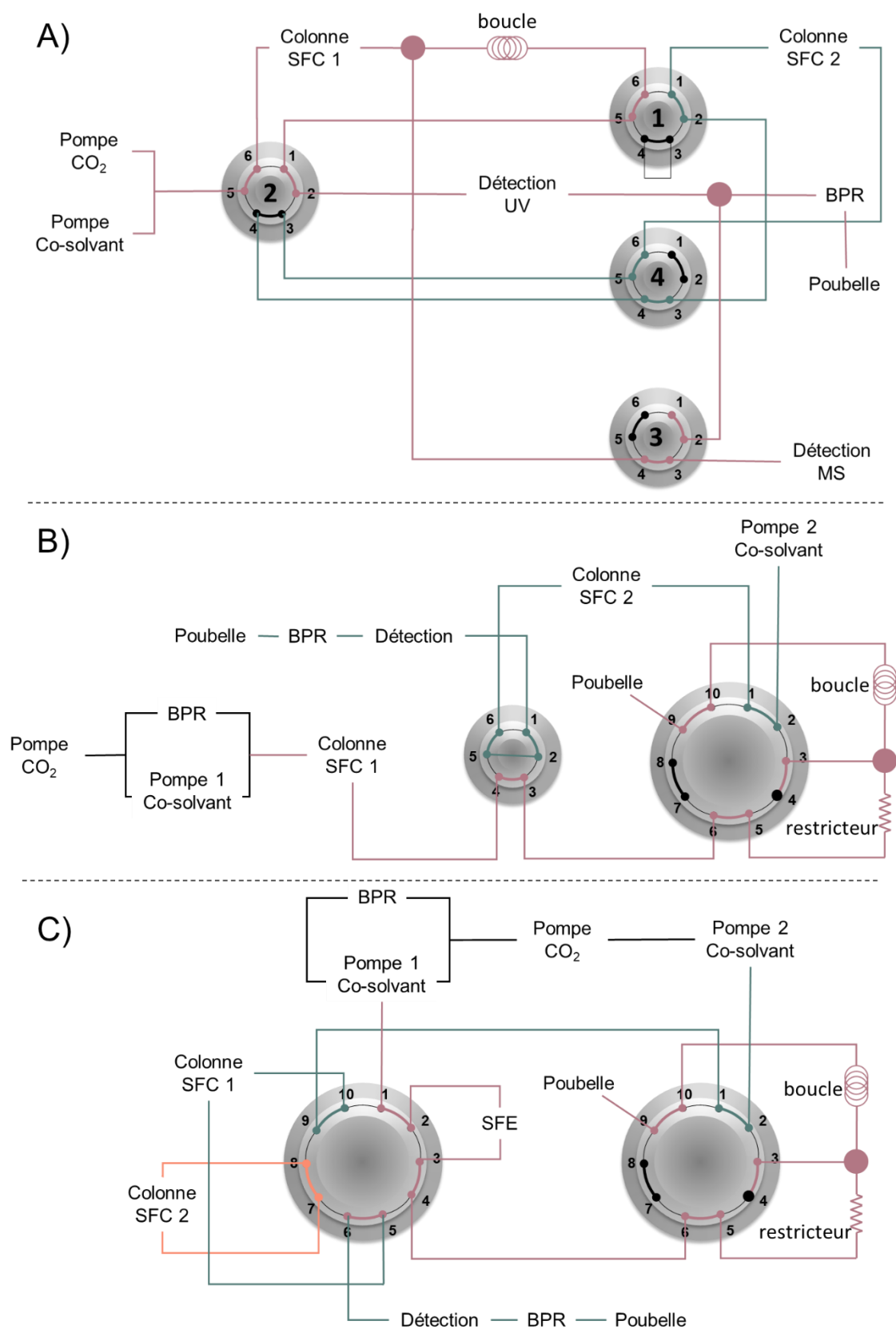


Figure I.15 – Configuration de systèmes SFC bidimensionnelle adaptées de la littérature. A) SFC-SFC [166] B) SFC×SFC [135,167] C) SFE-SFC×SFC [168]

Une autre configuration de SFC×SFC utilisée par Lavison *et al.* illustre une approche permettant de réaliser une séparation compréhensive sans recourir à une boucle de stockage (voir Figure I.16) [169]. Dans cette étude portant sur l'analyse d'additifs dans les lubrifiants automobiles, les auteurs exploitent un principe de rétro-élution à travers la colonne de dim1 afin d'éluer les composés vers la dim2. Cependant, cette configuration présente une limitation importante : elle n'est pas compatible avec toutes les phases stationnaires, ce qui restreint sa flexibilité d'application. En effet, la rétro-élution implique un flux inverse dans la colonne de première dimension, ce qui peut perturber les mécanismes de rétention ou endommager certaines phases stationnaires.

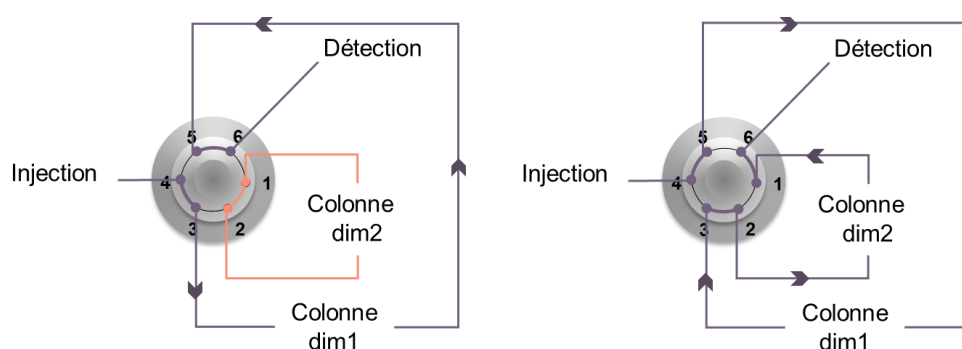


Figure I.16 – Configuration de systèmes SFC×SFC adapté de [169]

Ces avancées technologiques démontrent que la SFC peut jouer un rôle central dans l'évolution de la chromatographie bidimensionnelle.

Ses analyses à haute résolution, rapides et respectueuses de l'environnement montre son intérêt. Les efforts actuels visent à simplifier les dispositifs, à accroître leur robustesse, et à élargir leur champ d'application.

Conclusion

La chromatographie multidimensionnelle, et son utilisation en SFC s'impose ici comme une technique adaptée à l'étude d'échantillons complexes. En effet, la combinaison orthogonale de différentes dimensions de séparation et d'outils de modulations (vannes, modes heart-cut ou compréhensifs), permet d'atteindre des niveaux de sélectivité et résolution qu'il est difficile d'obtenir en chromatographie unidimensionnelle. Ces avancées ont permis d'étendre les domaines d'applications de la chromatographie, surtout dans les secteurs pharmaceutique, agroalimentaire ou des produits naturels.

C'est dans ce cadre que s'inscrit le chapitre suivant : l'étude des flavonoïdes chiraux. Ces composés possèdent des propriétés biologiques dépendantes de leur stéréochimie, ce qui rend leur séparation et leur caractérisation complexe. La chromatographie, qu'elle soit unidimensionnelle ou multidimensionnelle, s'est montrée adaptée à cette problématique. Une étude particulière est portée aux flavonoïdes issus du *Citrus aurantium* L. car ils sont très riches sur le plan structural et présentent de nombreuses propriétés bioactives.

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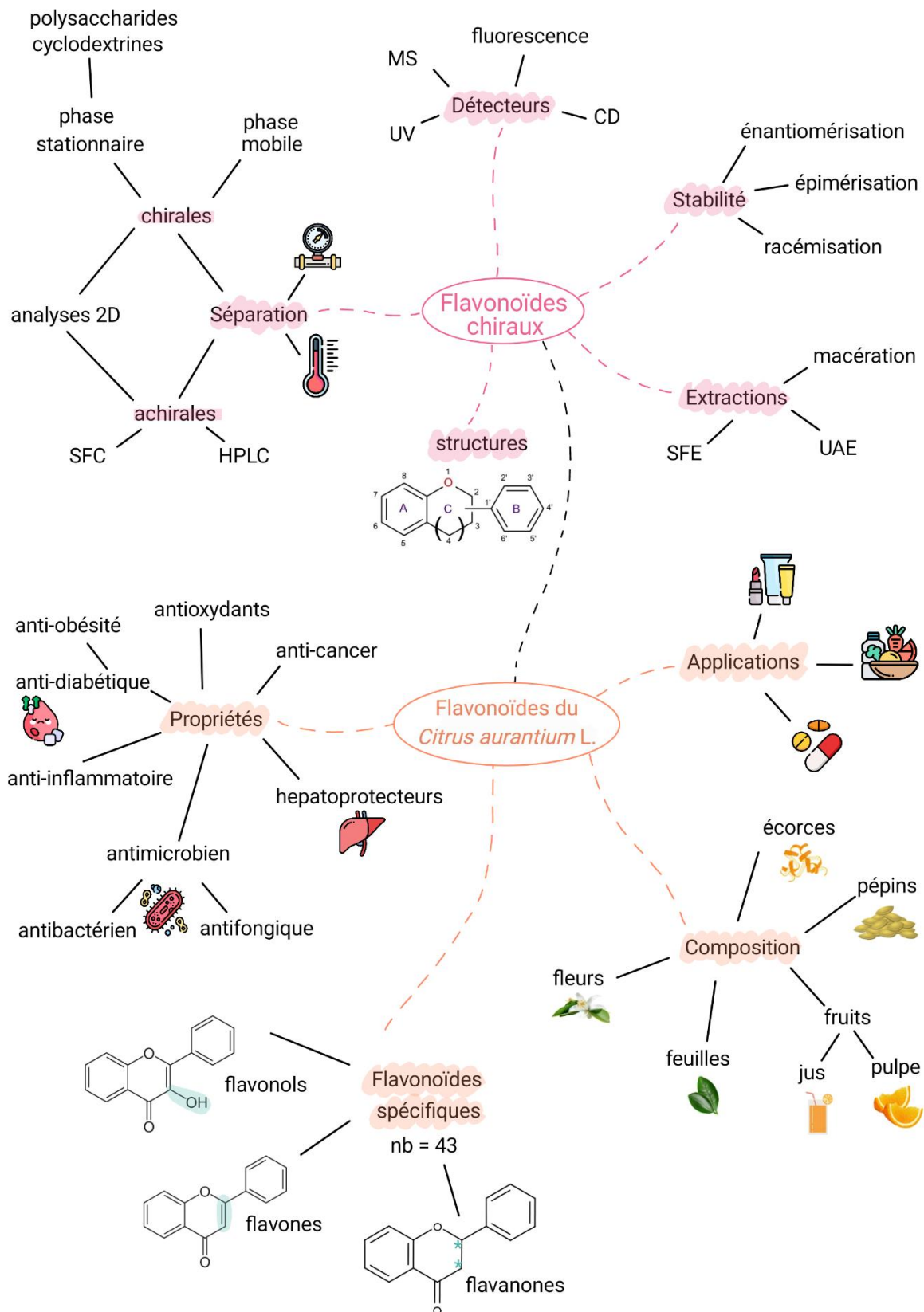
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Chapitre II

Citrus aurantium L. une plante riche en
molécules actives





Introduction

Les flavonoïdes, composés bioactifs présents dans de nombreuses plantes, sont connus notamment pour leurs propriétés antioxydantes et anticancéreuses. Leur analyse est essentielle pour comprendre leurs effets biologiques, en particulier en raison de la présence de centres chiraux qui influencent leur activité. Les techniques modernes de chromatographie, telles que la chromatographie liquide et supercritique, sont couramment utilisées pour séparer et identifier ces composés.

L'analyse des flavonoïdes est un domaine en constante évolution, avec des avancées significatives dans les techniques chromatographiques et de détection. L'évolution de l'instrumentation et des phases chirales ont amélioré la capacité à détecter et à quantifier ces composés à des niveaux de concentration plus faibles. Ces progrès sont cruciaux pour mieux comprendre les propriétés biologiques des flavonoïdes et leur potentiel dans les applications thérapeutiques.

C'est dans ce contexte que le *Citrus aurantium* L. s'inscrit dans notre étude. Le *C. aurantium*, communément appelé bigaradier ou oranger amer, est une espèce d'agrumes très répandue dans les régions méditerranéennes et subtropicales. Utilisé depuis longtemps dans la médecine traditionnelle, l'alimentation ou encore la parfumerie, le bigaradier est une ressource végétale d'un intérêt tant économique que scientifique : ses feuilles, fleurs, fruits et écorce en font une plante de choix.

Grâce à ses composés secondaires diversifiés, le *Citrus aurantium* L. joue à présent un rôle clé dans différents secteurs industriels. Dans le secteur pharmaceutique, des extraits en sont utilisés pour leurs propriétés apaisantes, digestives, mais aussi antimicrobiennes. Dans les cosmétiques, ses huiles essentielles sont prisées pour leurs effets tonifiants, purifiants et leur parfum caractéristique. Enfin, dans l'industrie alimentaire, le fruit entre dans la composition de confiseries, de liqueurs, ou comme arôme naturel.

L'objectif de cette étude est de passer en revue les méthodes d'analyse et d'extraction des flavonoïdes chiraux, en mettant l'accent sur l'utilisation de phases stationnaires chirales et de techniques de détection avancées. Cela inclut l'exploration des défis liés à la stabilité des molécules et à la sensibilité des détecteurs, ainsi que l'impact des conditions analytiques sur la résolution des énantiomères.

Dans un second temps, ce chapitre vise donc à explorer les flavonoïdes présents dans le *C. aurantium* comprendre leurs intérêts biologique et industriel, mais également leurs nombreux

bénéfiques pour la santé humaine, notamment grâce à leurs propriétés antioxydantes, anti-inflammatoires, neuroprotectrices, cardioprotectrices et anti-diabétiques.

Partie 1 : Les flavonoïdes chiraux

A. Structure et analyse des flavonoïdes chiraux : revue de la littérature

Article 2: Chiral and achiral analysis of chiral flavonoids with liquid and supercritical fluid chromatography – A review

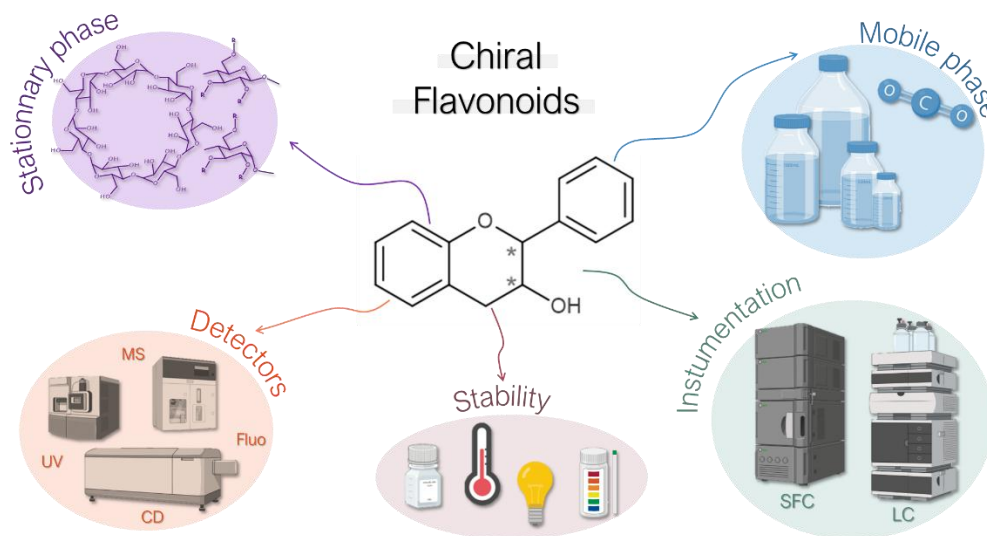
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Graphical abstract



Abstract

Flavonoids have been the topic of numerous studies for decades because they possess varied interesting bioactivities including e.g. antioxidant and anticancer properties. In particular, some flavonoids possess chiral features, due to the presence of one or two chiral carbon atoms on their aglycone root. They include flavans, isoflavans, flavanones, flavanonols, flavanols and auronols. In addition, other chirality centres may be observed on the carbohydrate groups attached to the aglycone root, but these will not be considered in the present paper. However, as stereoisomers usually have different bioactivity, analysis methods focusing on these specific flavonoid classes

should be of interest to better understand their mode of action and their biosynthetic pathways. In the present review, we have examined chromatographic analyses in the liquid phase (LC) and the supercritical phase (SFC) in achiral and chiral modes to analyse chiral flavonoids. One- and two-dimensional methods are reported, and the different detection modes are also examined. Note that this review did not aim at being comprehensive, but rather at displaying the diversity and main features of current methods.

1. Introduction to chiral flavonoids

Flavonoids belong to the polyphenol family. They are widely distributed in nature and form an integral part of our daily lives. They are found in many food products such as fruits, vegetables, cereals, herbs and spices. As a result, flavonoids are also present in drinks such as tea, red wine and fruit juice. They contribute to the colour of certain foods and flowers, but they also play a crucial role in the survival, growth, reproduction and defence of plants. In addition to their presence in the plant kingdom, flavonoids are present in certain marine organisms [1], but their function in marine biology is not yet fully understood. Nevertheless, they also appear to be important compounds for the survival and adaptation of marine organisms in their environment. In 1994, the number of flavonoids identified was estimated at around 4,000 [2], then increased over the years to reach 7,000 in 2005 [3]. To date, 9,000 flavonoids have been identified, which make up 60% of the known phenolic compounds [4]. This makes them one of the most important and diverse families of natural compounds yet identified. The major reason for this intensive research is that flavonoids have been associated with a number of beneficial properties to be exploited in a variety of fields, including pharmaceuticals, cosmetics and the food industry. In addition, some flavonoids have chiral features, which should contribute to differentiated properties between stereoisomers. Therefore, there is a great interest in developing analysis methods for flavonoids, and among them, for chiral flavonoids. Chromatography is one such method, on which the present review will particularly focus.

1.1. Flavonoid properties

Flavonoids have antioxidant properties that help protect body cells from damage caused by free radicals, which are responsible for diseases such as cancer [5–7] or cardiovascular disease [8–10], but also contributing to ageing [11]. They also have anti-inflammatory effects and have been used in traditional medicine to treat bacterial and fungal infections [12]. In 1936, Rusznyak and Szent-Gyorgyi demonstrated that flavonoids have the ability to strengthen the resistance of blood

vessels [13]. They improve the elasticity and tightness of blood vessels, reducing the risk of heart disease by lowering cholesterol levels and regulating blood pressure. In addition, they have promising potential in the fight against cancer by inducing programmed cell death and inhibiting the angiogenesis feeding cancer cells [14,15]. Finally, flavonoids appear to improve cognitive function by improving blood flow to the brain, which helps protecting against oxidative damage and inflammation, and may help prevent neurodegenerative diseases such as Alzheimer's [16]. Working with flavonoids is rendered difficult since they are produced in small quantities by living organisms, and are divided into so many different structures. Although flavonoids are widely distributed in nature, their availability in food and their bioavailability in the human body vary considerably [17]. This variation is due to numerous factors such as plant variety, growing conditions, maturity and processing method. Their complex chemical structures (see next section) mean that they cannot always be easily produced by conventional chemical synthesis. In fact, their physicochemical properties, such as sensitivity to pH, temperature [18] and light, or limited solubility [19], complicate their use and analysis.

1.2. General structure of chiral flavonoids

The biological properties of flavonoids stem from their chemical structure (Figure II.1). The core structure consists of two rings, A and B, which may be linked through a carbon chain (in chalcones) or through a third ring (ring C).

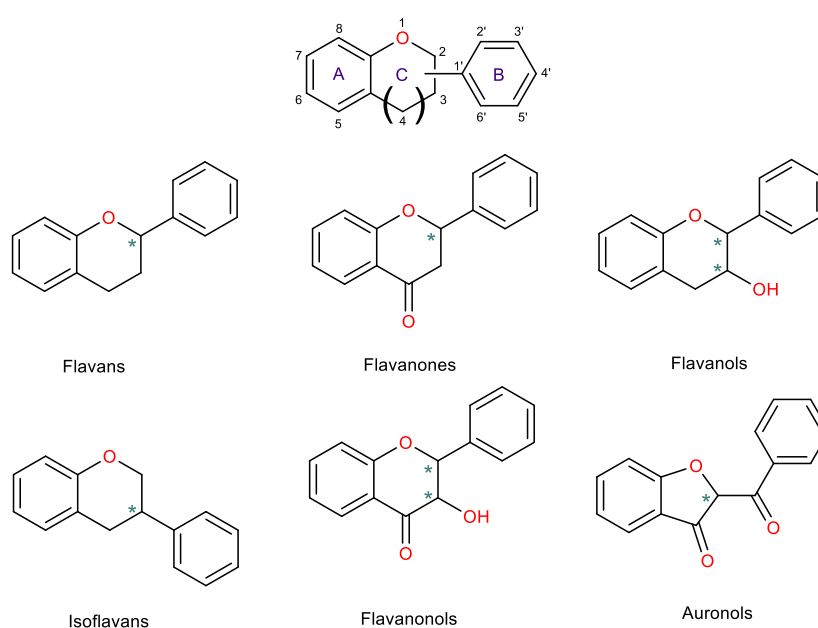


Figure II.1 - General structures of chiral flavonoids

Chemical modifications on this backbone notably include methoxyl groups and hydroxyl groups [20], as can be observed for example on Hesperetin in Figure II.2. Hydroxyl groups are also possibly modified with carbohydrate groups, as appears in Hesperidin (Figure II.2), which is the glycosylated form of Hesperetin, or in Naringin that is the glycosylated form of Naringenin. Hydroxyl groups may also be acylated with different acids. For instance, acetylation may be obtained with gallic acid, as appears in Epigallocatechin gallate (Figure II.2), which is an acetylated form of Epigallocatechin. The general classification of flavonoids into sub-families is based on the oxidation state and degree of unsaturation of the central heterocycle (ring C in Figure II.1). In other words, flavonoid classification depends on the presence or absence of a double bond between C2 and C3, the position of the bond between the B and C rings, the opening or closing of the C ring, and the nature and position of the substituents in the C ring [21].

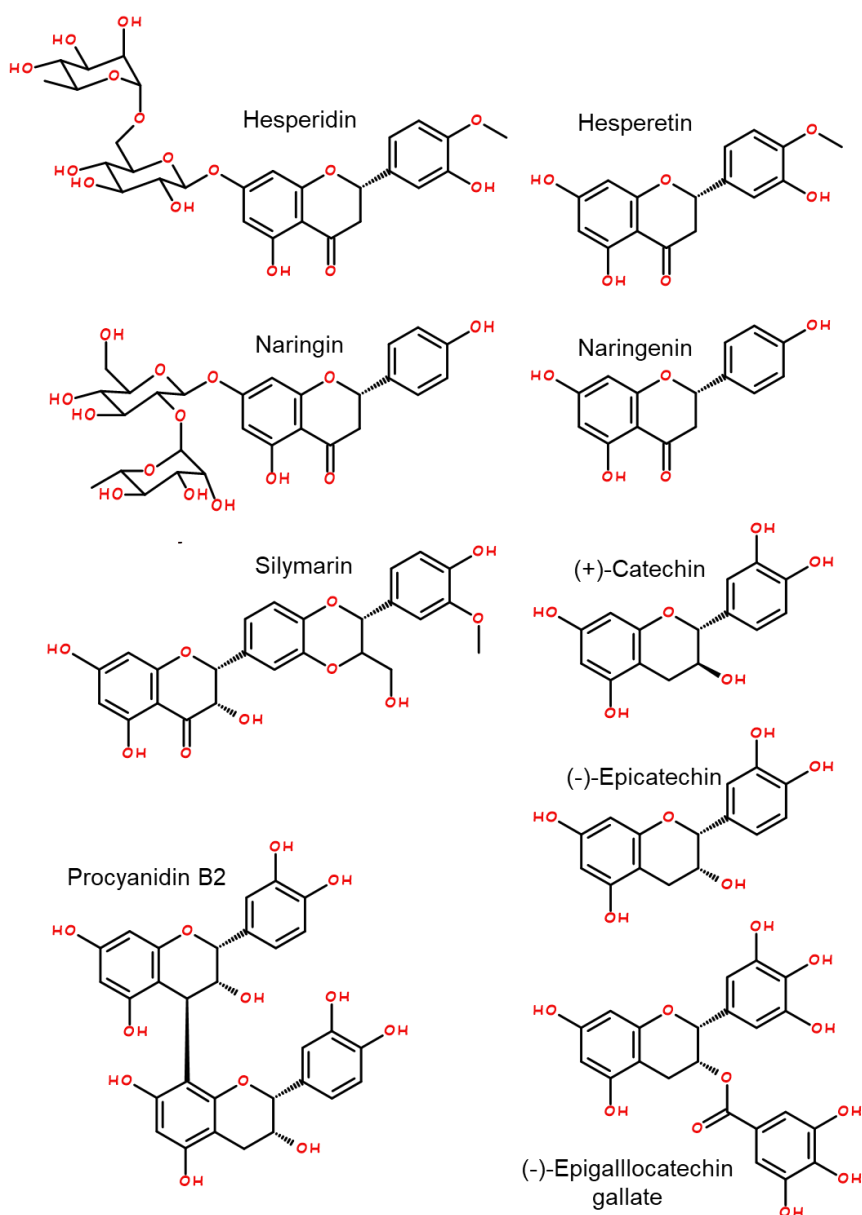


Figure II.2 - Examples of chiral flavonoids mentioned in this paper

Moreover, when no double bond is present between C2 and C3, a chiral centre is present at the C2 and/or C3 positions. This is the case for the flavan, isoflavan, flavanone, isoflavanone, flavanonol and flavanol families (Figure II.1). For instance, Hesperetin and Naringenin, together with their glycosylated forms Hesperidin and Naringin, all belong to the group of flavanones. Catechin and Epicatechin belong to the group of flavan-3-ols, while Silymarin is a flavanonol. Moreover, flavans, isoflavans and flavanols may bear a third chiral centre on C4 when a hydroxyl group or a larger group is present in this position, as is the case in procyanidin oligomers (Figure II.2). Finally, the C ring is most often a 6-membered ring (including one oxygen atom and 5 carbon atoms), but also exists as a 5-membered ring in the auronol family, which is less frequently encountered. In auronols, a single chiral centre exists on the C2 carbon atom (Figure II.1).

In this review article, we will focus solely on flavonoids bearing one or more chiral centres on the root structure (not taking account of carbohydrate chirality).

To better understand chromatographic methods applied to these compounds, it is also useful to examine the variations of size and polarity within these families. Figure II.3 represents the variation of log P (octanol-water partition coefficient) depending on molecular weight. The figure was drawn based on molecules found in the literature cited in Tables 1, 2 and 3 and using the same computation tool for all of them (molDESC.icoa.fr) because literature data were not available for all molecules. First, it can be easily noticed that no general trend can be observed between molecular weight and the hydrophobicity expressed by log P when all molecules are considered. However, a tendency can be observed with the white bubble points on the left side of the figure (low molecular weight) where increasing numbers of hydroxyl groups cause both an increase in molecular weight and decreasing hydrophobicity (decreasing log P). Another trend can be seen with increasing size of glycosylated groups: on the left are aglycon molecules, while the introduction of a monosaccharide unit or a disaccharide unit is causing again increased molecular weight and decreased hydrophobicity. Other points fall out of these trends: they are gallate acetylated molecules and dimers (*e.g.* procyanidin dimers). For such variations, the increase in molecular weight is not causing any significant change in log P values.

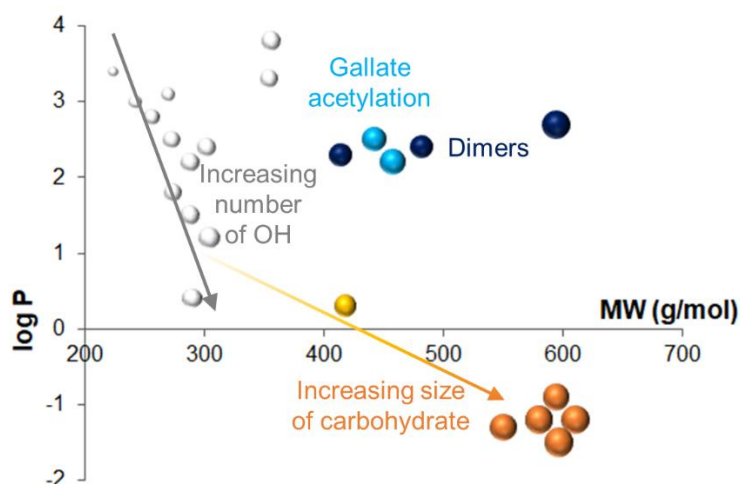


Figure II.3 - Relation between molecular weight (MW) and hydrophobicity (octanol-water partition coefficient) in chiral flavonoids cited in this review (Tables 1, 2, 3). Bubble size is related to the topological polar surface area (TPSA). All molecular descriptors were computed with [MolDesc \(icoa.fr\)](http://MolDesc.icoa.fr)

A better relation between molecular weight and polarity can be found when polarity is expressed by the topological polar surface area (TPSA), with a logarithmic tendency fitting all molecules (Figure II.S1). On Figure II.3, TPSA is represented by the bubble size. Quite logically, all points on the right side of the figure (large molecular weight) have large TPSA values, due to the presence of numerous oxygen atoms, although they exhibit a variety of log P values as the hydrophilicity of oxygen atoms is counterbalanced by the hydrophobicity of carbon atoms.

Understanding these structural variations will be useful in the next sections when discussing the choice of chromatographic methods and elution orders. Typically, log P values are useful to explain elution orders in reversed-phase liquid chromatography, where hydrophobic interactions dominate the retention mechanism, while TPSA values are useful to explain elution order in normal-phase elution modes where hydrogen bonding is most significant to explain retention.

1.3. Significance and difficulties of chirality

Chirality is a fundamental characteristic of nature [22] that is widely encountered in many classes of natural compounds as carbohydrates, amino acids, lipids, and of course flavonoids, to name a few. Chirality is well known to have a significant impact on bioactivity as stereoisomers (enantiomers and diastereomers) usually have different biological activity and toxicity [23]. Furthermore, they are usually metabolized at a different rate, or degrade at a different rate in the environment. As a result, while the chirality of natural products is not frequently considered, chiral separation is a primary concern in the pharmaceutical industry, where enantioseparation is considered crucial. Considering their different features, the resolution of chiral active compounds

should progressively become a concern to other application fields such as cosmetics and the food industry. The purpose of separating stereoisomers may be to control their proportions (analytical scale) or to purify single stereoisomers for activity testing (preparative scale). Chromatographic techniques are well suited to both purposes.

Regarding the specific case of chiral flavonoids, in recent years most chromatographic studies were related to two specific families of chiral flavonoids, the flavanones and flavan-3-ols. These studies focused on their quantification in natural products, as well as their enantioresolution on different chiral stationary phases [24].

Chiral separations are always challenging because they are difficult to predict. Furthermore, molecules with several chiral centres cause additional complications, because the number of stereoisomers increases with the number of chiral centres: with n chiral centres, there are a maximum of 2^n stereoisomers. In the case of chiral flavonoids, if one excludes the chirality on possible carbohydrate moieties that are generally enantiopure, there would most often be one or two chiral centres (on C2 and/or C3), resulting in two to four stereoisomers. A third chiral centre on C4 would induce eight stereoisomers.

Examining the conformation of chiral flavonoids is therefore important to understand their biosynthetic pathways, their interactions with other biological molecules, as well as their bioavailability and activities. Molecular modelling methods have been used to predict the physicochemical and biological properties of flavonoid enantiomers, providing a deeper understanding of their biological activity, but analytical methods are also useful in this respect, as will be further discussed in this review.

1.4. Stability of chiral flavonoids

Some chiral flavonoids are more stable than others, and this stability depends on the conditions to which they are exposed. Before and during chromatographic analysis, flavonoids can be subjected to various conditions such as temperature, pH, light and solvents [25]. These conditions can affect certain flavonoids, which have specific functional groups on their structure. This can lead to their degradation or transformation into other isomers, causing processes such as racemisation, epimerisation and enantiomerisation. It is important to identify such behaviours, as molecules that are sensitive to such changes of configuration should typically not be used in drug formulations. Thermodynamic studies and molecular modelling can be helpful to understand the stability of chiral flavonoids and their degradation and conversion processes [26]. The most stable isomeric forms generally have a lower free energy, indicating greater thermodynamic stability.

1.4.1. Racemisation

Racemisation occurs when a single enantiomer or scalemic mixture (non-equimolar mixture of the enantiomers) is transformed into a racemic mixture, *i.e.* an equimolar mixture of the two enantiomers. When only one chiral centre is present, it means that this chiral centre takes the opposite configuration. When more than one chiral centre is present, all of them take the opposite configuration to obtain the opposite enantiomer. In the case of chiral flavonoids, racemisation can only concern aglycon forms, since the carbohydrate groups are stable in this respect. Several factors favour the racemisation process, such as temperature and pH. The solvent can strongly influence racemisation, affecting the chiral purity of a mixture. During acid or enzymatic hydrolysis, an inappropriate solvent can accelerate the conversion of enantiomers between them, thus reducing the enantiomeric excess and the chiral purity of the final product. In addition, it seems that flavanones substituted with hydroxyl groups are more susceptible to racemisation than those substituted with methoxyl groups [27].

1.4.2. Epimerisation

Epimerisation is the process of reversible conversion of an enantiomer comprising more than one chiral centre into a diastereomer, thus impacting only one chiral centre. This can again occur under the influence of factors such as exposure to light, pH variations or chemical rearrangements. Many chiral flavonoids may be subject to this effect: it may occur in aglycon molecules, where more than one chiral centre is present, as is the case in flavanols and flavanonols, or in glycosylated forms, related to the configuration change of the C2, C3 or C4 chiral atom.

For instance, in 2005, Nahoko Uchiyama *et al.* showed that when a sample of commercial Hesperidin (Figure II.2), a glycosylated flavanone bearing the S configuration on C2, was kept in boiling water, the relative amount of Hesperidin decreased and the amount of epimer (2R) increased as a function of time [28].

In the case of aglycon flavanols, the catechin family (Figure II.2) was examined by different authors and for different purposes. As appears in Tables 1, 2 and 3 (-)-Epicatechin is present in many plants. However, it is more bioactive than (+)-Catechin [29,30] and therefore more desirable. Studies have shown that it is possible to obtain the (+) enantiomer of Epicatechin from (+)-Catechin using a catalysis reaction [30]. However, Catechin may degrade during the manufacturing process [29]. For instance, it is present in cocoa beans but can no longer be found after transformation to chocolate.

Hiroiyuki Terashima *et al.* studied the epimerisation of Catechin and Epicatechin [31]. Thanks to a chiral chromatographic method (see section 3), it was found that the epimerisation of (+)-Catechin

and (+)-Epicatechin could be suppressed by adding D-fructose, but not D-glucose and D-sucrose. Conversely, this epimerisation reaction is sometimes deliberately provoked. In nature, the (+)-Epicatechin enantiomer is not present or is present in too small quantities; the predominant form is 93% (-)-Epicatechin [29]. Using a native *C. sinensis* anthocyanidin reductase (CsANR), Yumei Qian *et al.* succeeded in producing (-)-Epicatechin and (+)-Epicatechin using a recombinant CsANR [30]. This provides a better understanding of the biosynthesis of these molecules. In a different study, Boulbekhari *et al.* examined epimerisation of naringin and hesperidin were analysed through a chiral chromatography method. Such methods will be further explained in section 3.3 below.

1.4.3. Enantiomerisation

Enantiomerisation refers to the reversible conversion of one enantiomer into another with an opposite spatial configuration. This process can be catalyzed by certain chemical agents or by specific reactions.

Regardless of the degradation process, the change in configuration can lead to a loss of biological activity or specific flavonoid properties. These phenomena can also affect the relative quantity of enantiomers in a final product, which can have consequences for the efficacy and safety of the product. It is important to note that these degradation and conversion processes may be reversible under certain circumstances. Moderate processing conditions or adjustments to environmental parameters can restore the original chirality of flavonoids. In order to minimize exposure of chiral flavonoids to conditions likely to degrade them or convert them into isomers, it is essential to use mild storage and analytical conditions, control temperature and pH, and protect samples from light. For instance, Roberto Cirilli *et al.* demonstrated the enantiomerisation of flavanone on a Chiralpak IA column in the presence of 2'-hydroxychalcone [25]. In their study, changing the pH of the borate buffer in the mobile phase (ethanol-borate) resulted in the disappearance of the interconversion zone, which appears as a plateau above the baseline between the peaks of the enantiomers (so-called “batpeaks”). In addition, increasing the percentage of aqueous solution in the mobile phase caused the order of elution to reverse.

2. Achiral analysis of chiral flavonoids

As mentioned above, over the last few decades, research into flavonoids has continued to grow due to their numerous bioactivities. As a result, the chromatographic methods to analyse flavonoids, and among them chiral flavonoids, have evolved to become more selective and

sensitive. Due to the limited volatility of most flavonoids, their analysis with gas chromatography (GC) would require derivatization procedures [32]. In the case of polyphenolic analytes, derivatization is often causing issues because multiple derivatives may be produced from a single analyte. GC will therefore not be discussed in the following, as we will focus on direct methods. Liquid chromatography (LC), whether as high-performance (HPLC) or ultra-high-performance (UHPLC), is the most widely used to separate and quantify flavonoids. The largest proportion of research papers is concerned with achiral analysis only (this section), while a smaller proportion of research papers is related to chiral analysis, *i.e.* the separation of stereoisomers (see section 3). In achiral and chiral LC methods, different elution modes may be applied, as further discussed below (section 2.1).

Recently, other techniques have been developed to improve selectivity in the analysis of chiral flavonoids, notably supercritical fluid chromatography (SFC, section 2.2) and two-dimensional (2D) chromatography (in the liquid or supercritical phase, section 4). SFC offers advantages such as highly-efficient fast separations and a complementary selectivity to the most used reversed-phase LC methods, while 2D methods improve peak capacity with complementary selectivity [33,34].

LC and SFC methods are both mainly used with UV detection at a single wavelength, or a diode-array detector (DAD) allowing to record the whole UV-visible spectrum of each analyte (see section 5.1). Alternately, higher sensitivity and some more selectivity may be obtained with fluorescence detection (FLD, see section 5.2). When a better selectivity and sensitivity is required, the chromatographic method is preferably hyphenated to mass spectrometry (MS). Furthermore, tandem mass spectrometry (MS/MS) provides more sensitive detection, and a chance for more detailed structural information (see section 5.3). Other, more specific detectors like circular dichroism (CD) may be useful when it is necessary to differentiate between two enantiomers (section 5.4).

2.1. Liquid chromatography

LC is by far the most common technique to analyse flavonoids. Achiral analysis of chiral flavonoids can be useful in many instances. In 2016, André de Villiers *et al.* highlighted the progress made in previous years in the achiral analysis of all flavonoids (not only the chiral ones) [35]. In many cases, it is necessary to rapidly assess the purity and composition of flavonoid samples, whether it be during (hemi)synthesis, following extraction [36,37] or monitoring of plant profiles during harvesting [38,39]. In such cases, extensive chiral separation is not always useful or necessary, and achiral analysis of flavonoids can provide sufficient information. For example, in oenology, it

is essential to analyse different phenolic compound profiles to select by-products and the appropriate grape variety depending on the phenolic compounds desired [40]. Furthermore, when the aglycon root is carrying a chiral centre, but also a carbohydrate moiety, because the carbohydrate group is usually enantiopure, the two opposite configurations of the chiral centre mean that the molecules obtained are diastereomers, thus may be resolved in an achiral environment. For instance, this would be the case for the (2S) and (2R) isomers of Hesperidin (Figure II.2). Also, when two chiral centres are present on the aglycon core structure, two pairs of enantiomers yield diastereomeric pairs. The best example of such case is that of (+)-Catechin and (-)-Epicatechin (see Figure II.2), which are diastereomers and therefore may be resolved in an achiral environment.

A summary table of the different stationary and mobile phases used in the recent literature on achiral analysis of chiral flavonoids is presented in Table II.1. As it will appear there, mostly flavanones and flavan-3-ols were reported.

Due to their structural similarities, the achiral separation of flavonoids can however be complex. In this context, an appropriate LC mode must first be selected. As is also the case for many applications, due to its separation capability and widespread availability, reversed-phase liquid chromatography (RPLC) is the most widely used for flavonoid analysis [37,41–43]. The reported RPLC methods ensured effective analysis of flavonoids in a variety of applications, such as the pharmaceutical industry, life science and foodomics.

In RPLC, a hydrophobic stationary phase is used, most often octadecyl-siloxane-bonded silica, although other non-polar stationary phases have been reported like phenyl-hexyl-siloxane-bonded silica. The mobile phase, on the other hand, is polar, mainly composed of a mixture of water or buffer (solvent A) and an organic solvent (solvent B), which is most often acetonitrile (ACN), or sometimes methanol (MeOH). Acidic additives such as formic acid (FA), trifluoroacetic acid (TFA) or phosphoric acid (H_3PO_4) are commonly introduced in the mobile phase to stabilize some of the flavonoids that are prone to degrade in aqueous solution, and therefore improve their elution with good peak shapes to favour resolution. However, the strongest acids are inappropriate for hyphenation to mass spectrometry.

RPLC is mostly appropriate for hydrophobic and moderately hydrophilic analytes. Judging from Figure II.3, this should be well applicable to flavonoids, since most of them have positive log P values. Only the glycosylated molecules are somewhat hydrophilic.

The elution order of flavonoids in RPLC depends on the position and number of hydroxyl groups on their structure. In general, increased hydrophilicity through increased number of hydroxyl groups or glycosylation logically causes earlier elution. On the contrary, increasing the number of methoxyl groups causes increased retention. The position of the hydroxyl groups also matters.

For instance, hydroxyl groups located at the ends of the molecule (as positions 3', 4', and 5' of B-ring) are more accessible for polar interactions (e.g. solvation by the polar mobile phase) than those located closer to the C ring, so some resolution of isomeric species is possible. In addition, intra-molecular hydrogen bonding would also occur between proximal polar functions, which may again cause different solvation by the polar mobile phase and/or different insertion in the non-polar stationary phase, resulting in different chromatographic retention. As is also the case for other applications fields, a trend towards ultra-high efficiency was observed in the recent applications to flavonoids analysis [40,44]. Ultra-high-performance LC (UHPLC) is best obtained with modern instruments offering reduced dead volumes combined with columns packed with sub-2 μm fully porous particles, or sub-3 μm superficially porous particles. However, many recent flavonoid analyses in RPLC were still based on larger particles (4-5 μm) and are thus still relevant of HPLC.

Ionic liquids are liquid salts with unique properties, including high thermal stability and the ability to form hydrogen bond. Ionic liquids were very trendy some 10-15 years ago, whether to be introduced in the mobile phase or to be bonded on the stationary phase. They are now less frequently observed in RPLC chromatographic methods [45]. However, the introduction of an ionic liquid in the mobile phase can improve the resolution of flavonoids: the selectivity of the separation depends on the hydrogen bonds formed between the ionic liquid and the hydroxyl groups present on the flavonoid structure.

Another LC mode used for flavonoid analysis is hydrophilic interaction liquid chromatography (HILIC). HILIC relies on a polar stationary phase (like bare silica gel, or polar-bonded silica), and a polar mobile phase comprising a major portion of ACN and a minor portion of aqueous buffer. Therefore, it is appropriate to retain the most polar flavonoids, *i.e.* those with glycosylated groups or flavonoid-derived polar metabolites. It was mostly employed to resolve non-chiral flavonoids that co-eluted in RPLC [43] and has been widely described for use in 2D-LC methods [44,46–48] (see section 4). A significant advantage of HILIC over RPLC is that it offers increased MS sensitivity with electrospray ionization mode (ESI), because the acetonitrile-rich mobile phase is easier to evaporate than the water-rich mobile phases of RPLC mode.

The choice between RPLC and HILIC therefore depends on the specific characteristics of the samples to be analysed (their polarity and the expected differences within the sample) and the analytical objectives.

2.2. Supercritical fluid chromatography

Over the last few decades, supercritical phase chromatography (SFC) has emerged as a possible alternative to LC to separate flavonoids. The specific properties of the mobile phase, mainly composed of pressurized CO₂, give SFC some advantages over LC. The most obvious is the significantly reduced consumption of organic solvents, making it more environmentally-friendly, especially when preparative-scale separations are desired. In addition, supercritical fluids offer high efficiency at high flow rate, which is often resulting in better resolution per unit time. Although known for over 60 years, in the past decade the manufacturing of new-generation SFC instruments has led to a renewed interest in the technique. Indeed, these modern instruments are more robust and provide higher efficiencies than older instruments [33], notably through reduced dead volumes, thereby favouring the use of small-particle stationary phases to provide ultra-high-performance SFC (UHPSFC). In addition, hyphenation to mass spectrometry is facilitated. All of this has led to an increase in the number of application articles published in the recent years, and flavonoids analysis is one example among them.

Developing and optimizing an SFC method involves various parameters, starting with the selection of stationary phase, which influence on the separation is paramount, followed by the nature and percentage of co-solvent in the CO₂-based mobile phase, to ensure the elution of all target analytes. The back-pressure applied after the separation and UV-visible detection to ensure that the fluid is maintained above its critical pressure, the oven temperature and the flow rate are fine optimization parameters. All of them influence the density of the mobile phase in SFC, making it possible to tune the method to the specific flavonoid separation requirements. Methanol is by far the most employed co-solvent in SFC. In the case of flavonoid analysis, isopropanol was also reported (see Table II.1).

Table II.1 - Achiral LC and SFC analyses of chiral flavonoids in the recent literature (from 2015). See main text for abbreviations of techniques, detectors, solvents and additives.

Sample	Flavonoid classes	Specific flavonoids examined	Technique	Detector(s)	Stationary phase and column dimensions	Mobile phases	Flow rate (mL/min)	Temperature / Pressure	Gradient	References
zhi-zhi Da Hiang (<i>G.jasminoides</i> Ellis, <i>C.aurantium</i> , <i>R.palmatum</i>)	Flavan-3-ols Flavanones	Catechin Epicatechin Naringenin Narirutin Naringin (Neo)hesperidin	UHPLC	MS	Kinetex C18 (100 x 4.6 mm – 2.6 µm)	A) H ₂ O + 0.1% FA B) ACN	0.8	35°C	5-95% B (55 min)	36
Herbal dietary supplements	Flavan-3-ols Flavanones	Hesperetin (+/-)-catechin. (neo)hesperetin (-)-epicatechin (-)-epicatechin gallate	UHPLC	UV MS/MS	Chromolith RP18 (50 x 2.0 mm)	A) H ₂ O + 0.05% TFA B) ACN	1.2	20°C	8-80% B (5 min)	43
Commercial chocolates	Flavan-3-ols	(+/-)-Catechin (+/-)-epicatechin	HPLC	DAD	Nova pak C18 (250 x 4.6 mm – 4 µm)	A) H ₂ O/ THF/TFA (92:2:0.1) B) ACN + 0.1% TFA	0.7	30°C	15-100% B (24 min)	29
Grape pomace	Flavan-3-ols	(-)-Epicatechin (-)-gallo catechin (-)-epigallocatechin (+)-catechin (-)-gallo catechingallate	UHPLC	DAD	Kinetex C18 (100 x 3.0 mm – 2.6 µm)	A) H ₂ O + 0.1% FA B) ACN	0.8	35°C	5-95% B (11.5 min)	40
<i>Saraca asoca</i>	Flavan-3-ols	Epicatechin Catechin Epicatechin gallate Epigallocatechin gallate Procyanidin	UHPLC	DAD	Acquity UPLC HSS T3 (50 x 2.1 mm – 1.8 µm)	A) H ₂ O + 0.1% H ₃ PO ₄ B) ACN	0.75	37°C	5-80% B (9 min)	37
<i>Sphaerophysa salsula</i>	Flavonoid glycosides	-	HPLC	UV	Unitary C18 (250 x 4.6 mm – 5 µm)	A) H ₂ O + 0.2% FA B) ACN	1	30°C	5-45% B (40 min)	98
Blackcurrant	Flavan-3-ols Flavanones	Fustin Catechin Epicatechin	HPLC	DAD MS	Synergi C18 Hydro-RP 80A (150 x 2.0 mm – 4 µm)	A) H ₂ O + 0.1% FA B) ACN + 0.1% FA	0.3	30°C	2-100% B (35 min)	39
Pera Rio orange juice	Flavanones	Hesperidin Narirutin Erioditrin	HPLC	DAD MS	BEH XBridge C18 (250 x 4.6 mm – 5 µm)	A) H ₂ O + 0.1% FA B) ACN	1	-	6-100% B (43 min)	38

Cramberry-based pharmaceuticals	Flavan-3-ols	Catechin Epicatechin Procyanidin	UHPLC	FLD	Kinetex C18 (100 x 4.6 mm – 2.6 µm)	A) H ₂ O + 0.1% FA B) MeOH	0.4	-	12-95% B (33min)	111
Tea Benifuuki (<i>Camellia sinensis</i>)	Flavanones	3'-O-methylated epigallocatechin gallate	HPLC	UV	XSelect CSH Phenyl hexyl XP (100 x 3.0 mm – 2.5 µm)	A) H ₂ O + 0.1% FA B) ACN + 0.1% FA	0.7	40°C	6-95% A	100
Propolis	Flavanones	Pinostrobin	HPLC	MS/MS FLD	Not mentioned	MeOH / H ₂ O (25/75) + 1.48 g KH ₂ PO ₃ , pH = 7.1	1	-	-	114
<i>Citri reticulatae</i> Pericarpium	Flavanones	Didymin Hesperidin	SFC	DAD	Zorbax RX-SIL (150 x 2.1 mm – 5 µm)	A) CO ₂ B) MeOH	0.8	45°C / 95 bar	8-36% B (11 min)	47
Synthetic compounds for pharmaceutical and cosmetic applications	Flavanones Flavan-3-ols	Hesperetin Naringenin Catechin Epigallocatechin	UC	DAD MS	Acquity Torus DEA (100 x 3.0 mm – 1.7 µm)	A) CO ₂ B) MeOH + 0.1 % MSA	2.0-0.7	25°C / 150-110 bar	20-100% B (12.5 min)	46
Bee pollen	Flavanones	Pinostrobin 2-hydroxyflavanone Flavanone 4-hydroxyflavanon 6-hydroxyflavanone 7-hydroxyflavanone Naringin Naringenin Hesperetin Taxifolin	SFC	DAD CD	Lichrospher 100 Diol (250 x 4.6 mm – 5 µm)	A) CO ₂ B) iPrOH	3	35°C / 150 bar	5-50% B (20 min)	48
Citrus	Flavanones	Hesperetin Naringenin	UHPSFC	DAD	Viridis BEH (100 x 3.0 mm – 1.7 µm)	A) CO ₂ B) MeOH + 1% FA	1	40°C / 140 bar	3-30% B (20 min)	118

In most cases, the percentage of co-solvent introduced in pressurized CO₂ remains below 50%, but the most polar flavonoids may require larger proportions of co-solvent at the end of an elution gradient. There is actually no limit to this proportion of co-solvent, although the fluid state may not be supercritical anymore when such large proportions are used. Similar to LC modes, additives are often introduced in the SFC mobile phase, especially to facilitate the elution of ionisable analytes. In the case of flavonoids, acidic additives like FA or TFA are preferred, for the same reasons mentioned in the previous section. A less common acid, methanesulfonic acid (MSA) was found to greatly enhance the peak shapes and resolution of flavonoids [46].

One of the special features of SFC is its ability to analyse a wide range of flavonoids, covering different polarities. A study carried out in 2020 [46] employed a broad gradient, covering a wide range of compositions in the mobile phase starting with CO₂-rich mobile phase and ending in pure liquid. Such wide gradients, covering SFC and LC conditions, are usually called “unified chromatography” analyses (UC). This approach offers great flexibility to analyse complex samples containing flavonoids with a wide range of polarities more completely and accurately.

Similar to LC, there are several retention modes in SFC, mostly depending on the stationary phase polarity, as the mobile phase is always composed of CO₂ and co-solvent. SFC can be similar to either the normal phase mode (NP), where a polar stationary phase retains polar compounds preferentially. In that case, similar elution orders to HILIC may be expected. Alternately, SFC can be similar to the reversed-phase mode (RP), where a non-polar stationary phase favours the retention of non-polar analytes. However, RPSFC may yield different elution orders from RPLC because the absence of water in the SFC mobile phase significantly changes the selectivity as no hydrophobic interactions occur. For instance, in one particular study, it was observed that using C18 columns in SFC yielded undesirable co-elution of some analytes and generally poor selectivity [47]. In the recent literature, flavonoids were mostly analysed on polar stationary phases, comprising a variety of chemistries (bare silica gel, diethyl-aminopropyl-bonded silica, propanediol-bonded silica or 2-ethylpyridine-bonded silica), thus these separations were all NPSFC. For instance, Ana M. Ares *et al.* were able to separate 10 flavonoids in a single analysis on a 5-μm propanediol-bonded silica stationary phase in 20 min [48].

Whatever the chromatographic technique (LC or SFC) or mode (RPLC, NPLC or HILIC) employed, it should be noted that a single achiral chromatographic method is not always sufficient, and that it may be necessary to use complementary approaches such as off-line or on-line two-dimensional chromatography to obtain a more complete and accurate resolution of complex flavonoid samples, which will be discussed in section 4.

3. Chiral analysis

As explained in section 1, chiral analysis is often necessary for in-depth characterization of a sample. This is particularly true when stereoselectivity of a newly found molecule must be determined, or when biological activities must be assessed.

The separation of enantiomers can be achieved with different chromatographic techniques. For a long time, the preferred method was the chemical derivatization with a chiral and enantiomerically pure agent, to form diastereomers that could be resolved in an achiral environment. A major advantage is the possibility to use classical achiral chromatographic methods like RPLC on a C18-bonded stationary phase. However, it requires highly pure derivatization agents and involves chemical reactions to produce the diastereomers [49], then possibly reversed reactions to recover the initial products when purification is desired, which can be lengthy and costly [44]. As a result, this method is no longer a favourite.

A second possible method is the use of chiral additives in the mobile phase, to form transient diastereomers. With this method, an achiral stationary phase can still be used. However, the consumption of chiral additive can be costly, unless some effort is done to recycle it. As a result, a single reference of such method was observed in the recent literature [31], employing cyclodextrins as mobile phase additives together with an achiral phenyl-bonded stationary phase to achieve the enantioresolution of Catechin and Epicatechin enantiomers in green tea. Cyclodextrins as chiral additives are also often used in capillary electrophoresis [50], as the quantities required are much lower than in chromatography, but this is outside the scope of the present paper.

Finally, the last and preferred method nowadays is the use of chiral stationary phases (CSPs), where a chiral selector is deposited, immobilized or bonded on a silica support, to form transient diastereomers with the chiral analytes. From the years 1980s', a variety of CSPs have been developed to achieve enantioseparations in the liquid phase, including brush-type phases, ligand and inclusion complexes (*e.g.* cyclodextrins), biomolecules (like proteins and macrocyclic glycopeptides) and synthetic or natural polymeric phases, with the most famous and versatile being polysaccharides. Depending on the choice of CSP, the same chromatographic modes as achiral chromatography may be used (NPLC, RPLC, HILIC, SFC, etc.).

Thanks to the wide possibilities offered by modern CSPs, chiral flavonoids are now commonly resolved on CSPs with LC methods [51]. As for SFC, although it is usually recognized as an excellent technique for chiral separations [52], making use of the same CSPs as enantioselective LC, its specific application to the enantioresolution of flavonoids was only little explored to this date, with only a few papers relating it.

Unlike achiral chromatography, where separation is based on differences in hydrophobicity or polarity, chiral chromatography exploits the specific interactions between the chiral selector of the CSP and the stereoisomers. Also, steric parameters are significant, as the chiral analyte must access the enantiorecognition sites of the chiral selector. As a result, the choice of CSP is highly significant to the success of a chiral separation. In addition, the choice of mobile phase composition (solvents and additives) and temperature conditions also have a significant influence on the enantioseparation and must be carefully optimized. The literature related to enantioseparations of flavonoids in the liquid and supercritical phase is presented in Table II.2.

3.1. Chiral stationary phases

In recent years, the development and marketing of new CSPs has continued apace. However, for flavonoid analysis, only two types of CSP stand out: polysaccharide-based stationary phases and CSPs based on native or modified cyclodextrins (Figure II.4). Note that other types of enantioselective stationary phases are employed in LC and SFC, like brush-type phases, macrocyclic glycopeptides, cyclofructans, calixarenes and others, but none of them were reported in the chiral analysis of flavonoids.

3.1.1. Polysaccharides

CSPs based on modified polysaccharides are by far the most widespread [53]. A first reason is their high success rate in a variety of applications. Indeed, polysaccharides include a large number of chiral centres and chiral cavities to interact with the analytes, and therefore have a high probability of interacting specifically with the analytes, thereby providing better chances to separate stereoisomers. Interactions between enantiomeric compounds and the modified polysaccharides occur mainly *via* hydrogen bonds and π - π interactions [54], which is perfectly adequate for flavonoids, as they contain both hydrogen-bonding groups (carbohydrate moieties, hydroxyl and methoxyl groups) and aromatic rings. Secondly, polysaccharide CSPs also have a high compatibility with a wide range of solvents, especially for the most recent immobilized versions. Actually, the initial versions of polysaccharide CSPs developed by Okamoto and co-workers [55] were simply coated on modified silica, which offered limited possibilities to optimize mobile phase composition, as strong solvents would have dissolved the polymer. Modern versions based on the works of Francotte and co-workers may be immobilized on silica through thermal or photochemical cross-linking of the polymer [56,57], offering the possibility to use a wider range of solvents to elute difficult compounds or adjust enantioselectivity. Coated phases are generally considered to be more versatile, while immobilized phases are more stable and can provide a

higher loading capacity [58], which is a useful feature when preparative-scale separations are desired. Also, in terms of silica support, some of these phases are now available in sub-2 μm particles, making it possible to reach high-efficiency enantioresolutions [59], although this possibility has not been reported yet for flavonoids. Some studies have also been achieved with nano LC, as it offers excellent resolution and sensitivity, thanks to the use of small columns and high-quality packing of the particles. This enabled efficient separation of flavonoid enantiomers, even at low concentrations [60]. This work also included chlorinated polysaccharide CSPs.

Summarizing Table II.2, it can be seen that the majority of recent separations were achieved on the older coated polysaccharides (68%), and a minority (32%) on immobilized phases.

We also note that two CSPs appear most frequently: amylose and cellulose modified with

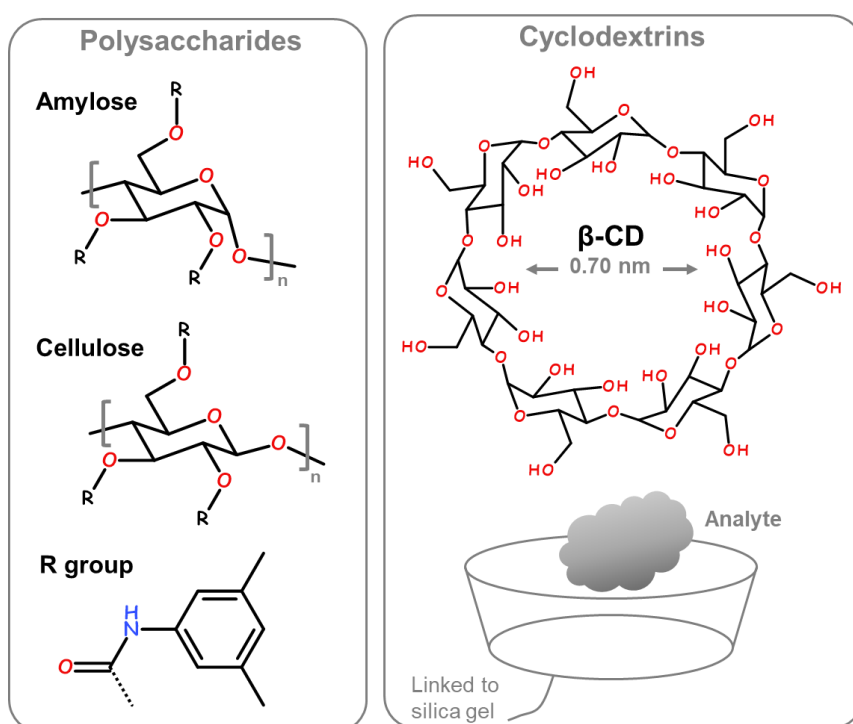


Figure II.4 - General structure of *tris*(3,5-dimethylphenylcarbamate) polysaccharides and β -Cyclodextrin (β -CD) chiral stationary phases

tris(3,5-dimethylphenylcarbamate) ligands (Figure II.4). As they are among the most versatile, they offer great flavonoid separation capacity. They are available in coated or immobilized versions from different manufacturers. In Table II.2, we can see the coated Chiralcel AD [27,48,61–66] and RegisPack [63], and the immobilized Chiralpak IA [25,28,30,67,68] for the amylose phase, and the coated Chiralcel OD [67,69–74] and Sepapak 1 [60], and the immobilized Chiralpak IB [49] for the cellulose phase.

Table II.2 - Chiral LC and SFC analyses of flavonoids

Sample	Types of flavonoids	Flavonoids	Mode	Detector(s)	Stationary phase (column dimensions)	Mobile phases	Flow rate (mL/min)	Temperature / Pressure	Gradient	MS mode	References
Standards	Flavanones	Flavanone	HPLC	UV CD	Chiralpak IA (250 x 4.6 mm – 5 µm)	A) MeOH B) H ₂ O + 10 mM Na ₂ HPO ₄ (pH=9)	1	25°C	-	-	25
Serum and Yerba Santa	Flavanones	Homoeriodictyol	HPLC	UV	Chiralcel OJ-RH (150 x 4.6 mm – 5 µm)	ACN/H ₂ O/H ₃ PO ₄ (22/78/0.5)	1	25°C	80 min	-	90
Biologic fluids (serum)				CD	Chiralcel OJ-RH						
Tutu ling (<i>Rhizoma Similacis glabrae</i>)	Flavan-3-ols	Taxifolin	HPLC	UV	(150 x 4.6 mm – 5 µm)	ACN/H ₂ O/H ₃ PO ₄ (15/85/0.5)	0.35	25°C	140 min	ESI(+)	113
Apple (<i>Malus domestica</i>)				MS							
<i>Launeae arborescence</i>	Flavanones	Naringin Hesperidin	HPLC	UV Polarimètre	Chiralpak IC (250 x 4.6 mm – 3 µm)	A) Hexane B) EtOH (70/30)	1	40°C	-	-	83
Albedo of pomelo and sour orange	Flavanones	Naringin	HPLC	CD	Chiralpak IB (250 x 4.6 mm – 5 µm)	A) n-hexane B) EtOH + 0.5% TFA (60/40)	1.2	-	-	-	84
Standards	Flavanones	Flavanone	HPLC	UV	B-CD (250 x 4.6 mm)	A) MeOH B) H ₂ O + 1% TEAA pH=5.5 (35/65)	0.5	-	-	-	78
Standards	Flavanones	Naringenin	HPLC	UV	Chiralpak AD-H (150 x 4.6 mm – 5 µm)	MeOH	1	30°C	-	-	63
Standards	Flavanones	Naringenin Eriodictyol	HPLC	CD	Chiralpak AD-RH (150 x 4.6 mm – 5 µm)	A) MeOH B) H ₂ O + 0.1% TFA					64
human plasma and urine	Flavanones	Hesperetin. Homoeriodictyol	HPLC	CD MS/MS	Chiralpak IA (150 x 2 mm – 3 µm)	A) H ₂ O + 0.1% FA B) ACN	0.4	30°C	33-100%B (8min)	ESI(+), MRM	68
rat serum	Flavanones	Liquiritigenin	HPLC	UV	Chiralpak AD-RH (150 x 4.6 mm – 5 µm)	ACN/H ₂ O/acetic acid (50:50:0.05)	0.6	25 °C	-	-	61
Canadian natural health product	Flavanones	Liquiritigenin Pinocembrin Pinostrobin	HPLC	MS	Chiralpak AD-RH (150 x 4.6 mm – 5 µm)	1) ACN/H ₂ O/FA (50/50/0.01) 2) MeOH/ + 0.1% AcNH ₄ 3) ACN/H ₂ O/FA (80/20/0.01)	1) 0.6 2) 0.5 3) 1.0	-	-	ESI	62
Cocoa-Based Ingredients	Flavan-3-ols	(+/-)-Catechin (+/-)-epicatechin	HPLC	Fluorescence	Astec Cyclobond I-2000 RSP) + B-CD (250 x 4.6 mm – 5 µm)	A) 20 mM AcNH ₄ buffer (pH=4) B) MeOH (70/30)	1	35°C	-	-	77

Tea (<i>Camellia sinensis</i>)	Flavan-3-ols	Epicatechin	HPLC	DAD	Chiralpak IA (250 x 4.6 mm – 5 µm)	A) 0.5% acetic acid + hexane B) 0.5% acetic acid + EtOH	1	-	15-50%B (45min)	-	30
Commercial chocolates	Flavan-3-ols	(+/-)-Catechin (+/-)-epicatechin	HPLC	DAD	Cyclobond I 2000 RSP (β-CD) (250 x 4.6 mm – 5 µm)	A) H ₂ O + 50mM NaH ₂ PO ₄ (pH=3) B) ACN/ H ₂ O + 30mM NaH ₂ PO ₄ (pH=3) 80/20	1	30°C	10-45%B (70min)	-	29
spices, roots plants	Flavanones	Naringenin Hesperitin	HPLC	MS/MS	Chiralpak AD-3R (150 x 2.1 mm – 3 µm)	MeOH	0.5	40°C	-	ESI+ (MRM) TQ	27
Bottled orange juice	Flavanones	Hesperitin Naringenin Flavanone	HPLC	CD DAD MS/MS	Chiralpak AD-3R (150 x 2.1 mm – 3 µm)	MeOH	0.5	40°C	-	API QTRAP ESI	65
Standards	Flavanones	6-methoxyflavanone	HPLC	UV	N-benzyl-phenethylamino-β- cyclodextrin- bonded ordered mesoporous SBA-15 (BZCDP) (150 x 4.6 mm)	A) H ₂ O + 1% TEAA B) MeOH (70/30) pH = 5	1	-	-	ESI	81
		7-methoxyflavanone		MS							
		2'-hydroxyflavanone									
		4'-hydroxyflavanone									
		6-hydroxyflavanone									
Synthetic compounds	Flavanones	4-iminonaringenin derivatives 2-6	HPLC	UV	Chiralpak IB (100 x 4.6 mm – 5 µm)	iPrOH	0.2	25°C	-	-	49
Pericarp	Flavanones	Naringin Flavanone-7-O- diglycosides	HPLC	CD	Chiracel OD-H (250 x 4.6 mm – 5 µm)	A) n-Hexane B) EtOH (70/30)	1	30°C	-	-	73
Standards	Flavanones	Flavanone	HPLC	MS	bridged bis(β- cyclodextrin) (150 x 4.6 mm)	ACN/H ₂ O + 0.1%FA (15/85)	0.8	30°C	-	ESI(+)	82
		6-methoxyflavanone									
		2-hydroxyflavanone									
		4-hydroxyflavanone									
		6-hydroxyflavanone									
	Flavanonols	Naringin Hesperidin Silymarin									

Citrus pulp and peel	Flavanones	Naringenin	HPLC	CD MS/MS	Chiralpak IG-3 (150 x 2.0 mm – 3 µm)	A) MeOH B) H ₂ O +0.1% FA (85/15)	0.5	40°C	-	ESI-	89
Urine	Flavanones	Flavanone Hesperetin	HPLC	DAD	Chiralcel OD-H (250 x 4.6 mm – 5 µm)	A) hexane B) iPrOH (70/30)	0.3	-	-	-	70
Green tea	Flavan-3-ols	Catechin Epicatechin	HPLC	DAD Fluorescence	Inertsustain phenyl (150 x 4.6 mm)	H ₂ O + 1M NaCl + 0.1% H ₃ PO ₄ (0.05%-0.6% B-CD)	1	35°C	-	-	31
Standards	Flavanones	Naringin	nano LC	UV	capillary column packed with phenyl carbamate 2-propyl-CD (100 µm x 220 mm)	EtOH/hexane/ TFA (60/40/0.5)	240 nL/min	-	-	-	79
		Naringenin									
		Hesperetin									
		Hesperidin									
		4'-methoxyflavanone									
		6-methoxyflavanone									
Standards	Flavanones	7-methoxyflavanone	nano LC	UV	capillary column packed with mesoporous silica 2-propyl-CD (100 µm x 220 mm)	A) ACN B) H ₂ O + 10 mM ammonium acetate pH=4.5 (40/60)	281 nL/min	-	-	-	80
		2'-hydroxyflavanone									
		4'-hydroxyflavanone									
		6-hydroxyflavanone									
		7-hydroxyflavanone									
Standards	Flavanones	Flavanone	SFC	UV	RegisCell (250 x 4.6 mm – 5 µm)	CO ₂ /MeOH with 25 mM isobutylamine	2	35°C/200 bar	4-40% B (14min))	-	86
Standards	Flavanones	Naringenin	SFC	UV	RegisPack (250 x 4.6 mm)	CO ₂ /MeOH (75/25)	4	30°C	-	-	63
Bee pollen	Flavanones	Pinostrobin	SFC	CD DAD	Chiralpak AD (250 x 4.6 mm – 10 µm)	A) CO ₂ B) EtOH/ MeOH (80/20) +0.1%TFA	3	30°C /150 bar	15-50%B (40min)	-	48
		2'-hydroxyflavanone									
		Flavanone									
		4-hydroxyflavanone									
		6-hydroxyflavanone									
		7-hydroxyflavanone									
Bee pollen	Flavanones	Naringin	SFC	CD DAD	Chiralpak AD (250 x 4.6 mm – 10 µm)	A) CO ₂ B) EtOH/ MeOH (80/20) +0.1%TFA	3	30°C /150 bar	15-50%B (40min)	-	48
		Naringenin									
		Hesperetin, taxifolin									

Polysaccharide CSPs have the advantage of being usable under normal-phase (NPLC), reversed-phase (RPLC) or polar organic (PO) LC conditions, as will be further discussed in section 3.2. The same stationary phases can also be used in SFC. However, as mentioned above, SFC has been little used to date in the enantioresolution of flavonoids.

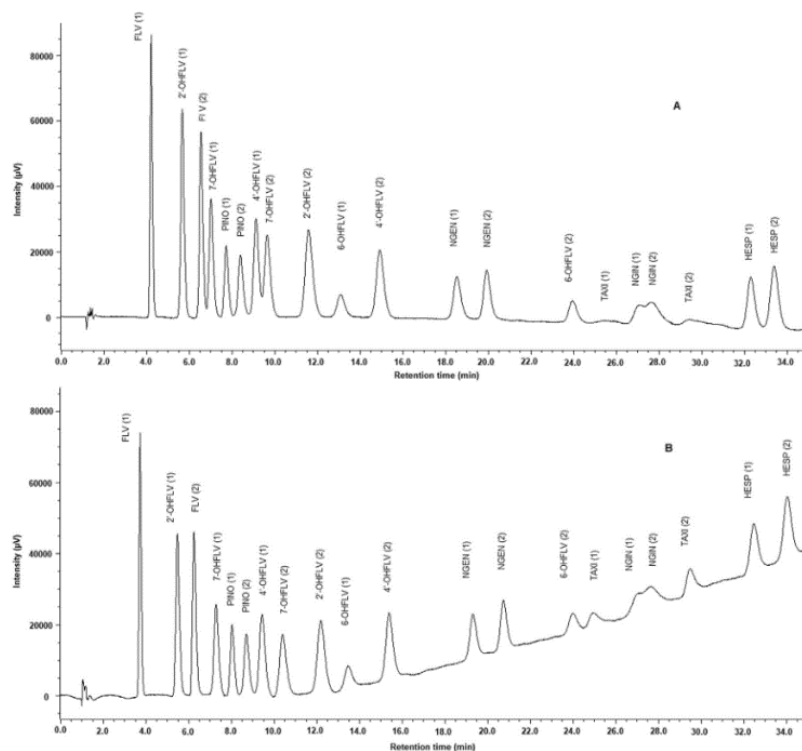


Figure II.5 - Chiral separation using the Chiralpak AD column. Chromatographic conditions 30 °C, 3mL/min, 150 bar. Gradient of ethanol/methanol (80:20): from 0.0 to 10.0 min it was held at 15%, from 10.0 to 23.0 min it was increased to 28% and from 23.0 to 40.0 min (A: without TFA and B: with 0.1% TFA) [48]

Enantiomeric separations of flavonoid samples were described in a single paper. In 2022, Ana M. Ares *et al.* examined the chiral separation of flavanones from a sample of bee pollen [48]. They found that retention on the Chiralpak AD column was higher than on achiral (diol) stationary phases, possibly resulting from the specific interactions in chiral binding sites, together with the added π - π interactions. On this column, they achieved the simultaneous enantioresolution of 10 pairs of flavanone enantiomers (Figure II.5). In this study, the 2R enantiomer eluted first for most analytes, except for Naringenin, 2-hydroxyflavanone and Taxifolin, for which the 2S enantiomer eluted first.

3.1.2. Cyclodextrins

Cyclodextrins are inclusion-type chiral selectors that can be used in different separation techniques, such as LC, SFC or GC, but also in capillary electrophoresis or in asymmetric synthesis [75]. Cyclodextrin-bonded stationary phases were often employed for the enantioresolution of flavonoids, as they offer an effective and selective method for these separations [51]. Cyclodextrins are macrocycles made of glucose units connected by glycosidic links, and they come in different sizes, with the most common being α -cyclodextrin (6 glucose units), β -cyclodextrin (7 glucose units) and γ -cyclodextrin (8 glucose units). This structure forms a basket-shaped hydrophobic cavity (Figure II.4) in which molecules can be inserted according to their size and shape. The analytes may also

interact with the secondary hydroxyl groups at the cyclodextrin “entrance”. These hydroxyl groups may also be modified to vary the possible interactions with analytes [70,76]. In the case of chiral flavonoid separations, native and modified β -cyclodextrins (β -CD) have been used [29,77,78].

Over the years, despite the strong interest in polysaccharide stationary phases, new β -CD-based stationary phases continue to be created [78–82]. Recently, Ning Zhang *et al.* prepared a new bridged *bis*(β -cyclodextrin) stationary phase that they compared with a native β -CD stationary phase [82]. This new phase showed a higher separation capacity than the native phase. In fact, with the chemical modification the new phase had two cavities instead of one in the native phase, which made it possible to accommodate larger chiral analytes, thereby improving selectivity.

As described above for polysaccharide stationary phases, nano-LC was also described with cyclodextrin stationary phases [79,80]. In 2017, Mariana Silva *et al.* used a β -CD-bonded stationary phase to achieve enantioseparation of 12 flavanones and flavanone glycosides by nano-LC with high efficiency [80].

3.2. Mobile phase composition

In addition to the choice of stationary phase, the selection of the mobile phase is of paramount importance in chiral analysis, as it can have a considerable impact on chromatographic enantioresolution. Firstly, as is also the case in achiral separations, the choice of mobile phase composition affects the possible interactions between the enantiomers and the chiral selector in the CSP as the chromatographic process relies on a balance of interactions between analyte and stationary phase on the one hand, analyte and mobile phase on the other hand. Secondly, and more specific to chiral separations, some stationary phases with flexible chiral selectors (typically, polysaccharide-based CSPs) may somewhat change their three-dimensional structure depending on the choice of solvent, opening some chiral cavities or, on the contrary, limiting access to them.

Finally, additives like acids, bases or salts can also contribute to the enantiorecognition process in different ways: limiting non-enantioselective interactions with the supporting phase, forming ion pairs with ionic analytes, etc. Thus, by modifying the composition of the mobile phase, it is possible to radically alter the separation performance and optimize chromatographic selectivity.

Keeping these elements in mind, the mobile phase can be adjusted by using different solvents and additives, or by modulating the pH in order to optimize the specific interactions between the enantiomers and the stationary phase. We will first discuss the choices of solvents, then the additives usually employed for flavonoids.

As appears in Table II.2, most flavonoid enantioseparations reported in the recent years were carried out in RPLC mode, where the interaction between the stationary phase and the target compounds is largely driven by hydrophobicity. Similar to achiral RPLC, a mobile phase composed of an alcohol and an aqueous buffer is used to achieve the separation. Widely used in chiral chromatography, the NPLC mode also remains a means of achieving flavonoids enantioresolution. In this mode, the mobile phase usually contains a non-polar solvent as a major component (typically hexane) and a polar co-solvent to adjust retention and enantioresolution [28,30,69–73,79,83,84]. While this type of solvent is convenient for preparative scale as they can be evaporated rather easily, they are however not environmentally friendly, especially as hexane is rather toxic. In this context, SFC is of great interest [52] as CO₂ would replace hexane [48,63,85–87]. As the eluent strength of CO₂ was often compared to that of alkanes, replacing hexane by CO₂ often enables enantioseparations to be carried out with comparable selectivity, although higher flow rates can be employed without damage to efficiency, so resolution per unit time is generally improved. The addition of an alcohol-type co-solvent increases the polarity of the mobile phase, as described above for achiral separations. Among the alcohols used in chiral analyses in RPLC, NPLC and SFC are isopropanol (iPrOH), EtOH and MeOH (Table II.2).

All separations reported on β -CD-based stationary phases employed H₂O / ACN [29,80] or H₂O / MeOH [77,81] mobile phases. For instance, Ning Zhang *et al.* studied the influence of the percentage of MeOH on resolution and retention [82] (Figure II.6). In this study, enantioseparations were examined on a modified β -CD-based stationary phase with a H₂O / MeOH mobile phase comprising 0.1% formic acid.

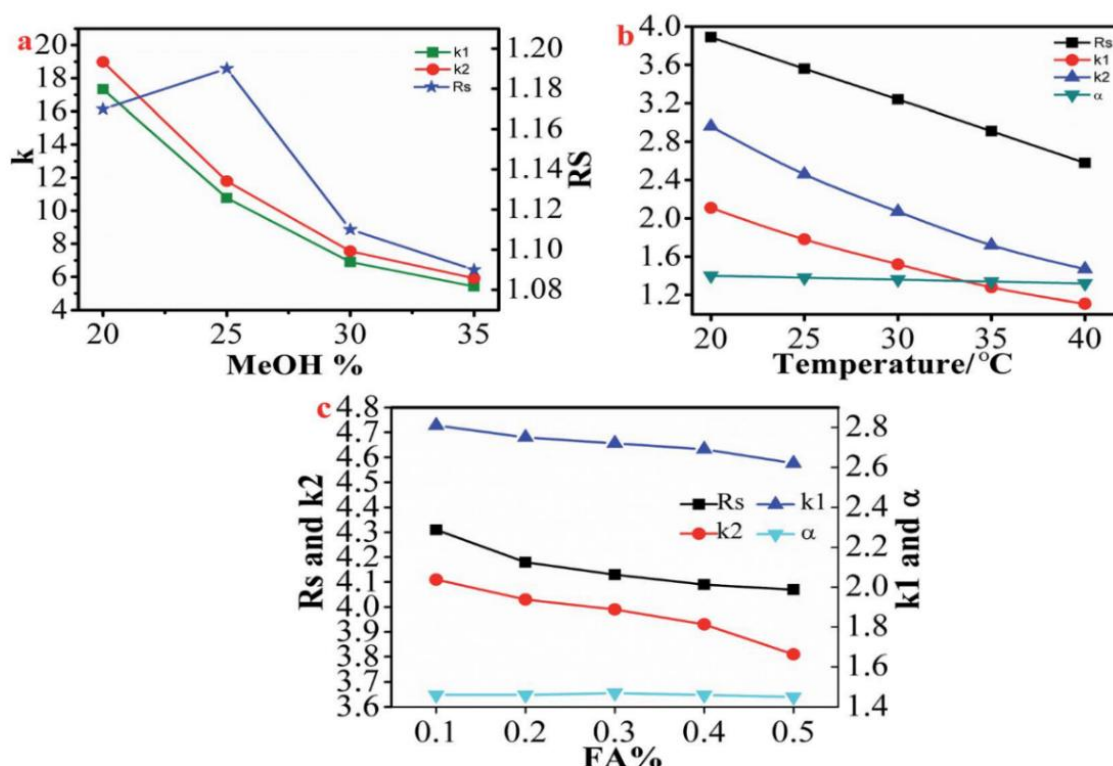


Figure II.6 - Effect of operating parameters on the chromatographic separation of flavanone enantiomers on a cyclodextrin-type stationary phase. (a) Effect of methanol content in the aqueous mobile phase on the retention factors and resolution of 6-methoxyflavanone enantiomers; (b) effect of column temperature on the retention factors, separation factor and resolution of 2-hydroxyflavanone enantiomers; (c) effect of the concentration of formic acid in the mobile phase on retention factors, separation factor and resolution of 4-hydroxyflavanone enantiomers [82]

They showed that, the higher the percentage of MeOH, the lower the retention and resolution, which corresponds to RPLC behaviour. A balance therefore needed to be found between analysis time and resolution.

As mentioned above, additives such as acids, bases or salts are also frequently added to the mobile phase to modify the interactions between the enantiomers and the stationary phase, which can impact the selectivity and resolution of the separation (Figures 5 and 6). In aqueous compositions, additives can also modify the pH of the mobile phase, thereby modifying the ionization state of acidic and basic analytes, or the ionization state of the stationary phase (silanol groups, ionizable bonded ligands). Flavonoids have alcohol and phenol functional groups that can be ionized at different pH levels, with pK_a values typically varying between 6 and 13 [88]. By adjusting the mobile phase pH, the ionization state of flavonoids varies and, consequently, their interactions with the chiral selectors also vary: when the alcohol and phenol functions remain

protonated, hydrogen-bonding with the chiral selector is favoured, which should be favourable to enantiorecognition. This can lead to significant changes in their retention and enantioseparation. However, as mentioned before, flavonoids have better stability in acidic conditions, so this parameter cannot be varied in a large range. In consequence, acidic additives are generally used in the mobile phase (Table II.2). These include formic acid [31,62,68,81,82,89], acetic acid [61], phosphoric acid [31,66,74,90,91] and trifluoroacetic acid [28,48,64,69,72,73,79,84]. In SFC as well, Ana M. Ares *et al.* as previously reported, carried out the separation of 10 flavanone enantiomeric pairs [48] (Figure II.5). Their work showed that the addition of an acidic additive, in this case 0.1% TFA, improved the symmetry of the peaks, particularly that of taxifolin, but also modified enantioresolution for several pairs of enantiomers.

When using β -CD as the stationary phase, a basic additive or a salt can be added (Table II.2) to promote the formation of an inclusion complex [75]. These additives can be ammonium acetate [77,80], triethylamine acetate [78,82], isobutylamine [86], or monosodium phosphate [25,29]. However, as mentioned earlier, basic conditions may cause analyte instability. None of the above references mentioned any such issue.

3.3. Operating parameters

3.3.1. Oven temperature

Temperature conditions can also have an impact on enantiomer separation through several modes of action. In liquid mobile phases, an increase in temperature reduces solvent viscosity, which is favourable to increased column efficiency, thereby possibly improving resolution. However, following van't Hoff law, increasing temperature in liquid phases usually yields lower retention, which is not so favourable to enantioresolution. In addition, lowering temperature is causing the analytes to be more rigid, which should be favourable to the enantioresolution of flexible molecules. But it may also cause the chiral selector to be more rigid, which can affect the enantiorecognition process in an unpredictable, positive or negative way. In this respect, it is frequent to observe that the effect of temperature on enantioresolution is not monotonous, possibly with different trends in different ranges of temperature. In supercritical fluids, the situation is even more complicated [48,63,85,86] as the fluid density decreases with an increase of temperature, which is changing the mobile phase elution strength and has several other consequences [92].

An example of the effect of temperature in LC mode can be seen in Figure II.6, where Ning Zhang *et al.* monitored retention, selectivity and resolution between flavanone enantiomers, when

increasing temperature on a CD-bonded stationary phase [81,82]. In that case, and in the range of temperature explored, retention and resolution decreased as temperature increased, while selectivity remained mostly unchanged.

We may note that the range of temperatures explored is usually rather small because the stability of most chiral stationary phases does not allow for temperatures higher than 40-45°C. In the lower range, reducing temperature below room temperature cannot always be achieved, depending on the type of oven available in the chromatographic system.

In addition to being an optimization parameter, temperature variation may also give access to thermodynamic parameters and is useful to understand enantiorecognition mechanisms [83,85]. Indeed, according to Van't Hoff law, the change in free energy (ΔG) accompanying the chromatographic separation of two enantiomers is directly related to the enantioseparation and is given by Eq. (1):

$$(\Delta G) = -RT \ln \alpha \quad (1)$$

where R is the gas constant, T is the absolute temperature and α is the enantioseparation factor. Plotting retention and separation factors according to the reversed temperature is thereby providing access to enthalpy and entropy changes. For the reasons explained above and unlike achiral separations, chiral separations often yield non-linear trends as the retention and enantioseparation mechanism are likely to vary over the range of temperatures examined.

For instance, in 2022, Ali *et al.* demonstrated a correlation between the free energy change, that is to say between the enantioseparation factor observed on a Chiralcel®OD-H column, and the carbon chain length of bis-asymmetric compounds derived from flavanes [70] (Figure II.S2). It was observed that higher molar mass molecules (long carbon chain lengths) had stronger interactions with the stationary phase, leading to increased retention and, in that case, to increased enantioseparation. However, it should be noted that the correlation between molecular weight and retention and/or separation can vary depending on the specific nature of the interactions and the chemical properties of the flavonoids studied. In other cases, increased molecular weight would cause reduced penetration of the analytes in the chiral cavities, thereby reducing enantioresolution, so no general conclusion should be drawn from this observation.

3.3.2. Pressure in compressible fluids

Unlike conventional liquid chromatography, SFC uses a compressible fluid as the mobile phase. Thus, pressure plays a key role in modifying the physicochemical properties of the mobile phase, as increasing pressure causes increased density, thereby increasing eluting strength, which can

influence the retention, separation and efficiency, all impacting enantioresolution [48,63,85,86]. This provides additional flexibility for adjusting separation conditions to achieve optimum results in chiral SFC.

4. Two-dimensional chromatographic analyses

When further increases in column efficiency are not possible and a single chromatographic analysis cannot achieve the full resolution of a sample, an extra dimension [93,94] should be added. Two-dimensional liquid chromatography [34] (2D-LC) is an advanced technique that combines two complementary separation dimensions to improve the resolution of compounds in a complex sample, thereby providing more detailed information about the composition of samples, and to identify compounds that would be difficult to separate using a single chromatographic dimension. However, certain combinations, although orthogonal, are not feasible due to the immiscibility of solvents (e.g. an aqueous fraction to be transferred to a hexane-based mobile phase) and incompatibility between the mobile and stationary phases (e.g. a strong solvent from the first dimension could not be introduced in a second dimension operating under HILIC mode). Consequently, particular care is required in the design of 2D systems, bearing in mind that the first-dimension eluent is also the second-dimension injection solvent. Recent literature on this topic is reported in Table II.3.

Complementarity may be achieved through different means. The simplest way is to maintain the same chromatographic mode in the two dimensions but changing the composition of the mobile phase [95]. For instance, RPLC in acidic conditions may be hyphenated to RPLC in basic conditions, ensuring facilitated transfer between the first and second dimensions as there would be no significant incompatibility of mobile phases. However, this solution is unfit for flavonoids analysis as they require acidic analytical conditions to ensure their stability. Hence another simple way to achieve complementarity is to use different stationary phases in the same chromatographic mode. For instance, both dimensions could use RPLC mode, but different stationary phases like octadecyl-bonded silica and phenyl-bonded silica, to obtain different selectivity. Here again, mobile phase compatibility remains excellent, but it is difficult to obtain high orthogonality with such strategies. To improve this point, different chromatographic modes (e.g. RPLC and HILIC) can be selected in the two dimensions [96–99]. The compatibility between RPLC and HILIC remains reasonably good as they may both employ mixtures of aqueous buffers and ACN.

Table II.3 - 2D analyses involving chiral flavonoids

Sample	Types of flavonoids	Flavonoids	Mode	Detector(s)	Dim	Stationary phase (column dimensions)	Mobile phases	Flow rate (mL/min)	Temperature / Pressure	Gradient	MS	References
Camellia sinensis in green and black tea	Flavan-3-ols	(+/-)-Catechin	SEC x RPLC	UV	1D	Ultra hydrogel -120 (300 x 7.8 mm - 5.10 ³ Da)	A) H ₂ O B) ACN	1	60°C	0-50% B	ESI (+/-) - TQ	100
		(+/-)-gallocatechin (+/-)-gallocatechin gallate (+/-)-catechin gallate		MS ELSD	2D	BEH-C18 (50 x 2.1 mm - 1.7 µm)	A) H ₂ O + 0.1% FA B) MeOH	1	80°C	0-100% B		
Denzhan Shennai	Flavanones	Eriodictyol	RPLC x RPLC	MS	1D	C8 XTerra MS (100 x 2.1 mm - 1.7 µm)	A) H ₂ O + 0.1% FA B) MeOH	0.1	40°C	10-100% B	QTOF	107
		Naringin-7-O-glucuronide			2D	Eclipse Plus C18 (50 x 4.6 mm - 1.8 µm)	A) H ₂ O + 0.1% FA B) MeOH	2	40°C			
		Homoisoflavanone			2D chirale	Chiralpak IC-3	A) H ₂ O + 0.1% FA B) MeOH	1	40°C			
		Catechin Epicatechin			1D	RP Amide (250 x 1.0 mm - 5 µm)	A) H ₂ O + FA (pH = 3) B) ACN + FA (pH = 3)	0.1	25°C	20-90% B (70 min)	-	
Red wine	Flavan-3-ols		RPLC x RPLC	UV	2D	Ascentis Express (30 x 4.6 mm - 2.7 µm)	A) H ₂ O + FA (pH = 3) B) ACN + FA (pH = 3)	4	40°C	0-100% B (1.7 min)	-	76
Citrus lemon peels	Flavanones	(Neo)hesperidin (iso)naringin	CCC x CCC	MS/MS	1D	n hexane/n butanol/MeOH/H ₂ O (0.23/1.00/25/1.13)		2.0	-		ESI (-)	108
		Neohesperidin			2D	n hexane/n butanol/MeOH/H ₂ O (0.47/1.00/38/1.46)						
Green tea	Flavan-3-ols	(Ep)gallocatechin gallate (Ep)gallocatechin (Ep)catechin	HILIC x RPLC	PDA MS Fluorescence	1D	Nomura Chemical Develosil Diol-100 (250 x 1.0 mm - 5 µm)	A) ACN + Acetic acid (99:1) B) MeOH/H ₂ O/Acetic acid (94.05/4.95/1)	0.05		4-40% B (55 min)	ESI (-)	97
					2D	2x Zorbax SB-C18 (50 x 4.6 mm - 1.8 µm)	A) H ₂ O + 0.1% FA B) ACN	0.8	50°C	2-100% (22 min)		
Rooibos (<i>Aspalathus linearis</i>)	Flavanones	Hesperidin Eriodictyol	NP-HPCCC x RP-UHPLC off line	UV MS	1D	EtOAc/H ₂ O (1/1) (A)	n-BuOH/H ₂ O (1/1) (B)	1.5	-	100-50% B (47 min)	QTOF, ESI	109
		flavanone-O-hexoside flavanone-O-malonylhexoside			2D	Poroshell 120 SB-C18 (100 x 2.1 mm - 1.8 µm)	A) H ₂ O + acetic acid 2% B) ACN	0.8	44.5°C	10-100% B (17 min)		

<i>Sphaerophysa safsula</i>	Flavonoids glycosides	RPLC x HILIC off line	UV	1D	Xaqua C18 prep (250 x 20 mm - 10 µm)	A) H ₂ O + 0.2% FA B) ACN	15	-	5-30% B (55 min)	98
				2D	Xanide prep (250 x 20 mm - 10 µm)	A) H ₂ O + 0.2% FA B) ACN	15	-	3-95% A (30 min)	
Grapevine canes (<i>Vitis vinifera</i> L.)	Flavan-3-ols. Flavanones	HILIC x RPLC	DAD MS	1D	Lichrospher diol-5 (150 x 1.0 mm - 5 µm)	A) ACN + 1% FA B) MeOH + 10 mM AcNH ₄ + acetic acid (95:4:1)	0.18	25°C	2-40% B (80 min)	96
				2D	Ascentis Express C18 (50 x 4.6 mm - 2.7 µm)	A) H ₂ O + 0.1% FA B) ACN + 0.5% FA	3	25°C	2-60% B (1 min)	
Licorice	Flavanones	RPLC x RPLC	MS	1D	Acquity CSH C18 (100 x 2.1 mm - 1.7 µm)	A) MeOH B) H ₂ O + 0.1% FA	0.1	50°C	50-90% A (40 min)	93
				2D	Poroshell Phenyl Hexyl (50 x 3.0 mm - 2.7 µm)	A) ACN + 0.1% FA B) H ₂ O + 0.1% FA	2	50°C	25-90% A (40 min)	
Quebracho, cocoa bean, grape seed	Flavan-3-ols	HILIC x RPLC	DAD MS Fluorescence	1D	Acquity UPLC BEH amide (150 x 1.0 mm - 1.7 µm)	A) ACN + 0.1% FA B) H ₂ O + 0.1% FA	0.08	25°C	5-100% B (150 min)	99
				2D	Kinetex C18 (50 x 3.0 mm - 1.7 µm)	A) ACN + 0.1% FA B) H ₂ O + 0.1% FA	2	40°C	0-95% B (0.8 min)	
Bitter orange (<i>Citrus aurantium</i>)	Flavanones	mSFC - SFC	MS	1D	Acquity Torus DEA (100 x 3.0 mm, 1.7 µm)	A) CO ₂ B) MeOH + 0.1% MSA	1.7-0.6	30°C / 150-110 bar	20-100% (8 min)	103
				2D	Chiralpak IB-3 (150 x 4.6 mm, 3 µm)	A) CO ₂ B) MeOH + 0.1% MSA	2	30°C / 150 bar	15-50% (17 min)	

Compatibility would however be much worse when RPLC is hyphenated to NPLC, which operates in purely organic solvents.

As a general rule, the second dimension is used to resolve co-elutions observed in the first dimension. The flavonoids that pose the greatest difficulty in terms of separation are the glycosylated forms because, as pointed out in section 1.2 and Figure II.3, they are larger and more polar than non-glycosylated forms. Increasing the size of the glycosylated group (monosaccharide, disaccharide...) further increases both molecular weight and polarity. Therefore, two different analyte features may be focused on: size and polarity. In this context, keeping RPLC as one of the two dimensions and introducing a different chromatographic mode in the other dimension seems a logical approach.

Considering the size feature, Scoparo *et al.* reported a 2D-LC method combining size exclusion chromatography (SEC) and RPLC in a comprehensive fashion (SECxRPLC), to analyse the flavonoids of tea leaves extracts [100]. While SECxRPLC is commonly used in the analysis of biopharmaceuticals, it was rarely described for natural products. This paper was the first to cover such a broad spectrum of compound polarity on a single plant extract [100].

Considering the polarity feature, there has been an ongoing development in the use of HILIC in 2D-LC, either in the first or second dimension. The high orthogonality between HILIC and RPLC, estimated around 80% in this case, shows the strong potential of this coupling [97,101]. Often used in off-line mode, this coupling has already proven to be very effective, both for analytical and preparative purposes [102].

Another complementarity that may be sought in the specific case of chiral flavonoids is that of stereoisomers. Réset *et al.* recently proposed to combine an achiral SFC method in the first dimension to a chiral SFC method in the second dimension in a multiple-heart cutting 2D-SFC system [103]. This allowed retrieving pairs of stereoisomers from the first dimension to be resolved in the second dimension. As both dimensions employed the same fluid (carbon dioxide and methanol co-solvent with acidic additive), no compatibility issues were encountered. In addition, the compressible nature of the supercritical fluid proved to be a great advantage in re-focusing the peaks upon entering the second dimension, which was favourable to resolution and sensitivity.

Besides complementarity of the two separation dimensions, in on-line analysis the way to transfer fractions from the first dimension to the second, and the number of fractions to be transferred may vary. For instance, a single fraction from a single peak can be transferred (heart-cut mode), or several separated fractions from different peaks (multiple heart-cut mode), or several fractions from a single peak (selective comprehensive mode), or all fractions from the first dimension (fully

comprehensive mode). Not all transfer modes have been applied to the analysis of flavonoids. In the multiple heart-cut approach to 2D, compounds are separated primarily in one dimension, with selective and targeted cutting of specific fractions in the first dimension, transferred to the second dimension for further analysis. This approach reduces sample complexity and focuses on specific analytes, while minimizing interference from other compounds [104,105]. In contrast, the comprehensive LCxLC approach transfers all fractions from the first dimension to the second dimension and offers a most complete view of the sample. To achieve this, it is necessary to have a fast analysis speed in the second dimension, which means using a shorter column, preferably packed with small particles to retain sufficient efficiency [93,99,101].

The purpose of 2D-chromatography is to increase peak capacities compared to one-dimensional analysis and thereby obtain a more complete view of a complex sample, which is generally the case. Off-line 2D methods usually pose few difficulties since the fractions obtained from the first dimension may be evaporated to dryness and reconstituted in an appropriate dilution solvent, but the situation is different for on-line coupling. In that case, the volume and mobile phase composition transferred from the first to the second dimension should be strictly controlled to avoid degrading the resolution or distorting the peaks [98,106]. When the first dimension is operated in gradient elution, shifting gradients can be used in the second dimension to progressively adapt the elution conditions in second dimension to the transferred fraction. Additionally, sensitivity may be poorer than in one-dimensional LC due to dilution caused by high flow rates in the second dimension [76]. Thus, it is important to take into account all the interfering parameters and optimize them. The group of André de Villiers is largely involved in the development of 2D-LC methods, particularly combining RPLC and HILIC. In 2022, they showed the limits of this coupling. In their approach, a solution proposed was to switch from one dimension to the other by stopping the first-dimension flow in multiple-heart-cut mode [99].

Ning Sheng *et al.* in 2017 also addressed the difficulties of 2D-LC to analyse flavonoids in a mixture of medicinal herbs [107]. By testing different gradient modes, they showed that adding a lag time before starting the second-dimension gradient was beneficial. As shown on Figure II.7 (Mode C) this strategy offered a good distribution of peaks in the two-dimensional space compared with conventional or segmented gradient modes (A and B modes). This served both for achiral-achiral and achiral-chiral analysis.

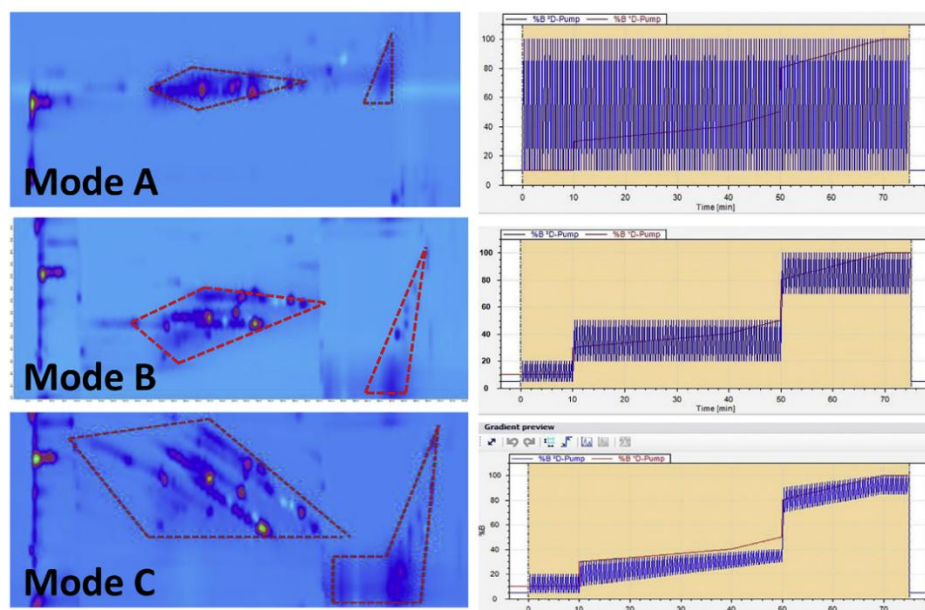


Figure II.7 - Comparison of contour plots from LC $\times$ LC analysis of a mixture of medicinal herbs under three types of gradient modes (contour plots with absorbance at 280 nm are shown on the left and the corresponding gradient program is shown on the right [107])

Two-dimensional methods may also be used at the preparative scale for the purpose of (i) purifying single flavonoids for accurate structure determination and/or activity testing or (ii) recovering flavonoid fractions to be further analysed with a high-resolution method like UHPLC-MS/MS. Counter-current chromatography (CCC) was reported for both purposes [108,109]. CCC is a liquid-liquid chromatographic technique relying on the partition of the target compounds between two non-miscible liquid phases. Although on-line hyphenation of CCC to HPLC is possible [110], in the case of flavonoids only off-line methods were reported. Using this technique, in 2017 Walters *et al.* demonstrated for the first time the presence of Hesperidin and 18 other compounds in Rooibos [109].

5. Detectors

Detection is essential for chromatographic monitoring and to characterize chiral flavonoids. In recent years, detection techniques have been improved to ensure greater sensitivity and selectivity of compounds.

Regardless of whether the chromatography is chiral or achiral, the detectors used are mostly the same. They include UV-visible single-wavelength or diode-array detectors (DAD), fluorescence detectors (FLD) and MS detectors. In addition, circular dichroism detector is useful when enantioseparation is achieved, or with achiral analysis when a single enantiomer is expected to be

present in the sample. By far the most commonly used detectors are DAD and MS detectors (Figure II.8).

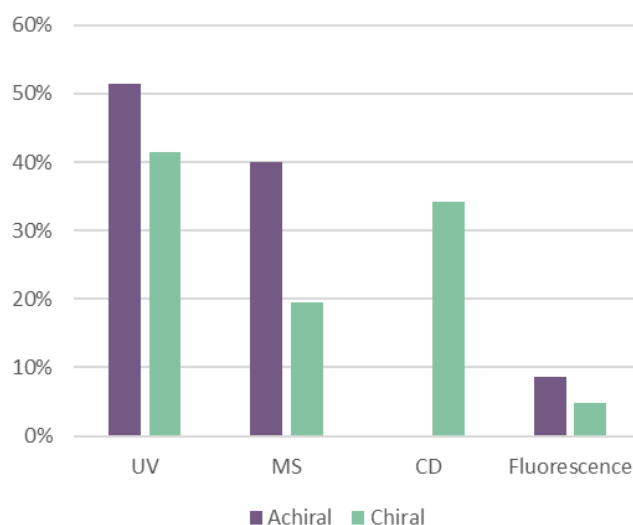


Figure II.8 - Comparison of the different detectors listed in tables 1, 2 and 3 for the analysis of chiral flavonoids

5.1. UV-visible single-wavelength and diode-array detectors

Single-wavelength UV-visible detector is widely used to detect and quantify chiral flavonoids in LC and SFC, as can be seen in Tables 1, 2, and 3. Although improved by electronic filters, sensitivity is still moderate, but linearity range is rather good and repeatability is usually excellent. Currently, it can accurately detect quantities of flavonoids ranging from a few nanograms to several milligrams. A major limitation is however its specificity, as it cannot differentiate between the stereoisomers of chiral flavonoids that possess similar UV absorption properties [48,64,65].

Nevertheless, the aromaticity of flavonoids is proving to be a real advantage for characterizing these compounds. Their absorptions at relatively long wavelengths improve the selectivity of qualitative and quantitative spectrophotometric methods, while the characteristic spectra of the different classes of flavonoids enable them to be distinguished from one another, and to be distinguished from other compounds that may be present in a complex sample of natural product. For this reason, DAD detectors are often preferred over single-wavelength detectors.

The UV spectra of flavonoids generally show two main absorption bands in the region of 240 to 400 nm. The absorption band I, attributed to ring B (Figure II.1), is located between 300 and 395 nm, while absorption band II, attributed to ring A, is located between 240 and 295 nm (Table II.4). Increasing the number of hydroxyl groups on the aglycone leads to a bathochromic shift of the absorption bands (increasing wavelength at maximum absorption). On the other hand, methylation or glycosylation, particularly on the hydroxyl groups in positions 3, 5, 7 and 4', causes a hypsochromic shift in the absorption bands (decreasing wavelength at maximum absorption). It

should be noted that the specific nature of the sugar moiety generally has no significant effect on these spectral shifts.

Table II.4 - UV absorption bands of the flavanol, flavanone and flavanonol families

Structure type	Band II (nm)	Band I (nm)
Flavonol (3-OH is substituted)	250-280	328-357
Flavonol (3-OH is free)	250-280	358-385
Flavanone and flavanonol	270-295	300-330

5.2. Fluorescence detection

The fluorescence detector (FLD) is a highly sensitive detector that can detect very small quantities of chiral flavonoids. Because it relies on light emission, it is typically more sensitive than the absorption-based UV detector [99,111]. For example, Inal Bakhytkyzy *et al.* have shown that the detection limit for FLD was 10 times better than for UV detection [111] (Figure II.S3). In addition, because not all molecules can emit fluorescence light, and because different fluorescent molecules would require different excitation wavelengths, FLD is also more specific than UV detectors, with fewer spurious signals. Indeed, in fluorescence there are always two wavelengths to be used: an excitation wavelength and an emission wavelength. In the case of flavanols, which were mostly studied in recent years, the excitation wavelength is between 270 and 280 nm and the emission wavelength between 315 and 347 nm (Table II.5). For instance, it appears in Figure II.S3 that the FLD chromatogram shows a cleaner trace than the DAD chromatogram, as the former allows focusing on selected compounds through excitation and emission wavelengths. However, FLD is usually more expensive than DAD.

Whether in chiral or achiral chromatography, FLD allows monitoring the separation but in no case provides information on the chiral identity of stereoisomers [31,77,97].

Table II.5 - Excitation and emission bands of flavanols for fluorescence detection

Type of Flavonoids	Flavonoids	Excitation (nm)	Emission (nm)	Reference
Flavanols	(Epi)catechin	276	316	77
	(Epi)catechin Procyanidin	280	347	111
	(Epi)gallocatechin gallate, (Epi)gallocatechin, (Epi)catechin	276	316	97
	(Epi)catechin (Epi)gallocatechin Fisetinidol Epifisetinidol	230	321	99
	(Epi)catechin	270	315	31

5.3. Mass spectrometry

The mass detector is a highly specific detector that allows both detection and quantification but may also provide some structural information. It offers both high sensitivity and high specificity. It can measure the exact molecular weight of chiral flavonoid molecules [35]. However, mass analysers are usually significantly more expensive than other detectors and often require more sample preparation prior to analysis. They may also impose specific mobile phase compositions as volatile components should be used, and corrosive additives should be particularly avoided. Hyphenation to SFC must be done with a little more caution. Solubility problems can arise due to the depressurization of CO₂ when entering the mass spectrometer. In addition, as CO₂ is not easily ionized in electrospray, it is useful to introduce an extra source of protons to favour analyte ionization. Both issues can be simply solved with the introduction of a make-up fluid prior to entering the MS [46].

When all the data in the tables 1, 2 and 3 are taken together, it can be seen that many analyses using UV detection were also coupled with simple or tandem MS. In all cases, the electrospray ionization mode was used in positive or negative mode [112]. For the analysis of chiral flavonoids, there was no preferred ionization mode, with positive mode being used for 48% and negative mode for 52% of the reported publications.

Although the ionization source was common to all the analyses of chiral flavonoids listed here, analysers were more varied [35]. The accessibility of low-resolution mass in terms of price, makes it a criterion of choice. In fact, the majority (72%) of reported analyses were done with a low-resolution analyser of single- [36,38,42,46,62,113] or triple-quadrupole [27,43,44,65,89,97,114] type. The remaining 28% were analysed with high-resolution mass spectrometry (HRMS, Figure II.9). HRMS is generally used for applications requiring high precision and selectivity, such as the precise identification of compounds and the analysis of complex mixtures [39,97,99,101,107,108]. Thanks to the high resolution provided by the Q-TOF analyser, Lidia Montero *et al.* in 2017 carried out the first determination of the presence of Hesperidin in grapevine cane [101].

In addition to identification, MS can also be used to quantify flavonoids. This quantification is possible in simple MS [36,62] or in MS/MS [27,44,89].

Finally, mass spectrometry is a powerful tool for characterizing molecules but is not always capable of differentiating between all flavonoids, as fragmentation patterns do not always allow differentiating between isomers, and especially not between stereoisomers.

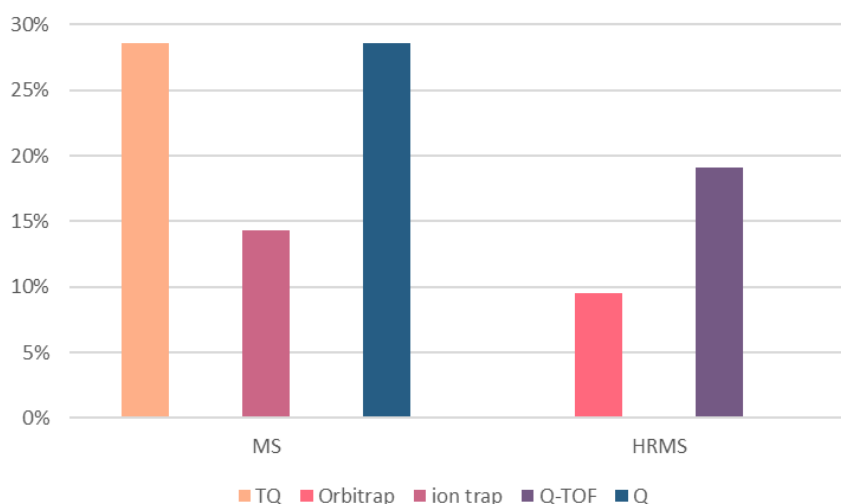


Figure II.9 - Comparison of MS analyzers used in low resolution and high resolution (HRMS)

5.4. Circular dichroism

Circular dichroism (CD) is often used as a detection method in enantioseparations, and in particular for chiral flavonoids (Figure II.10). Widely used in biochemistry for proteins and nucleic acids [115,116], the CD detector can be used to study the structure and conformation of molecules. It is based on the difference in absorption of left and right circularly-polarized light as it passes through a solution containing a chiral molecule [117]: enantiomers have opposite CD spectra, so the chromatographic peaks seen with CD detector are either positive or negative, as appears in Figure II.10. This detector is therefore specific of chiral molecules. It is very useful to detect and quantify flavonoid enantiomers, as it can distinguish between molecules with different spatial configurations [25,28,69,71,72,113].

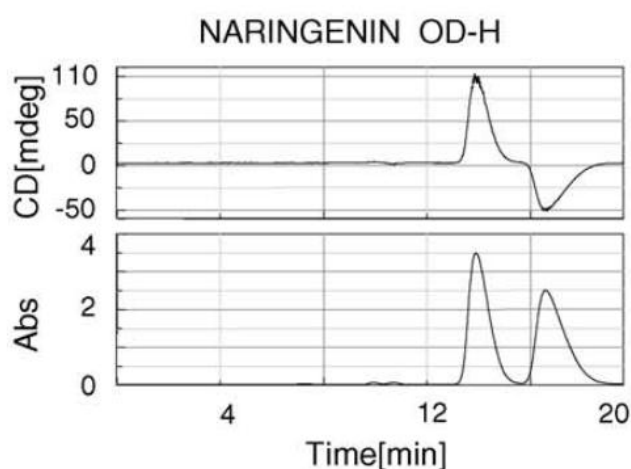


Figure II.10 - Chromatogram of Naringenin obtained by HPLC analysis on a Chiralcel OD-H column in UV and CD.

[72]

CD can provide information on the conformation of flavonoids, but it may have limited specificity for distinguishing between different types of flavonoids. Some flavonoids may have similar CD spectra, making it difficult to distinguish them on the basis of this technique alone. In such cases, another detector as MS or MS/MS may be required for accurate identification.

Used both in LC [25,28,49,64,65,69–73,78,113] and SFC [48,87], the CD detector is used for a variety of purposes. It can be used to monitor enantiomer configurations as a function of fruit maturity [69,84], to assess the optical activity of new compounds [73], or to determine the elution order of enantiomers [64,65]. For example, in 2005, Salvatore Caccamese *et al.* performed several chiral separations of flavonoids such as Naringenin (Figure II.10) in HPLC using CD detection [72]. Thanks to this detection, it is possible to determine which of the enantiomers elutes first. In cases where a scalemic mixture would be present, it would therefore be useful to determine which enantiomer is the most concentrated. By studying flavonoids that are widespread in the plant kingdom, they showed the potential of this detector and its great need for application examples such as natural products. However, at the time, the potential of CD was underestimated: it was mostly used as an independent spectroscopic technique to determine molecular structure, but not so much as a chromatographic detector.

5.5. Sensitivity

Detector sensitivity plays a crucial role in molecular analysis, as it determines the ability to accurately detect and measure low concentrations of target analytes. This makes the comparison of flavonoid profiles in different matrices more complete, to identify and quantify the different classes of flavonoids present. It also makes it possible to assess fine variations in composition as a function of various factors, such as fruit maturity, growing conditions or processing methods. In the case of chiral flavonoids, sensitive detectors would also provide a better view of isomer ratios and isomerisation effects when a chiral chromatographic method is placed in front of the detector. It is important to note that the sensitivity of a detector can be influenced by various factors such as background interference, sample matrix or experimental conditions. Philip Machonis *et al.* determined the presence of Catechin and Epicatechin in cocoa [77]. In this matrix, they showed the presence of the 2 enantiomers of each molecule. Measuring the limit of quantification (LOQ) with fluorescence detection for each enantiomer, they found that the two Catechin enantiomers had the same LOQ. However, for Epicatechin the LOQs were very different, with 0.6 µg/mL for (+)-Epicatechin and 12.1 µg/mL for (-)-Epicatechin. It is difficult to understand such a large difference, unless some other compound co-eluted with one of the two enantiomers.

Regarding two-dimensional chromatography, it is important to note that the limits of detection and quantification are often deteriorated compared to one-dimension separation, because this small quantity is divided into several fractions in the second dimension. For instance, in 2009, Paola Dugo *et al.* showed that adding an extra RPLC dimension increased the LOQ by around a factor of 5 [76]. However, in other cases, it could also be favourable to detectability, e.g. when a low-concentration compound co-eluted with a large-concentration compound in the first dimension. Figure II.11 shows representative values of LOQs reported in the literature with UV, FLD and MS detectors for different chiral flavonoids. Naturally, these values vary greatly from one detector to another. If we take a closer look at the Naringenin compound, we can see that the UV detector used with SFC [48,118] was not very sensitive compared with the same detector employed with an LC separation. This is a common observation, as SFC-UV is typically twice less sensitive than LC-UV, when all system dimensions are in the

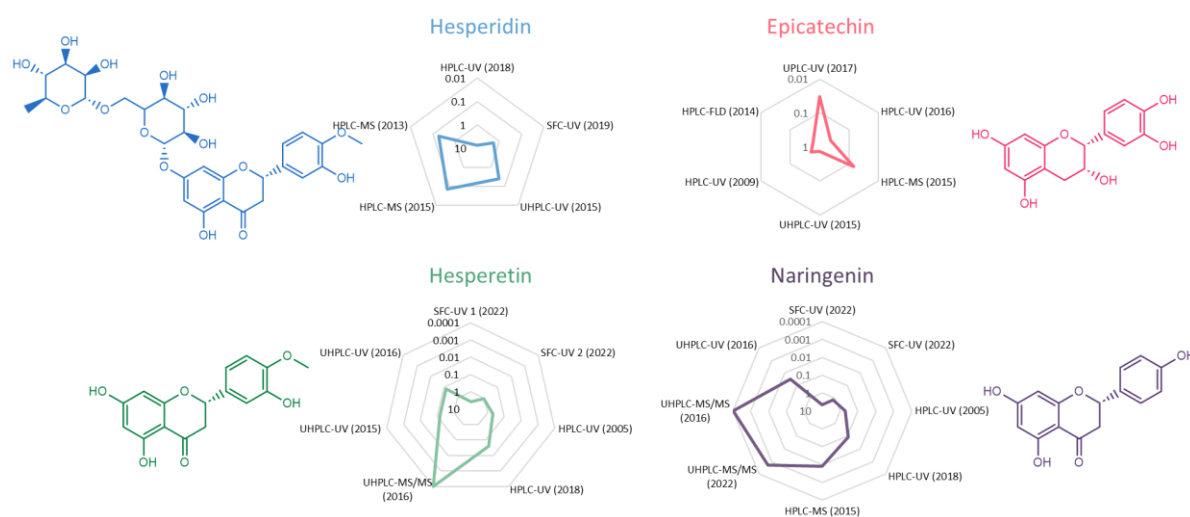


Figure II.11 - Representation of the sensitivities (LOQ in mg/mL) of the detectors (UV, MS, FLD) used in the analysis of hesperidin (blue), hesperetin (green), epicatechin (red), and naringenin (purple).

HPLC-UV (2018) [38]; SFC-UV (2019) [47]; UHPLC-UV (2015) [43]; HPLC-MS (2015) [36]; HPLC-MS (2013) [44]; SFC-UV 1 (2022) [118]; SFC-UV 2 (2022) [48]; HPLC-UV (2005) [66]; UHPLC-MS/MS (2016) [27]; UHPLC-UV (2016) [65]; HPLC-UV (2005) [74]; UHPLC-MS/MS (2022) [89]; UPLC-UV (2017) [30]; HPLC-UV (2016) [40]; HPLC-UV (2009) [76]; HPLC-FLD (2014) [77]

same scale. However, between a UV detector used in 2005 [66] and one used in 2018 [38], there was an increase in sensitivity of a factor of 5. If we now compare the difference between an HPLC-UV analysis [38] and an UHPLC-UV analysis [65], a lower limit of quantification was observed for Naringenin in UHPLC-UV than in HPLC-UV, which seems quite logical since ultra-high-performance should offer higher efficiency, thereby improving detectability.

MS detection naturally offers the best sensitivity whatever the year, compound or chromatographic mode [36,44]. The introduction of tandem MS/MS detection also increases sensitivity [27,89]. Whereas the performance of UV and FLD detectors can hardly be further increased, MS sensitivity continues to improve in new instruments.

Finally, and as is well known, the chromatographic technique used upstream of detection has a strong influence on sensitivity. Typically, HILIC mobile phases are more favourable to MS detection than RPLC, since mobile phases in HILIC contain a larger proportion of organic solvent thus are easier to evaporate. For the same reason, SFC is usually favourable to MS detection as most of the mobile phase is made of pressurized CO₂. Technical developments in chromatography and detection mean that we can now work with more sensitive instruments, which also makes it easier to identify and quantify samples.

6. Conclusions

Advances in liquid chromatography and supercritical fluid chromatography, in one or two separation dimensions and combined with the most sensitive detectors, have improved the detection, quantification and identification of flavonoids. Chiral flavonoids set specific issues related to their limited stability. However, the latter can be improved with optimised storage conditions, advanced sample preparation and analysis techniques to reduce degradation and/or isomerisation effects. In short, these advances are enabling more accurate and reliable analysis of chiral flavonoids, opening up new prospects for research and applications.

Overall, the evolution of chromatographic methods to analyse chiral flavonoids has led to a better understanding of the importance of stereochemistry in the molecular and biological interactions of flavonoids. These advances have major implications for drug design and understanding the interactions between natural compounds and biological processes.

B. Extraction des flavonoïdes chiraux

Dans le *C. aurantium*, nous nous intéressons en particulier aux flavonoïdes chiraux. Comme il apparaît dans la revue de la littérature présentée ci-dessus, les flavonoïdes chiraux sont susceptibles de se dégrader. L'extraction de ces flavonoïdes chiraux constitue donc une étape critique dans leur étude, car elle doit être réalisée de manière à préserver leur configuration stéréochimique et éviter toute racémisation ou dégradation susceptible de compromettre leurs propriétés.

1. Extraction par macération et infusion

Les méthodes d'extraction traditionnelles telles que la macération et l'infusion sont très utilisées pour isoler les flavonoïdes dans les matrices végétales. Ces techniques sont simples et ne nécessitent pas d'équipement particulier. De plus, ces techniques sont respectueuses des composés thermosensibles car généralement l'extraction s'effectue à température ambiante (macération) ou modérée (infusion).

Ces techniques sont très utilisées pour les plantes médicinales comme première extraction pour avoir des informations rapidement sur la composition chimique de la plante. Différentes revues de la littérature présentées par Sasidharan *et al.*, Dai *et al.* et Azmir *et al.* ont montré la facilité de mise en œuvre de la macération et l'importance du choix des solvants pour augmenter l'efficacité d'extraction [119–121]. Les solvants les plus couramment utilisés sont des solvants polaires comme l'éthanol, le méthanol, l'eau, purs ou en mélange. Leur polarité permet d'extraire efficacement les flavonoïdes. Par exemple, Quy Diem Do *et al.* ont montré que la nature du solvant influence significativement la teneur en flavonoïdes dans des extraits de *Limnophila aromatica* [122].

Ces approches restent pertinentes dans les cas où la préservation des structures thermolabiles est cruciale, mais elles sont généralement moins efficaces que les techniques modernes en termes de rendement et de rapidité.

2. Extraction assistée par ultrasons (UAE)

L'extraction assistée par ultrasons (UAE) représente une méthode innovante et écologique pour extraire efficacement les flavonoïdes. Cette technique améliore la pénétration du solvant dans la matrice végétale en réduisant le temps, la température et la consommation de solvant.

Comme évoqué dans la section 1.1., les flavonoïdes chiraux sont de plus en plus étudiés pour leurs nombreuses propriétés. Khan *et al.* se sont intéressés à ceux présents dans les agrumes et plus particulièrement à l'écorce d'orange [123]. Ils ont montré l'efficacité de l'UAE pour extraire la naringine et l'hespéridine, en optimisant les conditions pour avoir un bon rendement sans dégrader ces composés glycosylés.

De même, Yang *et al.* ont appliqué la méthodologie de surface de réponse pour maximiser l'extraction de flavonoïdes totaux, à partir de fleurs de *C. aurantium* [124]. Ces recherches soulignent l'intérêt de l'UAE pour conserver la structure stéréochimique des flavonoïdes, notamment dans les matrices complexes riches en composés thermosensibles.

Plus récemment, l'UAE a été utilisée avec des solvants eutectiques naturels (NADES), ce qui a permis d'extraire encore plus sélectivement les flavonoïdes glycosylés. Jiménez-Ortega *et al.* ont utilisé cette combinaison pour extraire des composés comme la quercitrine à partir de *Capsicum annuum* [125].

Ainsi, l'UAE est une technique clé pour l'extraction, rapide et sélective de flavonoïdes chiraux sans dégradations des composés.

3. Extraction par fluide supercritique (SFE)

L'extraction par fluide supercritique SFE est une méthode verte et sélective qui utilise principalement du CO₂ à l'état supercritique pour extraire les molécules d'intérêt. Dans le cas des flavonoïdes, qui sont des molécules très polaires, un solvant additionnel, généralement un alcool, est ajouté au CO₂ pour augmenter sa polarité et ainsi pouvoir extraire ces molécules polaires. Un contrôle de la température et de la pression est essentiel pour extraire efficacement les flavonoïdes.

En 2007, Wang *et al.* ont déterminé par un plan d'expérience les conditions optimales d'extraction des flavonoïdes dans la *Pueraria lobata* (90 min, 20 MPa et 50 °C) [126]. Plus récemment, Atwi-Ghaddar *et al.* ont mené deux études d'optimisation des conditions d'extraction également avec un plan d'expérience. Une de ces études met en avant l'extraction sélective des flavonoïdes dans le *Calendula officinalis* [127]. Il est ainsi possible d'extraire en une seule étape des molécules d'intérêt cosmétique comme la caféine et les catéchines. L'autre étude est basée sur l'utilisation d'un mélange éthanol/eau comme modificateur de polarité pour améliorer l'extraction de flavonoïdes dans *Camellia sinensis* [128].

L'étude comparative de Mora *et al.* en 2024, sur des écorces d'oranges montre que la SFE permet d'obtenir un meilleur rendement et une meilleure pureté des extraits de flavonoïdes obtenus comparée aux méthodes conventionnelles telles que la macération [129]. De plus, les progrès technologiques en matière de SFE permettent une optimisation des paramètres d'extraction et les diverses applications industrielles [130]. Un autre avantage de la SFE est qu'elle permet de faire de l'extraction verte avec des procédés durables du point de vue environnemental et toxicologique : elle utilise du CO₂ qui est non toxique, non inflammable, réutilisable et qui ne contamine pas les extraits obtenus [131].

Finalement, ces trois méthodes d'extraction offrent chacune des avantages spécifiques selon la nature des flavonoïdes d'intérêt, la matrice ou encore les moyens techniques à disposition. Les méthodes traditionnelles comme la macération ou l'infusion restent efficaces pour un grand nombre de cas. En revanche, les techniques plus avancées comme l'UAE ou la SFE montrent un avantage dans le cas d'extractions sélectives mais sont aussi avantageuses du point de vue de la consommation de solvant et donc d'approches éco-responsables.

Partie 2 : Les flavonoïdes dans le *Citrus aurantium* L.

Le *C. aurantium* est une espèce végétale largement étudiée pour la richesse et la diversité de ses métabolites secondaires. Ces composés, souvent impliqués dans diverses activités biologiques, sont répartis en plusieurs grandes familles chimiques, comme illustré dans la figure ci-dessous, construite d'après les données issues de la base de données LOTUS [132] répertoriant les produits naturels.

Cette figure se compose de deux diagrammes circulaires : le premier illustre la répartition générale des grandes classes de composés, tandis que le second détaille la composition spécifique de la famille des shikimates et phénylpropanoïdes, qui est l'une des classes les plus abondantes et inclut les flavonoïdes.

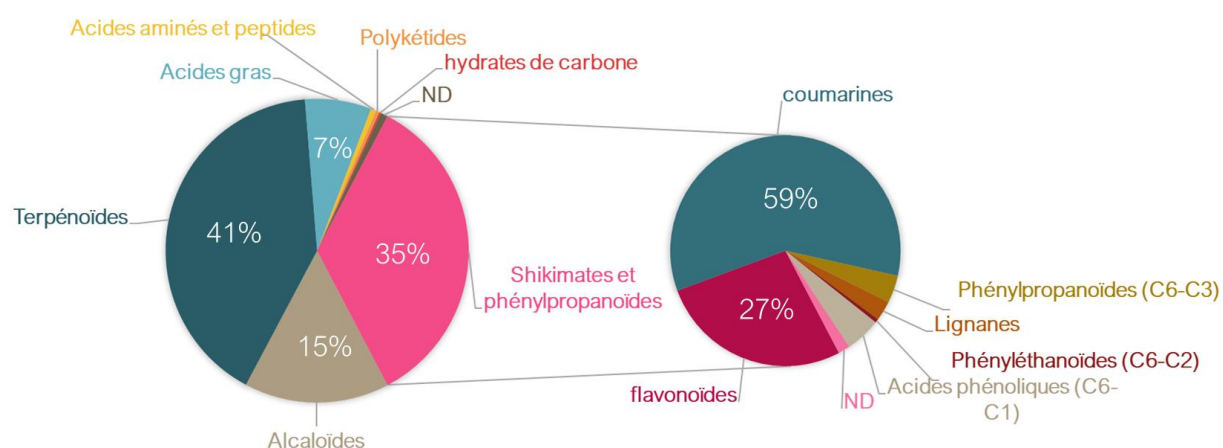


Figure II.12 - Représentation de la composition du *C. aurantium* indiquant les molécules retrouvées sur la base de données LOTUS avec comme mot clé « Citrus aurantium », consulté le 31 mai 2025. A gauche, diagramme de la composition générale et à droite la composition de la famille des shikimates et phénylpropanoïdes, incluant les flavonoïdes.

Comme il apparaît sur cette figure, les molécules principalement répertoriées dans le *C. aurantium* se composent de deux classes majoritaires : les terpénoïdes, représentant 41% des molécules répertoriées, et les shikimates et phénylpropanoïdes représentant 35% des molécules répertoriées. D'autres familles comme les alcaloïdes et les acides gras suivent avec respectivement 15% et 7%. Les acides aminés et peptides, polykétides, hydrates de carbone, ainsi qu'un petit pourcentage de composés non classés (ND) sont présents en proportions plus faibles.

C'est dans la classe des shikimates et phénylpropanoïdes que se situe la famille des flavonoïdes (second diagramme dans la Figure II.12). Malgré une dominance des coumarines (59%), les flavonoïdes sont largement présents (27%). On retrouve également, en proportions plus faibles, des phénylpropanoïdes de type C6-C3, des lignanes, des phényléthanoïdes (C6-C2) et des acides phénoliques (C6-C1) (Figure II.13). Là encore, une petite portion des composés de cette famille n'a pas pu être classifiée précisément.

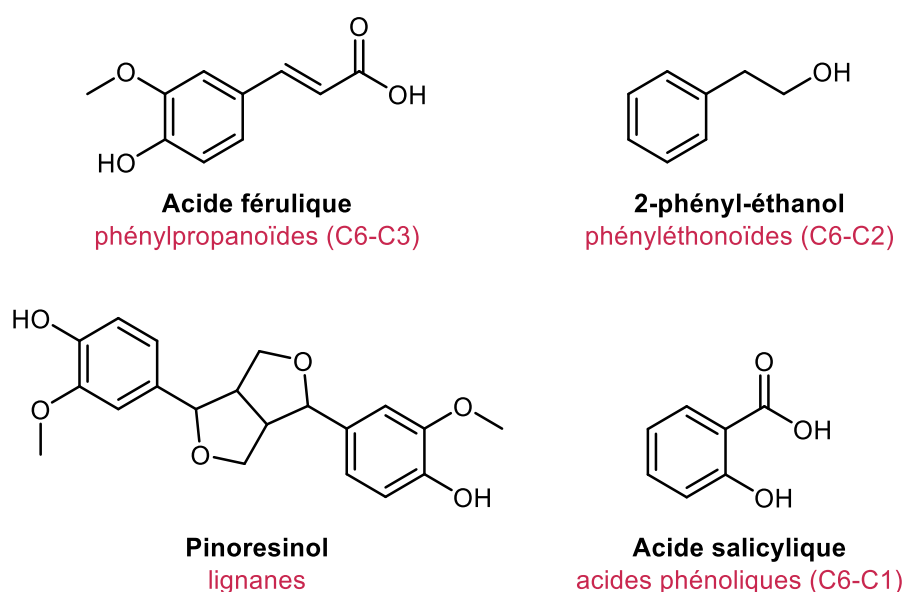


Figure II.13 - Exemples de molécules des familles minoritaires (phénylpropanoïdes, phényléthanoïdes, lignanes, acides phénoliques) de la classe des shikimates et phénylpropanoïdes

En résumé, *C. aurantium* se distingue non seulement par la richesse de ses métabolites issus de la voie des shikimates, mais aussi par la présence significative de flavonoïdes. Ces derniers, en plus d'être des marqueurs phytochimiques importants, sont également associés à de nombreuses propriétés pharmacologiques, faisant de cette famille un sujet d'intérêt majeur pour de nombreux domaines.

1. Flavonoïdes spécifiques du *Citrus aurantium* L.

Les flavonoïdes présents dans *C. aurantium* appartiennent majoritairement aux classes des flavanones, flavones, et flavonols [133]. Ils se présentent le plus souvent sous forme glycosylée, ce qui influence leur solubilité, leur absorption, et leur activité biologique. La chiralité joue un rôle important, notamment pour les flavanones, qui possèdent un carbone asymétrique en C-2,

rendant ces molécules chirales. La distribution de ces flavonoïdes varie significativement selon les organes de la plante [134].

1.1. Écorce

L'écorce est la partie de l'orange amère la plus concentrée en flavonoïdes (Table II.5). On y retrouve la naringine, la poncirine, la naringénine, la quercétine, l'hespéridine. C'est notamment une source en flavones polyméthoxylées comme la tangerétine, le nobiletine, la sinensétine, l'isosinensétine, la 3-hydroxynobiletine, l'heptaméthoxyflavone et la tetraméthyl-o-isoscutellaréine [135–147]. L'écorce contient également d'autres flavonoïdes moins abondants mais bioactifs, tels que l'auranétine ou la desméthylnobiletine [142].

1.2. Feuilles

Cette partie est moins étudiée que l'écorce mais contient tout de même plusieurs flavonoïdes (Table II.6). Ces derniers sont souvent sous forme de glycosides tels que la naringine, la néohespéridine, la narirutine, la néoéricitrine, la rhoifoline, la diosmine, et divers dérivés de l'isosakuranétine [148,149].

1.3. Fleurs

Les fleurs de l'orange amère, très utilisées en parfumerie, sont également une source riche en flavonoïdes glycosylés (Table II.6). Parmi eux la rutine, la quercétine, la rhoifoline, la naringine, la néoéricitrine, l'hespéridine, la néohespéridine ainsi que l'ériocitrine [146,150–153].

1.4. Fruits: pulpe, jus

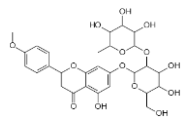
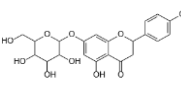
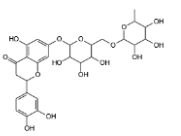
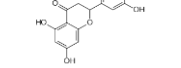
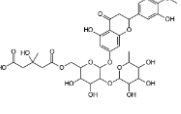
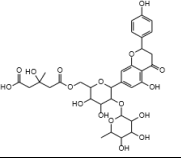
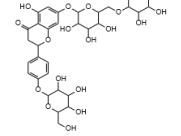
Le fruit dans son ensemble contient une variété de flavonoïdes, dont certains peuvent être responsables de l'amertume. Les principaux sont la naringine, la néohespéridine, l'hespéridine, la narirutine, l'hespérétine, la prunine, l'apigénine, l'isorhoifoline, et des flavanones spécifiques comme l'eriodictyol ou la brutieridine [154–162] (Table II.6). Ces composés sont souvent présents sous forme de glycosides et leur teneur peut varier selon le stade de maturation du fruit [154].

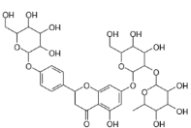
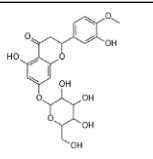
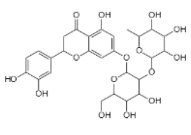
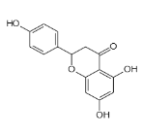
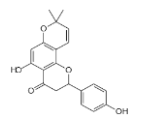
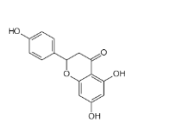
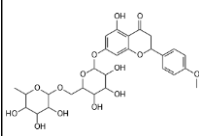
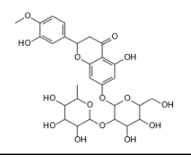
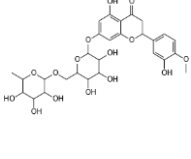
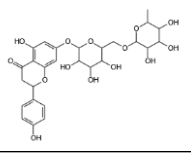
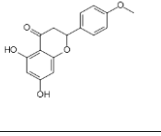
1.5. Pépins

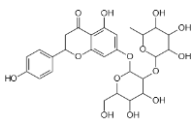
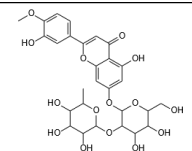
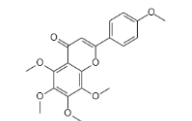
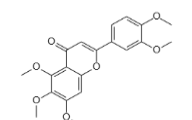
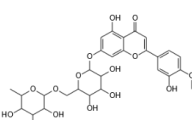
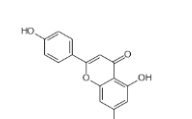
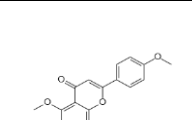
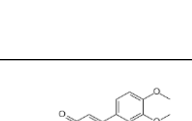
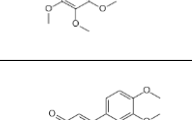
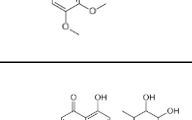
Les graines de *C. aurantium* contiennent principalement deux flavonoïdes majeurs : la naringine et la néohespéridine [163], bien que cette partie de la plante soit encore relativement peu étudiée par rapport aux autres.

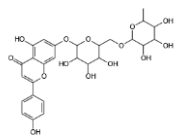
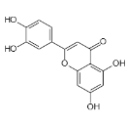
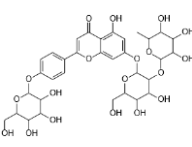
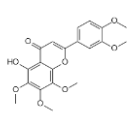
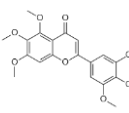
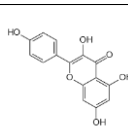
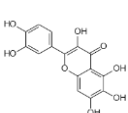
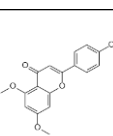
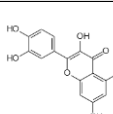
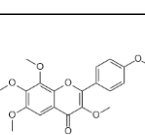
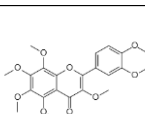
Le tableau suivant (Table II.6) recense les flavonoïdes majoritaires contenus dans le *C. aurantium* répertoriés dans la base de données LOTUS. Ce tableau n'est pas exhaustif et sa composition a été ajustée en vérifiant la présence de chacun de ces flavonoïdes dans la littérature.

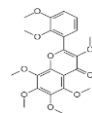
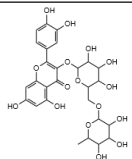
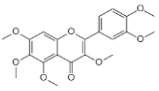
Table II.6 – Répertoire des flavonoïdes contenus dans le *C. aurantium* répertoriés sur LOTUS

	Molécules	Familles	Masse molaire (g/mol)	Structures	Matrices	Références	ID Lotus
1	Poncirine	Flavanones	594,56		écorce feuilles jus	135, 136, 137, 139 149 162	Q3388129 Q1043915
2	Prunine	Flavanones	434,39		fruit	134	Q1542479 Q1042530
3	Ériocitrine	Flavanones	596,53		écorce fleurs fruit jus	147 151 156 157	Q1043915 Q1356766
4	Biespérétine	Flavanones	302,28		écorce plante	147 133	Q2716696 Q1049128 Q411310
5	Brutieridine	Flavanones	754,69		écorce fruit jus	140 156 157	Q1052528 Q1053694
6	Mélitidine	Flavanones	708,66		écorce jus	140 157	Q1053023 Q1053463 Q1051779
7	Narirutine 4'-glucoside	Flavanones	742,68		jus	157	LTS00210

8	Naringine 4'-glucoside	Flavanones	742,68		feuilles	149	Q1049954
9	Hespérétine 7-O-glucoside	Flavanones	464,42		fruit	134	Q2305524
10	Néoériocitrine	Flavanones	596,53		écorce fleurs feuilles jus	140 151 149 157	Q1356478
11	Asahina	Flavanones	272,25		fruit	158	Q5782685
12	Citflavanone	Flavanones	338,35		fruit	161	Q1051347 Q1051347
13	Naringinine	Flavanones	272,25		écorce plante	135, 147 133	Q2712197 Q418374
14	Didymine	Flavanones	594,56		feuilles jus	149 162	Q6339932
15	Néohespéridine	Flavanones	610,56		écorce feuilles fruit pépins jus plante	140 149 134, 151 163 157, 162 133	Q1043906 Q1860945 Q1049173
17	Hespéridine	Flavanones	610,56		écorce fleurs feuilles fruit jus/pulpe plante	136, 137, 138, 139, 146, 154 146, 151 149 156 154, 162 133	Q1043883 07 Q385937
18	Narinrutine	Flavanones	580,54		feuilles fruit jus plante	149 156 157, 162 133	Q1050351
19	Isosakuranétine	Flavanones	286,28		fruit	159	Q3459109

20	Naringine	Flavanones	580,54		écorce feuilles fleurs fruit pépins jus/pulpe plante	135, 136, 137, 139, 146, 154, 138, 143, 140 149 146, 150, 151 134, 158 163 154, 157, 162 133	Q1042533 37 Q418469
21	Néodiosmine	Flavones	608,54		écorce feuilles	140 149	Q6339536 Q1052836 Q1043919 Q1052836
22	Tangéritine	Flavones	372,37		écorce feuilles plante	136, 137, 139, 140, 141, 143, 145, 147 149 133	Q1748737
23	Sinensétine	Flavones	372,37		écorce feuilles	136, 137, 139, 149	Q3484909
24	Diosmine	Flavones	608,55		écorce feuilles	147 149	Q2607865
25	Apigénine	Flavones	270,24		fruit plante fleurs	160 133 153	Q424567
26	Tétraméthyl-O-isoscutellaréine	Flavones	342,34		écorce	135, 136, 137, 139, 144	Q2711601 4
27	Nobilétine	Flavones	402,39		écorce feuilles plante	136, 137, 138, 149 133	Q2402963
28	Isosinensétine	Flavones	372,37		écorce	135, 136, 137, 139	Q2726366
29	Rhoifoline	Flavones	578,52		écorce fleurs feuilles jus	140, 146 146, 151 149 157	Q3429675

30	Isorhoifoline	Flavones	578,52		fruit	160	Q1042530
31	Lutéoline	Flavones	286,24		plante	133	Q415011
32	Rhoifoline 4'-glucoside	flavones	740,66		écorce	140	LTS00713
33	Déméthyl-nobilétine	Flavones	388,37		écorce fleurs	142 152	Q2728564
34	Hexaméthoxy-flavone	Flavones	402,40		écorce	136, 137, 139	Q8305401
35	Kaempférol	Flavonols	286,24		plante	133	Q393336
36	Quercétagétine	Flavonols	318,24		fleurs	153	Q5931085
37	Apigénine triméthyl éther	Flavonols	312,32		écorce	141	Q2727764
38	Quercétine	Flavonols	302,24		écorce fleurs jus fruit plante	135, 139 150 162 156 133	Q409478
39	Auranétine	Flavonols	372,37		écorce	142	Q8294898
40	3-méthoxy-nobilétine	Flavonols	432,42		écorce	145	Q2725429

41	Heptaméthoxy-flavone	Flavonols	432,42		écorce	136, 137,139,144	Q1049789
42	Rutine	Flavonols	610,52		écorce fleurs	147 150, 151	Q2716346
43	Hexamethyl-quercetagine	Flavonols	402,40		écorce	144	Q2716537

2. Propriétés des flavonoïdes

Les flavonoïdes présents dans le *C. aurantium* ont au-delà de leurs structures diverses, une grande variété de propriétés différentes. Une analyse des publications liées à la famille des Citrus réalisée en 2022 (Figure II.14), montre le grand intérêt de la recherche sur les propriétés biologiques de ces flavonoïdes [164].

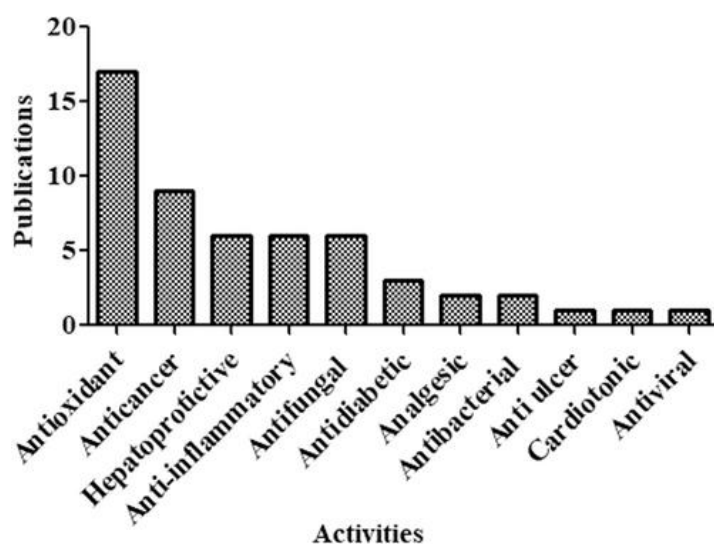


Figure II.14 - Histogramme des publications référent aux différentes propriétés des flavonoïdes dans les Citrus, selon Alam *et al.* [164]

L'activité antioxydante est la plus décrite, montrant leur rôle essentiel dans la neutralisation des radicaux libres et la protection cellulaire. D'autres propriétés sont beaucoup étudiées comme des

effets anticancéreux, hépatoprotecteurs, anti-inflammatoires, antimicrobiens (antifongiques, antibactériens), ou encore antidiabétiques. D'autres propriétés sont moins explorées mais prometteuses comme les effets antiulcéreux, cardiotoniques et antiviraux. L'ensemble de ces propriétés montre un potentiel multifonctionnel des flavonoïdes dans le Citrus dans les domaines agroalimentaire, cosmétique et pharmaceutique.

2.1. Activité antioxydante

Les flavonoïdes contenus dans *C. aurantium* ont été beaucoup étudiés ces dernières années pour leurs propriétés antioxydantes [133,165,166]. Ils agissent sur le piégeage des radicaux libres, sur le stress oxydatif mais aussi sur l'activité de certaines enzymes antioxydantes. Ces facteurs dépendent des parties de la plante, de la période de récolte, la variété ou encore de la maturité.

Tang *et al.* ont mis en avant l'impact de la période de récolte sur la teneur en composés bioactifs [148]. Cette teneur variable était liée directement à l'activité oxydante du *C. aurantium*. Lors de la période de récolte optimale, les extraits issus des fruits protègent mieux les cellules contre les agressions oxydatives notamment grâce à une augmentation de l'activité des enzymes antioxydantes (SOD, CAT, GSH). La période de récolte est étroitement liée à la maturité du fruit. En effet, Moulehi *et al.* l'ont montré sur les graines de *C. aurantium* et de *Citrus reticulata* [163]. Cette capacité a été mesurée à l'aide d'essais de piégeage de radicaux libres tels que le test DPPH (2,2-diphényl-1-picrylhydrazyle). Ce composé est d'une couleur violette et devient jaune lorsqu'il réagit avec les radicaux libres. Finalement, plus l'extrait a une bonne capacité à neutraliser les radicaux libres, meilleure est son activité antioxydante. Un autre test souvent utilisé est le test bêta-carotène/acide linoléique. Dans ce cas, on mesure la décoloration du bêta-carotène. Si le bêta-carotène garde sa couleur, cela signifie que l'extrait testé protège efficacement contre l'oxydation. Les résultats ont montré que *C. aurantium* présentait une activité antioxydante supérieure à celle de *Citrus reticulata*. De la même façon, grâce au piégeage des radicaux DPPH, Haraoui *et al.* ont montré l'impact de la variété des agrumes sur leur caractère antioxydant [167]. Le *C. aurantium* s'est distingué des autres variétés grâce à sa grande teneur en phénols totaux. En effet, la variation de la composition phénolique peut changer l'intensité de l'activité antioxydante [168,169].

Cependant, toutes les parties de la plante n'interviennent pas sur les mêmes mécanismes antioxydants évalués selon différents tests. Il est donc difficile d'avoir une efficacité de l'activité antioxydante globale de la plante [170].

2.2. Anticancer

Une deuxième propriété importante des flavonoïdes, en particulier ceux extraits de *C. aurantium*, est leur caractère anticancéreux. Ils agissent sur les voies de régulation du cycle cellulaire, l'induction de l'apoptose et la modulation du stress oxydatif [164,165].

Do-Hoon Lee *et al.* ont montré que les flavonoïdes isolés du *C. aurantium* peuvent bloquer la division des cellules cancéreuses mais aussi déclencher l'apoptose de celles-ci [136]. Ils agissent en perturbant certaines protéines (cycline B1 et la CDK1) qui sont essentielle à la division cellulaire. En empêchant ces protéines de fonctionner correctement, les cellules cancéreuses ne peuvent plus atteindre cette étape de division, ce qui freine leur multiplication. De plus, les flavonoïdes activent des enzymes spécifiques appelées caspases, qui déclenchent l'apoptose et permettent l'élimination des cellules cancéreuses.

De la même façon, Seung Hwan Lee *et al.* ont étudié l'effet des flavonoïdes de *C. aurantium* sur des cellules cancéreuses du foie (HepG2) [139]. Ils ont montré que des composés comme la naringine, l'hespéridine, la poncirine, et la nobiletine induisent une apoptose via la voie mitochondriale. Ce processus consiste à libérer une molécule appelée cytochrome c depuis la mitochondrie, ce qui déclenche ensuite l'activation de caspases, des enzymes responsables de la mort cellulaire. En ciblant directement les voies apoptotiques, les flavonoïdes sont efficaces quel que soit le type de tumeur.

Leur effet peut être aussi préventif. Lim *et al.* ont étudié un extrait d'écorce d'orange amère riche en flavonoïdes polyméthoxylés [171]. Cet extrait s'est montré protecteur du foie contre les ligatures du canal biliaire. Ces ligatures ne sont pas tumorales mais dans ce cas, l'extrait riche en flavonoïdes a un effet protecteur contre l'apoptose hépatique dû à ses propriétés antioxydantes, anti-inflammatoires et anti-apoptotiques.

En résumé, les flavonoïdes de *C. aurantium* possèdent une activité anticancéreuse par de nombreuses voies qui leurs permettent de toucher plusieurs types de cancers (gastrique, hépatique, ...).

2.3. Hépatoprotecteur

Le *C. aurantium* se distingue également par son activité hépatoprotectrice, qui a un intérêt pour la prévention et le traitement des maladies hépatiques d'origine toxique, inflammatoire ou métabolique.

Dans une revue récente, Gajender *et al.* ont mis en avant le potentiel des flavonoïdes dans l'alimentation comme de bons hépatoprotecteurs [172]. Cette analyse montre que les flavonoïdes interviennent dans divers mécanismes : limitation du stress oxydatif, inhibition de l'inflammation hépatique mais aussi amélioration des profils lipidiques hépatiques.

Dan He *et al.* se sont penchés sur les effets protecteurs d'un extrait phénolique de *C. aurantium*. Ils l'ont associé avec le méthotrexate (MTX) qui est un agent connu pour son hépatotoxicité chronique. Grâce à l'activité antioxydante du *C. aurantium* décrite précédemment l'insuffisance hépatique que le MTX avait induite a été réduite, montrant ainsi son intérêt comme agent hépatoprotecteur [173].

2.4. Anti-inflammatoire

Les flavonoïdes extraits de *C. aurantium* présentent également une activité anti-inflammatoire significative.

Kang *et al.* ont mis en évidence cette propriété pour trois flavonoïdes issus d'écorce de *C. aurantium*, la nobiletine, la naringine et l'héspéridine [138]. Pour cela, ils ont montré que ces composés inhibent la production de molécules pro-inflammatoires (TNF- α , IL-6, IL-1 β). Ces effets passent par l'inhibition de deux grandes voies de signalisation impliquées dans l'inflammation cellulaire : les voies NF- κ B et MAPK. Ces résultats sont confirmés par Shen *et al.* qui ont démontré sur un extrait de fleurs de *C. aurantium*, une inhibition significative de la production des mêmes molécules pro-inflammatoires et de l'expression du gène de l'iNOS, impliqué dans la production de médiateur clé de l'inflammation [151]. Cet extrait de fleurs est riche en flavonoïdes comme la rhoifoline, l'héspéridine, la naringine, mais aussi la rutine et la néohéspéridine. Finalement, différentes parties de la plante, ici écorce et fleurs peuvent partager des cibles moléculaires communes, ce qui renforce leur intérêt thérapeutique.

D'un autre côté, l'action anti-inflammatoire ne se limite pas qu'aux cellules isolées mais peut aussi se manifester au niveau de certains organes comme l'intestin. He *et al.* ont ainsi démontré que les flavonoïdes du *C. aurantium* (naringénine, nobiletine, héspéretine) étaient capables de réduire l'inflammation intestinale dans un modèle de colite (une pathologie analogue à la maladie de Crohn), tout en diminuant les contractions intestinales anormales [174].

Finalement, toutes ces études montrent que les flavonoïdes de *C. aurantium*, exercent une action anti-inflammatoire en agissent non seulement sur les médiateurs classiques de l'inflammation, mais aussi sur les réponses tissulaires locales, comme celles observées dans l'intestin ou le foie.

2.5. Antimicrobien

Les propriétés antimicrobiennes des flavonoïdes du *C. aurantium* peuvent être antibactériennes ou antifongiques, permettant ainsi de lutter contre les infections bactériennes. Dans un contexte où la résistance aux antibiotiques est un problème majeur, le *C. aurantium* pourrait représenter une piste intéressante pour lutter contre ces infections. Cependant, les premiers résultats proviennent d'études en laboratoire et doivent encore être confirmés avant toute utilisation médicale chez l'humain.

L'étude de Gopal *et al.* a analysé l'activité antimicrobienne des extraits de feuilles de *C. aurantium* contre plusieurs souches bactériennes par la méthode de diffusion sur gélose [175]. Grâce à un test de concentration minimale d'inhibition, Gopal *et al.* montrent une efficacité contre plusieurs souches bactériennes (Gram+ et Gram-). C'est également ce que montre Ben Hsouna *et al.* en étudiant l'huile essentielle des fleurs de *C. aurantium* [176]. Cette huile a montré une forte activité antibactérienne contre des espèces pathogènes, avec une élimination significative des bactéries en moins de 5 minutes.

L'étude de plusieurs variétés d'agrumes cultivés en Algérie par Haraoui *et al.* a montré l'importance de la teneur élevée en flavonoïdes totaux [167]. En comparant les extraits de jus et de feuilles, les auteurs ont mis en avant l'intérêt d'avoir une composition riche en composés phénoliques pour l'efficacité antimicrobienne. Une autre comparaison de variétés entre le *C. aurantium* et le *Citrus sinensis* étudiée par Chakroun *et al.* amène à la même conclusion [177]. Cette fois-ci ce sont les écorces qui sont comparées, ce qui suggère que même des sous-produits comme les écorces, souvent jetées, pourraient avoir une valeur ajoutée biomédicale, notamment dans les domaines alimentaire, cosmétique ou pharmaceutique.

2.6. Anti-diabétique et anti-obésité

Aujourd'hui, l'obésité et le diabète de type 2 constituent deux pathologies métaboliques principales, et représentent un vrai problème de santé publique à l'échelle mondiale. L'isolement de composés bioactifs d'origine naturelle ayant des propriétés d'une part anti-obésité et d'autre part anti-diabétique pourrait constituer une voie thérapeutique. Dans cette optique, des flavonoïdes issus du *C. aurantium* ont paru avoir un intérêt.

En effet, Lin *et al.* ont montré que les flavonoïdes extraits des fleurs de *Citrus aurantium* var. *amara* peuvent transformer de la graisse blanche, qui stocke de l'énergie, en une graisse brune plus active. Cette transformation est étudiée sur des souris soumises à un régime riche en graisses.

Elle permet d'augmenter la dépense énergétique, ce qui pourrait aider dans le combat contre l'obésité [178]. Ces résultats sont confirmés avec ceux de Park *et al.* montrant que l'extrait de fruit de *C. aurantium* active une voie métabolique particulière (via l'AMPK), impliquée dans la réduction du stockage des graisses (adipogénèse) et l'augmentation de la production de chaleur par le corps (thermogénèse) [179]. L'impact anti-obésité a été observé à la fois *in vivo* (diminution du poids corporel et des graisses dans le sang chez la souris obèse) et *in vitro* (diminution des gouttelettes de graisse sur des cellules adipeuses). Kim *et al.* précisent l'effet anti-adipogène des flavonoïdes du *C. aurantium* Ils empêchent la formation de nouvelles cellules graisseuses en bloquant une voie de signalisation (Akt-GSK3 β), qui joue un rôle important dans leur développement [180]. Ces extraits de citrus régulent à la baisse l'expression de deux protéines (PPAR γ et C/EBP α), deux facteurs favorisant la production de graisse dans l'organisme (lipogénèse).

Concernant la glycémie, l'étude de Jia *et al.* se concentre sur l'effet de la néohespéridine sur un modèle de souris diabétiques de type 2 [181]. Ce composé présent dans le *C. aurantium* provoque une chute de la glycémie et du taux de graisses dans le sang. Cet effet passe par l'activation de la voie métabolique via l'AMPK en agissant sur plusieurs cibles liées au métabolisme des graisses (SCD-1, FAS, ACOX). L'étude lie ainsi l'activité hypoglycémiante et hypolipidémique à une régulation de la vascularisation métabolique.

3. Applications et valorisation des flavonoïdes

Les flavonoïdes extraits de *C. aurantium*, reconnus pour leurs propriétés précédentes (antioxydantes, anti-inflammatoires, cardioprotectrices, neuroprotectrices et anticancéreuses) trouvent des applications concrètes dans plusieurs secteurs industriels.

3.1. Secteur de l'alimentaire

Le *C. aurantium* a plusieurs applications en secteur alimentaire grâce à sa richesse en flavonoïdes et composés phénoliques. Les antioxydants issus d'extraits d'écorce ou de jus ont montré une bonne activité antioxydante même si elle est légèrement inférieure à celle des antioxydants de référence comme le BHT ou l'acide ascorbique [182]. En Turquie, le fruit n'étant pas consommé directement en raison de son amertume, son jus est utilisé comme substitut du citron dans diverses préparations culinaires. Les graines, quant à elles, ont longtemps été négligées par rapport aux écorces. Aujourd'hui, elles sont étudiées pour leurs propriétés conservatrices et

fonctionnelles dans les aliments, ce qui en fait une excellente ressource pour des produits sains et durables [183]. Les diverses parties du fruit représentent ainsi une nouvelle source naturelle d'antioxydants pouvant enrichir les formulations alimentaires

3.2. Secteur pharmaceutique

Dans l'industrie pharmaceutique, le *C. aurantium* est un ingrédient d'un grand intérêt grâce à ses flavonoïdes comme l'hespéridine, la naringénine, la nobiletine et l'hespérétine. Ces composés sont étudiés pour leurs propriétés biologiques décrites dans la section 2, mais certaines applications sont déjà connues.

Le *C. aurantium* est utilisé en médecine traditionnelle, notamment en médecine chinoise (TCM) [184,185]. Deux formes du fruit sont couramment utilisées : Zhi shi (fruit immature) utilisé pour réguler le qi (terme désignant « l'énergie vitale » qui circule dans le corps selon la médecine traditionnelle chinoise), traiter les douleurs gastriques et abdominales. Zhi Qiao (fruit plus mûr) est quant à lui utilisé pour apaiser les troubles digestifs et les douleurs thoraciques [186]. Ces deux formes sont inscrites dans la Pharmacopée chinoise et sont intégrées dans de nombreuses formules traditionnelles. En médecine japonaise (Kampo), le Daidai qui est une variété du *C. aurantium* est utilisé pour traiter les troubles respiratoires, digestifs et psychosomatiques [187].

Certaines parties de l'orange comme les graines ont montré une efficacité supérieure à un médicament standard utilisé dans la prévention des dommages neurologiques liés à la maladie d'Alzheimer [188]. Récemment, des fleurs ont également été testées dans des essais cliniques et ont montré une réduction significative de la sévérité de la dysménorrhée primaire [189]. De plus, le *C. aurantium* est commercialisé comme alternative naturelle à l'éphédra – utilisée pour favoriser la perte de poids mais interdite par la Food and Drug Administration car elle provoquerait des accidents vasculaires cérébraux, des crises cardiaques, de l'hypertension et des troubles psychiatriques. Le fruit du *C. aurantium* est donc utilisé dans des compléments alimentaires pour la perte de poids, bien que certains effets secondaires potentiels aient également été signalés [190]. Cette plante présente aussi des propriétés anticancéreuses, anti-anxiété, anti-obésité, antibactériennes et antidiabétiques, ce qui en fait un excellent agent thérapeutique [133]. En plus de ses fruits, ses feuilles sont aussi prometteuses pour être intégrées dans des formulations pharmaceutiques [191].

3.3. Secteur de la cosmétique

L'industrie cosmétique est aussi intéressée par les avantages du *C. aurantium*, notamment dans le développement de produits naturels anti-âge. En effet, le vieillissement cutané est une préoccupation majeure dans le domaine des cosmétiques, ce qui pousse constamment la recherche à explorer de nouvelles solutions et de nouveaux actifs. Par exemple, l'huile essentielle extraite des feuilles d'orange amère a montré une bonne activité anti-oxydante permettant l'inhibition de l'élastase et de la collagénase, deux enzymes responsables du vieillissement cutané [192]. Les fleurs, lorsqu'elles sont soumises à une fermentation par *Lactobacillus brevis*, ont une augmentation de leurs effets physiologiques, notamment en matière de propriétés blanchissantes et anti-rides, ce qui les rend intéressantes pour les formulations cosmétiques [193].

4. Conclusion

L'analyse bibliographique des flavonoïdes chiraux issus de *C. aurantium* a mis en avant une grande variété structurale qui complique leur séparation. Cependant, cette séparation est nécessaire pour pouvoir accéder à leurs propriétés spécifiques. Cette revue a également souligné les limites des approches chromatographiques conventionnelles face à cette problématique, et l'intérêt croissant pour des techniques plus performantes, notamment en chromatographie chirale et multidimensionnelle.

Afin de répondre à ces exigences analytiques, un développement instrumental a été mis en place pour développer et optimiser un système bidimensionnel en chromatographie en fluide supercritique (SFC-SFC). Ce système a été conçu pour permettre une analyse plus fine des flavonoïdes chiraux.

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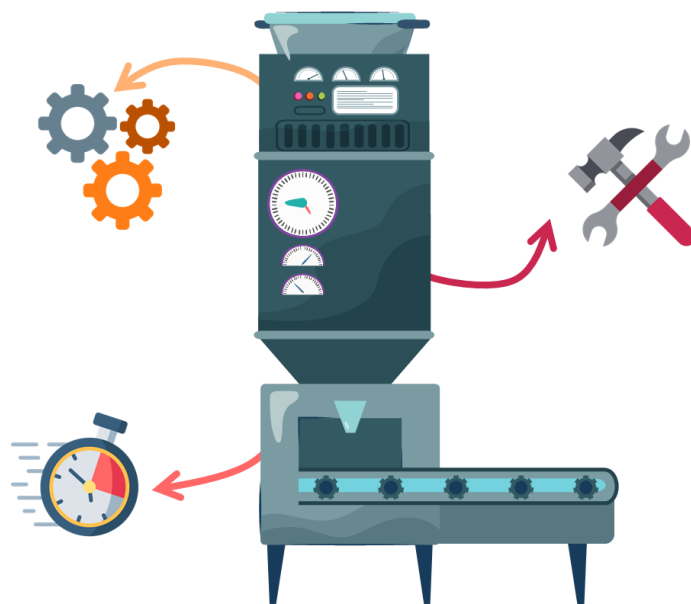
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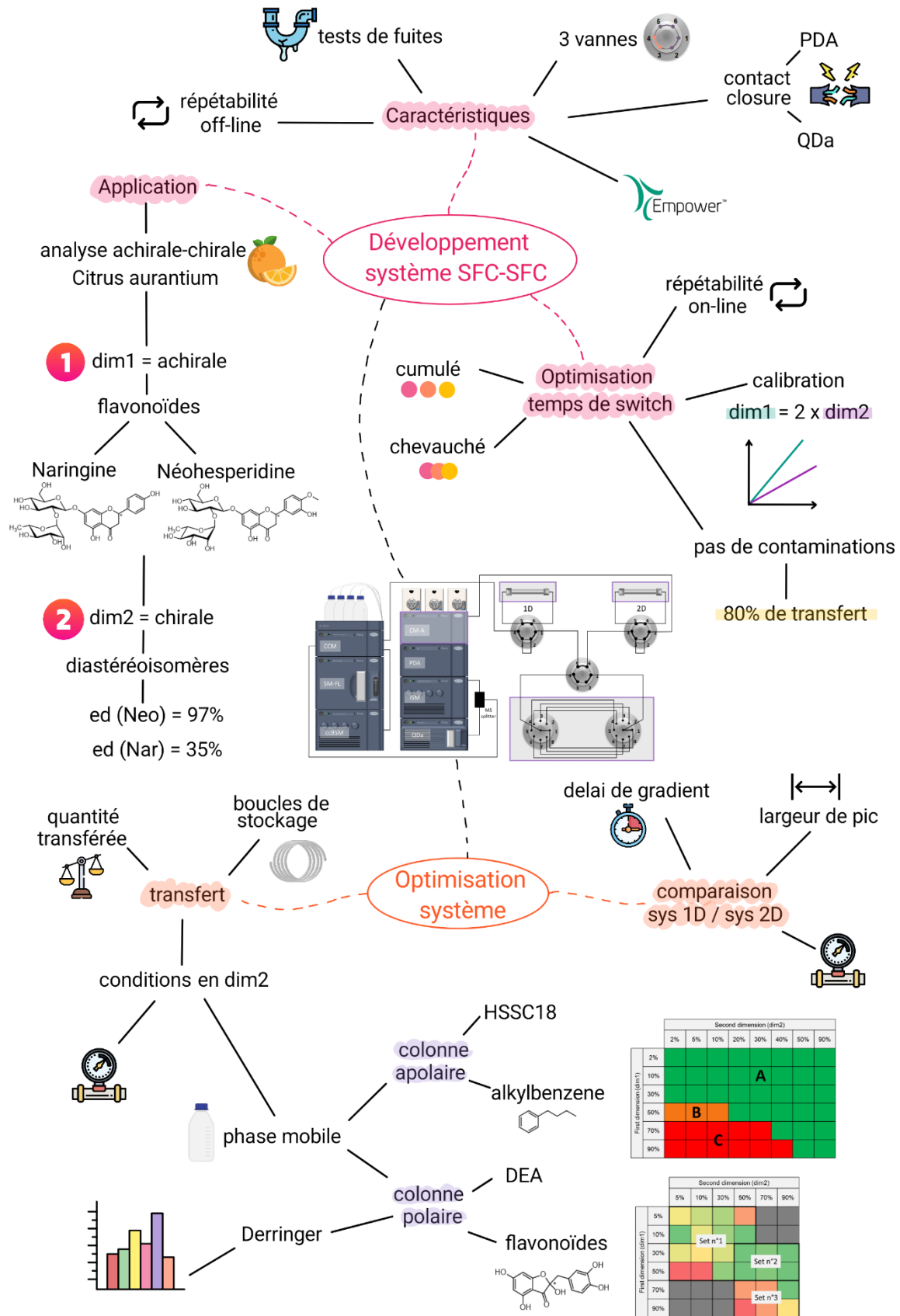
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Chapitre III

Développement et optimisations d'un système SFC-SFC en mode MHC





Introduction

Ce chapitre présente le développement d'un système de chromatographie en phase fluide supercritique bidimensionnelle en mode multiple heart-cut (SFC-SFC). L'objectif principal de cette conception instrumentale est de permettre un transfert efficace et contrôlé des pics entre les deux dimensions chromatographiques, en gardant une simplicité d'utilisation. Pour cela, des vannes automatiques ont été intégrées afin de gérer précisément le transfert des échantillons entre les dimensions.

Le système a été conçu à partir d'un instrument de chromatographie en phase fluide supercritique unidimensionnel, auquel trois vannes 2-voies et 6-positions ont été ajoutées pour faciliter le transfert des échantillons. Deux boucles de stockage (50 μ L et 114 μ L) ont été intégrées, pour assurer le stockage temporaire des pics issus de la première dimension avant leur injection dans la seconde. Les tests préliminaires ont permis d'optimiser les temps de commutation des vannes, pour garantir un transfert efficace des pics, indépendamment de la composition de la phase mobile.

Les performances du système ont été évaluées en analysant des flavonoïdes présents dans le *Citrus aurantium* L. Les résultats ont mis en évidence une excellente répétabilité des temps de rétention et des aires de pics, avec un taux de transfert des composés de l'ordre de 80%. Une séparation efficace des diastéréoisomères a également été observée dans la seconde dimension, montrant la pertinence du système pour des analyses stéréoisomériques complexes.

Le développement de ce système SFC-SFC représente une avancée instrumentale importante pour l'analyse de composés chiraux dans des matrices naturelles. Néanmoins, certaines optimisations restent nécessaires afin de mieux connaître les limites de ce système. La première étape est de comparer les performances de ce système bidimensionnel avec celles d'un système unidimensionnel. L'ajout des vannes et des tubings nécessaires à la modulation entraîne une augmentation de la pression ainsi qu'un effet mesurable sur les délais de gradient, ce qui entraîne un léger élargissement des pics, sans affecter significativement la qualité de la séparation.

Le point central d'un système 2D repose sur le transfert de fractions via des boucles. Le volume de ces dernières influence directement la quantité transférée ainsi que la pression du système, tout en ayant un effet limité sur la largeur des pics chromatographiques. À l'inverse, la quantité de solvant injectée dans la seconde dimension peut avoir un impact significatif sur la forme des pics. En effet, l'injection d'un volume important de solvant dans la deuxième dimension peut perturber la forme des pics, en particulier ceux faiblement retenus sur les colonnes apolaires. Dans le cas

des colonnes polaires, l'effet est différent puisque même les composés fortement retenus peuvent être affectés. L'ensemble de ces effets et de ces optimisations seront présentés dans deux articles.

Partie 1 : Développement du système SFC-SFC

Article 1: Development of a two-dimensional supercritical fluid chromatography system in multiple heart-cutting mode

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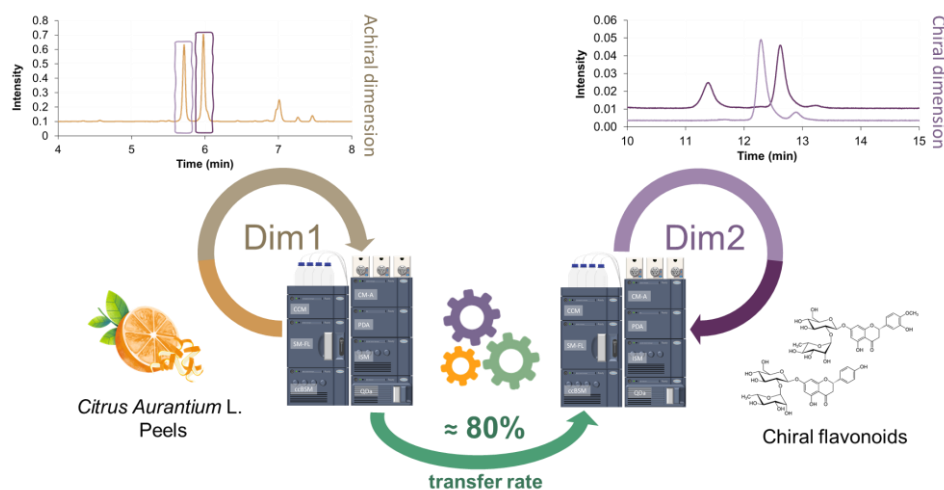
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Graphical abstract



Abstract

In this paper, we present the development of a loop-based two-dimensional supercritical fluid system in multiple heart-cutting mode (mSFC-SFC), with diode-array and mass spectrometric detection. The instrument design was developed to be as simple as possible, based on a single SFC instrument, with the sole addition of three external 2-port 6-position valves. The objective was

to achieve the most complete transfer of a peak from the first to the second dimension, whatever the composition of the mobile phase, i.e. whatever the proportion of carbon dioxide and methanol co-solvent along a wide gradient elution. Thanks to fine adjustment of the valve switching times, the first-dimension peaks were parked in 50- or 100- μ L loops and later discharged to the second dimension. The interest of this instrument was then demonstrated with a sample application on a natural product: an extract of *Citrus aurantium* L. bark was analyzed, with a particular focus on chiral flavonoids, neohesperidin and naringin. In this system, the first dimension was an achiral separation of the flavonoids, based on a polar diethylamine-bonded silica stationary phase (ACQUITY Torus DEA), while the second dimension used a stereoselective polysaccharide stationary phase (CHIRALPAK IB-3) to resolve flavonoid diastereomers. Excellent repeatability was demonstrated, with relative standard deviation values on retention times and peak areas all below 2%, together with excellent peak capacity and peak shapes (no distortion observed), making it possible to quantify diastereomers in the second-dimension separation. This good repeatability was also shown for the transfer rate between the two dimensions, which reached a value of 83%.

1. Introduction

Since its first applications in the 1970s', two-dimensional (2D) chromatography has evolved considerably [1]. The rapid development of analytical technologies has opened up new perspectives in chromatography, with significant advances in multidimensional chromatography [2]. By combining two chromatographic separation steps, this approach overcomes the limitations of one-dimensional (1D) chromatography. The advantages include superior peak capacity and the ability to separate closely related compounds by combining different selectivities in both dimensions.

From this perspective, 2D multiple heart-cut chromatography [3,4] is characterized by the application of an initial separation, followed by the selection and selective reinjection of specific fractions containing the compounds of interest into a second chromatographic column. This approach enables a more advanced separation of compounds, providing a more detailed view of the analyzed mixture. Over the years, two-dimensional liquid chromatography has been widely developed. In particular, the applications of 2D liquid chromatography (LC) are vast and diverse. It is widely used in fields such as pharmaceutical [5,6] research, food analysis [7,8], natural products[9,10], and many others.

Compared to LC, supercritical fluid chromatography (SFC) offers a number of advantages, such as high efficiency at high flow rates, environmentally friendly and economically advantageous solvent (mainly CO₂) [5]. However, coupling LC with SFC can be challenging [6,7,11–13] due to possible incompatibility of mobile phases and differences in pressure and flow rate between the two chromatographic systems. This typically causes broadening and distortion of the peaks.

Faced with these difficulties, the development of two-dimensional SFC (2D SFC) has emerged as a promising alternative. During the 1990s', capillary column systems developed and evolved over the years towards packed columns. The main difficulty in using SFC in two-dimensional systems was to successfully maintain back-pressure to keep CO₂ in its supercritical state. The first developments of these systems operated with neat CO₂, without any co-solvent [8,14–16]. However, the applicability of SFC is significantly improved by the addition of a polar co-solvent to the non-polar CO₂, thereby increasing the polarity of the mobile phase and solvating power to a wider range of molecules. A first approach to such a system was put forward in 2011 by Lu Zeng *et al* [17], focused on selecting a single pair of enantiomers from the first dimension, to resolve them in the second dimension, particularly for transfer to preparative scale.

In this study, we present the development of a 2D SFC-SFC system in multiple heart-cutting mode. A major challenge in this development was to maintain the instrument as simple as possible to favor its applicability in different laboratories, without the need for complex equipment. The implementation of a dedicated valve system using multiple 6-port, 2-position switching valves allowed maintaining the system pressure with a single back-pressure regulator (BPR). However, optimization of the valve switching times was necessary to ensure good peak harvest. Combined with repeatability tests, it was demonstrated that the composition of the transferred fractions did not impact the proper functioning of the system, not even when transferring fractions including high co-solvent percentages. Finally, the usefulness of this system was demonstrated with an achiral-chiral method applied to major chiral flavonoids in a sample of bitter orange bark.

2. Experimental

2.1. Chemicals and reagents.

HPLC-grade methanol used as the co-solvent and sample diluent, ethanol Hipersolv Chromanorm 99.7-100% and ammonium hydroxide were purchased from VWR (Fontenay-sous-Bois, France). CO₂ with a purity of 99.7% was delivered by Air Liquide (Paris, France). Methanesulfonic acid (MSA) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Ultra-pure water was supplied by a Milli-Q® IQ 7000 system from Merck (Darmstadt, Allemagne).

Reference compounds (naringin and neohesperidin) were purchased from Extrasynthese (Genay, France). Standards of epicatechin and caffeine were obtained from Sigma-Aldrich. *Citrus aurantium* L. peels were bought from Louis herboristerie (Charleville-Mézières, France).

2.2. Sample preparation.

Standard solutions were prepared at a concentration of 1 mg/mL in methanol (MeOH). Orange peels were finely ground, then 1 g of sample was extracted with 15 mL of ethanol (EtOH) for 30 min in an ultrasonic bath. The extract was then centrifuged (20 °C, 8000 rpm) for 5 min and filtered through 0.20 µm, 13-mm diameter PVDF syringe filters from Agilent Technologies (Les Ulis, France) and diluted by 4 in EtOH prior to analysis.

2.3. Chromatographic instrumentation.

The supercritical fluid chromatography system was a Waters Corporation (Millford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®). It was equipped with an autosampler with a partial loop volume injection system, a back-pressure regulator, a column oven allowing the implementation of two columns compatible with 150 mm length columns, via column switching valves. In the 2D mode, these column switching valves (V4, V5) were not used for column switching but for the implementation of multiple storage loops, as described in Section 3.1.1. The system also included a binary solvent delivery pump compatible with a maximum mobile phase flow-rate of 4 mL/min and a maximum pressure of 414 bar. Two detectors were available: a diode-array detector (ACQUITY PDA®) 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 and with a split, where one portion was sent to the back-pressure regulator and the other to the MS. The use of MSA in the mobile phase involved adapting the exhaust valve of the QDa system as MSA may cause corrosion on certain parts.

Empower® 3 was used for chromatographic acquisition and for data treatment.

The system was extended with three 6-port, 2-position automatic valves (V1-V3) from Rheodyne (MXT715-000), supplied by CIL Cluzeau (Sainte-Foy-la-Grande, France). The loops to park the fractions were created in-house using tubing also provided by CIL Cluzeau. They were 1/16" stainless steel tubes with internal diameters of 0.020 and 0.030 inches. The tubing was cut to a length of 25 cm using a tube cutter, resulting in 50 µL and 114 µL loops. The tubes were then sanded to even out the cut. Each tubing was then rinsed with ethanol to remove any dust from the sanding to prevent system clogging.

2.4. Chromatographic conditions

Preliminary tests were carried out using identical stationary phases in both separation dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm) from Waters. For applications on a real sample (bitter orange peel), two different columns were used. The first-dimension (dim1) column was again ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm). The second-dimension (dim2) column was CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 µm) from ChiralTechnologies (Illkirch-Graffenstaden, France). Additionally, a Torus DEA pre-column (2.1 × 5.0 mm) from Waters was placed in front of the Torus DEA column.

Based on previous investigations [18], the mobile phase used was CO₂ with MeOH containing 0.1% MSA. The first-dimension method (Table III.1.1a) was also adapted from J. Molineau *et al.* for flavonoid separation [18]. Only flow-rates were adapted due to the use of a pre-column causing additional pressure drop.

Table III.1.1 - Gradient profiles in the two dimensions

Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)
a. First dimension method			
0.0	20	150	1.7
0.5	20	150	1.7
8.0	100	110	0.6
10.0	100	110	0.6
10.5	20	110	0.6
12.0	20	150	1.7
12.5	20	150	1.7
b. Second dimension method			
0.0	15	150	2.0
0.5	15		
17.0	50		
19.0	50		
19.5	15		
22.0	15		

The column oven was heated to 30 °C and the sample compartment was kept at 10°C. All chromatograms were recorded in the 210-400 nm range, with 1.2 nm resolution. Scan rate was 20 Hz. Visualization and peak integration were done at 280 nm for standards and sample. The injection volume was 5 µL.

2.5. Mass spectrometry conditions

The optimal MS parameters were as follows: make-up fluid MeOH comprising 20 mM NH₄OH and 2% H₂O, pumped at 0.4 mL/min; probe temperature 600 °C; ion source temperature 120 °C; cone voltage 10 V; capillary voltage +/- 0.3 kV (both ionization modes examined in each run with voltage

switching); scan rate 3.7 Hz. The mass range for total ion recording was set from m/z 100 to 1000, with SIM m/z values as indicated in Table III.1.2.

Table III.1.2 – Mass spectrometry identification of target compounds

	MW (g/mol)	$[M + MSA]^-$	$[M - C_{12}H_{22}O_9 + H]^+$ (aglycone)
Neohesperidin	610.56	m/z 705	m/z 303
Naringin	580.54	m/z 675	m/z 273

3. Results and discussion

3.1. Characteristics of the 2D system

3.1.1. Description of operation

The 2D mSFC-SFC system was developed to allow for 4 possible configurations in a single instrument. It was based on a conventional one-dimensional SFC instrument. To add an extra dimension, three external 6-port, 2-position valves were added (Figure III.1.1). Valves 1 and 3 were used to manage the flow directed to the columns: valve 1 directs the flow to the dim1 column while valve 3 was used to send the flow to the dim2 column. Valve 2 provides access to the selectors carrying the storage loops. The selectors used for storage loops were already present on the column manager of the original system (valve 4 and valve 5). In its original configuration from the manufacturer, they were used to link the input (valve 4) and output (valve 5) of the column, and allow automated column switching. They were 9-port 8-position selectors. Thus, it was possible to install up to 8 storage loops. Because the minimum tubing length had to be 25 cm, the loops prepared had volumes of 50 or 114 μ L. This minimum length was imposed by the system because the distance between the two selectors was fixed. The choice of loop volume depends on the quantity of sample to be recovered from the first dimension.

All valves were controlled automatically by Empower software, using contact closure located at the rear of the diode-array (valve 2) and MS (valve 1 and 3) detectors. Simultaneous control of valves 1 and 3 prevented the two columns from being in series which avoided causing excess pressure drop in the system. By applying the right valve positions, this configuration allowed different modes of analysis without any manual handling. These different modes included: 1) one-dimensional analysis on dim1 column (Figure III.1.1A) 2) one-dimensional analysis on the dim2 column (Figure III.1.1C), 3) two-dimensional analysis using a successive combination of all four

settings (Figure III.1.1A, B, C, D). Whichever configuration was used, detection was carried out with a diode-array detector and MS placed in series at the outlet of valve 3.

The first stage of the development of a 2D multiple heart-cut method was to examine the dim1 separation so that appropriate fractions can be selected. The settings of the dim1 analysis are shown in Figure III.1.1A. To do this, valve 1 was in position 2 (V1-2) to direct the flow from the pump and injector towards the dim1 column. Valve 2 was in position 1 (V2-1) to bypass the storage loops, and stoppers were placed in ports 2 and 3 to avoid any leaks when switching this valve. Valve 3 was in position 2 (V3-2) to bypass the dim2 column and transfer the effluent to the detectors. This first step allowed the identification of the target peaks as well as their elution window. The second step was to repeat the dim1 analysis but now including the loading of the fractions into the storage loops. Valves 1 and 3 remained in positions V1-2 and V3-2, respectively (Figure III.1.1A). Valve 2 would switch during analysis depending on the peaks to be recovered from the loops: it would be positioned at V2-1 when not loading, and would switch to V2-2 when the peaks of interest started eluting from the column (Figure III.1.1B).

In this case, the flow was directed through valve 4, a selected storing loop and valve 5 before going back to valve 2. Indeed, the initial presence of valves 4 and 5 in the instrument means that they could also be controlled automatically from the software, making switch programming easier and enabling the recovery of peaks that were very close to each other, which would otherwise not be feasible. In fact, when a peak was targeted to be retrieved and stored in a loop, valve 2 was set to V2-2, granting access to the selectors. Prior to this event, the loop selectors were already placed in the correct position towards the loop that was intended to be loaded, hence when valve 2 was still in position V2-1. As a result, when the selectors were turning, there was no flow in this part of the system. This absence of flow ensured that the fractions collected in the loops were maintained there, until their discharge to the second dimension was desired. Otherwise, the mobile phase flow would have started eluting the fractions as they passed in front of the loaded loops, during the rotation of the valve as observed during initial experiments.

Important to note was the fact that although the detectors remained in the flow path of the eluent, the dim1 chromatogram was impacted as the target compounds were stored in the loops, thus were not sent towards the detectors.

The final step in the 2D method was to unload the loops and send the fractions consecutively to the second dimension. This time, valve 1 switched to position V1-1 to bypass the dim1 column and valve 3 to position V3-1 to unload the stored sample to the dim2 column. Valve 2 remained in position V2-2 to discharge the loop continuously (Figure III.1.1D), so the eluent

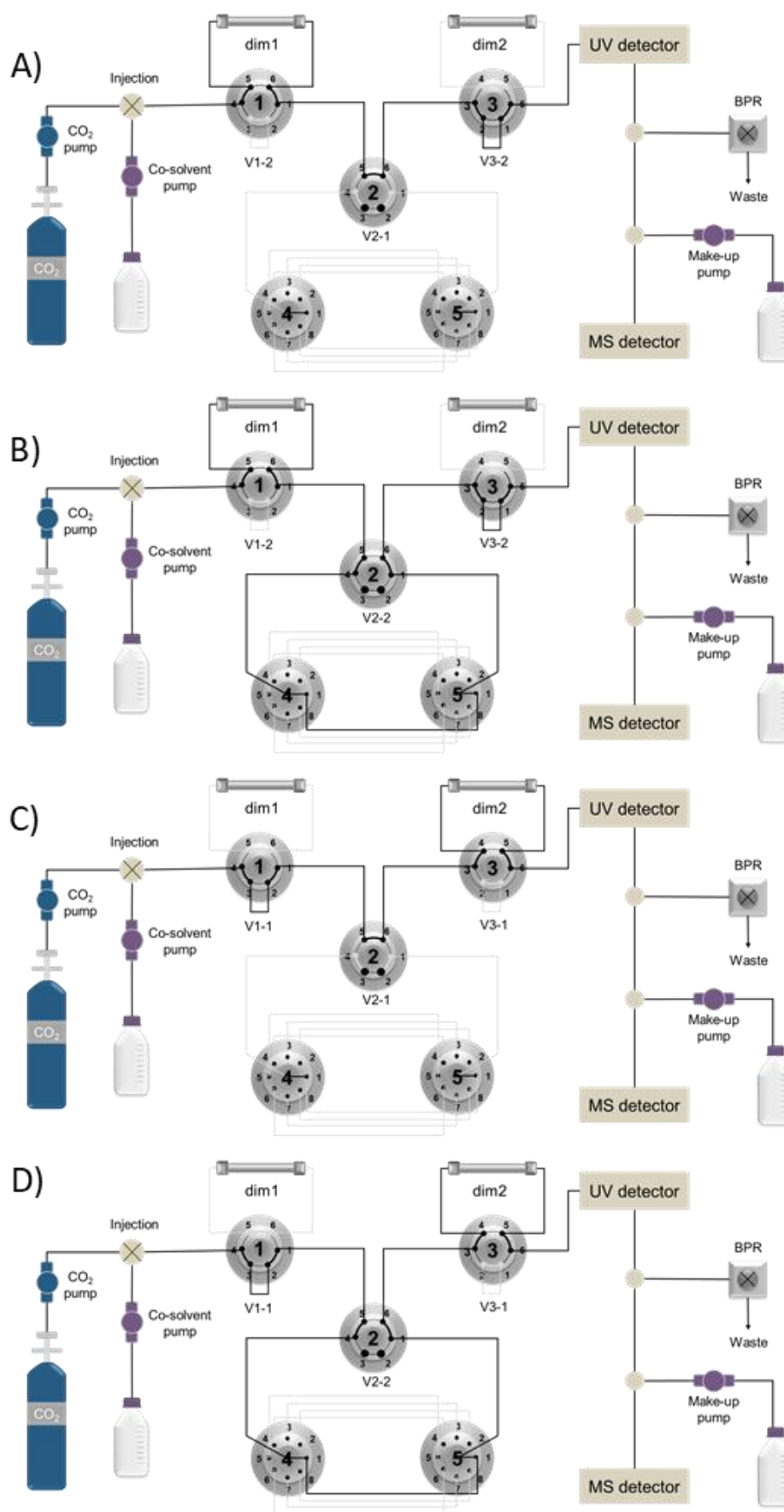


Figure III.1.1 - 2D system configuration. (A) First dimension only (dim1), (B) first dimension with loading of the loops, (C) second dimension only (dim2), and (D) second dimension with unloading of the loops

was flowing through valve 4, the selected storing loop and valve 5, before going back to valve 2. When several fractions had to be collected from dim1, the steps described in Figure III.1.1A and 1B were repeated, storing the fractions in different selected loops with the simple control of valves 4 and 5. Then the step described in Figure III.1.1D was repeated for as many times as the number of loops to obtain the dim2 chromatograms. Finally, by combining all these settings, it was possible to automatically perform several on-line separations with a single injection.

3.1.2. Preliminary tests

The main difficulty in developing this 2D system was to ensure good repeatability despite the modifications brought to the instrument. The addition of valves (1, 2 and 3) and numerous tubings can create significant variations in retention times. This phenomenon was observed during preliminary tests with a caffeine standard. In fact, significant variations in peak area were observed even for the analyses carried out in dim1 only. However, the retention times were highly repeatable. A leak test was then carried out at the level of each ferrule of each valve which led to the observation of the presence of micro-leaks throughout the installation.

Following these leak tests and appropriate handling of the tubings and fittings, new repeatability analyses were carried out in isocratic mode with a caffeine standard and in gradient mode with an epicatechin standard (flavonoid compound selected to obtain sufficient retention, comparable to the target compounds). In order to check whether the entire system was operating correctly, the analyses were carried out in dim1 only (Figure III.1.1A), in dim2 without passing through the loops (Figure III.1.1C), and in dim2 passing through different loops (Figure III.1.1D) of different volumes. Different features were measured (retention time, peak area, peak width, peak symmetry) and relative standard deviations (RSD) were computed from 20 and 10 replicates, for caffeine and epicatechin respectively. The full results are reported in Table III.1.S1 (supplementary information). Overall, they showed excellent RSD values, all below 2%. In conclusion, the modified instrument was repeatable, regardless of the mode and flow path used.

3.2. Sample application with achiral-chiral analysis

3.2.1. Preliminary study of the sample

Citrus aurantium L., also known as bitter orange, is a plant distinguished by its richness in various bioactive molecules, notably flavonoids. The latter are of particular interest for their potential medicinal properties [19].

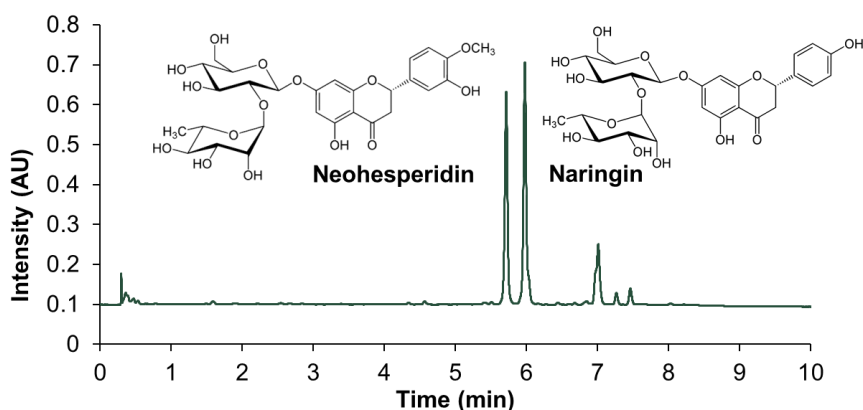


Figure III.1.2 - dim1 chromatogram of a sample of sour orange peel. Analysis conditions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 150 to 110 bar, 1.7 to 0.6 mL/min (gradient conditions in Table III.1.1.a)

When the extract of *Citrus aurantium* L. peels was analyzed in dim1, based on a previously developed method [18], two major flavonoids were identified, neohesperidin and naringin. As shown in Figure III.1.2, representing the analysis of the peel extract in dim1, they eluted between 5.5 and 6.0 min. These glycosylated flavanones have a chiral center on the aglycon part. The determination of diastereomeric excesses is of interest [20] thus the purpose of dim2 was to resolve the diastereomers, which could not be resolved with the first-dimension column but could be achieved with an enantioselective stationary phase.

For this purpose, the neohesperidin peak and the naringin peak were recovered in separate loops and re-analyzed in the second dimension. We aimed at collecting the full peak of each target compound in a single loop. Indeed, because diastereomers were not resolved in the first dimension, a more accurate diastereomeric excess measurement should be obtained if the whole peak from the first dimension was retrieved and re-analyzed in a single second-dimension analysis, especially when the diastereomeric excess would be large. So the largest 114 μL-loops were used. (See Table III.1.S2 in supplementary information explaining fraction volume calculation.)

3.2.2. Calibration

When a compound is eluted from different columns in different operating conditions, the peak area measured with any detector will vary. In fact, it makes no sense to compare the intensity of peaks obtained on different columns. The column internal diameter, the flow rate and mobile phase composition will impact the detector response. Even under the same chromatographic conditions, but with a different column, the cumulative area of the peaks cannot be compared.

In order to be able to evaluate the quantity recovered from dim1 to be sent to dim2, it was necessary to quantify this difference in response. This was achieved by running a calibration curve

for a neohesperidin standard on the two columns (Figure III.1.S1). This calibration was carried out with concentrations ranging from 0.1 to 0.7 mg/mL. Once the two linear regression equations were obtained, the response factor was obtained from the ratio of the two slopes. As a result, a factor of 2 was taken into account between the two dimensions.

3.2.3. Repeatability test

Assessing repeatability was important to ensure that the modified instrument was working properly. It was shown previously (section 3.1.2) that the system was repeatable in dim1 and dim2 when they were taken independently, but additional in-line tests with an automatic switch from dim1 to dim2 were necessary.

Sample analyses were made in triplicate with the extract of *Citrus aurantium* L. peels. Before each analysis, three blank analyses (injecting dilution solvent) were run to ensure that the column was conditioned correctly. A first analysis was carried out in dim1 to adjust the switching time as closely as possible to the retention time, as described above. A second dim1 analysis was then performed by loading two loops, one for the neohesperidin peak and one for the naringin peak. As the dim1 analysis was repeated 3 times, a total of six loops were loaded (3 for each target flavonoid). The dim2 configuration was then switched to unload the loops and analyze them in the second column. Prior to this, three blank runs were carried out using a bypass loop to condition the dim2 column. Six analyses were performed (one analysis per loop). In total, nine chromatograms were generated: three analyses in dim1 when the chromatogram was cut, three analyses in dim2 for the neohesperidin peak and three in dim2 for the naringin peak.

RSD values from triplicate analyses are shown in Table III.1.3 and include the values measured for retention time, peak area, width at half-height and asymmetry at 10% peak height. From the RSD values, it can be observed that the dim2 analyses were highly repeatable, especially as the most important RSD values for retention time and peak areas were both lower than 1.5%.

As described in Section 3.1.1, no data for areas or widths can be generated from this experiment for dim1, as the target compounds were removed after elution from dim1 and parked in the loop, thus not directed towards the detectors before elution from dim2. The data provided for retention time in dim1 correspond to two artefacts caused by the valves when they were switched, thus corresponding to the start of loop loading and end of loop loading. Indeed, turning the valves during analysis can create pressure gaps or slight overpressures that distort the baseline on the UV chromatogram. However, these baseline disturbances were also perfectly repeatable.

Table III.1.3 - Relative standard deviation in percentage of retention time, peak area, peak width at half height, asymmetry at 10% peak height, calculated after 3 replicates in dim1 and dim2 of a peel extract sample

	Peak	Retention Time	Peak Area	Width 50%	Asymetry 10%
Dim 1 - cut	1	0.04	-	-	-
	1'	0.00	-	-	-
	2	0.22	-	-	-
	2'	1.0	-	-	-
Dim 2 -Neohesperidin	1	0.2	1.0	1.5	0.9
	2	0.2	1.1	3.4	-
Dim 2 - Naringin	1	0.2	1.3	3.7	20.1
	2	0.2	0.2	2.0	10.9

3.2.4. Optimization of the switching time

According to the repeatability test (Table III.1.3), the dim1 analyses during loading, *i.e.*, when valve 2 was switched, appeared to be repeatable in terms of retention time. The RSD values were well below 2%. Prior to the optimization tests, the peak collection interval was determined by the delay between the column and the detector, the size of the tubing, the elution composition and the volume of the loop. Taking into account these factors and the exact retention times of the compounds, 5.71 min for neohesperidin and 5.98 min for naringin, the switch intervals for valve 2 could be determined. Initially, the valve was in position V2-1. To collect the neohesperidin peak at 5.71 min, valve 2 was switched to position V2-2 at 5.61 min and back to position V2-1 at 5.72 min. For the naringin peak at 5.98 min, the valve was switched in position V2-2 at 5.88 min and back to position V2-1 at 5.99 min.

At this stage, the fact that the system was known to be repeatable in 2D mode led to conclude that switch and collection times were also repeatable. However, despite this observed repeatability, optimum transfer was not guaranteed. Indeed, it was necessary to optimize the switch time to maximize the quantity of peak transferred to the second dimension. Two tests were carried out, focusing particularly on the first peak of interest, neohesperidin. The first test involved extending the interval already tested by sampling before and after. A single injection was required but with six loop loadings at 5.47, 5.58, 5.69, 5.80, 5.91 and 6.02 min. Six loops of 100 μ L were used and the interval duration remained constant, *i.e.*, 0.11 min. When the analysis in dim1 was complete, each loop was discharged to dim2 to be analyzed. It was then possible to calculate the transfer rate from the area of the peak in dim1 and the cumulative area of the peaks in dim2. As explained above, a response factor was observed between dim1 and dim2, which was important to take into account in this transfer calculation. Figure III.1.3A shows that, in the second fraction (cut2), which corresponds to the time interval from 5.58 to 5.69 min, more than 80% of the peak

was transferred. Before that, neohesperidin was not detected. No overlap between the neohesperidin and naringin peaks was observed, as the MS trace showed no sign of the other compound.

Despite a good transfer rate in cut2, 18% of neohesperidin was still observed in cut3. Therefore, precise determination of the time when the maximum amount of target compound could be retrieved was necessary. To achieve this, a second test was conducted, still centered on the neohesperidin peak, but this time overlapping the collection times. One injection per loop loading was then required, *i.e.*, six injections to fill six loops. Loops were loaded at 5.50, 5.55, 5.61, 5.66, 5.72 and 5.77 min. The loop size and the intervals remained the same, *i.e.* 100 μ L and 0.11 min.

When examining the results in Figure III.1.3B, representing the transfer rates per load, the maximum amount transferred was observed in cut3. This loading, done at 5.61 min (cut3), corresponds to the initial loading that was previously determined using calculations. Cut4 done at 5.66 min also had a high transfer rate because it overlapped the cut3 interval. By comparing cut3 and cut5, which were performed consecutively, the optimum of 83% was concluded to be in cut3, as cut5 had only a 2% transfer rate.

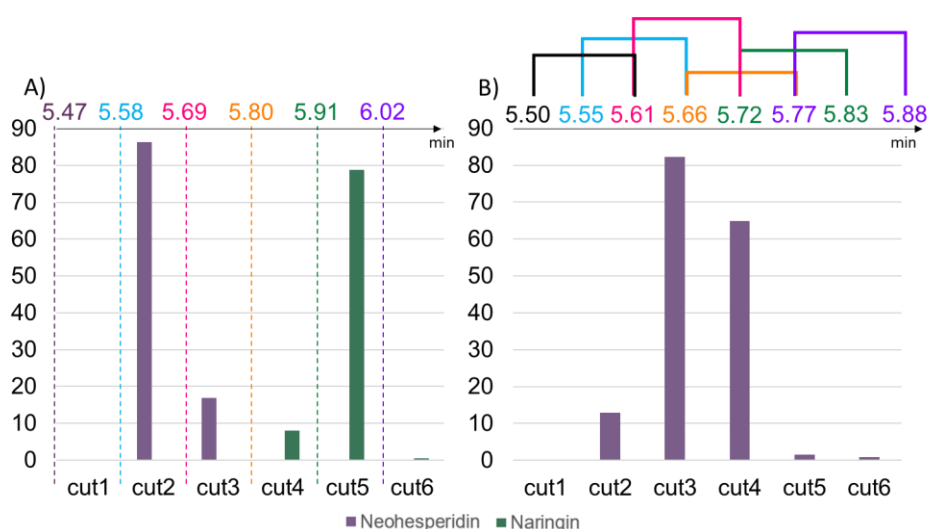


Figure III.1.3 - Transfer rate calculated on peak areas in dim1 during loop loading. A) Consecutive cuts with 1 injection and 6 loads B) Overlapping cuts with 6 injections and 6 loads

To confirm this conclusion, the MS response was observed, in order to ensure that a maximum amount of the target compound had been loaded into the loop. In Figure III.1.4A, the absence of UV peaks at the retention time of the compounds on the chromatogram of dim1 obtained when loading the loop confirms the previous results. The two spikes observed before and after the expected peak were the above-mentioned artefacts due to pressure changes. In addition, the

study of the different single-ion monitoring (SIM) signals in positive and negative ionization mode confirms this observation (Figure III.1.4B). In dim1, the compounds were present, and their diagnostic ions were easily detectable. On the other hand, in dim1 with loading, none of the target m/z values were detected, indicating that the compounds had been correctly extracted.

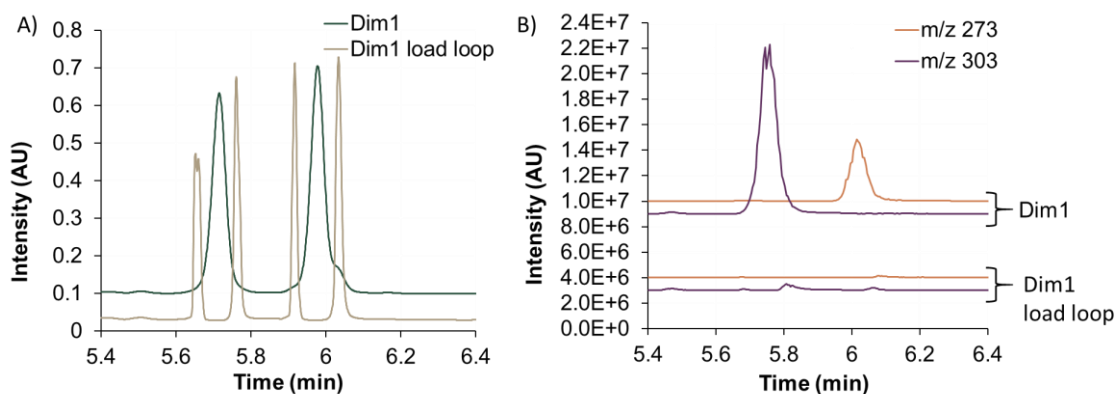


Figure III.1.4 - A) Chromatogram of neohesperidin and naringin extracted from bitter orange peel, peaks analyzed in dim1 with and without loop loading. Artefacts due to pressure variations are visible when the valves rotate to fill the storing loops. B) Chromatograms obtained with single-ion monitoring (SIM) in dim1 with and without loop loading

In conclusion, the major part of the peak of interest must have been recovered at the end of dim1, since it was no longer detectable thereafter. The loop unloading step in dim2 was very conclusive, transferring 83% of the peak.

These various system and method optimizations for loading and storage in loops enabled reliable 2D analyses of the bitter orange peel sample. The dim2 chromatograms for neohesperidin and naringin both show 2 peaks (Figure III.1.5). The diastereoisomers of neohesperidin and naringin were well resolved, with diastereoisomeric excesses of 97% and 35% respectively.

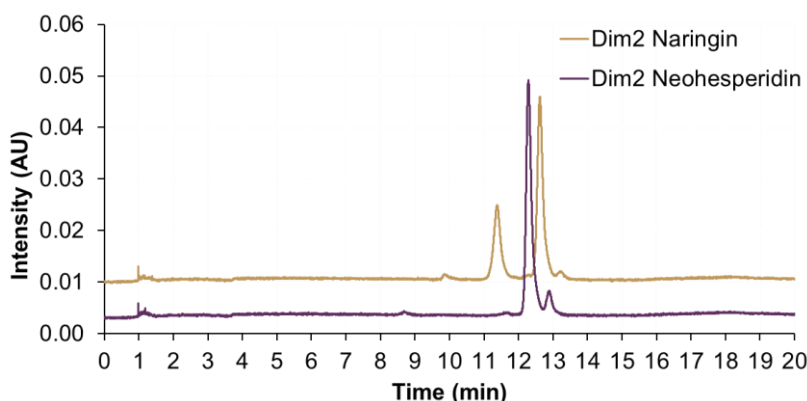


Figure III.1.5 - Dim2 chromatograms of neohesperidin and naringin extracted from bitter orange peel. Analysis conditions: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table III.1.1.b), 150 bar, 2.0 mL/min

Although the goal of this project was to demonstrate the feasibility and repeatability of a 2D mSFC-SFC set-up, another striking observation was worth noting. It is well known that the injection volumes in analytical 1D-SFC are limited to only a few μL s, particularly when using injection solvents of higher polarity [21,22]. When injecting larger volumes of “strong” solvent, peak distortion is usually observed. In the current research, however, storage loops of 50 and 114 μL were used. These storage loops were, in fact, the injection loops for the dim2 analyses in the 2D experiment. For both compounds, neohesperidin and naringin, which eluted from dim1 at co-solvent percentages of around 77%, excellent peak shapes were observed in dim2, despite the large injection volumes with large proportion of liquid co-solvent. Two factors can explain the good peak shapes: lower elution strength of the dim2 mobile phase (15% co-solvent at the gradient start), stacking the compounds at the column inlet, but also the higher pressure acting on the compressible fraction. Indeed, if the low elution strength was a sufficient explanation, injecting the same volume of a liquid sample would yield similar results. However, as can be seen in Figure III.1.S2, injecting the same volume (114 μL) of pure liquid in dim2 resulted in much lower chromatographic quality: pure methanol yielded strongly deteriorated peaks, while heptane-ethanol 75/25 (v/v), used to approximate the polarity of the CO_2 -methanol stored fraction, yielded slightly better peak shapes but still inferior quality to the injection of the compressible fluid sample. We must therefore conclude that the fact that the compounds were stored in supercritical/subcritical state prior to their injection onto the dim2 column clearly aided in ensuring good peak shapes. In addition, it is useful to examine the chromatographic features of “normal” and modified instrument (see Figure III.1.S3). In the first-dimension separation, adding valves and loops, thus increasing extra-column volume, caused a decrease in peak capacity of 11%. However, in the second-dimension separation, the decrease in peak capacity was only 5% when comparing a normal analysis on unmodified instrument to a dim2 analysis with loop-transfer injection. These observations clearly open perspectives for further research in this domain.

4. Conclusion

In this work the feasibility of a 2D mSFC-SFC system was demonstrated. This system only required the addition of three automatic valves to a conventional SFC system. This addition did not cause overpressure problems in the system, which was limited to a maximum pump head pressure of 414 bar, and a minimum back-pressure of 110 bar. Several configurations were then possible: a one-dimensional analysis with the first or the second column, and a two-dimensional analysis with loading of storage loops. The compliance of the repeatability in all these configurations proved the robustness of the set-up and the reliability of our results. In addition, the adjustment of valve

switching times to load a peak into a storage loop was fully optimized to ensure total collection of the peak of interest from the first dimension. This resulted in 83% of the peak being transferred to the second dimension. Sample application demonstrated the feasibility of determining stereoisomeric excesses of chiral flavonoids. This approach opens perspectives to any laboratory with an SFC instrument of which the users might be interested in occasionally and relatively easily implementing a second dimension to specific applications, without impacting the functionality of that system in routine 1D use.

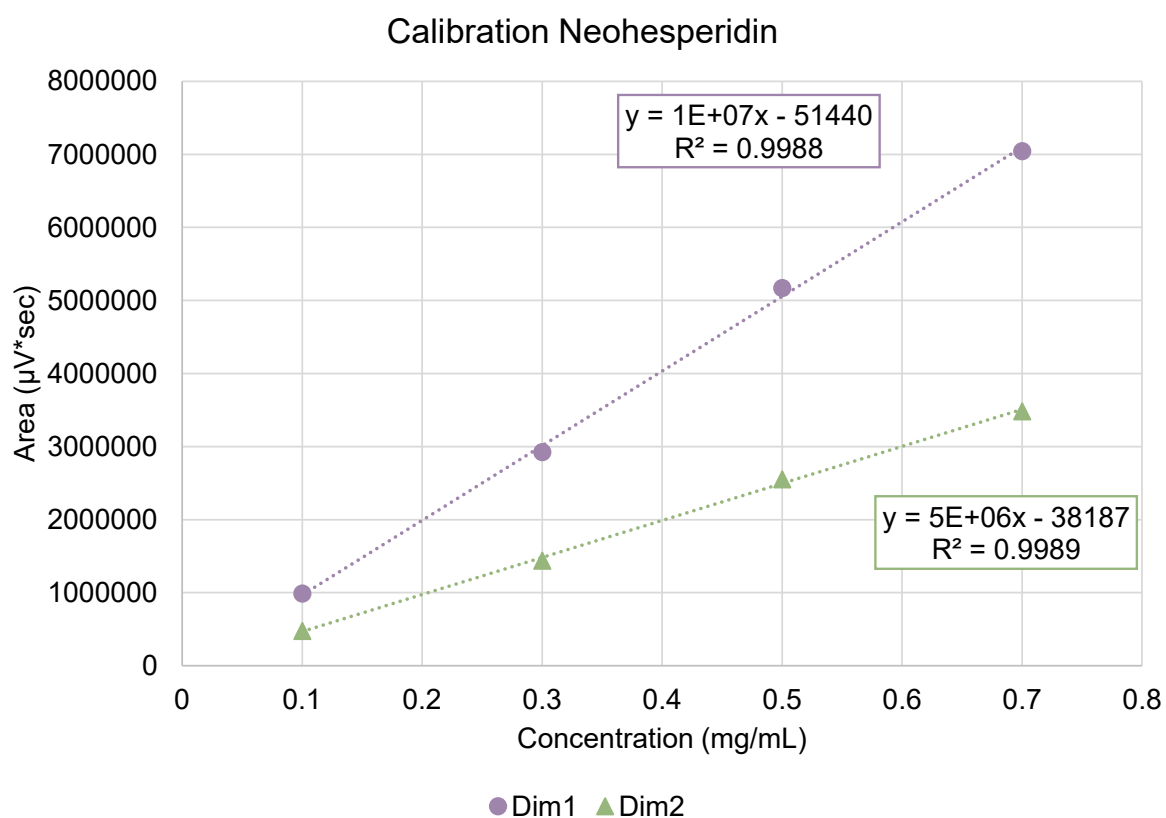
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Supplementary information

Figure III.1.S1 – Neohesperidin calibration curves obtained in dim1 and dim2. In dim1, the area of the whole neohesperidin peak comprising the two diastereomers was used. In dim2, the sum of areas of the two peaks (two diastereomers) was used.



Related data:

Dim1

Concentration (mg/mL)	Area ($\mu\text{V}\cdot\text{sec}$)
0.1	988301
0.3	2924641
0.5	5172910
0.7	7046371

Dim2

Concentration (mg/mL)	Area Peak 1 ($\mu\text{V}\cdot\text{sec}$)	Area Peak 2 ($\mu\text{V}\cdot\text{sec}$)	Sum Area
0.1	83840	392513	476353
0.3	267107	1174470	1441577
0.5	469406	2082581	2551987
0.7	644158	2839832	3483990

Figure III.1.S2 – Comparison between (A) normal injection of 5 μL liquid sample in unmodified instrument, (B) sub/supercritical injection and (C, D) liquid samples injected in dim2. Naringin diastereomers analyzed on Chiralpak IB with dim2 method.

(A) Direct injection of 5 μL methanolic sample in the dim2 column (Chiralpak IB) but in normal operation of the instrument (without the added valves).

(B) Fraction stored from the first dimension (injection of 5 μL methanolic sample) prior to transferring to dim2.

(C) and (D) Liquid samples prepared in methanol and in heptane-ethanol 25-75, introduced through port 3 of valve 2 to fully load a 114 μL storage loop, thus with the same volume as a sub/supercritical fraction. The concentration of samples in liquid solvent was adjusted to retain identical quantity injected in the dim2 column in all cases.

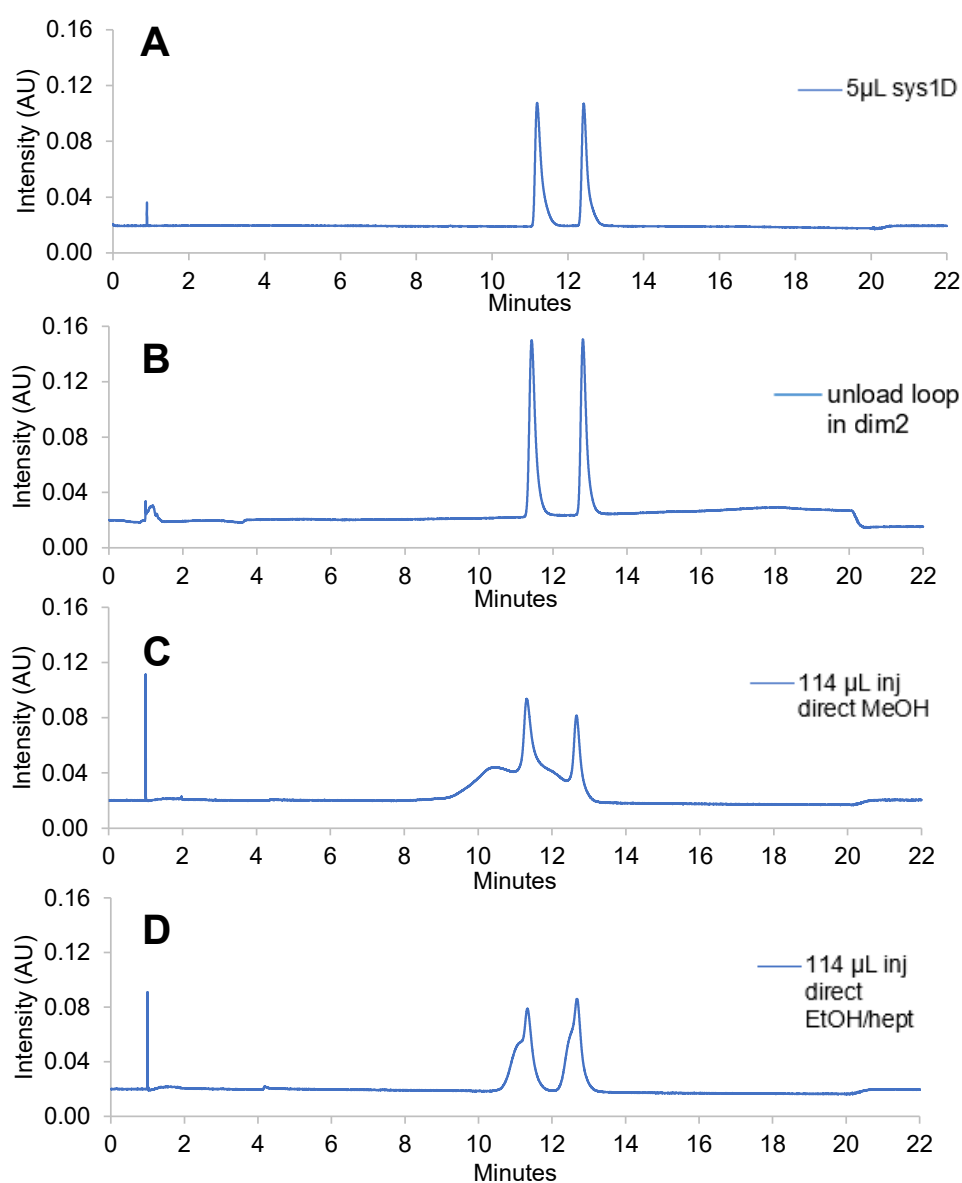


Figure III.1.S3 – Comparison between normal instrument and modified instrument.

(A, B) Analysis of naringin standard in the dim1 method (Torus DEA) with (A) the unmodified instrument and (B) modified instrument to see the impact of valves and tubings on efficiency.

(C, D) Analysis of naringin diastereomers in the dim2 method (Chiralpak IB) with (C) the unmodified instrument and (D) modified instrument operated in 2D, thus unloading the transfer loop to the second dimension.

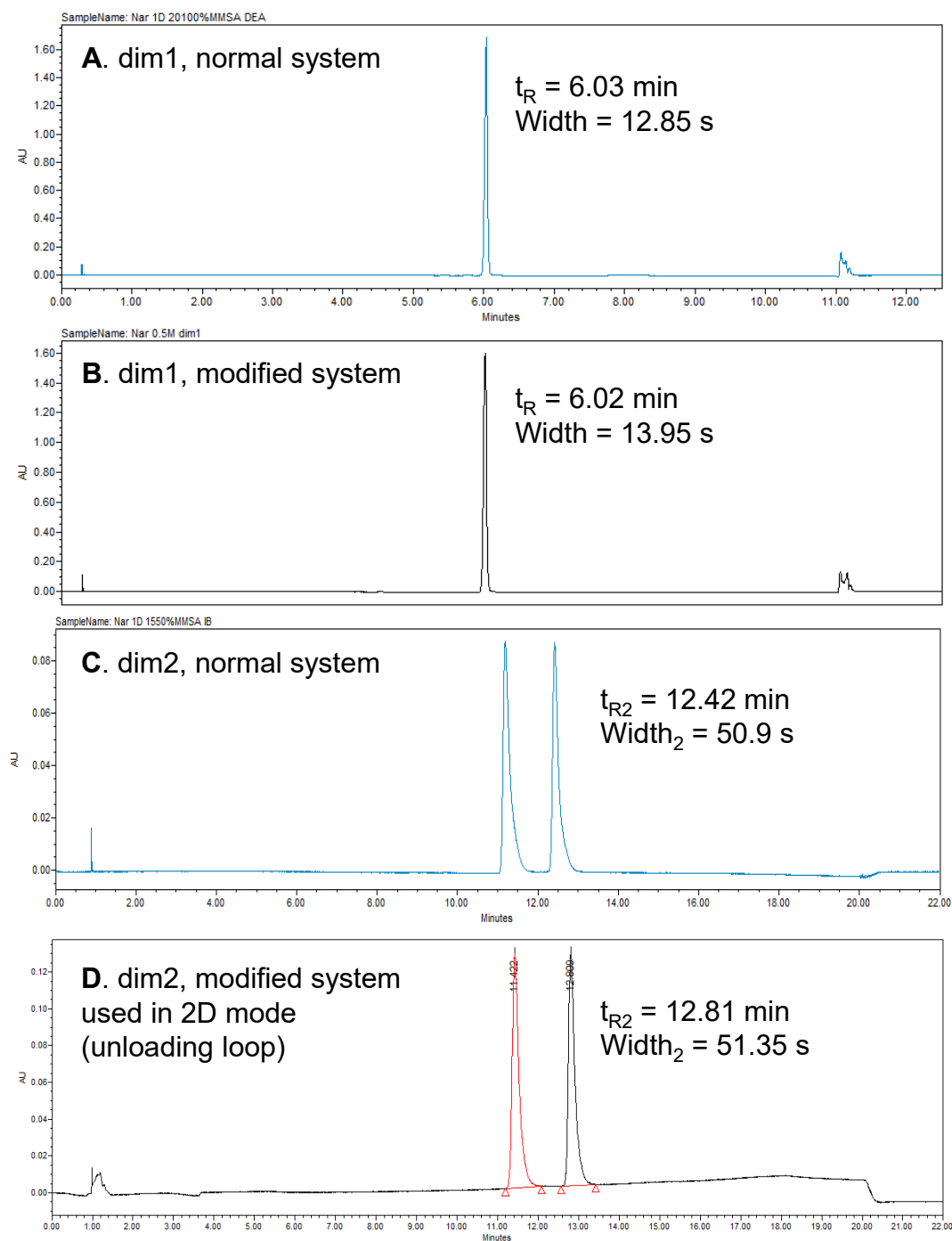


Table III.1.S1 – Relative standard deviation on significant features (retention time, peak area, width at 50% peak height, asymmetry at 10% peak height) calculated after analysis in different configurations (dim1, dim2 without loop, dim2 in 2 different loops of 100 μL and dim2 in a loop of 50 μL). A) 20 replicates of caffeine analysis in isocratic mode B) 10 replicates of epicatechin analysis in gradient mode. (i.e. P3 = position 3 of the selector and L3 = loop n°3)

	caffeine	retention time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Width 50%	Asym 10%
A)	Dim1	0.1	1.2	1.1	2.1
	Dim 2 – no loop	0.4	0.6	2.1	1.1
	Dim 2 – P3L3 100 μL	0.3	0.2	0.9	0.7
	Dim 2 – P4L4 100 μL	0.1	0.6	0.6	0.7
	Dim 2 – P5L5 50 μL	0.2	0.6	0.5	0.6
	Epicatechin	retention time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Width 50%	Asym 10%
B)	Dim1	0.0	0.8	0.4	0.4
	Dim 2 – no loop	0.0	1.9	0.3	0.5
	Dim 2 – P3L3 100 μL	0.0	0.9	0.1	0.5
	Dim 2 – P4L4 100 μL	0.0	0.6	0.3	0.3
	Dim 2 – P5L5 50 μL	0.0	0.4	0.1	0.4

Table III.1.S2 – Calculation of volume fraction retrieved from dim1 and cutting times depending on retention time (at peak apex)

Retention	Gradient	Dwell time	Co-solvent (%)		Flow rate (mL/min)	
t_R (min)	t_G (min)	t_D (min)	initial	final	initial	final
6	7.5	0.46	20	100	1.7	0.6
Elution composition						
1) Co-solvent (%)		2) Flow rate (mL/min)				
79.093		0.887				

Flow rate	Length	Radius	Volume
(mL/min)	l (cm)	r (cm)	V (mL)
0.887	169	0.009	0.043
3) Delays Column 1/PDA (min)			
0.048			

V_{loop} (μL)	100	50
4) t_{loop} (min)	0.113	0.056
Switch valve	5) ON	5.92
PDA	6) OFF	5.98

$$1) \%Cs_{elution} = \%Cs_{initial} + \frac{\%Cs_{final} - \%Cs_{initial}}{t_G} \times (t_R - t_D)$$

$$2) Fr_{elution} = \%Fr_{initial} + \frac{\%Fr_{final} - \%Fr_{initial}}{t_G} \times (t_R - t_D)$$

$$3) Delay_{column1/PDA} = \frac{V_{column}}{Fr_{elution}}$$

$$4) t_{loop} = \frac{V_{loop} \times 10^3}{Fr_{elution}}$$

$$5) Switch_{valve PDA (ON)} = t_R - delay_{column1/PDA} - \frac{t_{loop}}{2}$$

$$6) Switch_{valve PDA (OFF)} = t_R - delay_{column1/PDA} + \frac{t_{loop}}{2}$$

Partie 2 : Optimisations du système SFC-SFC

Article 2: Examining the conditions to achieve successful fraction transfer in two-dimensional supercritical fluid chromatography

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Abstract

In this study, the optimization of the conditions necessary to ensure successful fraction transfer in two-dimensional supercritical fluid chromatography (2D-SFC) were examined. Particular attention was placed on the influence of instrumental parameters, including the addition of valves, tubes, and loop volumes, on the chromatographic performance in both dimensions. The impact of these modifications on retention times, gradient delays and peak shape was evaluated. While the first dimension remained unaffected, a slight broadening of the peaks was observed in the second dimension, which did not significantly compromise resolution.

Optimizing the loop volume revealed no benefit in reducing the loop dimensions, as dividing the peaks into several fractions did not improve resolution. Furthermore, combining different proportions of CO₂ and methanol between the two dimensions showed that most combinations guaranteed satisfactory performance on polar and non-polar columns. Only the cases where the methanol proportion was greatly different between the first and second dimensions, yielded unsatisfactory peak shapes, but these are quite unlikely in normal SFC operation. Finally, these results provide indications of the operational limits of 2D-SFC systems to guarantee stable and efficient performance.

Keywords: gradient delay; injection effect; injection solvent; multidimensional chromatography; supercritical fluid chromatography

1. Introduction

Following the current trend on expanding two-dimensional liquid chromatography (2D-LC) applications, two-dimensional supercritical fluid chromatography (2D-SFC) is emerging as an innovative approach to improve the separation of analytes in complex matrices [1,2]. By combining the advantages of supercritical fluid chromatography (SFC) with the increased peak

capacity offered by two-dimensional (2D) chromatography, this technique provides better resolution of compounds that would be difficult to separate in one-dimensional (1D) chromatography [3]. One of the main advantages of 2D-SFC lies in the possibility to obtain high orthogonality through the simple use of different stationary phases in both dimensions while maintaining the same mobile phase composition, offering increased selectivity and therefore increased practical peak capacity compared with conventional methods.

However, the development of 2D-SFC presents major challenges, particularly with regard to managing the transfer of analytes between the two dimensions [4–7]. As 2D LC [3,8,9] and in LC-SFC or LC×SFC coupling [10–13], the transfer conditions directly influence the quality of the separations obtained in the second dimension. Optimization of valves, loop dimensions and storage volumes is essential to minimize dilution and peak distortion [14]. In addition, potential incompatibility between the conditions of the first (dim1) and second (dim2) dimensions, particularly with regard to the percentages of co-solvent [15], may compromise the detection and quantification of analytes.

Another limiting factor is related to the SFC-specific injection effects, which can alter the reproducibility of analyses and quantification of analytes. As observed in 1D-SFC and LC×SFC, significant pressure variations can occur during the injection of polar solvents, particularly those containing water [16–18]. These fluctuations may impact not only the separation performance, notably through alteration of peak shapes, but also the system stability. The volume and composition of the injection solvent must be carefully controlled to ensure optimum resolution [19–21].

The coupling of reversed-phase liquid chromatography (RPLC) and SFC has already demonstrated its potential by improving analytical coverage and exploiting the complementarities between the two techniques [3]. Technical developments have been proposed to improve RPLC and SFC hyphenation, which facilitate the transfer of analytes while taking advantage of the low viscosity of the SFC mobile phase, enabling faster analyses and improved peak capacity [14]. However, unlike traditional liquid-phase systems, transferring a compressible supercritical fluid from the first to the second dimension in a 2D-SFC system sets additional challenges compared to LC×SFC systems, particularly to maintain pressure during the fraction transfer. This difference requires careful optimization of injection strategies, solvent compatibility and transfer conditions to ensure robust and reproducible performance in 2D-SFC instruments.

In this context, our study aimed at optimizing the transfer of analytes in an SFC-SFC system used in multiple heart-cutting mode, paving the way for future developments of comprehensive 2D-

SFC. First, a comparison was made between a 1D and a 2D system. Then the impact of elution conditions in the first dimension (dim1) and the influence of the quantity of solvent stored and transferred to the second dimension (dim2) were thoroughly examined, using two sets of analytes (non-polar and polar) and different chromatographic conditions. Parameters were optimized, considering peak shape and bandwidth. Among the parameters studied were the size of the storage loops, the system pressure and the composition of the mobile phase in order to improve compatibility between the two dimensions and ensure the best possible transfer of analytes in a 2D-SFC system.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol (MeOH) used as the mobile phase and sample diluent, and ethanol (EtOH) Hipersolv Chromanorm 99.7–100 % used for sample preparation were purchased from VWR (Fontenay-sous-Bois, France). CO₂ with a purity of 99.7 % was delivered by Air Liquide (Paris, France). Methanesulfonic acid (MSA) and acetone were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

A naringin standard containing identical proportion of the (+) and (-) diastereomers and standards of pinocembrin, pinocembroside, hesperetin, naringenin, taxifolin, alphononin, epigallocatechin and epicatechin gallate were all provided by Extrasynthese (Genay, France). Standards of caffeine, n-butylbenzene, n-hexylbenzene, n-octylbenzene, n-decylbenzene and n-dodecylbenzene were obtained from Sigma-Aldrich. A standard of n-tetradecylbenzene was purchased from Thermo Fisher (Illkirch, France).

Citrus aurantium L. peels were bought from Louis herboristerie (Charleville-Mézières, France).

2.2. Standards and sample preparation

Standard solutions of naringin, other flavonoids and alkylbenzenes were prepared respectively at the concentration of 1 mg/mL, 0.5 mg/mL and 0.5 mg/mL in MeOH.

Orange peels were finely ground, then 1 g of sample was extracted with 15 mL EtOH for 30 min in an ultrasonic bath. The extract was then centrifuged (20 °C, 8000 rpm) for 5 min and filtered through 0.20 µm, 13-mm diameter PVDF syringe filters from Agilent Technologies (Les Ulis, France) and diluted by 4 in EtOH prior to analysis.

Caffeine was directly introduced in the mobile phase co-solvent (MeOH) at different concentrations: 0.025 mg/mL and 0.05 mg/mL.

Figure III.2.1 – Multiple heart-cut SFC-SFC system configuration. The elements in orange rectangles are all contained in the column oven (CM-A). See text for complete description.

Briefly, the system was equipped with three Rheodyne 6-port, 2-position automatic valves (MXT715-000), supplied by CIL Cluzeau (Sainte-Foy-la-Grande, France). The fraction storage loops were manufactured in-house from tubes also supplied by CIL Cluzeau. They were made of stainless steel (OD 1/16") with internal diameters of 0.50 and 0.80 mm. They were cut into 25 cm or 56.5 cm segments using a tube cutter and then sanded to obtain smooth cuts. Finally, each tube was rinsed with ethanol to remove particles from sanding and prevent any obstruction of the system.

Between valves 1 and 2, the tubing was 10 cm x 0.18 mm. Between valves 2 and 4 and 2 and 5, the tubings were 20 cm x 0.18 mm.

Empower® 3 was used for chromatographic acquisition and for data treatment.

2.4. Chromatographic conditions

For applications on a real sample (bitter orange peel) and for the analysis of naringin standard, two different columns were used. The first-dimension (dim1) column was ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm). The second-dimension (dim2) column was CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 µm) from ChiralTechnologies (Illkirch-Graffenstaden, France). Additionally, a Torus DEA pre-column (2.1 × 5.0 mm) from Waters was placed in front of the Torus DEA column. The mobile phase used was CO₂ with MeOH containing 0.1 % MSA. The injection volume was 5 µL. The gradient used, comprising variation of mobile phase composition, but also back-pressure and flow-rate (for the first-dimension analysis) is presented in Table III.2.1 (gradient 1). This method was used for the flavonoid samples in: bitter orange peel, naringin and neohesperidin standards. For the measurement of gradient delay, the tests were carried out on the same columns as those mentioned above, but with MeOH containing 0.1 % acetone as mobile phase, and using the gradient 2 presented in Table III.2.1. Back-pressure was set at 150 bar, and flow-rate was respectively 2.0 and 1.5 mL/min in dim1 and dim2 methods.

For the tests with the caffeine standard, the same columns as before were used. The co-solvent for the first dimension was MeOH, containing dissolved caffeine, and for the second dimension MeOH containing 0.1 % MSA. The step gradient involving several isocratic steps is shown in Table III.2.1 (gradient 3). Back-pressure was set at 110 bar and 150 bar, and flow-rate was 0.6 and 2.0 mL/min respectively in dim1 and dim2 methods.

Table III.2.1 – Gradient profiles in the two dimensions

Gradient 1				Gradient 2				Gradient 3			
Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)	Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)	Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)
a. First-dimension method				a. First-dimension method				a. First-dimension method			
0.0	20	150	1.7	0	20	150	1.5	0 to 2	20	110	0.6
0.5	20	150	1.7	0.5	20			2 to 4	40		
8.0	100	110	0.6	1.44	30			4 to 6	60		
10.0	100	110	0.6	2.50	30			6 to 8	80		
10.5	20	110	0.6	2.60	20			8 to 10	100		
12.0	20	150	1.7	3.0	20						
12.5	20	150	1.7								
b. Second-dimension method				b. Second-dimension method				b. Second-dimension method			
0.0	15	150	2.0	0.0	15	150	2.0	0.0	15	150	2.0
0.5	15			0.5	15			0.5	15		
17.0	50			17.0	50			17.0	50		
19.0	50			19.0	50			19.0	50		
19.5	15			19.5	15			19.5	15		
22.0	15			22.0	15			22.0	15		

Tests with alkylbenzene standards were performed with similar stationary phases in both dimensions: XSelect HSS C18 (150 × 4.6 mm, 5.0 µm) and HSS C18 (150 × 4.6 mm, 5.0 µm) from Waters. Isocratic elution with MeOH percentage ranging from 2 % to 90 % in CO₂ were examined in the first and second dimensions.

Tests with flavonoids standards were performed with the same stationary phases in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm) from Waters. Isocratic elution with MeOH containing 0.1 % of MSA, with percentage ranging from 5 % to 90 % in CO₂ were examined in the first and second dimensions.

For both alkylbenzenes and flavonoids, the back-pressure was set at 110 bar, the flow rate was 1.0 mL/min and the injection volume was 2 µL.

In all cases, the column oven was heated to 30 °C and the sample compartment was kept at 10 °C. All chromatograms were recorded with PDA in the 210–400 nm range, with 1.2 nm resolution, with a scan rate of 20 Hz. Visualization and peak integration were performed at 280 nm for flavonoids standard and peel extract, 272 nm for caffeine and 210 nm for alkylbenzenes.

2.5. Mass spectrometry conditions

Mass spectrometry (MS) only served to confirm peak identity, especially for the citrus samples to distinguish target compounds from other peaks. The MS parameters were as follows: make-up fluid MeOH comprising 20 mM NH₄OH and 2 % H₂O, 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 3.7 Hz. The mass range for total ion recording was set from *m/z* 100 to 1000.

3. Results and discussion

3.1. Comparison of two-dimensional system and one-dimensional system

As appears in Figure III.2.1 describing the modified instrument, the insertion of additional valves and tubing in the 1D instrument shown in Figure III.2.S1 (noted as “sys1D” in the following) to obtain a 2D instrument (noted as “sys2D” in the following) results in the addition of dead volumes, which can impact retention time and peak shape in both dimensions. It was therefore essential to assess the impact of these structural modifications on the overall performance of the system.

3.1.1. Gradient delay

To compare the performance of sys1D and sys2D, the impact on gradient delay was first studied. To measure this delay, a low concentration of acetone (0.1 % in methanol) was added to the mobile phase and the UV trace was recorded during gradient operation.

The calculation of the dwell volume (D) is based on several parameters: the flow rate (F), the predefined gradient duration (t_G) and the time $t_{0.5}$, corresponding to the moment when the UV absorbance has reached a half of the maximum absorbance that is measured on the final plateau [22]. The formula used is as follows:

$$D = (t_{0.5} - 0.5 \times t_G) \times F \quad (1)$$

In liquid conditions, the usual practice is to use a wide gradient, covering most of the possible solvent proportions, i.e., from 1 to 99 %, to achieve this measurement. The objective is to have a large variation of UV absorbance to offer a good precision in the measurement. However, it is important to note that the choice of conditions to achieve a reliable measurement with supercritical or subcritical fluids is not trivial. Indeed, since the fluid is compressible, the amount of fluid that can be pumped to fill in a defined volume varies with the operating conditions, where the latter include the co-solvent nature and proportion, the temperature, pressure and flow rate [23]. Typically, the apparent void volume was observed to vary depending on operating conditions. Similarly, and relevant to the present case, the measured dwell volume varies depending on the conditions employed at the beginning of the gradient program. More precisely, as compressibility is reducing when the proportion of co-solvent increases, the measured dwell volume will also reduce when the proportion of co-solvent at the start of the gradient increases. In other words, the amount of fluid that is necessary to fill in the volume is increasing when the fluid is more compressible, that is to say when the proportion of co-solvent is less.

In previous works, Sarrut *et al.* had shown that a wide co-solvent gradient (1–99 %) in SFC made it impossible to obtain a precise measurement of the dwell volume [22], because the slope of the UV absorbance was changing along the gradient. Instead, they chose to measure the dwell volume based on a gradient program where the proportion of co-solvent was large (69 to 99 %), to offer conditions of low compressibility, close to a liquid. In the present study, we chose, on the contrary, to measure the dwell volume with low starting proportion of co-solvent (20 %), identical to those chosen for analysis, hoping to provide a value that would be more relevant to our analysis conditions, and to use only a narrow range of co-solvent proportion (20 to 30 %) to preserve a constant slope of the UV absorbance.

In sys1D, the normal Acquity UPC² instrument was used, without any modification (Figure III.2.S1). In sys2D, the mobile phase would go through different pathways when eluting the samples in dim1 or dim2 (Figure III.2.1).

For dim1 operation, the mobile phase would go through valve 1, the dim1 column, valve 1 again, valve 2 and valve 3. Note that the mobile phase would not go through the loops at this stage since this is the dim1 analysis, that serves to measure collection times. When performing sample collection, a second analysis of the samples is done, going through a different path to store the fractions in the storage loops between valves 4 and 5.

With the initial method we had developed for flavonoids analysis (gradient 1.a in Table III.2.1) [24], accurate determination of the gradient delay in dim1 was not possible due to the complex combination of increasing co-solvent on a large concentration range (20 to 100%), decreasing flow rate and decreasing back-pressure. Therefore, a second elution gradient (gradient 2.a in Table III.2.1) was used to facilitate the measurement of gradient delay, where only the proportion of co-solvent was varied: methanol increased from 20 to 30% with the same slope as in gradient 1.a. These conditions were chosen to be similar to the gradient start in method 1, and should therefore adequately represent the case of experiments done with gradient 1, although the back-pressure and flow-rate remained constant.

Comparing the results obtained with this method for sys1D and sys2D, the measured gradient delay remained identical for the first dimension (dim1), with a value of around 1.7 mL (see Figure III.2.S2.A in supplementary information).

For dim2 operation, as the fluid must go through a storage loop to transfer the fraction to the second column, the mobile phase would go through valves 1, 2, 4, a 50- μ L storage loop, then valves 5, 2 again and 3, the dim2 column, then again valve 3.

For the second dimension (dim2), gradient 2.b, identical to gradient 1.b (Table III.2.1) was used. Although a significant amount of tubing and valve volume was added in sys2D, the measured

delays were not significantly different: 3.8 mL for the one-dimensional system and 3.7 mL for the two-dimensional system. It can be observed in Figure III.2.S2.B that the curves are nearly superimposed.

The reader may however be surprised that a larger extra-column volume would not yield a larger dwell volume. To understand this point, it is worth reminding that the fluid is compressible and that the dwell volume was measured as the volume of compressible fluid required to fill it, not as the true volume that could result from a measurement in liquid conditions. The longer pathway taken by the fluid in the modified system may yield a slightly higher internal pressure, resulting in a less compressible fluid in this system. However, the higher the compressibility of the fluid, the larger the volume of fluid you would need to pump in the system to fill in the available space. Hence, even a larger empty volume could yield a lower measured volume of compressible fluid to fill it in. Nonetheless, these observations showed that adding the second dimension to the chromatographic system had no significant impact on the gradient delay, confirming compatibility between the two configurations under the conditions tested.

3.1.2. Pressure

Because the mobile phase is a compressible fluid, where density may impact chromatographic retention and band width, internal pressure is an important parameter that may be impacted by the system modification. The pressure variation induced by the addition of new components (valves, tubings and second-dimension column) was measured through the captors of the pumping system during the previous analyses. In the sys2D, in first dimension (dim1), the addition of components to build up the two-dimensional system generated no measurable pressure increase (Figure III.2.S3.A) compared with the initial configuration. In the second dimension (dim2), the pressure variation reached 3 bar (Figure III.2.S3.B). Such increase can be considered as negligible in most usual operating conditions and is therefore unlikely to affect the correct operation or overall performance of the system. However, it can be related to the observations in previous section that the fluid may be slightly less compressible in the 2D configuration.

3.1.3. Peak width

Most importantly, it was essential to assess the impact of instrument changes on chromatographic efficiency, as this would have further consequences on overall system performance, notably affecting resolution and sensitivity. As all experiments were done in gradient elution mode, peak width was used as key metrics.

For this study, a solution of naringin standard (containing two diastereomers in identical proportions) was used. In dim1, an achiral stationary phase that is not resolving the diastereomers of naringin (Torus DEA) was used with gradient 1.a (Table III.2.1). In dim2, a chiral stationary phase (CHIRALPAK IB) was used to resolve the diastereoisomers of naringin, with gradient 1.b (Table III.2.1).

The results obtained with sys1D and sys2D are shown in Figure III.2.2.

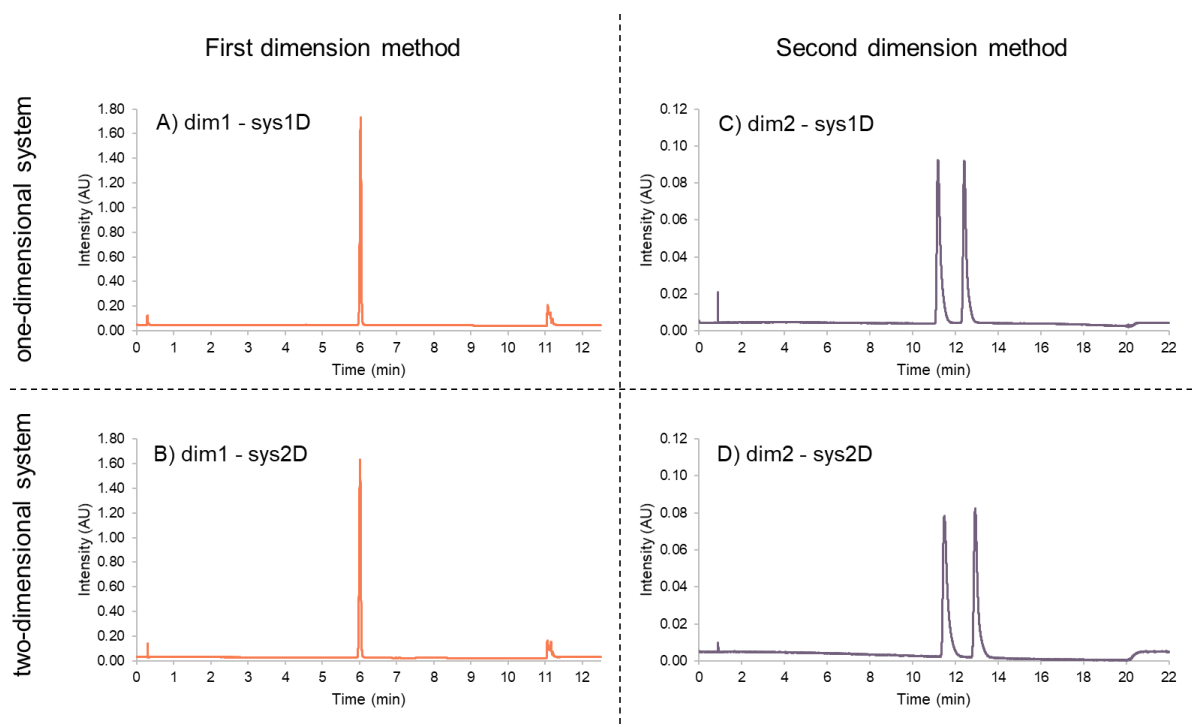


Figure III.2.2 – Chromatograms of dim1 and dim2 showing the analysis of a naringin standard performed on a one-dimensional system (sys1D) and a two-dimensional system (sys2D).

A) Analysis using the dim1 method with sys1D. B) Analysis using the dim1 method with sys2D. C) Analysis using the dim2 method with sys1D. D) Analysis using the dim2 method with sys2D, where the flow is going through a storage loop to mimic the real pathway of a sample when operating the SFC-SFC system. UV detection 280 nm.

In the dim1 method, the width at half-height of the naringin peak remained very narrow in both configurations, close to 0.04 min. In the sys2D configuration, a 12 % increase in peak width was observed.

However, in dim2, a larger increase in peak width was observed in the two-dimensional system compared with the one-dimensional system. Specifically, the width at half-height of peak 1 was 0.165 min with sys1D and 0.209 min with sys2D (Table III.2.S1), which represents a 27 % increase in peak width. For the second peak, the increase in peak width was slightly less (18 %). However,

this reduction did not impair the resolution of the diastereomers, which was 4.67 in sys1D compared to 4.46 in sys2D.

This small increase of peak width in dim2 was not unexpected and can naturally be attributed to the addition of dead volumes (tubings and valves) in the two-dimensional system. Although this difference is significant, it appears that sufficient resolution between the diastereomers was maintained.

3.2. Transfer conditions

3.2.1. Storage loop dimensions

In two-dimensional chromatography systems operating in multiple heart-cutting mode, storage loops play an essential role in ensuring accurate transfer of analytes between dim1 and dim2. These loops first serve as storage loops, to collect specific fractions from dim1, then serve as injection loops to re-inject the fractions into dim2 for further analysis. In liquid chromatography, the loop dimensions have a direct impact on band broadening in the second-dimension analysis, since an incompressible fluid is transferred so the loop length has a direct impact on band width, unless some peak compression may be obtained from the eluting phase. However, transferring a compressible fluid may result in different effects. Thus, in this section, we intended to examine how loop dimensions would influence band dispersion in dim2, and therefore dim2 resolution. Three loop configurations were tested: a 114- μ L loop (25 cm $\times$ 0.762 mm id), a longer but narrower loop with the same 114 μ L volume (56.5 cm $\times$ 0.508 mm id), and two identical 50- μ L loops (25 cm $\times$ 0.508 mm id). The interest in smaller loops was also to examine the possibility of fractionating a large peak into two smaller-volume fractions, as would be done in selective comprehensive 2D methods.

These tests were carried out on a sample of orange peel extract, which we had previously examined [5], to offer a representative example of a complex matrix. The neohesperidin peak (containing two diastereomers and some other compounds) was collected from dim1 and re-analyzed in dim2 to resolve its two diastereomers and the other co-eluting compounds.

The results presented in Figure III.2.3 show that the width of the dim2 peaks remained constant, with an average value of 0.17 min, whatever the loop dimensions. This indicates that, under the operating conditions of this study, neither the volume nor the dimensions of the loops introduced measurable dispersion changes or altered the dim2 resolution. This would probably be a surprising observation for 2D LC chromatographers, but it is important to remember here that the fluid is compressible, so the internal volume of the storage loop does not necessarily translate to the

expected band width in the dim2 chromatogram. Indeed, the fluid is compressed again during the transfer, and the analytes get stacked at the column entrance. We had previously demonstrated that transferring a volume of supercritical fluid resulted in much reduced band broadening compared to the same volume of a liquid with comparable polarity [5,25].

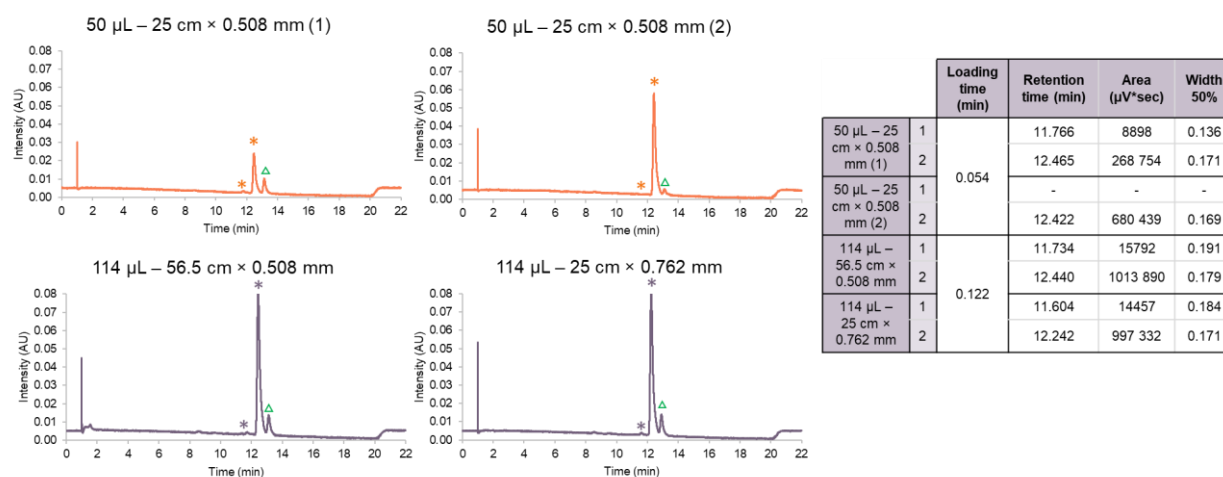


Figure III.2.3 - Second-dimension analysis of dry peel of Citrus aurantium, where the peak of neohesperidin was selected from the first dimension and re-analyzed in second dimension. Influence of the dimensions of the storage loop on the peak area and peak width. Green triangles indicate the co-eluting peak in dim1.

Analysis conditions: CHIRALPAK IB-3 (150 $\times$ 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1 % MSA (gradient conditions in Table III.2.1, gradient 1.b), 150 bar, 30 $^{\circ}\text{C}$, 2.0 mL/min. UV detection 280 nm. MS detection was used to confirm peak identity.

The impact of loop volume (50 or 114 μL) and added tubings (12.5 μL) was however visible in the values of measured peak areas. First, and as could be expected, the large-volume loops resulted in larger peak areas. In addition, the two loops with 114 μL volume but different dimensions yielded very close peak areas, suggesting that the whole target peak had been correctly collected from dim1, whatever the loop dimensions. Second, and also quite logically, the peak areas from the two 50- μL loops were smaller and different from each other, since they corresponded to two fractions of one large peak from dim1. Furthermore, the peak areas from these two fractions added up to a total amount that was nearly equal to the values measured from the large loops. This again suggests that two small loops recovered the whole target peak from dim1.

In other words, the division of the large peak into two fractions did not improve resolution in the second dimension but only divided peak areas into different fractions. This is an important conclusion from this experiment as it suggests that there would be little interest in further reducing the volume of fractions in a comprehensive 2D-SFC system, as it would mostly impair the method sensitivity. At least, such is the case for a whole peak retrieved from dim1. Naturally, when partial

separation occurs in dim1 within the collection time, it could still be a better option to fractionate it in smaller collection loops.

Finally, the reduced internal diameter of some of the loops (0.508 mm vs. 0.762 mm) or their longer length (56.5 cm vs. 25 cm) did not cause any significant changes in system pressure as observed in the pump recordings (Figure III.2.S4). This suggests that the loop dimensions cannot cause any significant change in fluid density, thus no significant change in analyte elution either and further confirmed that the loop dimensions could not influence the results obtained in this study. It is however possible that different operating conditions, where the fluid would be more compressible (closer to the critical point or with lower proportions of co-solvent), would have shown more impact of loop dimensions.

In conclusion, loop dimensions had no significant impact on analyte dispersion or dim2 resolution under the specific conditions examined here. Also, loop dimensions showed no impact on system pressure. Furthermore, dividing one peak into two smaller-volume loops did not appear to improve efficiency in dim2 analysis and would therefore bring no benefit in terms of resolution nor sensitivity.

3.2.2. Quantity transferred

Transfer issues between the two dimensions can lead to significant biases in quantitative results. In particular, with the present system, it is possible that some portions of a collected peak would remain in the intermediate tubings between valves 2 and 4 and between 5 and 2 thus would not be transferred to the second dimension. To avoid this issue, valve switching must be accurately controlled to ensure that the target fraction from dim1 is entirely stored in the storage loop. In our previous works, we had examined this point and demonstrated that no loss or mixing of successive fractions occurred [5]. However, in our previous works, the transfer conditions were always in the same range of co-solvent proportion. In the present study, we wanted to assess the impact of a wide range of co-solvent on the amounts collected and transferred, to guarantee the reliability of quantitative analyses.

The compressibility of the CO₂-solvent mobile phase, which is present as a subcritical fluid in the system, is again a crucial element of this investigation. Depending on the proportion of co-solvent, the fluid is more or less compressible and sensitive to operating conditions [23]. While the loops have a fixed volume, different operating conditions resulting in different fluid density could influence the quantity of analyte transferred from one dimension to another.

In this study, caffeine was chosen as the model compound to assess the efficiency and reproducibility of fraction transfer. A step-gradient elution method was used in the first dimension

(Gradient 3.a), with increasing proportion of methanol co-solvent containing a fixed concentration of caffeine, resulting in increasing quantity of caffeine being stored in a fixed-volume transfer loop. This method was chosen over gradient elution in order to ensure that the composition of the mobile phase was known precisely throughout collection in the transfer loop. The caffeine collected and transferred to dim2 was then eluted with the same gradient program as previously (gradient 3.b, identical to gradient 1.b and 2.b).

More precisely, caffeine was dissolved in the mobile phase cosolvent of pure methanol (MeOH) and examined at two different concentration levels: 0.025 mg/mL (Figure III.2.S5, shown in supplementary information) and 0.05 mg/mL (Figure III.2.4). Co-solvent steps were applied at increasing percentages of methanol in the mobile phase, i.e., 20 %, 40 %, 60 %, 80 % and 100 %. For each step, a fraction was collected, with a constant loop volume of 114 μ L and a fixed loop loading time of 0.19 min. The fraction was then transferred to dim2 to observe the caffeine peak and measure peak area. The chromatograms obtained in the second-dimension analysis, showing the caffeine peaks in all conditions, are shown in Figure III.2.4A and Figure III.2.S5A. Moreover, the peak area was plotted against the theoretical amount of caffeine transferred, computed from caffeine concentration in the mobile phase and loop volume (Figure III.2.4B and Figure III.2.S5B).

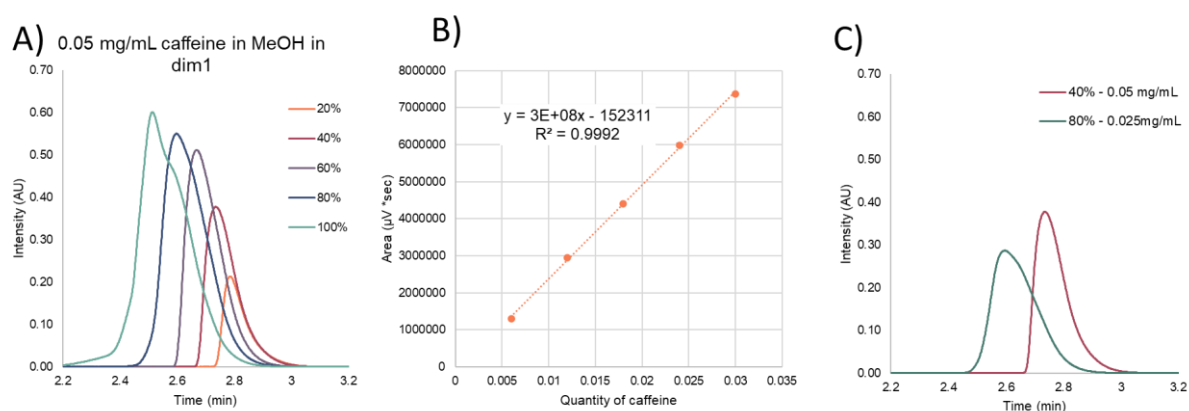


Figure III.2.4 - Observation in dim2 of the transfer of caffeine contained in the mobile phase from dim1. A) Peak of caffeine observed in dim2, with loop loading in isocratic conditions (step gradient) at 20, 40, 60, 80, 100 % MeOH containing 0.05 mg/mL caffeine. B) Relation between the area of caffeine peak obtained in dim2 and the actual amount transferred from dim1. C) Superposed peaks of identical quantities of caffeine introduced with different proportions of co-solvent: 40 % MeOH containing 0.05 mg/mL caffeine or 80 % MeOH containing 0.025 mg/mL caffeine. UV detection 272 nm.

Analysis conditions dim1: ACQUITY UPC² Torus DEA (100 $\times$ 3.0 mm, 1.7 μ m), co-solvent: MeOH containing 0.025 or 0.05 mg/mL caffeine, 110 bar, 30 $^{\circ}$ C, 0.6 mL/min (gradient conditions in Table III.2.1, gradient 3.a)

The results show that, for a constant caffeine concentration, increasing the percentage of co-solvent in the mobile phase, thereby increasing the concentration of caffeine in the collected fraction, has a direct and linear effect on the amount transferred to dim2. The linear correlation coefficient was excellent for both concentrations of caffeine (Figure III.2.4B and Figure III.2.S5B). This confirms the consistency of the quantities transferred between the two dimensions, independently of the composition of the mobile phase in dim1. Thus, under the present experimental conditions, the compressibility of the mobile phase had no measurable impact on the quantity collected and transferred in dim2. This is also confirmed by the comparison of points measured with the two different concentration levels of caffeine. For instance, introducing the 0.025 mg/mL solution at 40 % methanol should yield an identical quantity to the 0.05 mg/mL solution at 20 % methanol. Similarly, introducing the 0.025 mg/mL solution at 80 % methanol should yield an identical quantity to the 0.05 mg/mL solution at 40 % methanol. Indeed, the peak areas were very close between the compared experiments. However, the peak shapes were different, as can be observed in Figure III.2.4C and is further discussed below.

This lack of significant effect of fluid compressibility can be explained by the stability of the experimental parameters (pressure, temperature and flow rate) during the collection and transfer stages, as well as by the robust configuration of the transfer loop, designed to minimize volume fluctuations due to the compressibility of the fluid. These observations confirm that, although CO₂ compressibility can be a potential disturbance factor in some systems, it did not interfere with transfer efficiency under the specific conditions of this study.

Another observation from Figure III.2.4A and Figure III.2.S5A is that an increase in the percentage of co-solvent appeared to cause a broadening of the caffeine peak in dim2, possibly due to a greater quantity transferred, but also probably due to the composition of the fluid containing an increasing proportion of MeOH. This hypothesis is supported by the shoulder observed on the fronting of the peaks retrieved with the large proportions of MeOH, and by the comparison of peak shapes in Figure III.2.4C. In the latter, identical quantities of caffeine were compared, but the peak shape was much more distorted when the proportion of methanol co-solvent was larger. This highlighted the need to further study the injection effects in the second dimension.

3.3. Effect of collection conditions on second-dimension injection

3.3.1. Pressure

As mentioned above, pressure is an essential parameter when operating with supercritical, or in this case, subcritical fluids. Internal pressure should be maintained above the critical pressure to maintain a high density and thus preserve analyte solubility. While this is normally the case during

the chromatographic separation, where the fluid state is maintained with the back-pressure regulator, it is also essential to keep the pressure constant during fraction transfer, to prevent variations that may result in peak distortion or analyte loss.

In storage loops, the compressibility of the fluid varies depending on the composition of the fluid entering the loop [26]. This composition would differ for different fractions collected from dim1 when a gradient elution is employed. Note that the storage loops were always conditioned before the dim1 transfer with the starting conditions of dim2 gradient. However, different conditioning fluids were explored, and appeared to have no significant effect on the final result.

A naringin standard, analyzed in dim1 with gradient 1 mobile phase conditions, was used in a series of tests to evaluate the effect of pressure on analytical performance. Initially, the dim1 approach relied on a reversed pressure gradient, where back-pressure varies from 150 down to 110 bar. In this program, as naringin had a retention time of around 6 min (the elution composition was around 79 % of MeOH), the back-pressure at the moment of its collection was about 120 bar. In a first series of experiments, the back-pressure in dim1 was modified to assess its effect on collection. In the first test, the back-pressure was kept constant at 110 bar throughout the analysis, meaning that the collection of the naringin peak also took place at this pressure. The second test maintained the initial back-pressure gradient (150 to 110 bar), therefore corresponded to collection at 120 bar. For both configurations, the constant back-pressure applied in dim2 was also varied, and three values were tested: 110 bar, 150 bar (the standard pressure in this method) and 190 bar, allowing a range of experimental conditions to be examined. The results, summarized in Table III.2.2, indicated that the area and width at half-height of the naringin peak in dim1 remained constant, regardless of the pressure conditions applied (constant back-pressure of 110 bar or gradient from 150 to 110 bar). In dim2, the widths at half-height of the peaks did not vary significantly either, regardless of the pressure conditions when the loop was loaded in dim1 or the changes in parameters in dim2. These widths were measured at around 0.2 min for the first peak and 0.17 min for the second, confirming the excellent stability of the system under the experimental conditions evaluated. The corresponding chromatograms are shown in Figure III.2.S6.

Table III.2.2 - Pressure variation in dim1 and dim2. Effect on peak area and peak width at half-height of naringin standard. Analysis conditions dim1: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 1.7-0.6 mL/min (gradient conditions in Table III.2.1, gradient 1.a). Analysis conditions for dim2: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table III.2.1, gradient 1.b), 2.0 mL/min

Method	Pressure (bar)	Peak	Retention time (min)	Area (μV*sec)	Width @50%
dim1	110	1	6.063	4524873	0.041
dim2	150	1	11.288	1035833	0.207
		2	12.581	925503	0.173
dim1	150-110	1	5.979	4459290	0.042
dim2	150	1	11.260	1331295	0.214
		2	12.551	1229501	0.179
	110	1	11.783	1054112	0.277
		2	13.100	961896	0.171
	190	1	10.832	1044838	0.212
		2	12.113	943477	0.176

These observations highlight the robustness of the pressure conditions tested but also underline the importance of the composition of the mobile phase in the storage loops, which was the next parameter examined.

3.3.2. Mobile phase composition

The influence of elution conditions in the first and second dimensions on analytical performance in the second dimension was evaluated. For this purpose, we examined the effect of the proportion of co-solvent (MeOH) at the moment of peak collection, which is the same as the proportion of co-solvent stored in the loops, on the transfer of analytes to the dim2 column. The aim of this study was to determine the limits of compatibility of co-solvent percentages in order to ensure optimal transfer of analytes between the two dimensions. Since the proportion of methanol co-solvent may impact polar and non-polar compounds differently, two sets of experiments were conducted. The first set of experiments was done with non-polar compounds (alkylbenzenes), and therefore required a non-polar stationary phase (C18-type) to ensure adequate retention. The second set of experiments was done with polar compounds (flavonoids) and therefore required a polar stationary phase (diethylaminopropyl-bonded silica).

3.3.2.1. Non-polar compounds on non-polar column

A mixture of alkylbenzenes (with chain lengths of C4, C6, C8, C10, C12, C14) was injected in dim1 on an XSelect HSS C18 column (150 × 4.6 mm, 5.0 μm) in isocratic mode, with different

percentages of methanol in the mobile phase. The conditions tested were 2%, 10%, 30%, 50%, 70% and 90% MeOH.

Analysis of the alkylbenzene mixture under these mobile phase conditions showed little impact of methanol proportion on separation and retention times from 2 to 50% MeOH (Figure III.2.5).

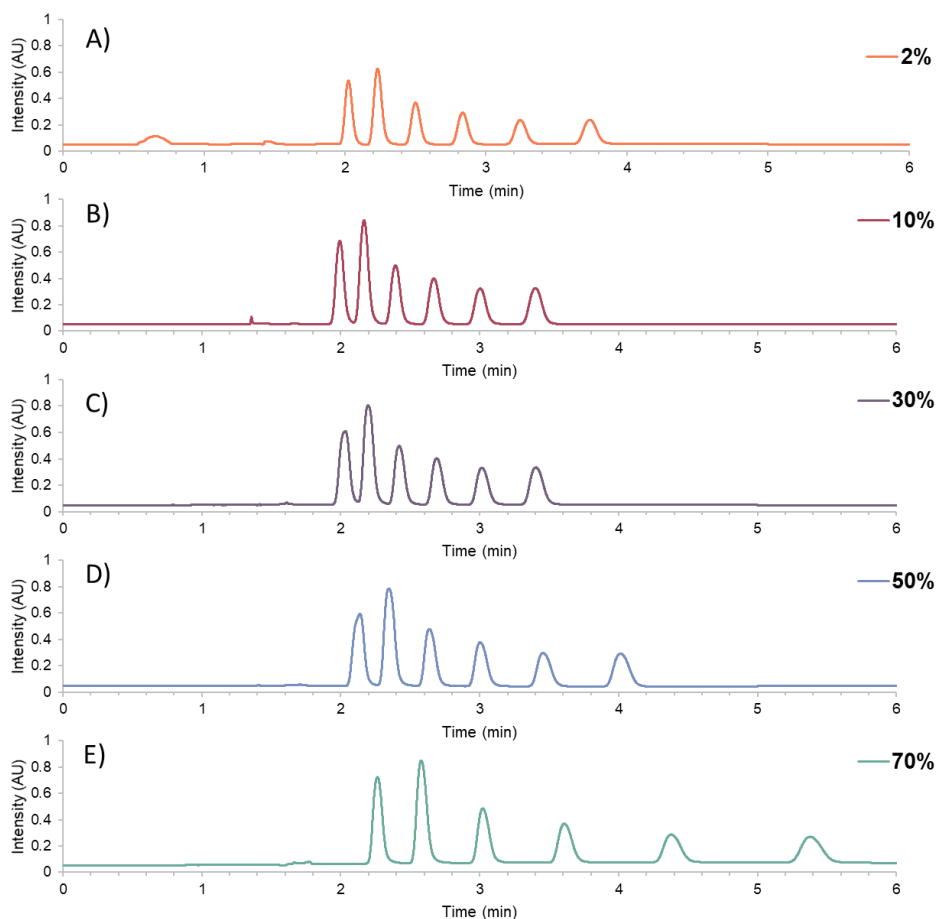


Figure III.2.5 - Analysis of the alkylbenzene mix on XSelect HSSC18 column (150 × 4.6 mm, 5.0 µm) with varying percentages of MeOH co-solvent in isocratic conditions. 110 bar, 30°C, 1 mL/min.

Only the largest portions of MeOH (70 and 90%) showed a significant retention increase. The U-shaped retention curves observed are quite typical of non-polar compounds analysis in SFC, where retention first decreases upon addition of a polar co-solvent, then increases again when solubility decreases with higher portions of polar co-solvent. This effect was previously described by Lesellier and co-workers [23,27]. In the present case, the minimal retention variation indicated that all alkylbenzenes had adequate solubility in all mobile phase compositions examined, which made this set ideal to explore the impact of mobile phase composition on peak collection and re-injection in isocratic mode.

For each condition employed in dim1 elution, each alkylbenzene peak was collected in a transfer loop then re-injected and analyzed in dim2 on a very similar stationary phase, HSS C18 with

identical dimensions from the dim1 column. The isocratic conditions tested in dim2 were 2%, 5%, 10%, 20%, 30%, 40%, 50% and 90% MeOH. In total, each alkylbenzene molecule was thus analyzed in 48 different conditions (6 conditions of dim1, times 8 conditions of dim2).

First, it is important to note that most of these paired conditions yielded dim2 analyses with adequate chromatographic quality. This is illustrated in Figure III.2.6 as a summary Table III.2. showing the areas of compatibility between dim1 and dim2.

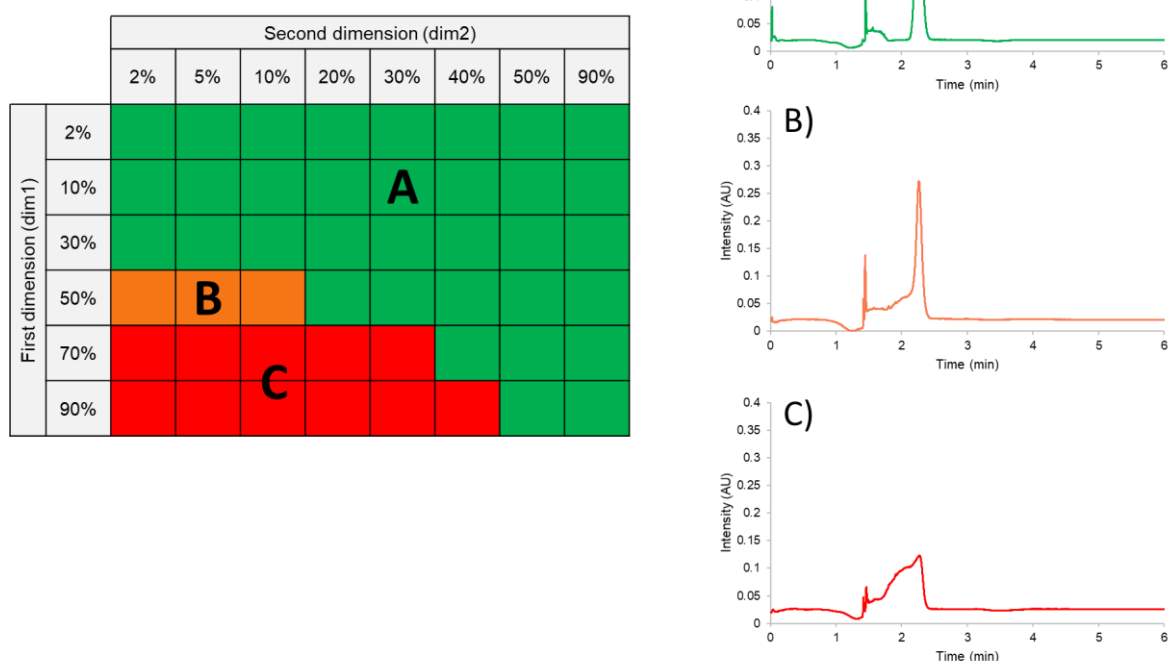


Figure III.2.6 - Mobile phase composition compatibilities between dim1 and dim2 and typical chromatograms obtained in the different situations. Green = the peak was well resolved and could be integrated (chromatogram A), orange = the peak was visible but could not be integrated (chromatogram B), red = no peak was observed (chromatogram C)

Three cases were distinguished and are illustrated with the chromatograms in Figure III.2.6:

- case A: all peaks were visible and could be integrated;
- case B: all peaks were visible, but not all could be properly integrated;
- case C: some peaks were strongly distorted and could therefore not be properly integrated.

As appears in the table, chromatographic quality was always adequate when the conditions of first and second dimension were close (diagonal area of the table). The quality was also always good when the proportion of methanol in the second dimension was larger than the proportion of methanol in the first dimension. However, issues occurred in the opposite case, namely when the proportion of co-solvent in the first dimension was very much larger than the proportion of co-

solvent in the second dimension. It is important to note that the dim2 conditions are actually the ones used to push to the dim2 column the fraction that was stored in the loop with the dim1 conditions. Therefore, solvent mismatch is particularly important when a large plug of methanol must be transferred and eluted from the dim2 column with a CO₂-rich mobile phase.

For instance, using 50 % MeOH in dim1, the compounds eluted in dim2 with MeOH percentage of 2 to 10 % yielded visible peaks, but that could not be integrated due to some baseline disturbance at the peak front (case B). In such a case, the analyte could be detected but could not be quantified. However, all dim2 compositions from 30 to 90 % yielded good chromatographic quality (case A). Similarly, for fractions collected with a high percentage of co-solvent (70 % or 90 %), it was necessary to use a sufficiently high percentage of co-solvent in dim2 (≥ 40 % when dim1 is eluted at 70 %, ≥ 50 % when dim1 is eluted at 90 %) to guarantee the elution of the analyte with a good peak shape, thus making it detectable and quantifiable.

Further illustration of these effects is provided in Figure III.2.7, with several chromatograms of butylbenzene analyzed in dim2 after being collected in a storage loop under isocratic conditions with 30%, 70% or 90% MeOH. When large proportions of MeOH were used in the first dimension, *i.e.*, when large quantities of MeOH were transferred to the dim2 column, the baseline was strongly distorted at the beginning of the chromatogram. Viscous fingering and methanol adsorption on the stationary phase must be largely responsible for this [28–32]. In the worst cases, when the difference in mobile phase composition was the largest, the analyte could not be seen as it was included in the distorted band. On the other hand, when the composition of mobile phase between the two dimensions got closer, visible and measurable peaks were observed.

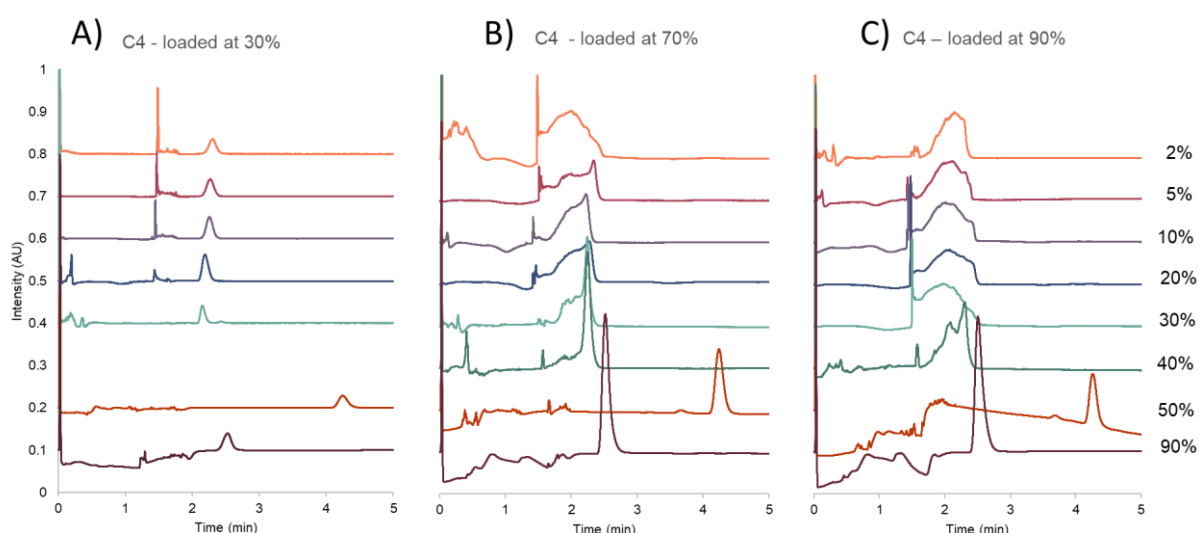


Figure III.2.7 - Analysis of butylbenzene, loaded from dim1 with A) 30% MeOH, B) 70% MeOH C) 90% MeOH, and eluted in dim 2 with different co-solvent percentages in isocratic conditions (2, 5, 10, 20, 30, 40, 50, 90%). Other analysis conditions in dim2: HSSC18 column (150 × 4.6 mm, 5.0 μm), 110 bar, 30 °C, 1.0 mL/min

As is usually the case in LC and SFC, these effects of injection solvents were of primary importance for the low-retention analytes, but became insignificant when analyte retention increased. This is illustrated in Figure III.2.8, which represents chromatograms of the 6 alkylbenzenes that were eluted from dim1 and collected in the loops with 90% MeOH, but transferred and eluted from the dim2 column with 2% MeOH. It appears from these chromatograms that the short-chain alkylbenzenes yielded strongly distorted baselines where no peak could be discerned, while the long-chain alkylbenzenes yielded perfectly acceptable peaks. With these loop dimensions and this set of operating conditions, for a peak to be visible, the retention factor (k) in dim2 had to be superior to 1, and for integration to be dependable, it had to be more than 1.33. Therefore, it is preferable for the analyte to be properly held on the column to reduce the solvent effect in order to provide accurate analysis with large percentages of co-solvent in dim1. Finally, and opposite to previous observations [32], possible breakthrough elution of the analytes, where the analyte elutes near dead volume with the solvent front, was not obvious in these experiments.

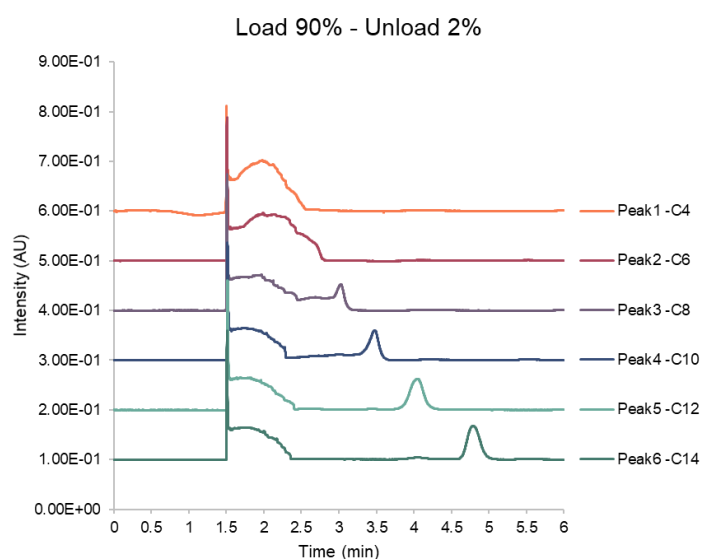


Figure III.2.8 - Analysis of individual alkylbenzenes (from C4 to C14), loaded from dim1 with 90% MeOH in the storage loop then eluted in dim2 with different co-solvent percentages (2, 5, 10, 20, 30, 40, 50, 90%). Analysis conditions in dim2: HSSC18 column (150 × 4.6 mm, 5.0 μm) with 2% MeOH co-solvent, 110 bar, 30 °C, 1.0 mL/min

A peculiar behavior was observed for butylbenzene (C4), and for this compound only. In Figure III.2.7A, when the analyte was loaded in the storage loop with 30 % MeOH, the peak was detectable and the peak area was measurable whatever the composition of the mobile phase in dim2. However, when it was eluted in dim2 with 50 % MeOH, an unexpected effect was observed: butylbenzene was much more retained than under any other condition. In Figure III.2.7B

and III.2.7C, the same retention shift was observed. This strange phenomenon was confirmed by several repetitions of the analyses. No plausible explanation could be found for this peculiar behavior. Note that all other alkylbenzenes followed the trend observed in Figure III.2.5, with no anomalous behavior.

To conclude on this set of experiments, a large part of the mobile phase combinations yielded perfectly acceptable chromatographic quality. Only the largest discrepancies between the mobile phase compositions in dim1 and dim2, where the proportion of co-solvent in dim1 is much larger than the proportion of co-solvent in dim2, caused severe baseline distortion and affected the peaks of the least retained analytes. It is important to note that such large differences in mobile phase composition are unlikely to be useful in normal SFC practice. Large portions of co-solvent (above 50 %) are only employed to achieve the elution of most polar compounds, when solubility in CO₂-rich mobile phases is not sufficient. In such cases, it would make no sense to elute them with a low proportion of co-solvent in the second dimension. Another important conclusion to draw from these experiments is that problems should be easily avoided when sufficient retention is obtained in dim2, which can usually be solved by an adequate choice of stationary phase.

3.3.2.2. Polar compounds on polar column

While the above results were highly informative, they may be limited in their applicability since the chosen test set was only composed of non-polar analytes, and the non-polar column employed may not be representative of the majority of current SFC applications [33]. Therefore, we considered repeating these experiments with polar compounds on a polar stationary phase.

Definition of the test set

The ideal behavior observed above, with low retention variation over a wide range of mobile phase compositions (Figure III.2.5) was unlikely to be observed again with polar compounds. Indeed, polar compounds are much more sensitive to mobile phase composition because the methanol co-solvent is interacting with them through hydrogen bonding. This is usually increasing analyte solubility all along, thereby decreasing its retention continuously when the proportion of methanol increases. As a result, it would be impossible to cover the entire range of co-solvents tested (5 to 90%) with a single series of compounds: the low-polarity compounds would soon be eluted in the dead volume when the proportion of co-solvent increases, while the high-polarity compounds would never elute with low proportions of co-solvent. To overcome this difficulty, three sets of test compounds were defined: low, average and high polarity, yielding high, average and low solubility in CO₂, therefore resulting in low, average and high retention on a polar stationary phase. The sets

were divided according to the co-solvent combinations between dim1 and dim2, as shown in Figure III.2.9.

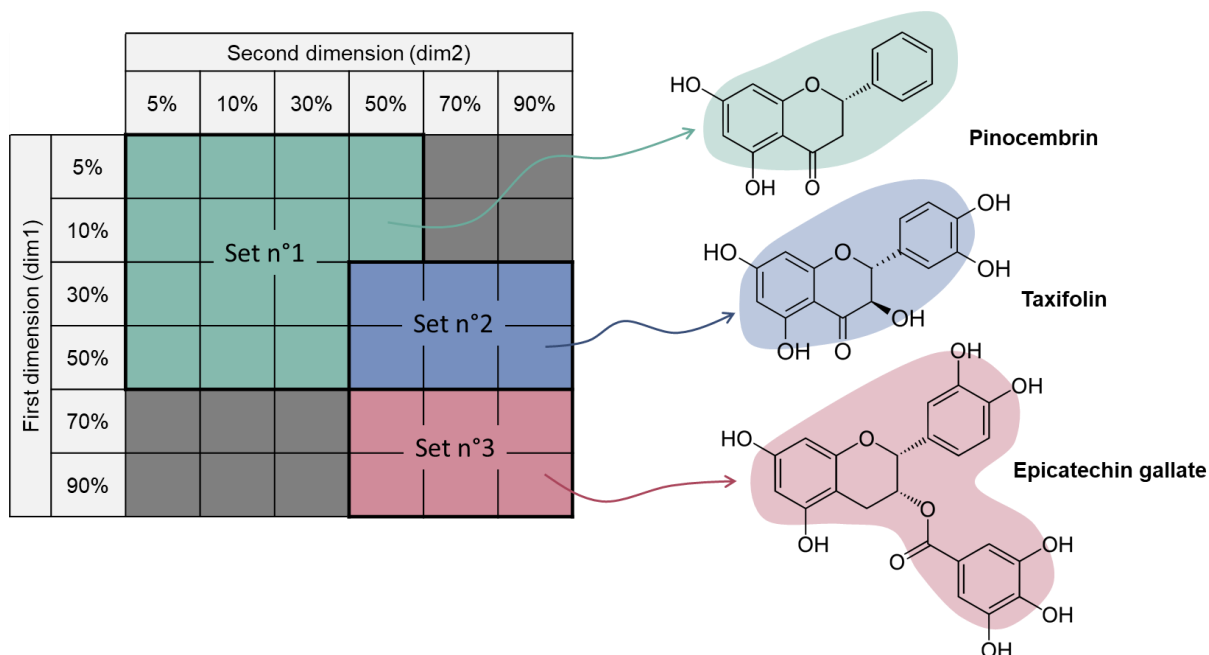


Figure III.2.9 - Distribution of the different flavonoid sets analyzed in different conditions between dim1 and dim2.

Set no. 1 = pinocembrin, hesperitin, pinocembroside

Set no. 2 = naringenin-7-O-glucoside, taxifolin, naringenin, alphononin

Set no. 3 = alphononin, epigallocatechin, epicatechin gallate

Because flavonoids comprise a large variety of molecules with a range of polarities and solubilities in CO₂-methanol mixtures, they were selected for this second set of experiments. The diethylaminopropyl-bonded silica stationary phase employed in our previous 2D analysis of flavonoids in the first dimension (Acquity Torus DEA) was used here in the two chromatographic dimensions (dim1 and dim2).

Set no. 1: low retention

The first set consists of pinocembrin, hesperitin and pinocembroside (Figure III.2.S7). These compounds have low polarity, and are therefore easily eluted in CO₂-rich mobile phase compositions. For example, pinocembrin (Figure III.2.9) is a simple flavanone with only 2 hydroxyl groups where one is involved in intra-molecular hydrogen bonding, and therefore has good solubility in CO₂ and rather low affinity with the polar stationary phase. At high co-solvent percentages, these three compounds are therefore eluted in the dead time, without significant interaction with the stationary phase. These compounds need to be analyzed at low co-solvent percentages (5-30%) to allow for sufficient retention in a reasonable analysis time (no more than 30 min).

Set no. 2: moderate retention

The second set consists of naringenin-7-O-glucoside, taxifolin, naringenin and alphononin. Some of these compounds contain several hydroxyl groups, like taxifolin, or sugar moieties, like naringenin-7-O-glucoside, which increase their polarity. This would both reduce their solubility in CO₂ mobile phases and favor retention on the polar stationary phase. These compounds require a minimum proportion of 30% co-solvent to elute with a reasonable analysis time. In dim1, where they are injected as a mixture, a large proportion of co-solvent (above 50%) was inadequate as they all eluted in the same retention window and could therefore not be transferred individually to the second dimension. However, in the second injected one by one, it was possible to use high percentages of co-solvent (up to 90%).

Set no. 3: high retention

This third set comprises alphononin, epigallocatechin and epicatechin gallate. These compounds are highly hydroxylated, notably due to the trihydroxylated gallic ester function present as on epicatechin gallate. These characteristics greatly decrease their CO₂ solubility and increase their affinity for the polar stationary phase, resulting in very high retention. If they were analyzed at low percentages of co-solvent, elution may require several hours, or would never happen if they would not be properly dissolved. They were therefore analyzed only at high co-solvent percentages (70 to 90%) to limit analysis times.

Inaccessible combinations

Two combination zones (in gray, Figure III.2.9) were not accessible as we could not identify compounds that would be both sufficiently soluble in low methanol proportion and sufficiently retained at high methanol proportion. At low dim1 and high dim2 percentages, set n°1 compounds would elute in dead time in dim2, and thus yield no measurable peak. Conversely, at high dim1 and low dim2 percentages, the compounds in set n°3 would have excessive retention in dim2 (or even remain on the column), making analysis unfeasible within a reasonable time. To see the impact of different combinations of dim1 and dim2, it is essential that retention remains in a balanced zone, neither too low (co-elution) nor too high (too long analysis time and detection issues). Perhaps these missing combinations are not an issue from a practical point of view, since it is unlikely that any polar compound would require such different mobile phase compositions in a 2D SFC system.

Definition of desirability functions and application to different conditions

With the non-polar compounds examined previously, the behaviors were rather consistent between different analytes, and it was thus easy to draw a general conclusion as was presented in Figure III.2.6. With the polar compounds however, different behaviors could be observed between the different analytes eluted in the same mobile phase conditions. Therefore, a new strategy needed to be developed to draw generally applicable conclusions. Derringer's desirability functions seem relevant in this case [34]. These functions make it possible to compare different co-solvent conditions on the basis of selected criteria. The first step was to choose different criteria that would be relevant to our study. Then the 3 selected criteria had to be translated into desirability functions (d_1 , d_2 , d_3). These functions are described in Figure III.2.10.

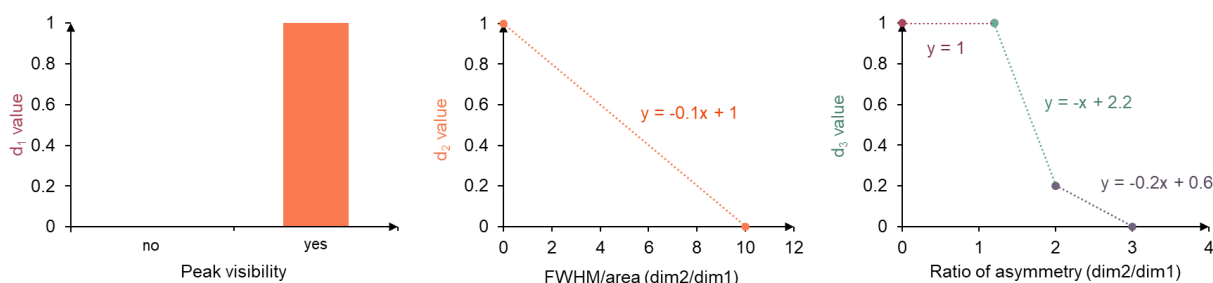


Figure III.2.10 - Derringer functions based on: visibility of a peak shape (d_1), ratio of peak width at half height and area in dim2 relative to dim1 (d_2) and ratio of asymmetry at 10% peak height in dim2 relative to dim1 (d_3)

Visibility of peaks (d_1)

The first criterion is the visibility of the peak represented by the d_1 function (Figure III.2.10). This criterion evaluates the presence of an identifiable peak in the chromatogram, regardless of its width or symmetry. For example, some compounds that were highly impacted by the solvent effect were hardly discernible from the baseline, making identification impossible. This criterion is the most important because it determines the ability to detect and possibly quantify the compound of interest. Thus, the presence of a peak was assigned a value of 1 and the absence of a peak a value of 0. All the values for each analyte in each pair or elution conditions are presented in Table III.2.S2.

Ratio of peak width at half height and area in dim2 to dim1 (d_2)

The evaluation of the quality of the peaks obtained in dim2 cannot be based exclusively on the width at half height of the peak. Indeed, this measurement loses its significance in the case of partial transfer between dim1 and dim2. In the case of large peaks, at the time of transfer, only a

fraction of the peak is collected, and therefore comparing peak width between the two dimensions makes no sense.

This can be explained by the way the instrument operates: the compounds were stored between the two dimensions in 114 μL loops. For example, when hesperetin was analyzed at 5% co-solvent and a flow rate of 1.5 mL/min (Figure III.2.10), the peak width at baseline was about 2 min, whereas the volume sampled corresponded to only 0.08 min, *i.e.*, a very small portion of the initial chromatographic signal. Thus, the widths measured in dim2 represented only a small part of the collected peak, making any direct comparison of peak width irrelevant.

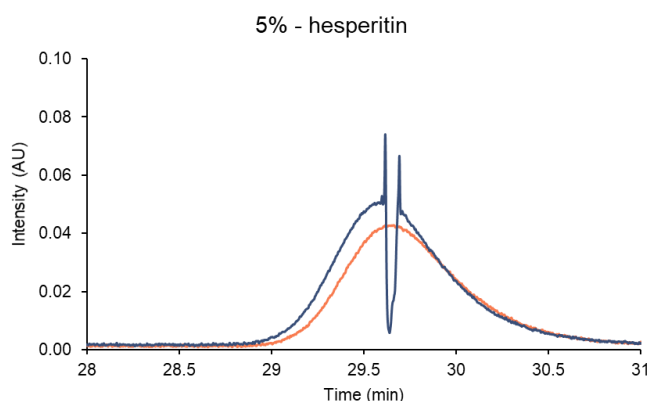


Figure III.2.11- Chromatogram of dim1 analysis (orange) of the hesperetin peak eluted with 5 % co-solvent (isocratic conditions). The superposed chromatogram (blue) shows the same dim1 analysis recorded when the peak is cut to be loaded in a storage loop.

Analysis conditions: ACQUITY UPC² Torus DEA (100 $\times$ 3.0 mm, 1.7 μm), co-solvent: 5 % MeOH comprising 0.1 % MSA, 150 bar, 30 $^{\circ}\text{C}$, 1 mL/min. UV detection 280 nm.

To overcome this bias, the width at half-height was normalized by the area of the peak, in order to calculate a width-to-area ratio. This approach makes it possible to compare peaks corresponding to similar relative quantities, while incorporating a correction linked to the signal intensity.

The ratios obtained for one analyte were then compared between dim2 and dim1. This ratio was integrated into the Derringer desirability function according to the following principles: a low ratio between dim2 and dim1 indicated good focusing of the compound in dim2, and a desirability value close to 1 was then assigned. Conversely, a high ratio indicated broadening or poor focusing, leading to a desirability value close to 0. All measured values are presented in Table III.2.S2.

Ratio of asymmetry at 10% peak height in dim2 to dim1 (d3)

Chromatographic peak asymmetry is another important indicator to consider, in order to evaluate the quality of the peak shape. To assess the impact of the mobile phase compositions on dim2

peak shapes, a ratio of asymmetry values was calculated by comparing the asymmetry at 10 % peak height in dim2 to that measured in dim1.

However, in some cases, the peaks were too distorted (highly flattened or irregular) for the asymmetry to be calculated reliably. In such cases, a zero value was assigned to the d3 criterion. The desirability value d3 was defined according to the ratio of asymmetry values (dim2/dim1). When asymmetry was unchanged or slightly improved, the ratio was less than or equal to 1 and a desirability value of 1 was assigned. When the ratio increased, indicating a deterioration in symmetry in dim2, the desirability value decreased, tending towards 0 and being less restrictive between 1 and 2 (Figure III.2.10).

This criterion thus makes it possible to integrate the quality of the peak profile into the overall assessment of the system's performance. All values are presented in Table III.2.S2. Figure III.2.11.

Total desirability

In the end, a total desirability value (D) was calculated as the geometric mean of the three functions:

$$D = \sqrt[3]{d_1 \times d_2 \times d_3} \quad (1)$$

It is possible to add weighting coefficients to add importance to certain criteria. In this case, the choice was made not to add any and instead to assign the same contribution to each criterion. It would also have been possible to calculate an arithmetic mean, but this was considered not restrictive enough. In fact, with the geometric mean, the absence of a peak (d1) is prohibitive, so when d1 is 0, D is also 0.

The desirability value (D) was calculated for each analyte eluted in each dim1/dim2 combination (Table III.2.3). Areas of high desirability (in green) correspond to optimal combinations between the two dimensions. Similar to the observations on non-polar compounds, the diagonal line where the compositions of dim1 and dim2 eluents are close, yields the best results. However, the best results are mostly located around intermediate percentages of co-solvent in the first dimension (10 to 50%). Conversely, some areas show low or zero desirability (orange or red) and are mostly encountered in cases of strong solvent mismatch, e.g., 50% in dim1 with 5 or 10% in dim2, or 90% in dim1 with 50% in dim2. These conditions should be avoided as they yielded invisible, poorly defined or highly asymmetric peaks.

In addition, again solvent effects were more critical to the least retained compounds. For instance, the combination of 5% in dim1 and 5% in dim2 showed a lower desirability for the first eluted analyte than for the second, due to the high asymmetry of the first. Similarly, the configuration of 70% in dim1 and 50% in dim2 showed a zero-value for total desirability for the first two peaks, but

high desirability for the last. So again, and consistent with the literature, most retained compounds are less affected by the injection solvent composition.

Table III.2.3 – Table of total desirability values for each % of co-solvent in dim1 and dim2, and for each flavonoid standard examined and on Acquity Torus DEA columns in both dimensions

		Second dimension (dim2)																				
		5%			10%			30%			50%				70%				90%			
		1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3	4	1	2	3	4
First dimension (dim1)	5%	0.40	0.68		0.95	0.40		0.97	0.55		0.52	0.00										
	10%	0.95	0.95		0.93	0.81	0.00	0.90	0.84	0.68	0.97	0.97	0.90									
	30%	0.28	0.89		0.71	0.70	0.00	0.69	0.72	0.47	0.96	0.96	0.94	0.82	0.94	0.95	0.92	0.76	0.97	1.00	0.97	0.85
	50%	0.00	0.00		0.00	0.00	0.00	0.91	0.85	0.77	0.90	0.91	0.90	0.90	0.85	0.93	0.90	0.89	0.96	1.00	0.98	0.93
	70%										0.00	0.00	0.73		0.42	0.00	0.39		0.96	0.71	0.98	
	90%										0.00	0.00	0.00		0.63	0.00	0.00		0.39	0.56	0.51	

1

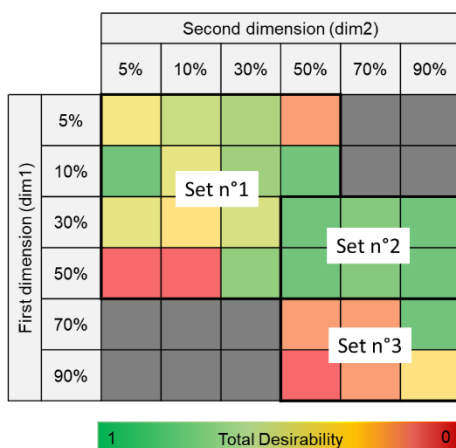
Total Desirability

0

Empty cells (light grey) correspond to non-existing cases. For instance, in the 70/50 area, the set no. 3 had only 3 standards, so the 4th column is empty. The analysis of pinocembroside with 5% co-solvent (peak 3 in set no. 1) was not evaluated because its retention was too high (over 35 min) to be relevant in this study.

Overall, it appears that the three series of flavonoids allowed a wide range of dim1/dim2 combinations to be explored. To summarize the observations from Table III.2.3, Table III.2.4 was obtained by averaging the total peak desirabilities for each condition. It shows a clear trend: when dim1 used a higher percentage of co-solvent than dim2, peak quality was significantly degraded. This was the case, for example, for the combination of 50% in dim1 and 5% in dim2. On the contrary, a reverse transfer, with a lower percentage of co-solvent in dim1 and a higher percentage in dim2 (e.g., 30% in dim1 and 70% in dim2), gave good results. This is again similar to the observations from the non-polar test compounds.

Finally, although some red zones remain, indicating incompatible conditions for transfer between dimensions, it is important to remember that these configurations are rarely used in SFC. In addition, the incompatibility mostly applies to the least retained compounds.

Table III.2.4 - Table of arithmetic average of total desirability for each co-solvent % combination


4. Conclusion

This study enabled a detailed assessment of the impact of different parameters of the two-dimensional system on its analytical performance. The addition of extra valves and tubing was examined, to ensure that the additional dead volumes did not significantly increase retention times or alter chromatographic quality in both dimensions. The results show that the gradient delay varies somewhat between the one- and two-dimensional systems, possibly due to changes in the internal pressure, but that the chromatographic quality was well preserved. Actually, the evaluation of peak widths revealed that, although dim1 was not affected by the switch to a two-dimensional system, dim2 showed a slight increase in widths at half-height. This phenomenon, although measurable, remains within acceptable limits and does not alter the overall resolution of the system.

Regarding the size of transfer loops, it was observed that there would be little interest in reducing the loop volume, as no resolution gain was observed when dividing a peak in two sections.

A thorough examination of all possible pairs of dim1/dim2 elution conditions on the performance of dim2 showed that most combinations of CO₂–MeOH compositions offered satisfying chromatographic quality, but that strong solvent mismatch could disrupt the detection and integration of the analytes that were least retained on the column. This effect was particularly marked when peaks were collected and stored in the loop with a high percentage of MeOH, followed by analysis in dim2 at a much lower percentage. However, this strong solvent mismatch should have little interest in practical operation of a 2D-SFC system where (i) the proportion of co-solvent is rarely raised above 50 % in usual practice and (ii) solubility issues would make it difficult to elute an analyte in both dimensions with strongly different mobile phase compositions. The case of alkylbenzenes being nicely eluted with a wide range of methanol (5 to 90 %) is quite exceptional.

Furthermore, adjusting operating conditions in the second dimension to obtain sufficient retention should largely mitigate this effect in displacing the analyte elution band out of the injection solvent disturbances.

In conclusion, the two-dimensional system set up is reliable and optimized for a wide range of analytical conditions. A good understanding of the interactions between the two dimensions means that undesirable effects can be anticipated and corrected, ensuring reliable separation of the analytes studied.

Although not explored in this paper, combining different polarities of stationary phases in the first and second dimensions should yield comparable conclusions, since the results obtained with non-polar and polar compounds basically yielded the same observations. Preliminary assumptions suggest that similar performance could be achieved on different stationary phases, provided that the retention behavior is well understood and the solvent effects are properly managed.

Finally, these results pave the way for the optimization of two-dimensional chromatographic systems in SFC, guaranteeing stable and efficient performance.

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Supplementary information

Table III.2.S1 - Data measured in dim1 and dim2 from the analysis of a naringin standard performed on a one-dimensional system (sys1D) and a two-dimensional system (sys2D).

Analysis conditions for dim1: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm), co-solvent: MeOH + 0.1% MSA, 150-110 bar, 1.7-0.6 mL/min (gradient conditions in Table III.2.1, gradient 1.a)

Analysis conditions for dim2: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 µm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table III.2.1, gradient 1.b), 150 bar, 2.0 mL/min.

Method	system	Peak	Retention time (min)	Area (µV*sec)	Width @50%	Resolution (min)
dim1	sys1D	1	6.032	4491427	0.0410	-
	sys2D	1	6.017	4772346	0.0461	-
dim2	sys1D	1	11.188	1080833	0.165	4.67
		2	12.418	967313	0.146	
	sys2D	1	11.484	1178090	0.209	4.46
		2	12.928	1054079	0.173	

Table III.2.S2 - Synthesis of the different criteria (d1, d2, d3) of the Derringer functions for each flavonoid in dim1 and dim2 condition. Data are calculated per peak in each condition. d1 = peak visibility, d2 = ratio of peak width at half height and area in dim2 relative to dim1, d3 = ratio of asymmetry at 10% peak height in dim2 relative to dim1.

		Second dimension (dim2)																					
		5%			10%			30%			50%				70%				90%				
		1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3	4	1	2	3	4	
d1	5%	1.00	1.00		1.00	1.00		1.00	1.00		1.00	1.00											
	10%	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00										
	30%	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
	50%	0.00	0.00		0.00	0.00	0.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
	70%											1.00	1.00	1.00		1.00	1.00	1.00		1.00	1.00	1.00	
	90%											0.00	0.00	0.00		1.00	1.00	0.00		1.00	1.00	1.00	
d2	5%	0.84	0.31		0.87	0.08		0.91	0.17		0.58	0.00											
	10%	0.87	0.86		0.80	0.56	0.00	0.87	0.63	0.31	0.92	0.91	0.72										
	30%	0.05	0.71		0.35	0.34	0.00	0.75	0.75	0.72	0.88	0.88	0.82	0.56	0.82	0.85	0.77	0.45	0.91	0.99	0.92	0.61	
	50%	0.00	0.00					1.00	1.00	1.00	0.73	0.76	0.73	0.73	0.67	0.79	0.73	0.72	0.88	0.99	0.93	0.80	
	70%										0.00	0.00	0.38		0.68	0.79	0.74		0.87	0.51	0.96		
	90%										0.00	0.00	0.00		0.42	0.00	0.00		0.63	0.99	0.91		
d3	5%	0.08	1.00		1.00	0.76		1.00	1.00		0.25	1.00											
	10%	1.00	1.00		1.00	0.96	1.00	0.84	0.96	1.00	1.00	1.00	1.00										
	30%	0.40	1.00		1.00	1.00	1.00	0.44	0.51	0.14	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
	50%	0.00	0.00		0.00	0.00	0.00	0.75	0.62	0.45	1.00	1.00	1.00	1.00	0.92	1.00	1.00	1.00	1.00	1.00	1.00		
	70%										0.17	0.55	1.00		0.11	0.00	0.08		1.00	0.72	1.00		
	90%										0.00	0.00	0.00		0.60	0.22	0.00		0.10	0.17	0.15		

Table III.2.S3 - Complete data from the analyses of set n°1 under dim1 and dim2 conditions: retention time, peak area, peak width at half-height (w50%), peak width at half-height/area ratio (w50%/area). peak 1 = pinocembrin, peak 2 = hesperitin, peak 3 = pinocembroside.

	dim1		pic1	pic2	pic3	dim2		pic1	pic2	pic3
Set n°1	5%	tR	4,00	28,58	pic3	5%	tR	3,80	23,81	
		area	2760728,00	2505869,00			area	640972,00	110453,00	
		w50%	0,25	1,20			w50%	0,09	0,44	
		asym 10%	0,85	1,22			asym 10%	3,07	1,77	
		w50%/area	0,00	0,00			w50%/area	0,00	0,00	
		tR	4,15	29,66		10%	tR	1,67	6,24	
		area	1117047,00	997584,00			area	645243,00	99437,00	
		w50%	0,11	0,66			w50%	0,03	0,10	
		asym 10%	1,20	1,49			asym 10%	1,52	1,36	
		w50%/area	0,00	0,00			w50%/area	0,00	0,00	
		tR	4,07	29,15		30%	tR	1,13	1,65	
		area	1123859,00	1004745,00			area	1017293,00	102663,00	
		w50%	0,11	0,67			w50%	0,02	0,02	
		asym 10%	1,16	1,49			asym 10%	1,52	1,46	
		w50%/area	0,00	0,00			w50%/area	0,00	0,00	
		tR	4,07	29,15		50%	tR	0,97	0,92	
		area	1123859,00	1004745,00			area	181766,00	24314,00	
		w50%	0,11	0,67			w50%	0,02	0,02	
		asym 10%	1,16	1,49			asym 10%	1,36	1,01	
		w50%/area	0,00	0,00			w50%/area	0,00	0,00	
	10%	tR	1,53	6,91	17,92	5%	tR	3,77	23,99	
		area	2913601,00	2635776,00	2606872,00		area	1749131,00	668936,00	
		w50%	0,10	0,26	0,53		w50%	0,21	0,55	
		asym 10%	2,14	0,71	0,75		asym 10%	1,08	1,38	
		w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	
		tR	1,53	6,91	17,92	10%	tR	1,63	6,09	15,52
		area	2913601,00	2635776,00	2606872,00		area	774721,00	233040,00	86462,00
		w50%	0,10	0,26	0,53		w50%	0,06	0,11	0,30
		asym 10%	2,14	0,71	0,75		asym 10%	1,42	1,17	0,61
		w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00
tR		1,60	7,01	18,17	30%	tR	1,12	1,65	2,28	
area		1081386,00	965078,00	960267,00		area	851464,00	257777,00	145951,00	
w50%		0,05	0,12	0,36		w50%	0,02	0,03	0,03	
asym 10%		1,22	1,17	0,78		asym 10%	1,94	1,67	1,26	
w50%/area		0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	
tR		1,60	7,01	18,17	50%	tR	0,96	1,08	1,18	
area		1081386,00	965078,00	960267,00		area	1686765,00	632807,00	248106,00	
w50%		0,05	0,12	0,36		w50%	0,02	0,02	0,02	
asym 10%		1,22	1,17	0,78		asym 10%	1,58	1,56	1,41	
w50%/area		0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	
30%	tR	0,89	1,53	2,23	5%	tR	3,17	24,32		
	area	1618543,00	1513334,00	1551876,00		area	959051,00	902875,00		
	w50%	0,03	0,04	0,05		w50%	0,85	1,54		
	asym 10%	1,42	1,34	1,10		asym 10%	2,11	0,31		
	w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00		
	tR	0,89	1,53	2,23	10%	tR	1,51	5,96	15,30	
	area	1618543,00	1513334,00	1551876,00		area	915552,00	445454,00	125200,00	
	w50%	0,03	0,04	0,05		w50%	0,22	0,31	0,45	
	asym 10%	1,42	1,34	1,10		asym 10%	1,18	0,52	0,65	
	w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	
	tR	0,88	1,51	2,19	30%	tR	1,07	1,58	2,22	
	area	1570452,00	1459786,00	1490986,00		area	1473408,00	1327649,00	1007673,00	
	w50%	0,03	0,04	0,05		w50%	0,08	0,09	0,10	
	asym 10%	1,42	1,35	1,13		asym 10%	2,51	2,28	2,53	
	w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	
50%	tR	0,68	0,85	0,96	5%	tR	No peak			
	area	1650917,00	1302196,00	1344524,00		area				
	w50%	0,04	0,03	0,04		w50%				
	asym 10%	0,62	1,49	1,49		asym 10%				
	w50%/area	0,00	0,00	0,00		w50%/area				
	tR	0,68	0,85	0,96	10%	tR	1,49	5,95		
	area	1650917,00	1302196,00	1344524,00		area	810872,00	536986,00		
	w50%	0,04	0,03	0,04		w50%				
	asym 10%	0,62	1,49	1,49		asym 10%				
	w50%/area	0,00	0,00	0,00		w50%/area				
	tR	0,69	0,86	0,97	30%	tR	1,01	1,45	2,03	
	area	1825579,00	1536434,00	1590126,00		area	3228821,00	3765773,00	3698277,00	
	w50%	0,04	0,03	0,04		w50%	0,18	0,30	0,40	
	asym 10%	0,86	1,52	1,59		asym 10%	2,06	2,13	1,95	
	w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	

Table III.2.S4 - Complete data from analyses of set n°2 under dim1 and dim2 conditions: retention time, peak area, peak width at half-height (w50%), peak width at half-height/area ratio (w50%/area). peak 1 = naringenin-7-O-glucoside, peak 2 = taxifolin, peak 3 = naringenin, peak 4 = alphonitin.

	dim1		pic1	pic2	pic3	pic4	dim2		pic1	pic2	pic3	pic4
Set n°2	30%	tR	1,67	5,27	6,91	16,35	50%	tR	1,09	1,45	1,85	2,89
		area	1962938,00	1096118,00	1264545,00	1600717,00		area	1705395,00	770202,00	664185,00	346852,00
		w50%	0,05	0,10	0,12	0,29		w50%	0,03	0,03	0,04	0,05
		asym 10%	1,38	0,86	1,02	1,03		asym 10%	1,61	1,41	1,39	1,34
		w50%/area	0,00	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	0,00
		tR	1,67	5,27	6,91	16,34	70%	tR	1,51	1,64	1,87	2,21
		area	1961726,00	1086993,00	1264096,00	1636887,00		area	2821080,00	1334018,00	1286208,00	678036,00
		w50%	0,04	0,10	0,12	0,29		w50%	0,04	0,03	0,04	0,04
		asym 10%	1,31	0,85	1,04	1,02		asym 10%	1,30	1,39	1,34	1,26
		w50%/area	0,00	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	0,00
		tR	1,67	5,27	6,90	16,33	90%	tR	1,42	1,42	1,52	1,61
		area	1948432,00	1032679,00	1234330,00	1518817,00		area	2813697,00	1234412,00	1177637,00	639109,00
		w50%	0,05	0,10	0,12	0,34		w50%	0,03	0,03	0,03	0,03
		asym 10%	1,41	0,91	1,03	0,82		asym 10%	1,41	1,56	1,45	1,40
		w50%/area	0,00	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	0,00
	50%	tR	0,86	1,32	1,76	2,93	50%	tR	1,03	1,41	1,78	2,82
		area	2004977,00	1168716,00	1385011,00	1888954,00		area	1727685,00	1042625,00	1073405,00	1118849,00
		w50%	0,03	0,04	0,04	0,06		w50%	0,07	0,08	0,09	0,10
		asym 10%	1,47	1,34	1,26	1,16		asym 10%	3,27	2,90	2,75	2,30
		w50%/area	0,00	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	0,00
		tR	0,86	1,33	1,77	2,95	70%	tR	1,47	1,60	1,84	2,20
		area	2029178,00	1173391,00	1397001,00	1897748,00		area	2981837,00	1594578,00	1669474,00	1821120,00
		w50%	0,03	0,04	0,04	0,06		w50%	0,07	0,05	0,05	0,05
		asym 10%	1,51	1,35	1,28	1,19		asym 10%	1,67	1,41	1,46	1,37
		w50%/area	0,00	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	0,00
		tR	0,87	1,35	1,80	2,99	90%	tR	1,41	1,41	1,52	1,62
		area	2035816,00	1184709,00	1401511,00	1922355,00		area	2859533,00	1574145,00	1483391,00	1347361,00
		w50%	0,03	0,04	0,04	0,06		w50%	0,05	0,04	0,04	0,04
		asym 10%	1,50	1,36	1,29	1,18		asym 10%	1,43	1,49	1,45	1,46
		w50%/area	0,00	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00	0,00

Table III.2.S5 - Complete data from analyses of set n°3 under dim1 and dim2 conditions: retention time, peak area, peak width at half-height (w50%), peak width at half-height/area ratio (w50%/area). peak 1 = alphonin, peak 2 = epigallocatechin, peak 3 = epicatechin gallate.

	dim1		pic1	pic2	pic3	dim2		pic1	pic2	pic3
Set n°3	70%	tR	1,87	3,22	5,61	50%	tR	2,67	1,39	2,21
		area	3332511,00	101308,00	1505052,00		area	1357998,00	913106,00	1300667,00
		w50%	0,06	0,08	0,12		w50%	0,28	0,39	0,49
		asym 10%	1,38	1,29	1,30		asym 10%	2,68	2,13	1,49
		w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00
		tR	1,84	3,18	5,52	70%	tR	2,11	1,52	1,81
		area	3380455,00	100961,00	1503558,00		area	2624408,00	2388646,00	1950776,00
		w50%	0,06	0,07	0,11		w50%	0,13	0,19	0,16
		asym 10%	1,32	1,19	1,29		asym 10%	3,29	nd	3,36
		w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00
		tR	1,84	3,18	5,52	90%	tR	1,58	1,89	2,14
		area	4991911,00	5624298,00	8280789,00		area	3729625,00	23766,00	3279327,00
		w50%	0,06	0,07	0,12		w50%	0,07	0,05	0,05
		asym 10%	1,32	1,19	1,29		asym 10%	1,79	2,03	1,71
		w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	0,00
	90%	tR	1,22	1,48	1,83	50%	tR	peak in the baseline		
		area	3372597,00	124763,00	1665280,00		area			
		w50%	0,05	0,05	0,06		w50%			
		asym 10%	1,62	1,40	1,51		asym 10%			
		w50%/area	0,00	0,00	0,00		w50%/area			
		tR	1,22	1,48	1,83	70%	tR	1,26	1,51	not integrable
		area	3406766,00	122165,00	1638520,00		area	1139748,00	23191,00	
		w50%	0,05	0,05	0,06		w50%	0,10	0,05	
		asym 10%	1,55	1,35	1,47		asym 10%	2,15	2,42	
		w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	
		tR	1,22	1,48	1,83	90%	tR	1,46	1,28	2,00
		area	3307975,00	104252,00	1609410,00		area	2878092,00	4174333,00	
		w50%	0,05	0,05	0,06		w50%	0,15	0,17	
		asym 10%	1,55	1,35	1,47		asym 10%	3,95	2,92	
		w50%/area	0,00	0,00	0,00		w50%/area	0,00	0,00	

Figure III.2.S1 -System configuration of the normal Acquity UPC²

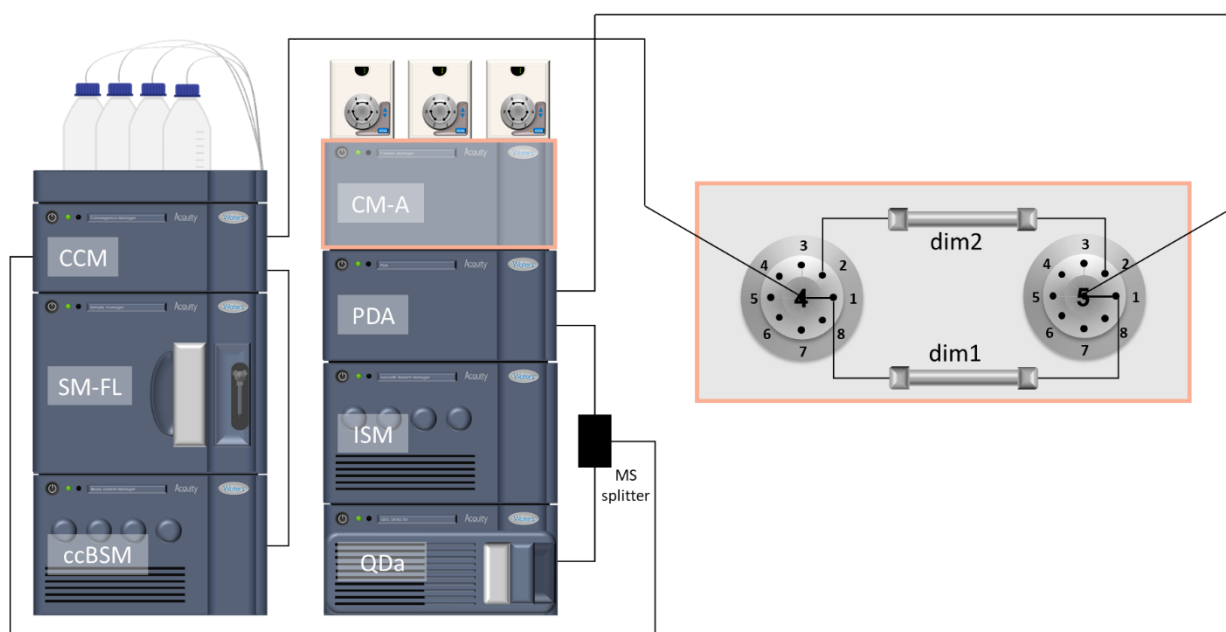


Figure III.2.S2 - Measurement of gradient delay. Comparison of a one-dimensional SFC system (sys1D, in blue) and a two-dimensional SFC-SFC system (sys2D, in black). A) Gradient delay in dim1. Analysis conditions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% acetone, 150 bar, 1.5 mL/min (Table III.2.1, gradient 2.a). B) Gradient delay in dim2 Analysis conditions: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1% acetone, 150 bar, 2.0 mL/min (Table III.2.1, gradient 2.b).

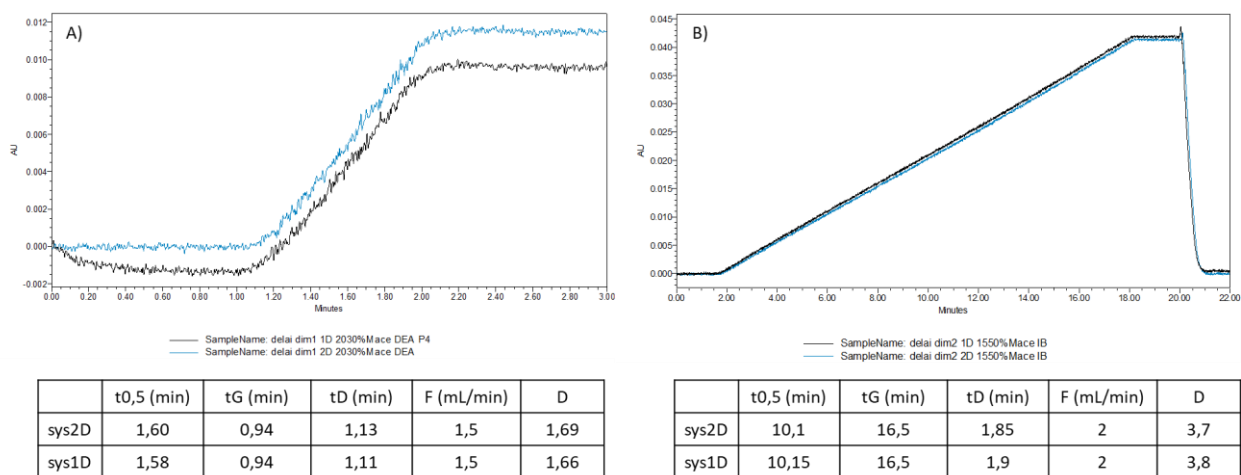


Figure III.2.S3 - Pressure monitoring during the analysis of a naringin standard on a dim1 and a dim2 method performed on a one-dimensional system (syst1D) and a two-dimensional system (syst2D).

A) dim1 method. Analysis conditions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 150-110 bar, 1.7-0.6 mL/min (gradient conditions in Table III.2.1, gradient 1.a)

B) dim2 method. Analysis conditions: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table III.2.1, gradient 1.b), 150 bar, 2.0 mL/min.

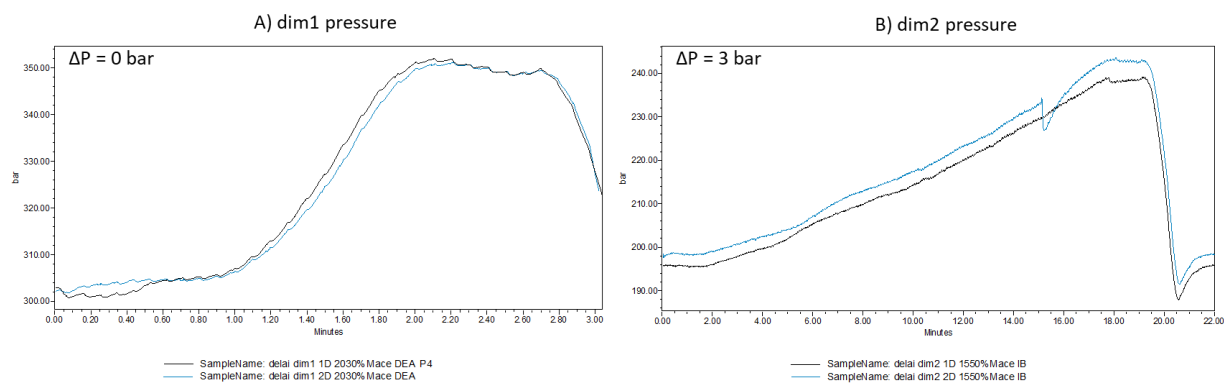


Figure III.2.S4 - System pressure monitoring during the analysis with the flow going through different storage loops of 50 μL and 114 μL volume and going through the dim2 column. Analysis conditions: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1% acetone (gradient conditions in Table III.2.1, gradient 2.b), 150 bar, 2.0 mL/min.

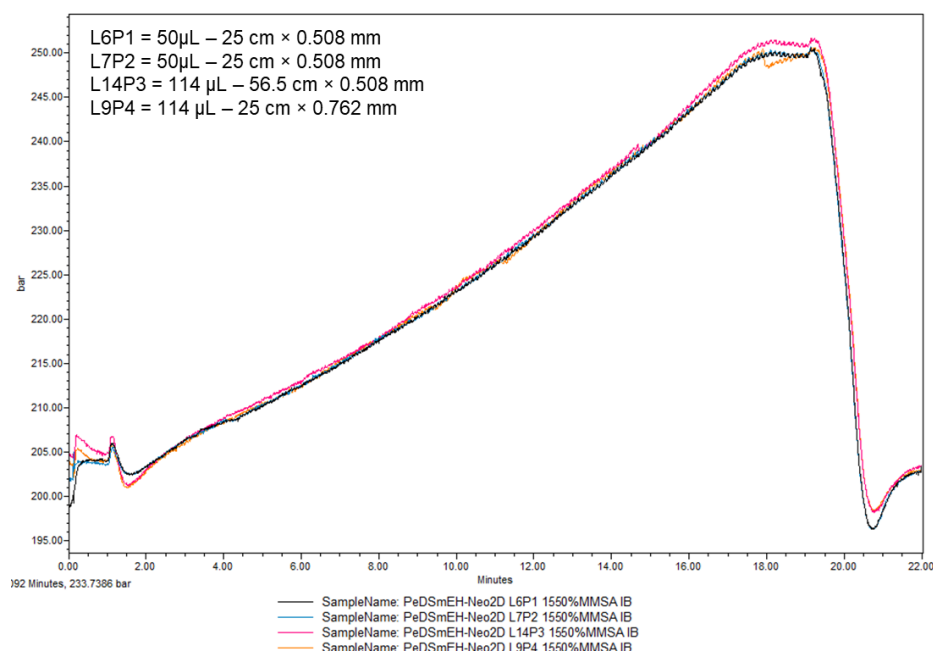


Figure III.2.S5 - Observation in dim2 of the transfer of caffeine contained in the mobile phase from dim1. A) Peak of caffeine observed in dim2, with loop loading in isocratic conditions (step gradient) at 20, 40, 60, 80, 100% co-solvent in the mobile phase. B) Relation between the area of caffeine peak measured in dim2 and the actual amount transferred from dim1.

Analysis conditions dim1: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH containing 0.025 mg/mL caffeine, 110 bar, 0.6 mL/min (gradient conditions in Table III.2.1, gradient 3.a.)

Analysis conditions dim2: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table III.2.1, gradient 3.b), 150 bar, 2.0 mL/min.

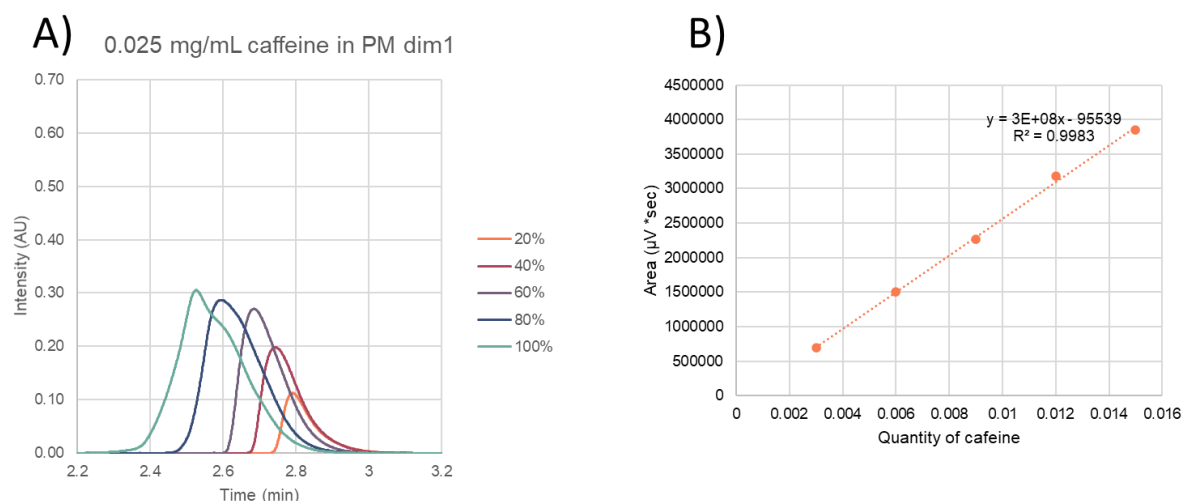


Figure III.2.S6: Pressure variation in dim1 and dim2 observed with naringin standard.

A) Analysis of dim1 with a pressure of 110 bar and analysis of dim2 at 150 bar.

B) Analysis of dim1 with a gradient pressure of 150 to 110 bar and analysis of dim2 at 150, 110 and 190 bar.

Analysis conditions dim1: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm), co-solvent: MeOH + 0.1% MSA, 1.7-0.6 mL/min (gradient conditions in Table III.2.1, gradient 1.a)

Analysis conditions for dim2: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 µm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table III.2.1, gradient 1.b), 2.0 mL/min.

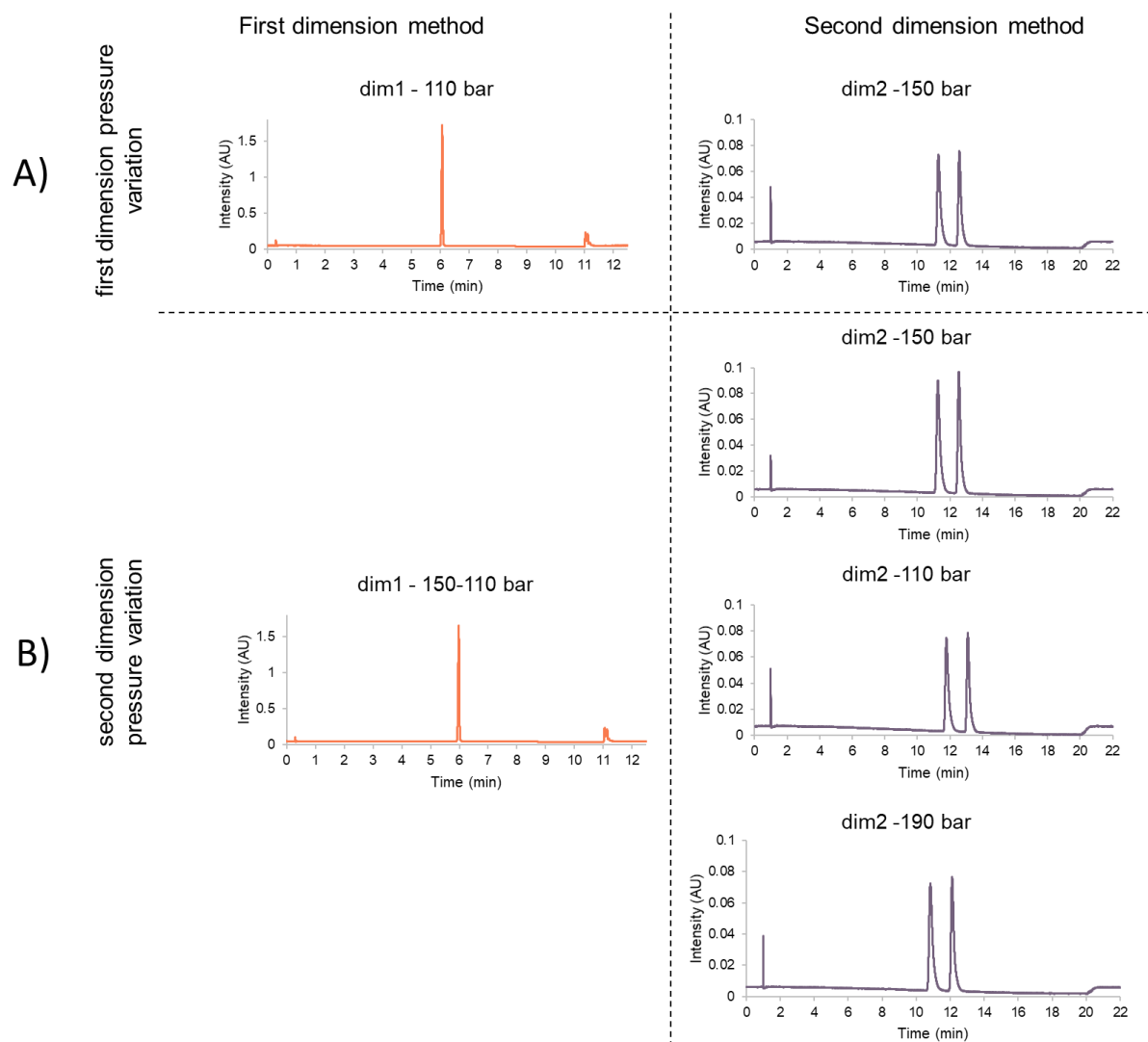


Figure III.2.S7 - Structure of flavonoids used for screening chiral columns, divided into 3 sets. Set no. 1: Pinocembrin, Hesperetin, Pinocembroside. Set no. 2: Naringenin-7-O-glucoside, Taxifolin, Naringenin, Alphononin. Set no. 3: Alphononin, Epigallocatechin, Epigallocatechin Gallate. (TPSA calculated with MolDesc)

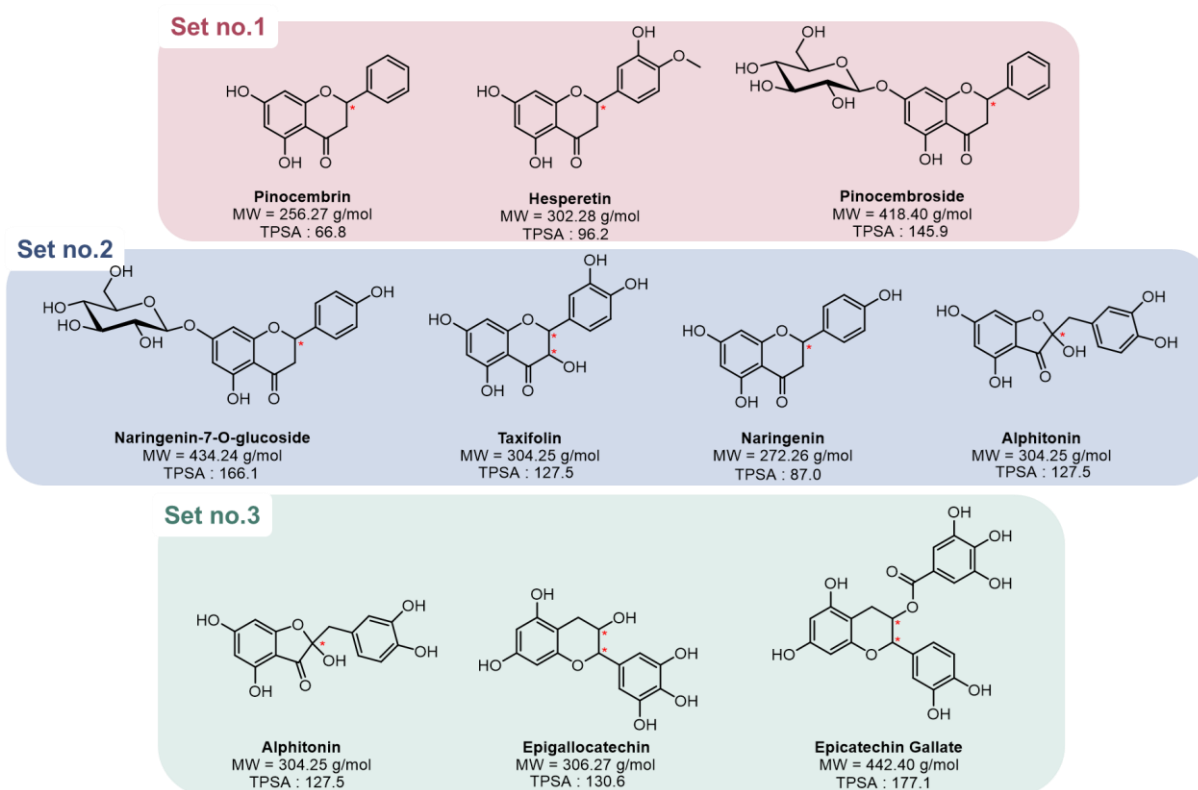


Figure III.2.S8: Chromatogram of SFC-SFC analysis of flavonoid set n°1 (pinocembrin, hesperitin, pinocembroside) loaded with 5% co-solvent (CS) in dim1.

A) dim1 analysis at 5% CS (black) or 10% CS (blue).

B) dim2 analysis at 5% CS.

C) dim2 analysis at 10% CS.

D) dim2 analysis at 30% CS.

E) dim2 analysis at 50% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm), co-solvent: MeOH + 0.1% MSA, 1.5 mL/min.

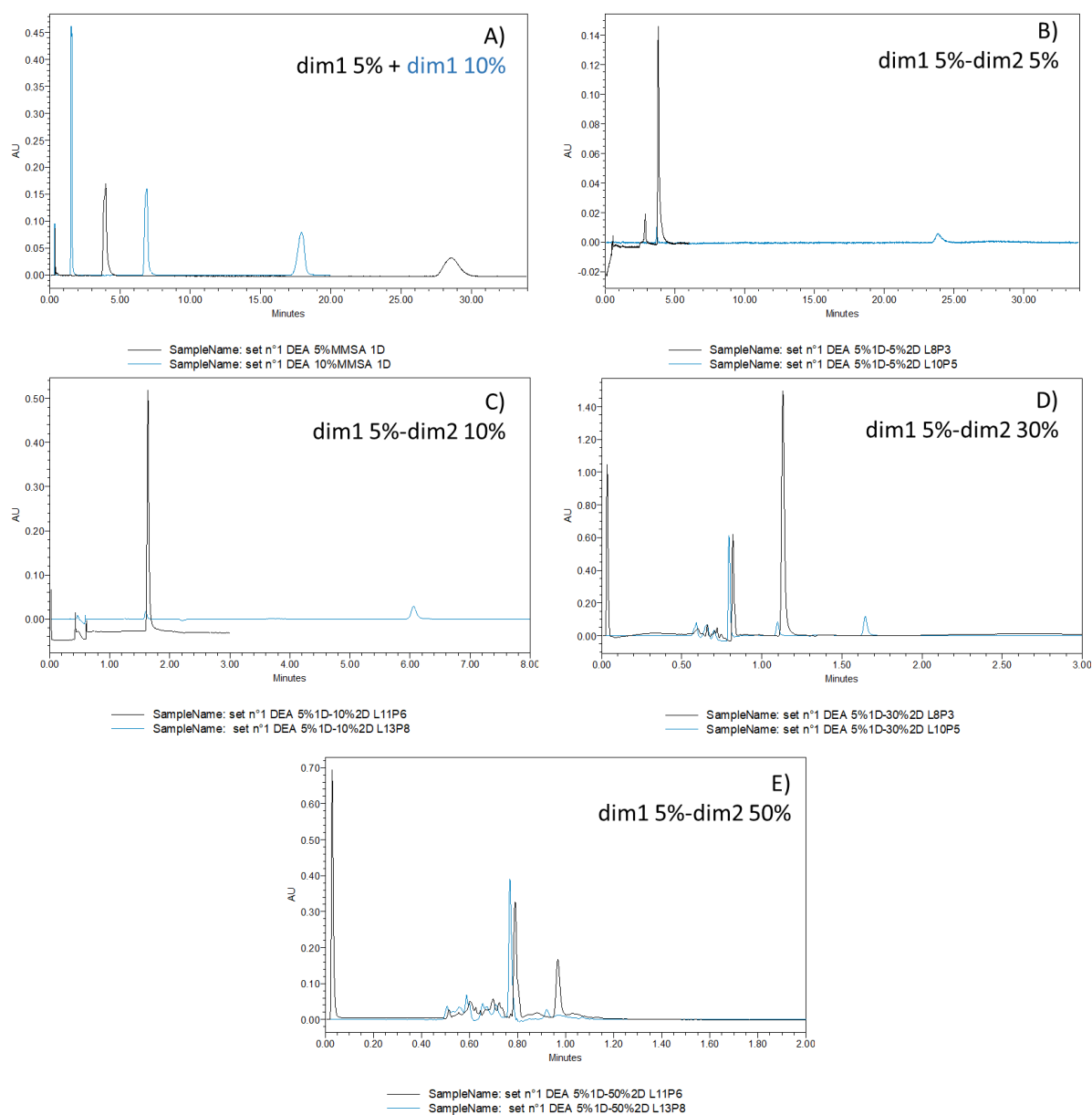


Figure III.2.S9 - Chromatogram of SFC-SFC analysis of flavonoid set n°1 (pinocembrin, hesperitin, pinocembroside) loaded with 10% co-solvent (CS) in dim1.

A) dim1 analysis at 5% CS (black) or 10% CS (blue).

B) dim2 analysis at 5% CS.

C) dim2 analysis at 10% CS.

D) dim2 analysis at 30% CS.

E) dim2 analysis at 50% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm), co-solvent: MeOH + 0.1% MSA, 1.5 mL/min.

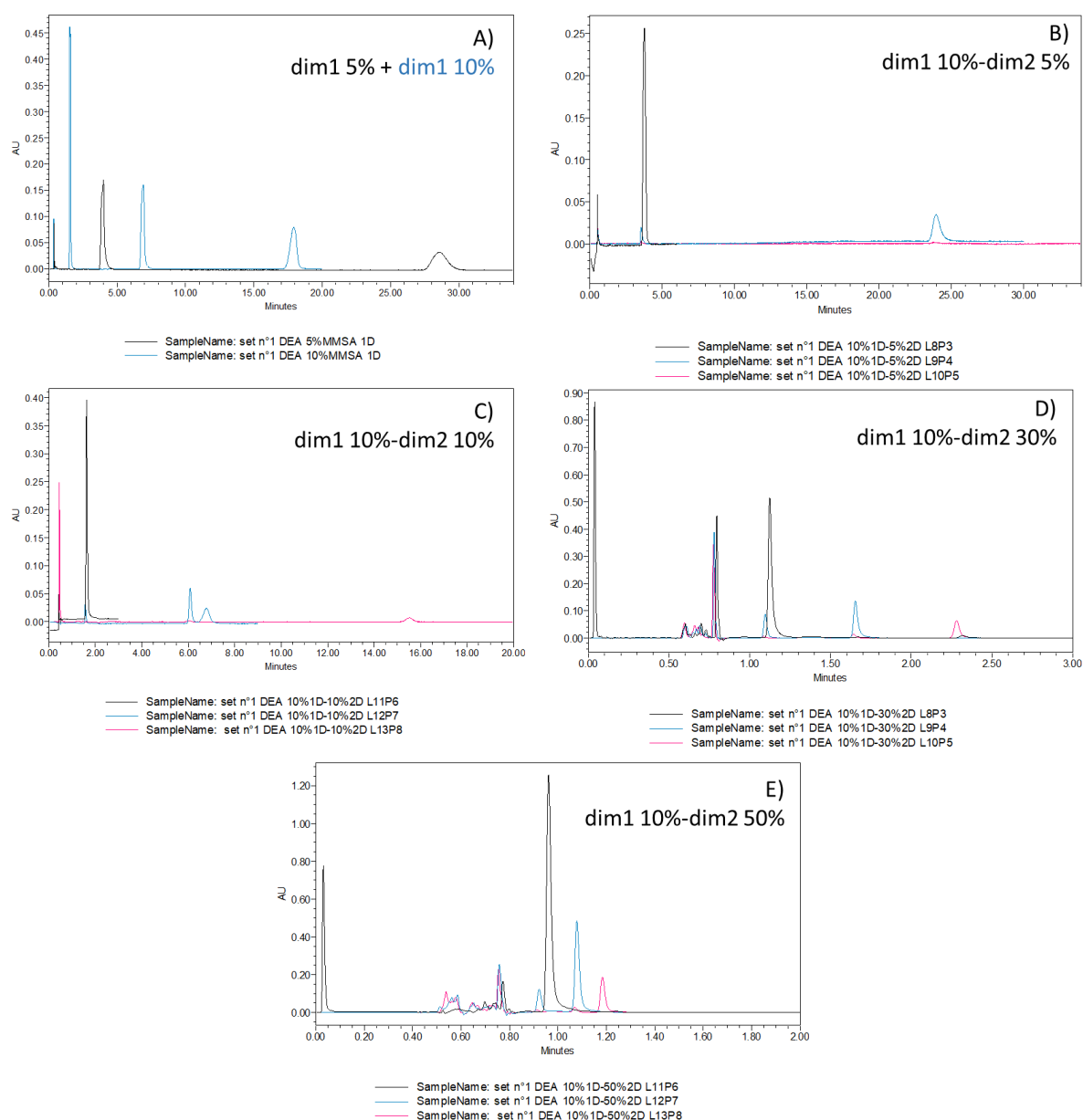


Figure III.2.S10 - Chromatogram of SFC-SFC analysis of flavonoid set n°1 (pinocembrin, hesperitin, pinocembroside) loaded with 30% co-solvent (CS) in dim1.

A) dim1 analysis at 30% CS (black) or 50% CS (blue).

B) dim2 analysis at 5% CS.

C) dim2 analysis at 10% CS.

D) dim2 analysis at 30% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm), co-solvent: MeOH + 0.1% MSA, 1 mL/min.

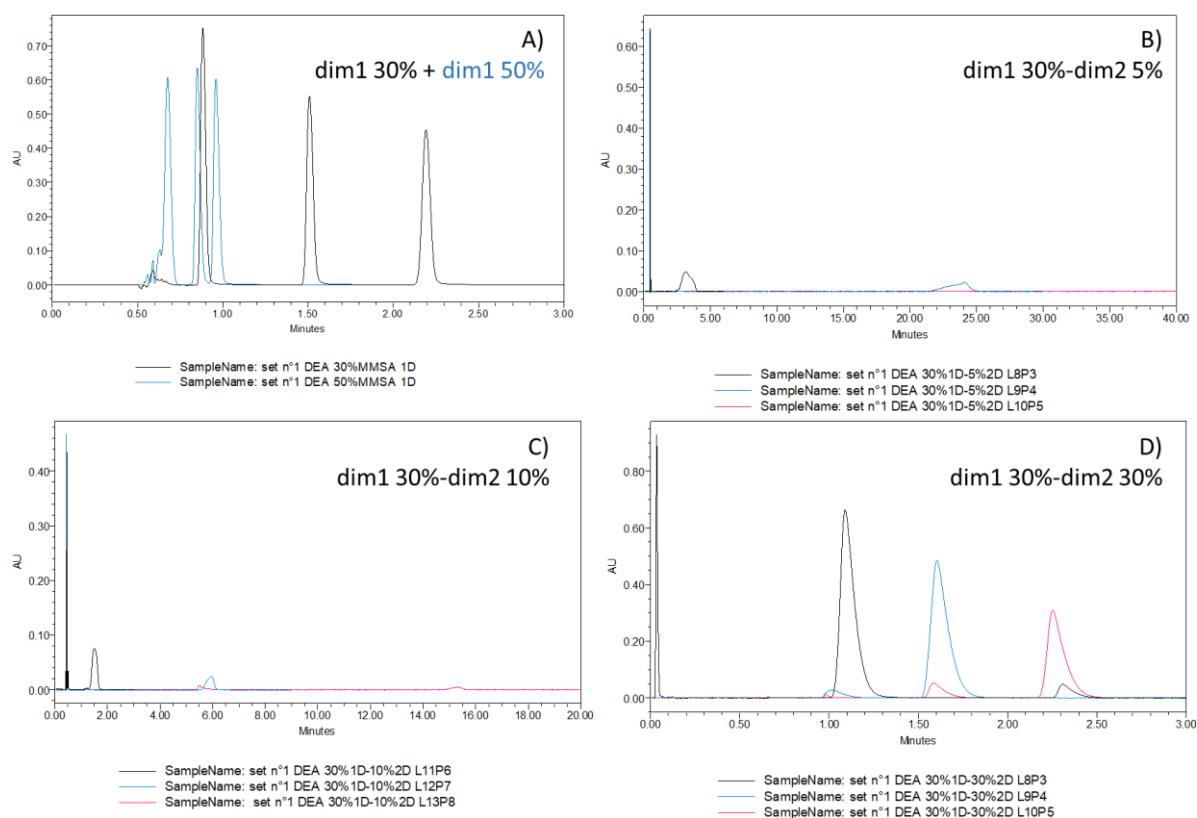


Figure III.2.S11 - Chromatogram of SFC-SFC analysis of flavonoid set n°1 (pinocembrin, hesperitin, pinocembroside) loaded with 50% co-solvent (CS) in dim1.

A) dim1 analysis at 30% CS (black) or 50% CS (blue).

B) dim2 analysis at 5% CS.

C) dim2 analysis at 10% CS.

D) dim2 analysis at 30% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 1 mL/min.

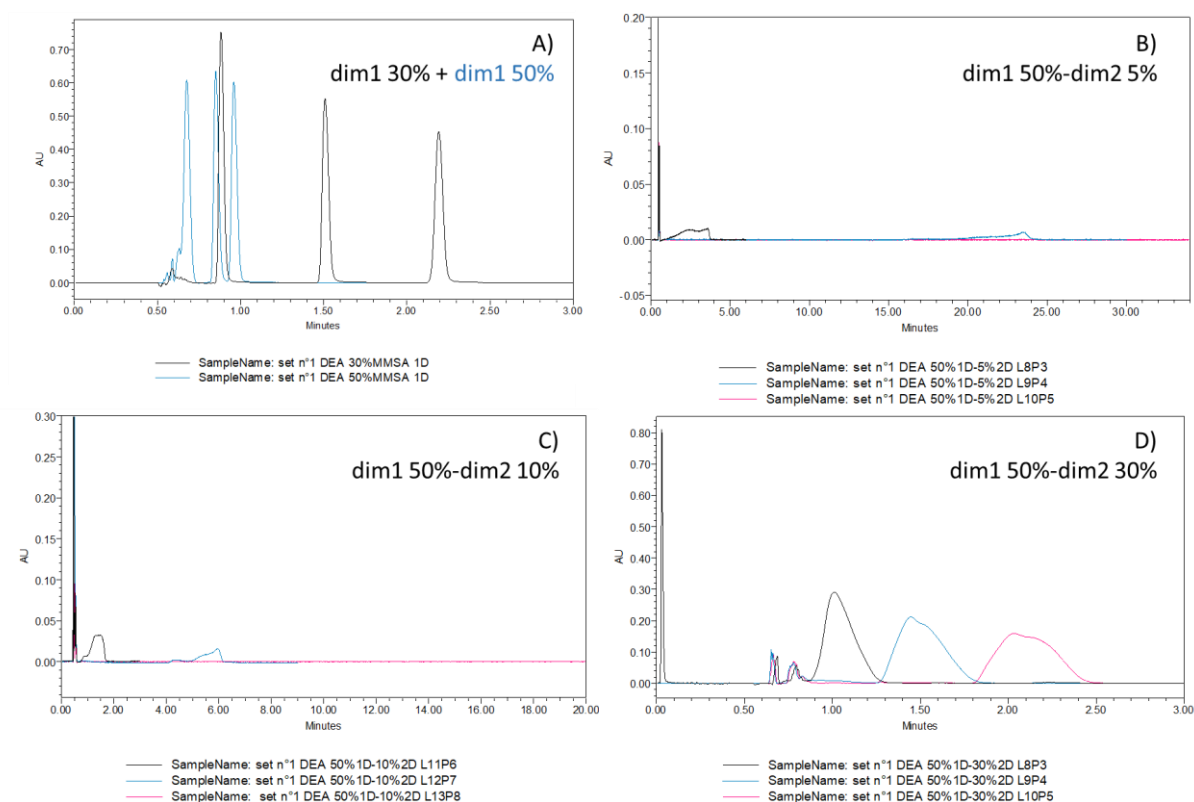


Figure III.2.S12 - Chromatogram of SFC-SFC analysis of flavonoid set n°2 (naringenin, naringenin-7-O-glucoside, taxifolin, alphononin) loaded with 30% co-solvent (CS) in dim1.

A) dim1 analysis at 30% CS (black) or 50% CS (blue).

B) dim2 analysis at 50% CS.

C) dim2 analysis at 70% CS.

D) dim2 analysis at 90% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 1 mL/min.

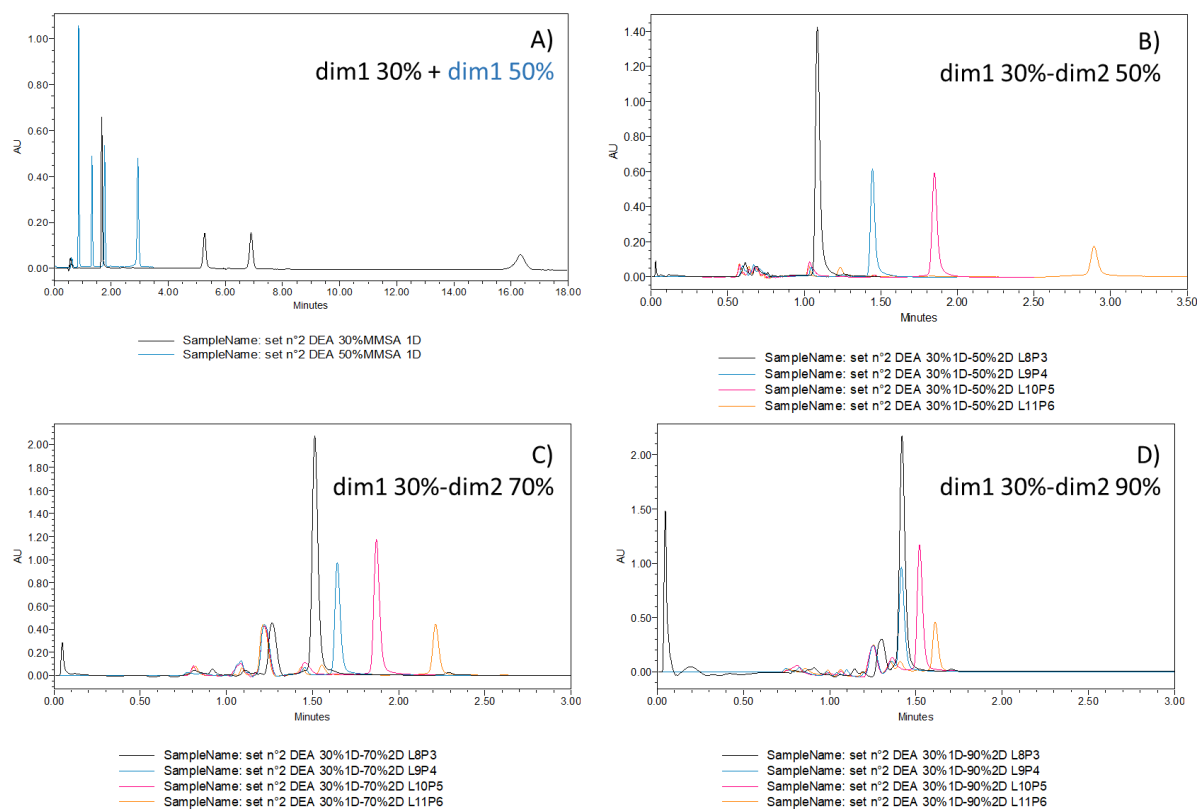


Figure III.2.S13 - Chromatogram of SFC-SFC analysis of flavonoid set n°2 (naringenin, naringenin-7-O-glucoside, taxifolin, alphononin) loaded with 50% co-solvent (CS) in dim1.

A) dim1 analysis at 30% CS (black) or 50% CS (blue).

B) dim2 analysis at 50% CS.

C) dim2 analysis at 70% CS.

D) dim2 analysis at 90% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm), co-solvent: MeOH + 0.1% MSA, 1 mL/min.

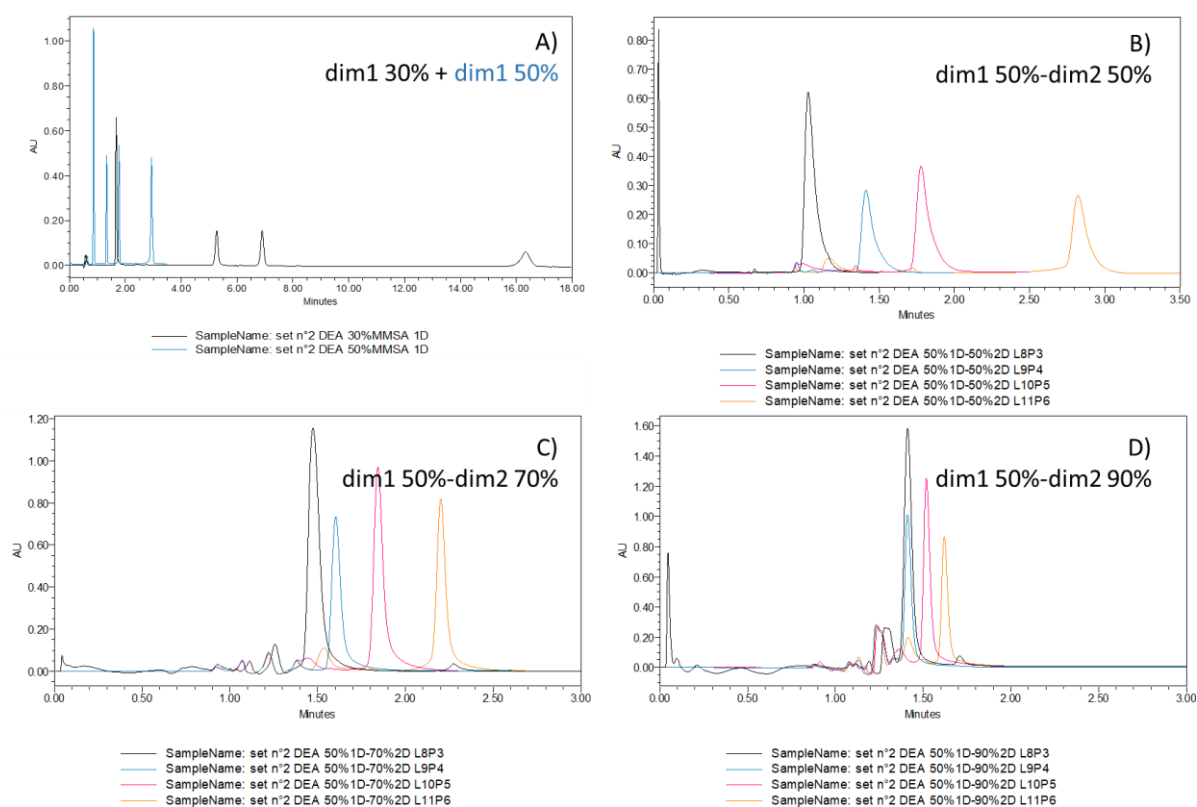


Figure III.2.S14 - Chromatogram of SFC-SFC analysis of flavonoid set n°3 (alphononin, epigallocatechin, epicatechin gallate) loaded with 70% co-solvent (CS) in dim1.

A) dim1 analysis at 70% CS (black) or 90% CS (blue).

B) dim2 analysis at 50% CS.

C) dim2 analysis at 70% CS.

D) dim2 analysis at 90% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 0.6 mL/min.

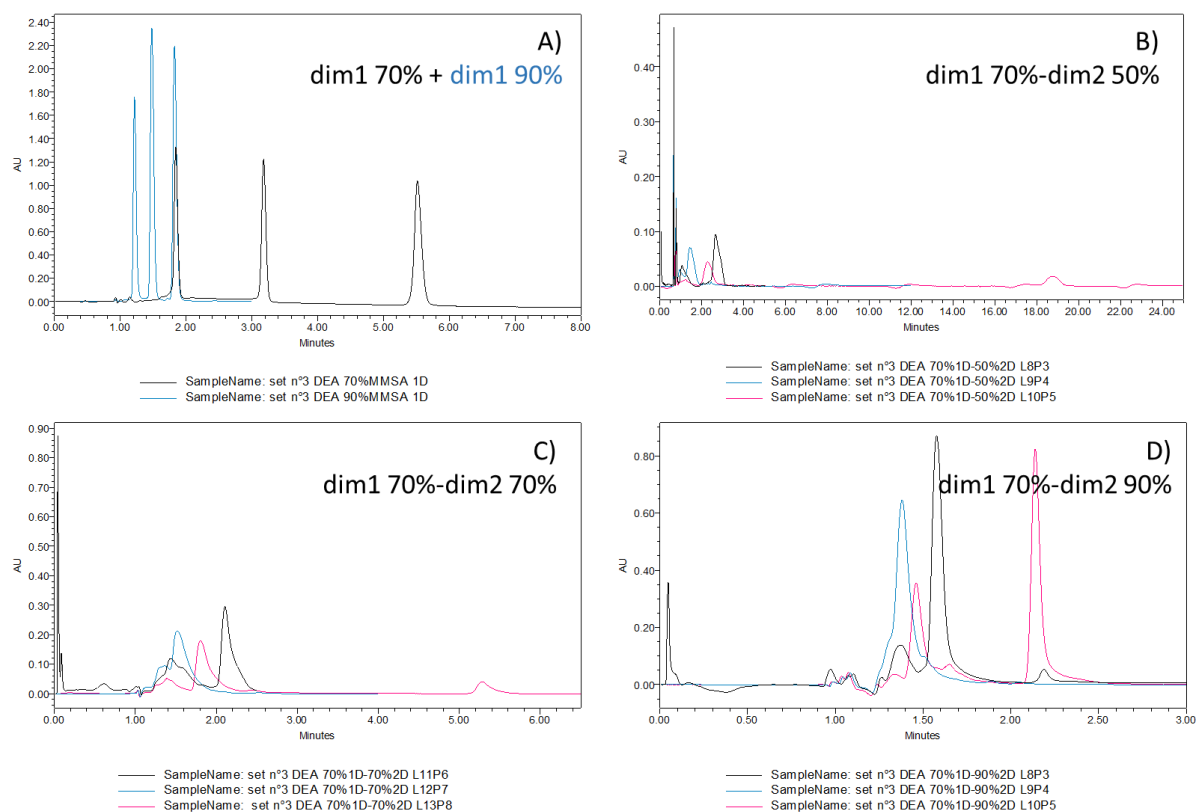


Figure III.2.S15 - Chromatogram of SFC-SFC analysis of flavonoid set n°3 (alphitonin, epigallocatechin, epicatechin gallate) loaded with 90% co-solvent (CS) in dim1.

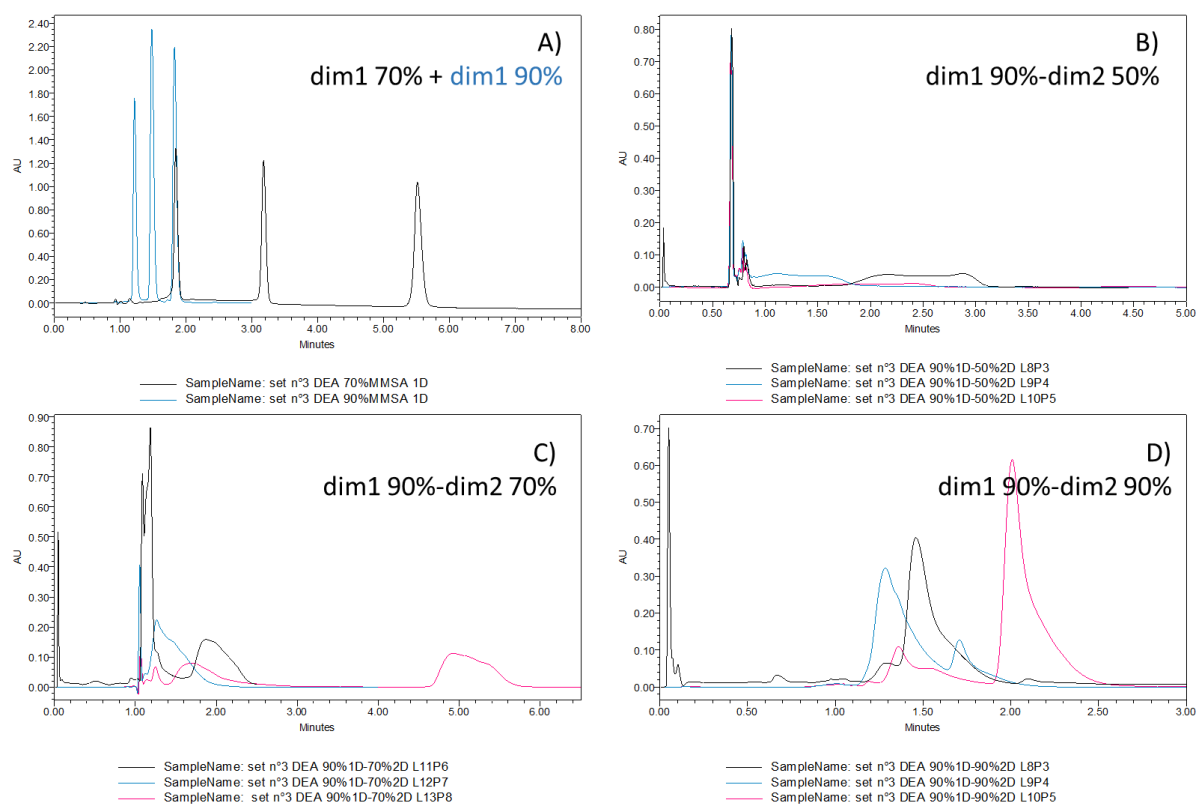
A) dim1 analysis at 70% CS (black) or 90% CS (blue).

B) dim2 analysis at 50% CS.

C) dim2 analysis at 70% CS.

D) dim2 analysis at 90% CS.

Analysis conditions in both dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 0.6 mL/min.



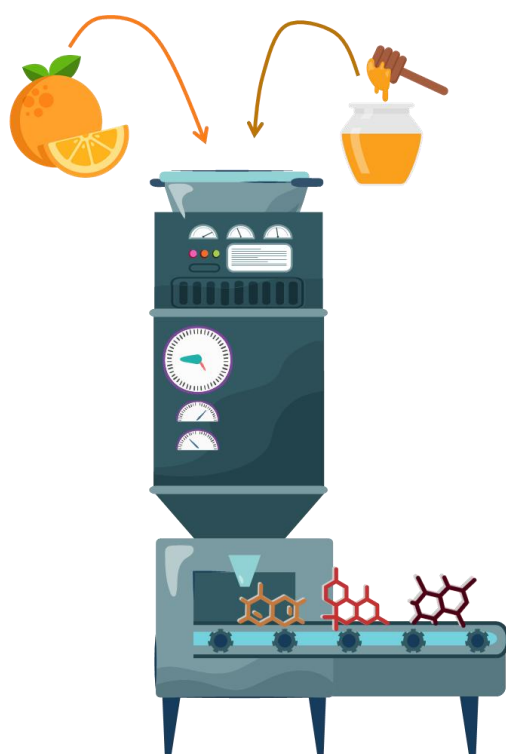
Conclusion chapitre

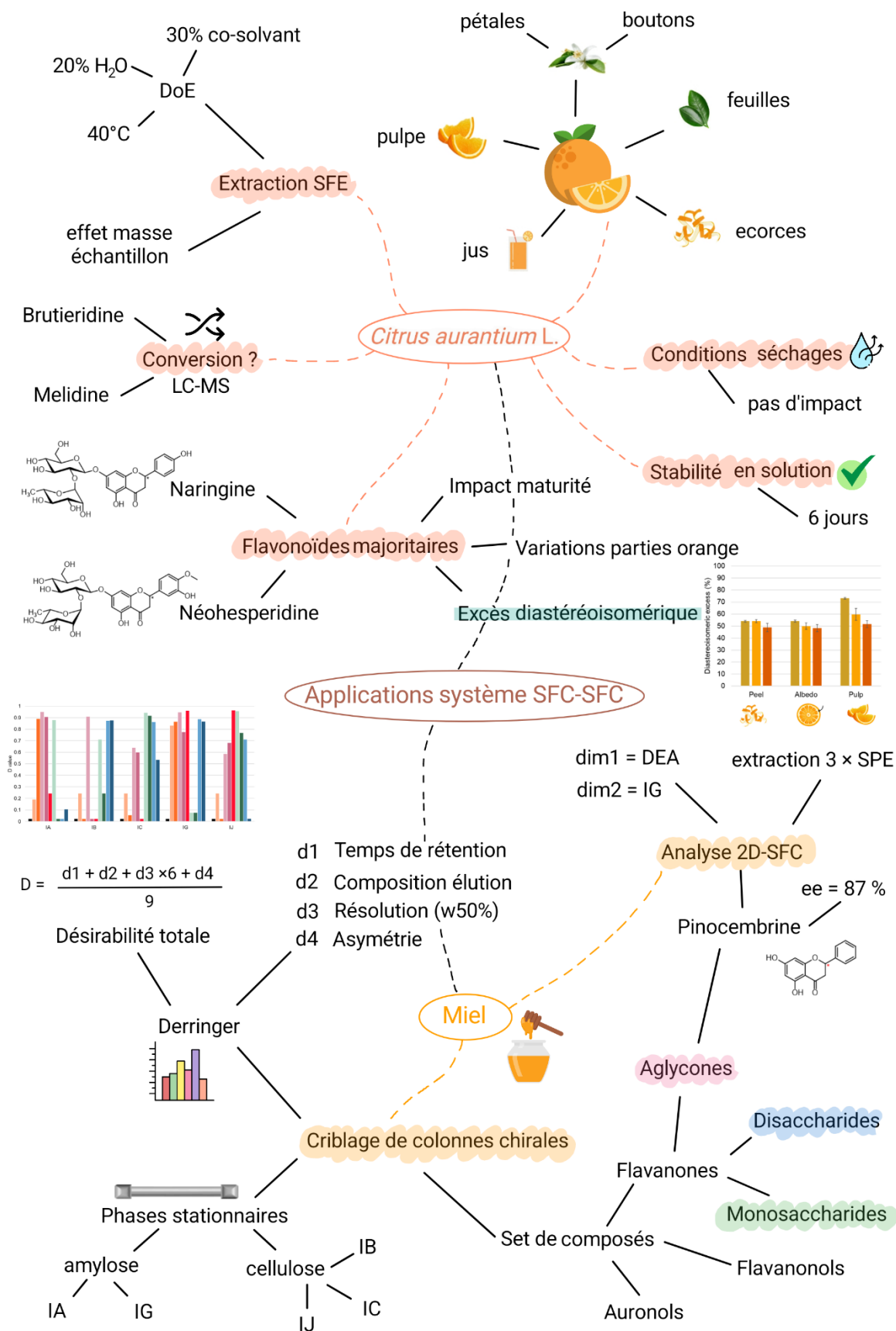
Ce chapitre a présenté le développement d'un système bidimensionnel SFC-SFC en mode multiple heart-cut, à partir d'un système unidimensionnel modifié par l'ajout de vannes. L'optimisation des temps de commutation a joué un rôle important pour garantir un transfert efficace entre les dimensions. Différents paramètres ont été étudiés, notamment les effets des boucles de transfert, de la pression et de la phase mobile. Des différences significatives sur l'allure des pics, la rétention et la compatibilité des dimensions ont été observées entre les colonnes polaires et apolaires lors du transfert de différentes quantités de co-solvant. Le développement et l'optimisation de ce système ont mis en évidence des limites et défini une zone d'applicabilité pour assurer les meilleures conditions de couplages.

Le chapitre suivant utilise ces avancées pour explorer l'application de la SFC-SFC à la séparation et à la quantification des stéréoisomères de flavonoïdes, dont la chiralité a un impact sur leurs propriétés biologiques. Ainsi, le système développé permet d'accéder à une caractérisation fine des stéréoisomères dans des échantillons complexes comme le *Citrus aurantium* L. ou le miel.

Chapitre IV

Analyses stéréoisomériques par SFC-
SFC de flavonoïdes chiraux issus de
produits naturels





Introduction

Après le développement d'un système SFC-SFC en mode multiple heart-cut, décrit dans le chapitre précédent, ce nouveau chapitre illustre son application à l'analyse de stéréoisomères de flavonoïdes dans des matrices naturelles complexes. La caractérisation de composés chiraux dans les produits naturels tels que ceux du *Citrus aurantium* L. ou du miel, peut être difficile. Comme évoqué au chapitre 2 (article publié dans J. Chromatogr. Open), la chiralité des flavonoïdes peut influencer directement leurs propriétés biologiques. L'utilisation d'un système bi-dimensionnel SFC-SFC comprenant des colonnes chirales pourrait être une bonne solution pour élucider la composition en stéréoisomères dans les mélanges complexes.

Ce chapitre comprend deux études complémentaires. La première s'intéresse aux organes du *C. aurantium* et à la détermination des excès diastéréoisomériques (d.e.) de leurs flavonoïdes extraits par SFE. Elle a mis en évidence une grande stabilité du diastéréoisomère majoritaire de la néohespéridine, avec de très hauts d.e., tandis que la naringine présente une variabilité importante liée notamment à des facteurs environnementaux. L'étude a aussi mis en avant l'importance de la masse de l'échantillon pour garantir la représentativité et la reproductibilité des mesures stéréospécifiques. Ces résultats ont montré la nécessité de bien maîtriser les conditions de culture, de récolte et de séchage pour préserver la stéréochimie des flavonoïdes. Cette étude sera présentée dans la partie 1.

Le second article se concentre sur la question du choix des colonnes chirales en fonction de la structure des flavonoïdes. En combinant différents polysaccharides (amylose ou cellulose) et différentes modifications de ces polymères de phases stationnaires chirales, une approche multicritère (fonctions de désirabilité) a permis de déterminer les colonnes les plus performantes pour chaque classe de composés. Cette méthodologie a été utilisée sur un miel de Guyane française, où le système SFC-SFC-DAD-MS a permis d'isoler puis de séparer les énantiomères de pinocembrine, montrant les capacités du système à décrypter la chiralité au sein de matrices naturelles complexes. Cette étude sera présentée dans la partie 2.

Ce chapitre présente donc l'application du système SFC-SFC à la séparation stéréospécifique des flavonoïdes, l'optimisation des conditions analytiques et de l'exploration structurale de produits naturels.

Partie 1 : Excès diastéréoisomérique des flavonoïdes du *Citrus aurantium* L. dans les différentes parties de l'orange

Article 3: Determination of the diastereomeric excesses of chiral flavonoids in organs of *Citrus aurantium* with two-dimensional supercritical fluid chromatography

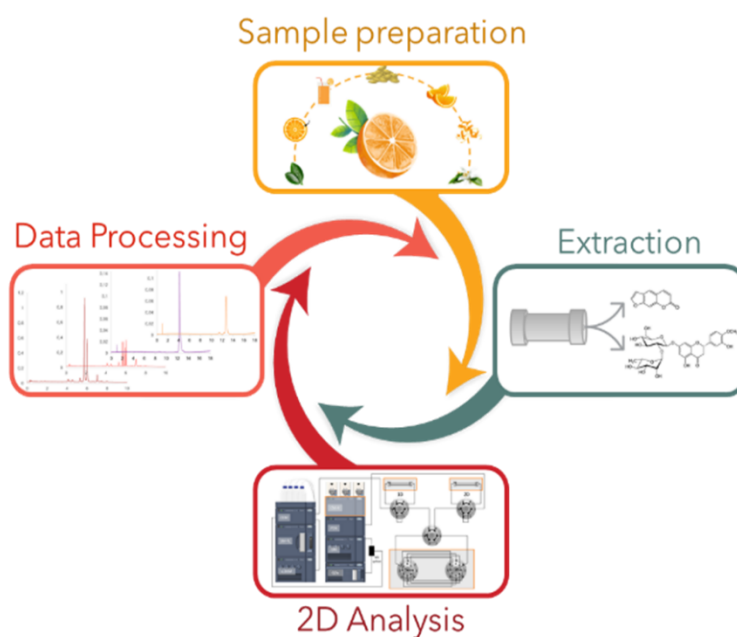
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Graphical abstract



Abstract

Citrus aurantium (bitter orange), is widely used in culinary preparations, food supplements, cosmetics and traditional medicine. Its bioactivity is mainly associated to two major glycosylated flavanones, naringin and neohesperidin, each comprising two diastereoisomers having different bioactivities. To possibly relate bioactivity to stereoisomeric composition, an analytical method was necessary.

A two-dimensional supercritical fluid chromatography (SFC) method was used to measure diastereomeric excesses of the flavonoids in bitter orange organs, including flowers, leaves and fruits at different stages of maturity. The extracts were prepared with supercritical fluid extraction (SFE) with CO₂, ethanol and water. SFE conditions were defined following a 3-factor Box-Behnken design of experiments. While neohesperidin was highly stable, with circa 100 % stereomeric excess in all samples, naringin was prone to racemization, especially in mature fruits.

This study also examined the importance of using a sufficient mass of sample to obtain accurate and meaningful measurements, particularly when stereoisomers are concerned.

Keywords: bitter orange; *Citrus aurantium*; flavonoids; stereoisomers; supercritical fluid extraction; supercritical fluid chromatography; two-dimensional chromatography

1. Introduction

Flavonoids are phenolic compounds well known for their diverse biological effects such as antioxidant activity [1,2], making them promising agents for the prevention and treatment of chronic and neurodegenerative diseases such as Alzheimer's [3,4]. In addition to their antioxidant properties, flavonoids have been demonstrated to have anti-inflammatory [5,6], anti-cancer [7,8], anti-microbial [9,10] and cardiovascular effects [11,12].

Flavonoids are broadly distributed in natural products, including *Citrus aurantium* L. (bitter orange). In addition to being used in culinary preparation as a substitute to lemon juice, or in food supplements [13], where it is supposed to contribute to weight loss, *C. aurantium* is widely used for aromatic and medicinal applications [14]. The bioactivity is mainly related to flavonoids, with naringin and neohesperidin being the most abundant [15]. In addition, several of these flavonoids, including the latter two, contain stereocenters in their aglycon root, leading to the presence of stereoisomers [16]. In the case of aglycon flavonoids, the isomers are enantiomers, while in the case of the glycosylated flavonoids (like naringin and neohesperidin), because the sugar moieties also contain stereocenters, the isomers are diastereomers. Whatever the isomer type, these stereoisomers may differ in their bioavailability and biological activity [17]. Consequently, achieving a chiral separation of these flavonoids is important to characterize the bioactivity of individual stereoisomers and to better understand their individual roles in various fields of application [18]. For instance, while it is well known that flavonoids are among the main molecules responsible for the bitterness of citrus fruits [19], Gaffield *et al.* observed that the 2S isomer of naringin yielded a lower bitterness in grapefruit than the 2R isomer [20]. The determination of

enantiomeric (e.e.) or diastereomeric excesses (d.e.) is therefore of interest to compare the distribution of flavonoid stereoisomers in samples from different parts of *C. aurantium* (leaves, peel, pulp, flowers, albedo) and at different stages of maturity [21]. For this purpose, we proceeded to develop an analytical method for e.e. or d.e. measurement in complex samples of plant extracts [22]. Such a method would also be useful when measuring the bioactivity of extracts, to possibly relate the activity to the observed stereoisomeric composition.

In recent years, two-dimensional chromatography has become an effective method to separate complex mixtures, especially in natural product analysis [23]. For stereoselective separations, one of the most useful techniques is supercritical fluid chromatography (SFC) [24]. This technique enables rapid separation and analysis of flavonoids using a low-viscosity fluid (supercritical CO₂) as the major component of the mobile phase. SFC uses low volumes of organic solvents, making it a greener analytical alternative to traditional techniques such as liquid chromatography (LC) [25]. When SFC is used in both dimensions of a two dimensional separation system, it can provide orthogonal separation mechanisms simply through an adequate selection of stationary phases, making it even more powerful. With the addition of mass spectrometry (MS), this setup should be useful to separate isomers that are difficult to distinguish and to reduce problems with overlapping peaks, which are frequent in complex mixtures [26].

Using the same supercritical CO₂ as major fluid, added with a small portion of ethanol, supercritical fluid extraction (SFE) is ideal for the extraction of low- to medium-polarity flavonoids from natural products [27,28]. This technique is efficient, selective and environmentally friendly. However, sample preparation is critical to analyte stability and extraction yield. In addition, combining SFE with SFC provides a green approach for the analysis of natural products [29].

In this study, we have examined SFE of the major flavonoids of *C. aurantium* and determined the diastereomeric excesses of naringin and neohesperidin with SFC-SFC-UV-MS analysis in a multiple heart-cutting mode [30]. In this system, the first SFC dimension allowed isolating two target flavonoids from the whole extract, while the second SFC dimension resolved their diastereomers. In particular, sample preparation raised many questions to achieve reliable results. Different organs of the plant were examined (leaves, peel, flowers, albedo and pulp) and at various maturity stages. The main goal was to observe the effects of ripening stages, organ samples, drying procedure and geographical origin of the fruit on the stereoisomeric profile of major flavonoids in this plant.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade methanol used as the mobile phase co-solvent and sample diluent, ethanol Hipersolv Chromanorm 99.7–100 % used as extraction co-solvent, ammonium hydroxide, LC-MS-grade water and formic acid with a purity of 99–100 % were purchased from VWR (Fontenay-sous-Bois, France). CO₂ with a purity of 99.7 % was delivered by Air Liquide (Paris, France). Methanesulfonic acid (MSA) and acetonitrile LC-MS grade were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Ultra-pure water was supplied by a Milli-Q® IQ 7000 system from Merck (Darmstadt, Allemagne). Naringin and neohesperidin standards were acquired from Extrasynthese (Genay, France). Individual stereoisomers were not available.

C. aurantium flowers, peels, and leaves were bought from Louis Herboristerie (Charleville-Mézières, France). Fresh *C. aurantium* fruit was purchased from Vergers de Boirie (Menton, France). Other leaves were kindly supplied by Prof. Mohamed Addi from University Mohammed Premier, Oudja, in Morocco.

2.2. Sample preparation and sonication

Fresh *C. aurantium* fruits were manually prepared. Three stages of fruit ripeness were observed: ripe oranges with an orange color, half-ripe oranges with a yellow-orange color and unripe oranges with a green color. For each stage of maturity, the different parts of the orange were separated: peel, albedo, un-seeded pulp, juice and leaves. Samples of albedo, pulp and leaves were freeze-dried. For the peel, one part was placed in the oven and the other in the freeze-dryer. All samples were ground into powder. Since the flowers, skins and leaves from Louis Herboristerie and the leaves from Morocco were supplied as dry material, they were simply ground without further drying.

Samples extracted by sonication were prepared as follows: 1 g of sample was extracted with 15 mL of ethanol (EtOH) or EtOH/H₂O (80/20) for 30 min in an ultrasonic bath. The extract was then centrifuged (20 °C, 8000 rpm) for 5 min and filtered through 0.20 µm, 13-mm diameter PVDF syringe filters from Agilent Technologies (Les Ulis, France) and diluted by 4 in EtOH or EtOH/H₂O 80/20 (depending on extraction conditions) prior to analysis.

2.3. Supercritical fluid extraction (SFE)

2.3.1. Instrumentation

A Nexera UC system from Shimadzu Corporation (Kyoto, Japan) was used for all extractions. The system was composed of a CO₂ pump (LC-30ADsf), a co-solvent pump (LC-30AD) for extraction, and an additional solvent pump (LC-20ADXR) to introduce make-up fluid after the extraction. The instrument included an extractor (SFE-30A) connected to a Rack Changer II, which housed 48 slots for 5-mL extraction vessels, as well as a back-pressure regulator (BPR) to control pressure within the auto-extractor (SFC-30A). The extraction system was directly linked to a fraction collector (FRC-40SF). Instrument control was managed using LabSolutions LC-MS version 5.120 from Shimadzu Corporation.

2.3.2. SFE conditions

Samples were introduced in 5-mL stainless steel extraction vessels. The sample weight varied between 50 mg and 1 g, mixed with dispersant (diatomaceous earth). A cellulose filter was placed at the bottom of the vessel, and cotton was placed at the top, respectively to filter the extract and fill the cell entirely.

Based on previous works that examined SFE of flavonoids [28], the co-solvent used was a mixture of ethanol and water, as further described below. A constant flow rate of 3 mL/min was used. A make-up flow rate of EtOH/H₂O 80/20 was set at 0.7 mL/min to avoid sample precipitation after CO₂ depressurization at the BPR outlet.

The fractions recovered from SFE were evaporated to dryness under a nitrogen flow, then resolubilized in 4 mL of 80/20 EtOH/H₂O and filtered through 0.20 µm, 13-mm diameter PVDF syringe filters.

2.3.3. SFE Design of experiment (DoE)

To optimize the SFE conditions, a DoE was developed. A Box-Behnken design (BBD) was previously described for the extraction of polar flavanol molecules from a different plant [28]. The same design was reproduced here to extract glycosylated flavanones. The three factors used in this study were :

- temperature (T), varying from 40 to 80 °C,
- total co-solvent (ethanol-water) percentage (C), varying from 10 to 30% in pressurized CO₂,
- water percentage added to ethanol (W), varying from 0 to 20%,

with three levels coded as (+1) for the highest, (0) for the middle point and (-1) for the lowest level (Table IV.1.S1). Two responses were considered: peak areas measured in the first-dimension SFC analysis for the two target compounds (naringin and neohesperidin) and the corresponding mass of flavonoids extracted, determined from calibration curves constructed with standard compounds [30]. A total of 17 experiments were carried out in a random fashion, including 5 replicates at the central point (see supplementary information, Table IV.1.S2 and Figure IV.1.S1). 50 mg of bitter orange dry flower sample were used for each experiment in this design.

Data reprocessing, model calculation and graph visualization were performed with the XLSTAT 19.03 software (Addinsoft, Paris, France).

2.4. SFC-MS analysis

2.4.1. Instrumentation

The supercritical fluid chromatography system was based on a Waters Corporation (Millford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®). It was equipped with an autosampler with a partial loop volume injection system, an automated back-pressure regulator (BPR), a 2-columns oven compatible with 150 mm-length columns, via column switching valves. The system also included a binary solvent delivery pump compatible with a maximum mobile phase flow-rate of 4 mL/min and a maximum pressure of 414 bar. Two detectors were available: a diode-array detector (ACQUITY PDA®) and a single-quadrupole mass spectrometer (MS) with an electrospray ionization (ESI) source (ACQUITY QDa®). An isocratic solvent manager was used as a make-up pump and was positioned after the diode-array detector and before a flow splitter, where one portion was sent to the BPR and the other to the MS. The use of MSA in the mobile phase involved adapting the exhaust valve of the QDa instrument, as MSA may cause corrosion on certain parts.

The instrument was modified in-house to achieve a multiple-heart-cut SFC-SFC configuration, with the addition of three 2-position 6-port valves and loading loops, as previously described [30].

Empower® 3 was used for chromatographic acquisition and for data treatment.

2.4.2. Chromatographic conditions

Two different columns were used. The first-dimension (dim1) column was ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm) from Waters, preceded with a Torus DEA pre-column (5.0 × 2.1 mm). This served to resolve naringin and neohesperidin from the sample and collect them individually for further analysis in the second dimension. The second-dimension (dim2) column

was CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 µm) from Chiral Technologies (Illkirch-Graffenstaden, France). This served to separate the diastereoisomers of naringin on the one hand, neohesperidin on the other hand. Thus, diastereomeric excesses (d.e.) could be measured.

The mobile phase used was CO₂ with MeOH containing 0.1% MSA. The injection volume was 5 µL. The gradient programs, including variation of solvent composition, back-pressure and flow-rate, are presented in Table IV.1.S3.

The column oven was heated to 30 °C and the sample compartment was kept at 10 °C. All chromatograms were recorded in the 210-400 nm range, with 1.2 nm resolution. Scan rate was 20 Hz. Visualization and peak integration in UV were done at 280 nm for standards and sample.

The SFC dim1 method used was previously developed by Molineau *et al.* to separate a large number of flavonoids, including regio-isomers [31]. The SFC dim2 method was optimized to resolve glycosylated flavonoid diastereoisomers [22]. Briefly, several chiral flavonoids from different classes including auronols, flavanonols and flavanones, including aglycon, monosaccharide or disaccharide forms, were examined on 5 different immobilized polysaccharide stationary phases. CHIRALPAK IB-3 appeared to provide the best performance for flavanonones with disaccharide groups. As a result, combining the two methods of dim1 and dim2, the risk of co-elution of isomeric species after two complementary methods should be minimal.

Sample chromatograms can be observed in Figure IV.1.1, showing the analysis of standards and of an extract of orange peel with UV and MS detection.

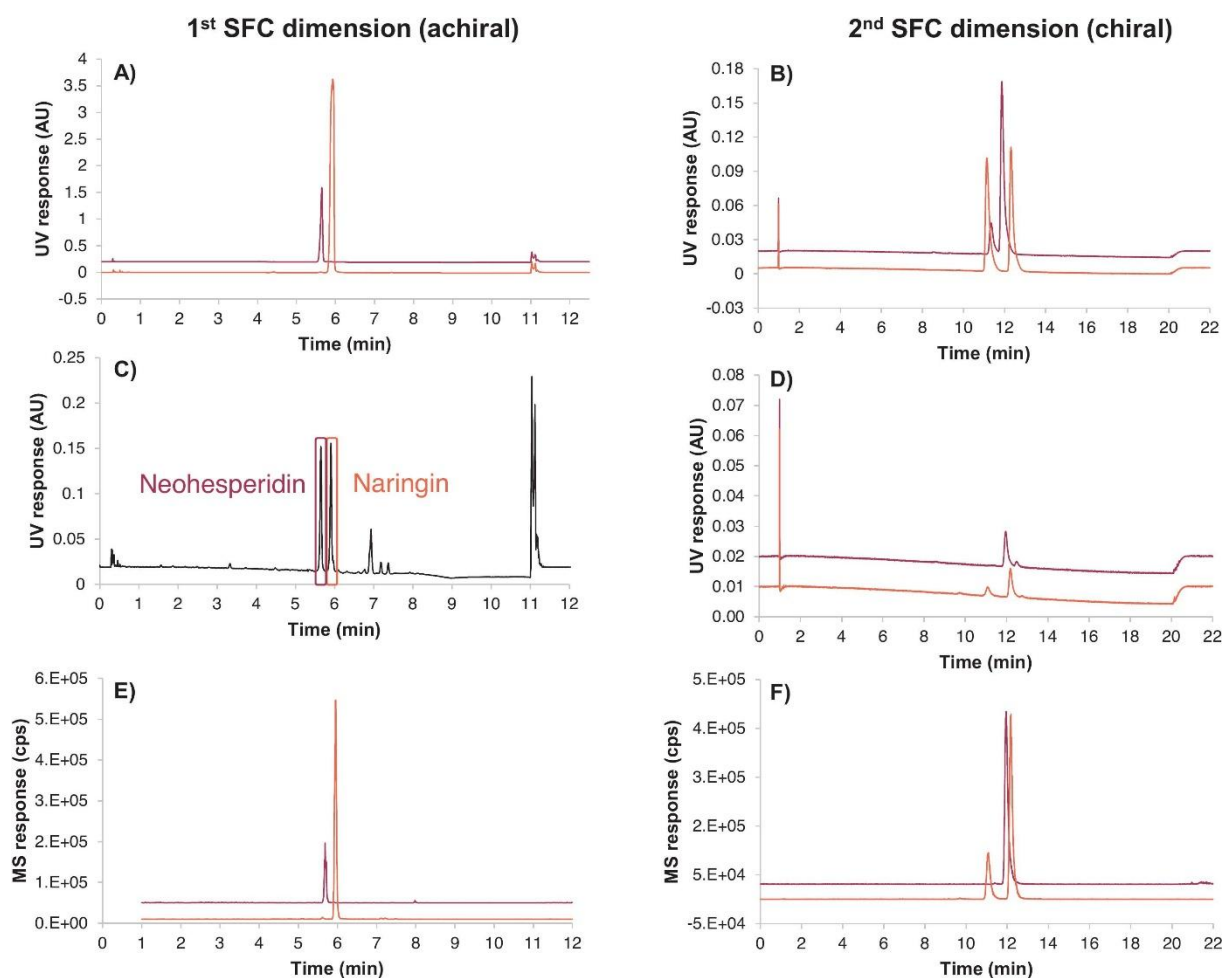


Figure IV.1.1 – Sample chromatograms obtained in the first SFC dimension (A, C, E) and second SFC dimension (B, D, F) for neohesperidin and naringin standards (A, B) and for a bitter orange peel extract (C, D, E, F) with UV detection at 280 nm (A, B, C, D) and MS detection with m/z 705 for neohesperidin and m/z 675 for naringin (E, F).

Analysis conditions for dim1: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH +0.1 % MSA, 150–110 bar, 1.7–0.6 mL/min (gradient conditions in Table IV.1.S3, gradient a). Analysis conditions for dim2: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 μm), co-solvent: MeOH +0.1 % MSA (gradient conditions in Table IV.1.S3, gradient b), 150 bar, 2.0 mL/min.

2.4.3. Mass spectrometry conditions

The optimal ESI-MS parameters were as follows: make-up fluid was either MeOH (section 3.4), or MeOH comprising 20 mM NH₄OH and 2% H₂O (section 3.2, 3.3), 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 3.7 Hz. The mass range for total ion recording was set from m/z 100 to 1000. The m/z values followed in single-ion monitoring (SIM) were: m/z 303 and m/z 273 corresponding to the aglycone protonated ion $[M - C_{12}H_{22}O_9 + H]^+$ for the positive mode, m/z 705 and m/z 675

corresponding to the MSA adduct $[M + \text{MSA}]^-$ for the negative mode. Negative mode was used for processing SFC-SFC-MS data.

Diastereomers may have different ionization efficiency. However, we had previously verified that the d.e. values obtained by UV and MS on neohesperidin and naringin standards were identical. Therefore, either one or the other detector could be used. However, since co-elution of non-isobaric compounds was observed in real samples of *C. aurantium*, UV was inadequate in this case and the MS data was retained for the calculations of d.e. values. Only for the reprocessing of the other cases, when no co-elution was observed, the UV data was examined.

2.5. LC-MS analyses

LC-MS analyses were performed on an Ultimate 3000 RSLC system, coupled with a TSQ Endura triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA), equipped with an electrospray ionization source (H-ESI). The column was SymmetryShield RP18 (150 x 3 mm, 3.5 μm) from Waters. A binary solvent system was used as mobile phase, solvent A consisting of water with 0.1% (v/v) formic acid and solvent B consisting of acetonitrile with 0.1% (v/v) formic acid. The flow rate was 0.5 mL/min, with a gradient elution where solvent B was increased from 5% to 100% in 5 min. The optimal MS parameters were as follows: spray voltage 3500 V in positive mode, 3200 V in negative mode; ion transfer tube temperature 356 °C; vaporizer temperature 420 °C; scan range m/z 100-1000; scan rate 1000 Da/sec. For product ion scan: collision gas pressure 1.5 mTorr; collision energy 30 V for the negative mode, 15 V for the positive mode.

3. Results and discussion

3.1. SFE conditions optimization

To optimize SFE conditions, a DoE was conducted on dry flowers petals. This sample was available in large quantities (larger than many of the other parts), making it ideal for the DoE. The three parameters varied and their levels are presented in Table S1.

Firstly, from the examination of samples, it soon appeared that high temperatures (60 and 80 °C) were inappropriate for these samples, since they resulted in a brown and viscous extract, probably containing burnt sugars, which tended to clog the system. Although temperature may be a significant parameter in the extraction of flavonoids in different samples, this caramel-like extract

was inadequate for further redissolution and analysis. Note that previous samples examined in comparable conditions had not necessarily shown any such issue.

Secondly, from the observation of DoE responses (peak areas of the target flavonoids and mass of naringin and neohesperidin in the extract), it appeared that experimental variability was high, with relative standard deviation at the central point reaching about 50 % in terms of peak area variability, and about 30 % for the mass of target flavonoids extracted. This high variability may be partly due to the SFE process, where significant variability is often observed, but may have been aggravated here by the reduced amount of sample used (50 mg). Another hypothesis may have been related to sample degradation during the experiments, but this can be easily ruled out, as no regular decrease was observed among the five central point replicates that were spaced out in the DoE experiments, rather a random variation.

However, as the total variance was still much larger than the variance at central point (over 100% for the peak areas and about 70% for the total mass of flavonoids extracted), the results were still amenable to interpretation.

Different models were examined to fit the data, considering linear and non-linear models, with or without interaction terms. In all cases, temperature was observed to be non-significant, possibly related to the above-described difficulties. The best fits were obtained with quadratic models, with only significant terms being reported (see Figure IV.1.S2 for further details on the models). The model obtained for the extracted mass is presented in Eq. (1):

$$\text{Extract. mass} = 11.80 + 5.18 \times C + 7.46 \times W - 2.68 \times C^2 + 2.65 \times W^2 + 6.50 \times C \times W \quad (1)$$

With $n = 17$; $R^2 = 0.823$; RMCE = 4.165.

From the models obtained and the observation of response curves, it appears that maximum extraction was obtained with the largest overall proportion of co-solvent (30% in CO₂), comprising the largest proportion of water (20% in ethanol). This is further illustrated in Figure IV.1.S3, representing the response curves depending on co-solvent proportion and water proportion. Note that the optimum point may be situated outside the design space, but we were unwilling to further increase the proportion of co-solvent, or the method would lose its ecological advantage. As observed above, low temperature should be preferred to avoid burning sugars, especially as other plant organs to be evaluated later were suspected to contain much more of them. Consequently, the extraction conditions retained for further experiments were: 40 °C, with 30% co-solvent comprising ethanol and water in 80/20 proportions.

Three experiments were repeated in these conditions. The variance observed was lower than observed at the central point (relative standard deviation was about 10%), suggesting that the

larger temperature (60 °C) may have contributed to the experimental variability at the center of the experimental domain. However, further experiments with different organs of the plant still showed significant variability so other causes of variance had to be examined.

3.2. Examination of sample mass

As mentioned above, SFE extraction conditions were optimized for dried flowers sample with a constant mass of 50 mg. However, a high variability was obtained for the central point replicates, and was further observed in the conditions of maximum extraction for other plant organs, prompting further examination of the best sample mass to introduce for accurate measurements. For this purpose, extractions were performed using increasing quantities of orange peel (PeDS), ranging from 25 mg to 1 g. Two independent samples (sample 1 and sample 2) were prepared and analyzed for each mass level. The extracts were all analyzed with the SFC-SFC-DAD-MS method, where the first dimension served to measure the quantity of naringin and neohesperidin in the extract, then, after loading them in storage loops, the second dimension provided a stereoisomeric separation for each of them to measure diastereomeric excess (d.e.).

First, it was important to determine whether the sample mass introduced in the extraction vessel had an impact on the extraction yield. Indeed, when large quantities are used and solubility of the target analytes in the extraction fluid is low, the quantity extracted may not be proportional to the quantity introduced in the extraction vessel. For this purpose, the UV peak areas observed in the first-dimension separation were measured. From the extracts, two distinct solutions were prepared: constant volume (blue) and constant concentration (green) (Figure IV1.2).

In the “constant volume” configuration, each extract was redissolved in an identical volume of dissolution solvent, so that the concentration increased proportionally to the mass introduced. The results showed a linear increase in peak area with sample mass for both compounds analyzed. This relationship was characterized by high determination coefficients ($R^2 = 0.998$ for neohesperidin and $R^2 = 0.995$ for naringin), confirming an excellent correlation between the mass of sample introduced in the extractor and the amount of flavonoids extracted. This also indicates that the solubility limit was not reached during these experiments.

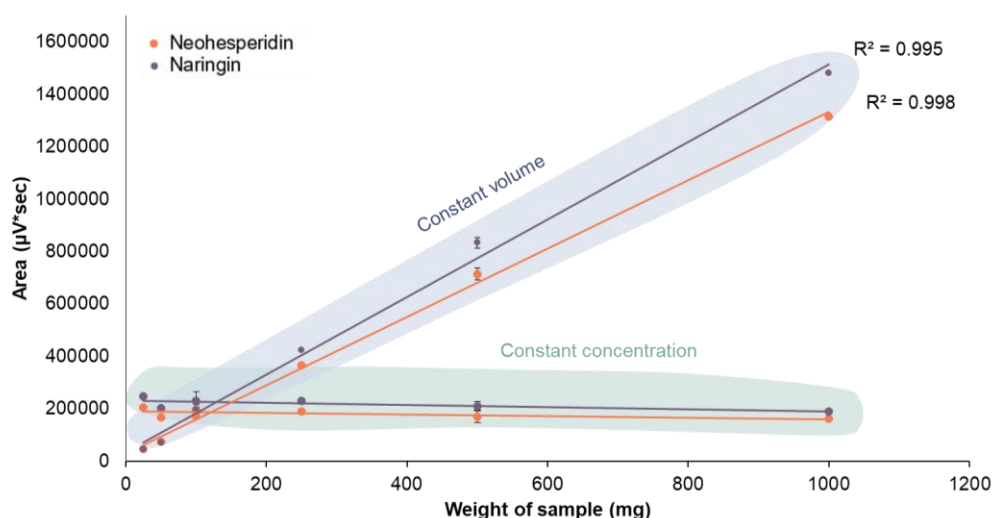


Figure IV.1.2 – Peak areas obtained from neohesperidin and naringin during the 1D analysis of a bitter orange peel sample (PeDS) extracted by SFE on the first SFC analysis dimension (dim1). Analysis with samples taken at constant volume (blue) and analysis at constant concentration (1 mg/mL). Analysis conditions were identical to Figure IV.1.1.

In the “constant concentration” configuration, the dissolution volume was adjusted depending on the mass of extract, in order to obtain identical concentration in all vials. As observed in Figure IV.1.2., the measured peak areas remained relatively stable despite the increase in bulk mass. This approach allowed samples to be compared on an equivalent basis, regardless of their mass. With this method, it appeared that the extraction yield was stable, allowing to conclude that the experiments conducted at different sample masses were comparable.

In addition to the extraction yield, a significant response to consider at this stage was the diastereomeric excess (d.e.) measured for the two target flavonoids. Indeed, diastereoisomers may have different solubility in the extracting fluid, and they may thus have been differently impacted by the sample mass. The results are shown in Figure IV.1.3, in the form of histograms comparing the d.e. (%) of neohesperidin (Figure IV.1.3A) and naringin (Figure IV.1.3B) for each mass level and for the two samples extracted individually.

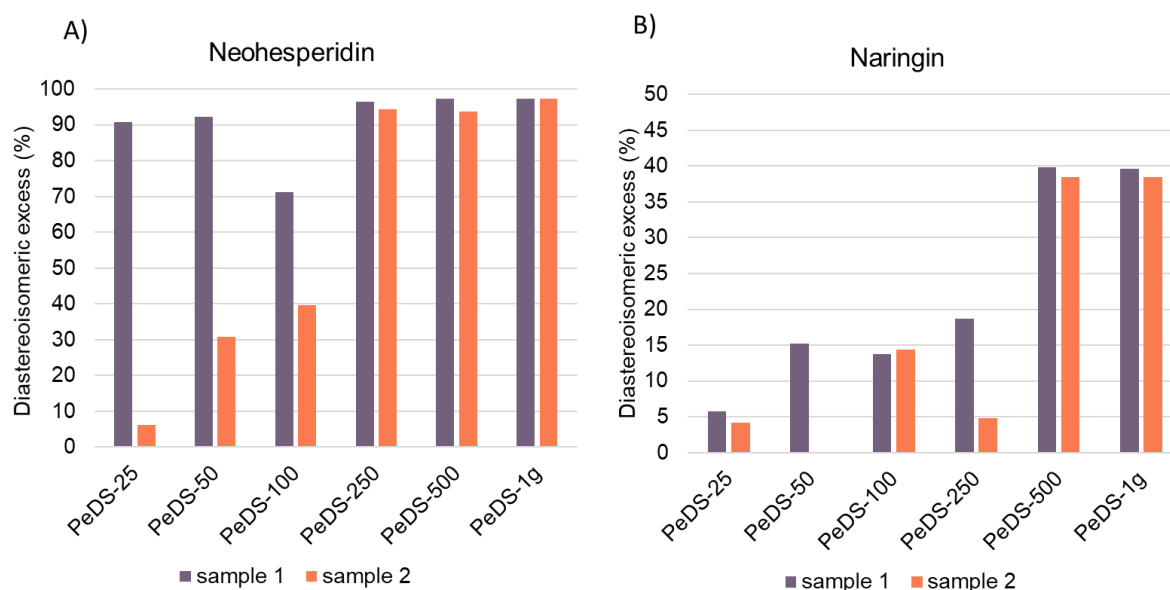


Figure IV.1.3 – Diastereomeric excess of Neohesperidin and naringin measured in SFC-SFC analysis of a bitter orange peel sample (PeDS) at different mass levels (25 mg to 1 g). Analyses performed in duplicate (sample 1 and sample 2). Analysis conditions were identical to Figure IV.1.1.

For both target compounds, it appears that the results were not repeatable when the sample amount was lower than 250 mg for neohesperidin and 500 mg for naringin. At low sample masses (from 25 to 100 or 250 mg), a large difference in d.e. was observed between the two samples. Note that the system was thoroughly rinsed between two samples, therefore we cannot suspect any carry-over effect. The increase in d.e. with extracted mass suggested that a minimum threshold was required to obtain a reliable representation of the stereoisomers. For neohesperidin, a high d.e. (above 90%) was observed in repeatable conditions. For naringin, the d.e. was lower, reaching 40% in repeatable conditions.

Thanks to the observations in Figure IV.1.2, we may conclude that the variations observed in the d.e. values (Figure IV.1.3) were not due to a difference in extraction efficiency (related to analyte solubility), but rather to secondary analytical effects (matrix effect, detection bias, or different isomer proportions in small samples). However, the use of 1 g of sample in SFE proved technically problematic, leading to recurrent clogging of the system. For this reason, a compromise had to be found between analytical reproducibility and technical feasibility. For subsequent experiments, it was determined that low sample masses (100-200 mg) were acceptable when d.e. values were not measured and only the extraction yield was of interest, while a minimal 500 mg mass should be used to determine d.e. values.

3.3. Analyte stability

To further investigate variability in lower extracted masses, stability studies were carried out on a sample of bitter orange peel.

3.3.1. Possibility of conversion

In the second-dimension SFC-UV chromatogram obtained for a peel sample, partial co-elution was observed in the retention windows corresponding to the neohesperidin diastereomers and the naringin diastereoisomers (Figure IV.1.4).

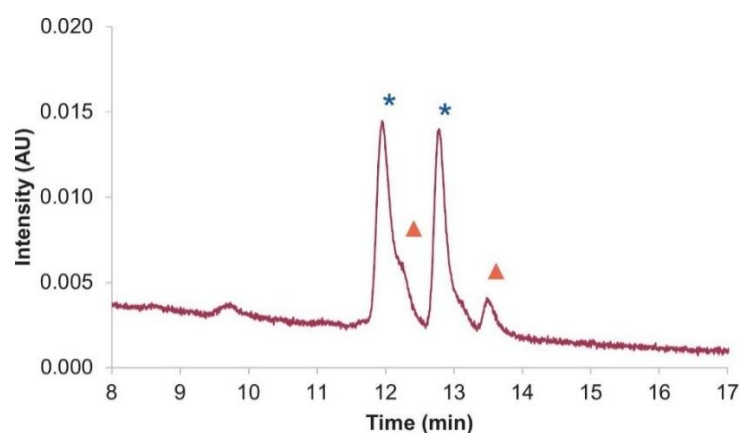


Figure IV.1.4 – dim2 SFC-SFC-UV chromatogram showing the co-elution of target flavonoids with suspected esterified flavonoids in a bitter orange peel extract where d.e. values were close to 50 %. The blue asterisks indicate the peaks of neohesperidin and the orange triangles indicate the peaks of brutieridin, attributed with MS single-ion monitoring.

Analysis conditions for dim1 were identical to Figure IV.1.1.

Mass spectrometry was used to determine the identity of the coeluted peaks, and suggested that these co-eluting species would correspond to brutieridin and melitidin, respectively (Figure IV.1.5). These two compounds are structurally close to neohesperidin and naringin, as they share the same aglycon root and the same sugar moiety, but have an additional 3-hydroxy-3-methyl-5-oxopentanoate group grafted onto the disaccharide skeleton via an ester function. Due to its possible lability, this bond could be hydrolyzed in acidic media during SFE extraction or SFC analysis and be a cause for peak area variability. Indeed, the combination of CO₂ and short-chain alcohols like methanol and ethanol produces a somewhat acidic environment through the formation of alkoxycarbonic acid [32]. In addition, the extraction conditions included water, further acidifying the medium with the formation of carbonic acid, while the analysis conditions included

methanesulfonic acid, which is a strong acid. Thus, conversion of brutieridin to neohesperidin and conversion of melitidin to naringin could occur either during the extraction, or during the analysis.

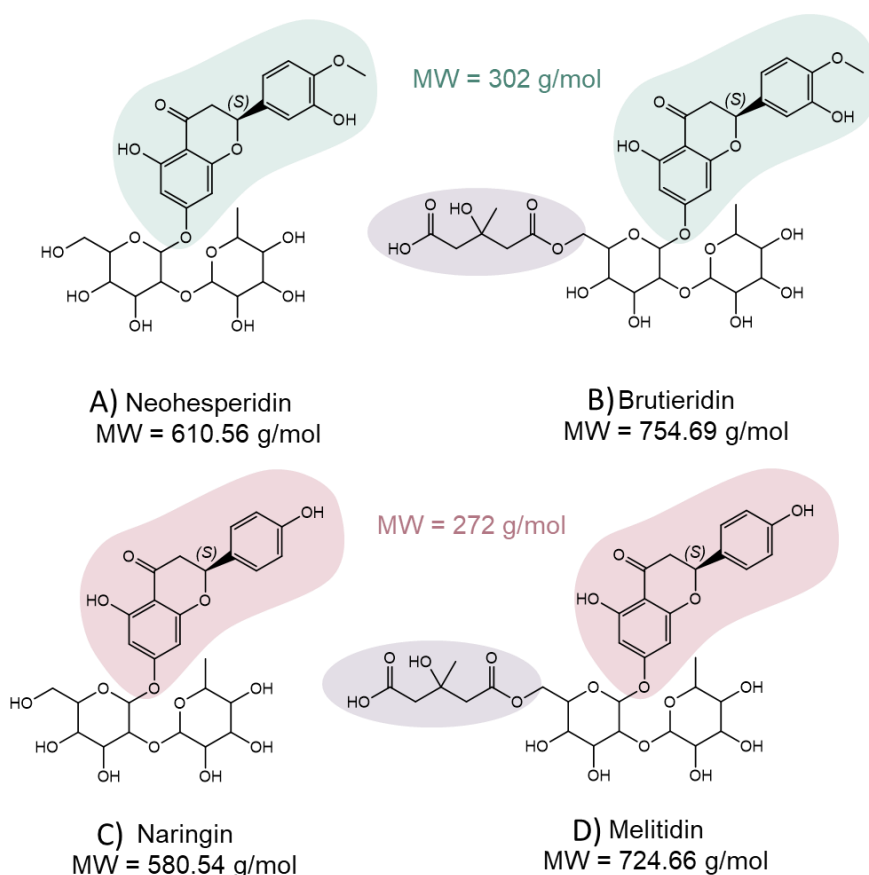


Figure IV.1.5 - Structures of the major isomers: Neohesperidin (A), Brutieridin (B), Naringin (C) and Melitidin (D)

First, two orange peel extracts (200 mg) prepared by SFE on the one hand and sonication on the other hand were subjected to LC-MS analysis in order to evaluate the possible contribution of extraction conditions on analyte degradation. The aim was to detect any conversion of brutieridin to neohesperidin, resulting from a break in the ester group. The $[M-H]^-$ deprotonated molecular ions were followed with single-ion monitoring, with values of m/z 609 for neohesperidin and m/z 753 for brutieridin. The areas of the corresponding peaks were measured (Table IV.1.S4), and the intensity ratios calculated. The results show that the ratios were comparable between the two extraction methods, indicating that the SFE step did not cause any significant degradation of brutieridin to neohesperidin.

The next step was to assess the potential impact of the acidic SFC conditions on brutieridin stability. For that, a peel extract obtained by SFE was analyzed in triplicate using the gradient method (Table IV.1.S5) in SFC-SFC-MS. The brutieridin/neohesperidin peak area ratios were calculated for each analysis (Table IV.1.S4). Although these ratios were higher than those observed in LC-MS, it was not possible to conclude on the impact of SFC conditions, as the ionization sources used for the two analyses were not identical, and the fluid composition entering the ionization source was different between LC-MS and SFC-MS experiments. Hence the ionization yield of the two analytes may have been different between the two conditions. However, the repeatability of the ratios between replicates suggested that, even if there was some conversion of brutieridin to neohesperidin during the SFC analysis, this remained constant and controlled.

3.3.2. Stability in solution

When samples were analyzed with the SFC-SFC-UV system in a multiple heart-cut configuration, the compounds of interest were temporarily stored in transfer loops before being transferred to the second dimension. This delay, which could last several hours when several fractions were stored for second-dimension analysis, could be a critical factor in terms of sample stability. Furthermore, although extraction replicates were prepared back-to-back, only the first one to be dried down was injected immediately. Subsequent samples remained in the cooled (10 °C) automated injector rack, sometimes for one or more days, before being analyzed. It was therefore essential to determine the stability window of the samples, in order to assess any degradation and adjust the preparation and storage conditions as best as possible.

For this stability study (Figure IV.1.S4), a sample of orange peel was extracted by SFE, then solubilized in an ethanol/water mixture (80/20, v/v), and analyzed repeatedly over a period of six days. To validate the results, a mixture of neohesperidin and naringin standards was subjected to the same conditions and process. During the whole stability study, the samples and standards were simply stored in the automated injector rack at 10 °C.

The results showed that after six days, no degradation was observed for either the peel sample or the standards. In the peel sample, the d.e. values of neohesperidin were stable at 100%, indicating that only one diastereomer was observed. On the other hand, some fluctuations of around 5% were observed for naringin. However, these variations do not follow a trend, suggesting that they are not the result of sample degradation, but probably of analytical variability. This is probable since the excess is small, the composition of naringin diastereoisomers being close to 50/50.

3.4. Determination of diastereomeric excess in *C. aurantium* samples

Having thoroughly examined the extraction conditions and stability of the stereoisomers, we were confident that the SFE and SFC-SFC-MS methods provided reliable results, and could then proceed to examine a variety of samples.

3.4.1. Fruit parts

In this section, three stages of fruit maturity were studied: ripe, half-ripe and unripe. For each of these maturity stages, different parts of the fruit were extracted: peel, albedo and pulp, then analyzed with SFC-SFC-MS to determine the d.e. values of neohesperidin and naringin.

In the case of neohesperidin, whatever the stage of ripeness, the d.e. remained very high, between 98% and 100% for all three parts of the orange. In other words, neohesperidin showed very high stereochemical stability (Figure IV.1.6A), with one diastereoisomer being largely dominant. The lack of variability may mean that neohesperidin biosynthesis is little affected by developmental or environmental processes such as UV exposure. This suggests that the downstream enzymes involved in its biosynthesis (such as flavanone 3-hydroxylase or other glycosyltransferases [33,34]) maintain high stereochemical selectivity, independently of maturity, and/or that neohesperidin and its aglycon precursor hesperitin are both rather stable and unaffected by other non-enzymatic ring-opening reactions (Figure IV.1.7).

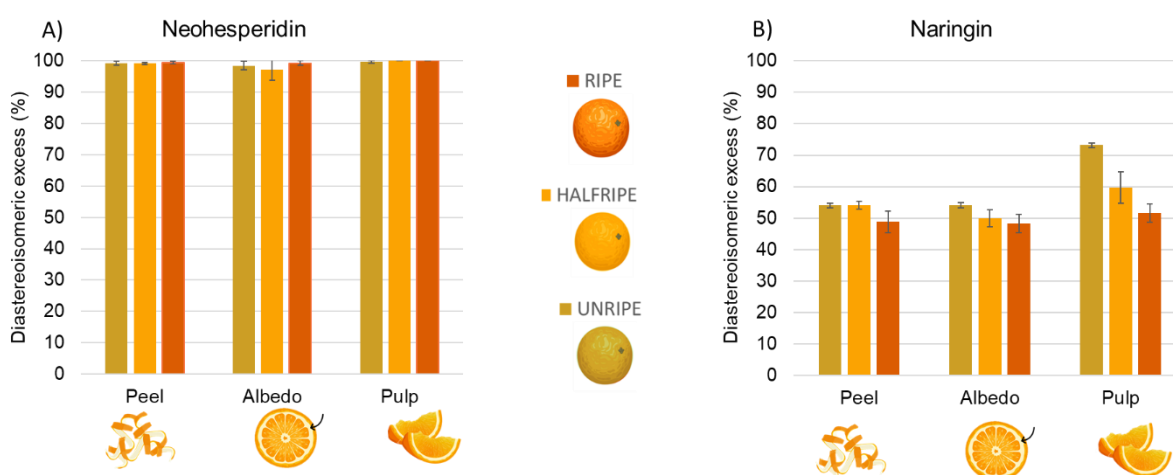


Figure IV.1.6 - Comparison of the d.e. of neohesperidin (A) and naringin (B) for 3 maturity stages (unripe, half-ripe and ripe) and for 3 parts of the fruit (peel, albedo and pulp). Error bars are based on two replicates. SFC-SFC conditions are identical to Figure IV.1.2.

On the other hand, the d.e. values observed for naringin were lower (Figure IV.1.6B), in the 45 to 75 range, thus indicating that both diastereomers were present with a significant concentration. In addition, some variation was observed with the stage of maturity, with bigger variations observed for the pulp samples, showing a decrease of about 70% to 50% between the unripe and ripe stages, indicating progressive racemization of naringin. To understand the differences between naringin and neohesperidin and the variations with maturity, the enzymatic and non-enzymatic pathways must be better explained. They are detailed in Figure IV.1.7.

First, starting from a chalcone precursor, the chalcone isomerase (CHI) catalyzes the stereospecific cyclization into the sole 2S-naringenin isomer, the aglycone form of naringin [35]. The 2S enantiomer of hesperitin, the aglycon form of neohesperidin, is subsequently formed from the 2S-enantiomer of naringenin, through the action of downstream enzymes: flavonoid 3'-hydroxylase (F3'H), flavonoid 4'-O-methyltransferase (F4'OMT) and further glycosyltransferases. Furthermore, the 2S-naringenin enantiomer may also face ring-opening and non-stereospecific closing of the cycle through non-enzymatic processes [36], thereby causing progressive racemization through the formation of both R and S enantiomers (see "non-enzymatic racemization" pathway in Figure IV.1.7). This may be favored by UV exposure and high temperature [37]. Finally, the 2R-enantiomer of naringenin may also yield a parallel cascade to produce 2R-naringin, 2R-hesperitin and 2R-neohesperidin.

The CHI enzyme-catalyzed step has been estimated to be 107 times faster than spontaneous cyclization [38]. This supports the initial formation of 2S enantiomers with a large enantiomeric excess. Thus, in the absence of further metabolism, the accumulation of the S-isomer of naringin is thermodynamically favored by enzymatic production, while the R-isomer will be formed over time by the non-enzymatic and non-stereospecific racemization as described above. The gradual racemization of naringin with increasing maturity observed in the present study is consistent with previous literature related to the maturation of citrus fruits [20,36,39], in particular in pummelo and sour oranges [36,40].

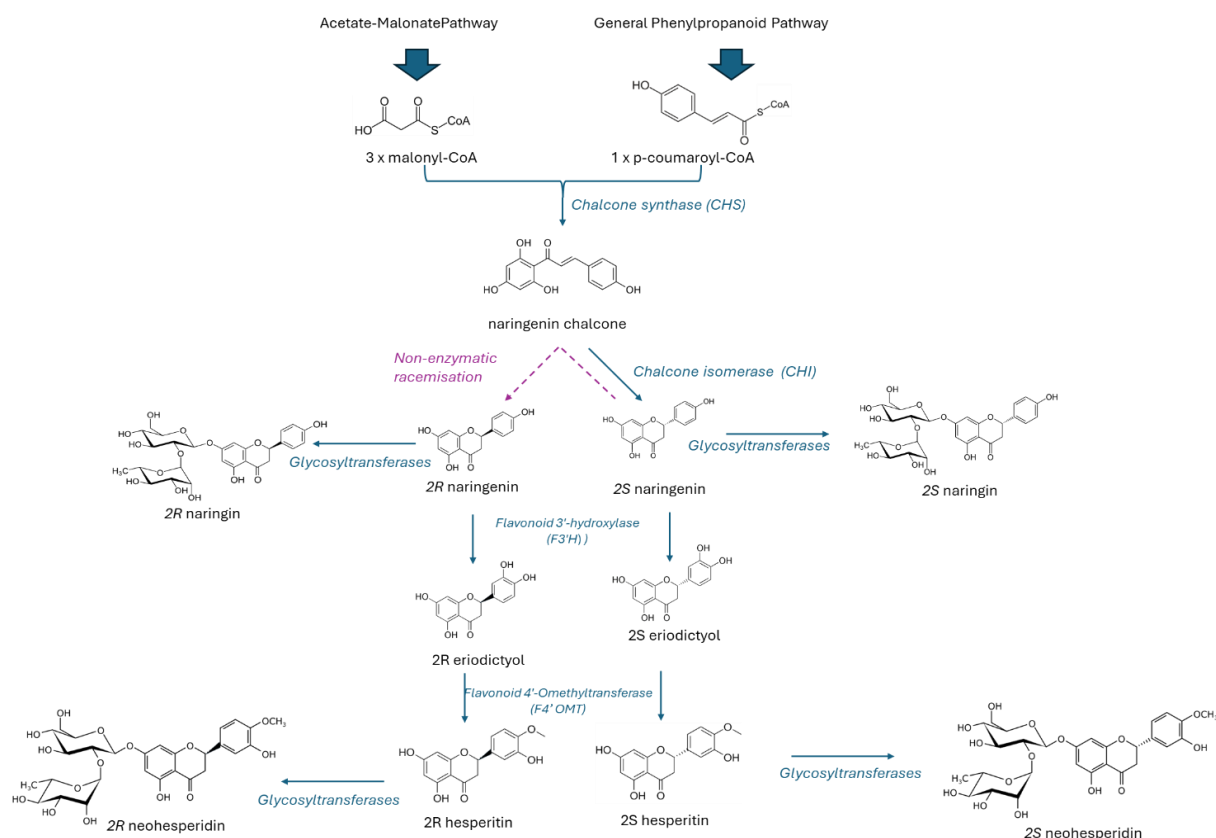


Figure IV.1.7 - Biosynthesis of 2R-2S-neohesperidin and 2R-2S-naringin

Since the enzymes downstream CHI to produce subsequent intermediates do not exhibit as strict a stereochemical preference as CHI, the stereochemistry of these intermediates therefore depends on the efficiency (substrate binding and turnover) of the subsequent steps, in particular the following F3'H step. The S-isomer versions of the subsequent intermediates therefore result from the use of the S-naringenin precursor having been formed both non-enzymatically during the racemization step as well as by stereospecific enzymatic production from CHI (the latter being favored from a thermodynamic point of view), while the R-intermediates are formed after conversion of the R-naringenin produced solely by non-enzymatic formation.

In our case, we can assume that the relative concentrations of R- vs. S-naringenin across plant organs and maturation stages will depend on the binding affinity and catalytic turnover of F3'H toward the R- and S- isomers, as well as the thermodynamic reaction equilibrium of this enzymatic step at cellular level in these different plant organs. A low conversion rate of F3'H from S-naringenin formed by CHI allows for a higher formation of R-naringenin, and other subsequent R-intermediates, such as the here studied hesperidin, by giving more time to the non-enzymatic racemization reaction to occur. It is noteworthy that in the case of hesperidin, binding affinity and catalytic turnover of F3'H to S-naringenin formed by CHI will favor the accumulation of S-hesperidin by limiting the slower non-enzymatic racemization reaction.

In other words, the fact that neohesperidin retains a high d.e. value in all samples and conditions (Figure IV.1.6A) suggests the sole use of the 2S isomer of naringenin for the downstream biosynthetic steps, leading to the accumulation of 2S-neohesperidin.

Moreover, it is worth mentioning the d.e. may also be influenced by the possibility of physical interactions between enzymes. Indeed, CHI together with other enzymatic partners involved in flavonoid biosynthesis have been shown to form a metabolon [41], a multi-enzyme complex in which soluble enzymes, including CHI, are anchored to the endoplasmic reticulum by association with membrane enzymes belonging to the cytochrome P450 (CYP) family such as F3'H, which serve as a stabilizing platform for this supramolecular enzymatic complex, allowing rapid channeling of intermediates between active sites, increasing local concentrations and preventing the formation of side-products. Thus, in our case, the possible existence of such metabolon could favor the accumulation of S-hesperidin and so the depletion of S-naringenin.

Finally, to explain the differences between the fruit organs, another option is that the naringin diastereomers have differential mobility in the fruit, resulting in varying stereoisomeric compositions when a single part of the fruit is observed. As the pulp contains a liquid portion (or juice), the analyte mobility may be higher there than in dryer tissues like albedo and peel.

3.4.2. Diastereomeric excess of other plant organs

After characterizing the d.e. of neohesperidin and naringin in the different fruit fractions (peel, albedo and pulp), the analysis was extended to other vegetative organs (leaves and flowers) and to compare samples from various geographical origins.

3.4.2.1. Leaves of different geographical origins

Three leaves samples were compared (Figure IV.1.8A): fresh leaves from the harvest of oranges at Vergers de Boirie and that were dried in-house, leaves bought as dried material from Louis Herboristerie, and leaves received as dried material from Oujda in Morocco.

In Figure IV.1.8A, neohesperidin showed a high d.e. comprised between 85 and 98% in the three samples, confirming its stability already observed in fruit tissues, which showed its resistance to variations related to the environment or post-harvest treatments. In contrast, naringin showed marked variations depending on the origin. Moroccan leaves showed a high d.e. (around 75%), close to that observed in fresh orange pulp and peel. The d.e. values were lower (around 40%) for the leaves from Vergers de Boirie and even lower (around 30%) for the leaves of Louis Herboristerie, accompanied by greater inter-sample variability.

These differences could reflect less controlled drying or storage conditions, greater exposure to UV light, or pre- or post-harvest degradation. Furthermore, commercial samples did not come from the same harvest, thus climatic or agronomic variations may also have impacted the results.

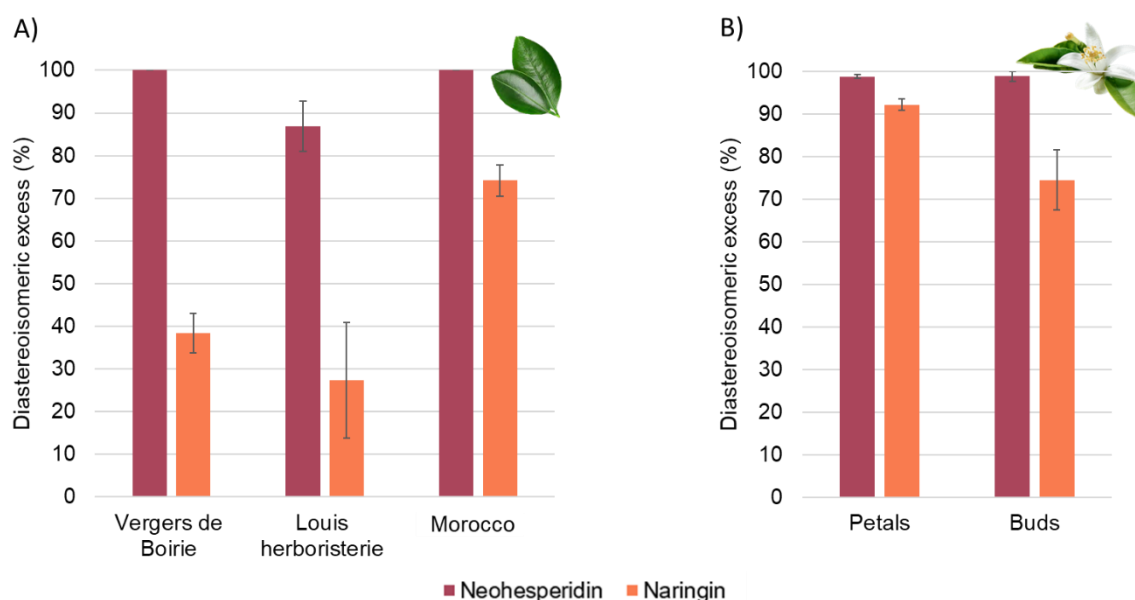


Figure IV.1.8 - Comparison of the d.e. values of neohesperidin (purple) and naringin (orange) for two plant organs: leaves (A) and flowers (B). The leaves come from different sources (Vergers de Boirie, Louis herboristerie and Oudja in Morocco). Error bars are based on two replicates. SFC-SFC analysis conditions are identical to Figure IV.1.2.

3.4.2.2. Flowers: comparison between petals and flower buds

Two types of tissue were studied: flower petals and flower buds (Figure IV.1.8B), both provided by Louis Herboristerie. As with the previous samples, the d.e. of neohesperidin was greater than 95%, once again demonstrating its stability whatever the nature of the plant tissue. For naringin, the d.e. was also high in petals (around 90%), but lower in buds (around 70%), with larger variability.

3.4.3. Influence of drying method on orange peels

Following the observations on dry leaves, the influence of drying method on flavonoid d.e. values may be questioned. Two drying methods were applied on the peels of ripe oranges harvested at Vergers de Boirie. The first method was based on freeze-drying, a cold vacuum dehydration process, while the second was a conventional oven drying. The samples obtained in this way were compared with a commercial sample from Louis Herboristerie, delivered as dry material, and for

which the post-harvest treatment conditions (drying, storage, exposure to light) were not known. The results are presented in Figure IV.1.S5.

Neohesperidin had a very high and constant d.e. close to 100% in all three types of samples. This stability suggests that this molecule is not very sensitive to drying conditions or heat treatments, which indicates strong resistance to isomerization.

On the other hand, naringin always showed a significantly lower d.e. than neohesperidin. Although the freeze-dried and oven-dried samples showed comparable values of around 50%, the commercial sample showed a significantly lower d.e. (around 30%). This decrease in d.e. could be explained by increased exposure to degrading factors such as light (particularly UV), humidity, unsuitable temperatures or possible metabolization by associated bacteria such as Actinobacteria, known to be able to act on naringenin derivatives [42], during the drying or storage stages, which favored isomerization or partial degradation of naringin. It should also be noted that the samples from Louis Herboristerie did not come from the same harvest as the other peels tested. Consequently, variables such as geographical location (edaphic and climatic variations) and agronomic practices could also have influenced the stereochemistry of the compounds analyzed.

Thus, although the drying method seemed to have little influence on the stereochemistry of neohesperidin, naringin appeared to be more vulnerable, particularly under post-harvest conditions. These observations underline the importance of controlling the processing methods used to preserve the flavonoids of interest.

4. Conclusion

This study showed that SFE extraction from low sample masses results in consistent results in terms of overall extraction yield, indicating consistent extraction efficiency, but high variability in the ratios of stereoisomers, probably resulting from local variability in the different matrices examined. To achieve a reliable measurement of stereoisomeric ratio, a minimum sample mass of 500 mg was required to achieve a good balance between analytical reproducibility and technical constraints. In addition, it was demonstrated that the extraction conditions did not significantly degrade the flavonoid compounds examined. The impact of acidic analysis conditions could not be ascertained but appeared to be repeatable.

The various plant parts studied showed that neohesperidin was particularly stable and unaffected by UV, enzymatic or non-enzymatic reactions, or fruit ripening, as the major diastereoisomer was present in all conditions, with d.e. values nearing 100%. Naringin, on the other hand, appeared

more sensitive to these effects, with varying stereoisomeric ratios observed among the samples, ranging from 30 to 90%.

These variations can affect the taste, bioavailability, and biological characteristics of flavonoids. Finally, it is important to regulate cultivation and processing conditions to maintain flavonoid stereochemistry in plant matrices, especially when application for therapeutic purposes is desired.

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Supplementary information

Table IV.1.S1 - Three factors and levels in the Box-Behnken design of experiments (DoE) used to optimize extraction. Oven temperature (T), total percentage of co-solvent (C) and water percentage introduced in the co-solvent (W), with three levels coded as (+1) highest level, (0) middle level and (-1) lowest level. See Table IV.1.S2 in supplementary information for detailed experiments.

Factors	levels		
	-1	0	1
T (°C)	40	60	80
C (%)	10	20	30
W (%)	0	10	20

Table IV.1.S2 - Box -Behnken design of experiments to optimize SFE conditions. T is the oven temperature, C is the percentage of co-solvent introduced in pressurized CO₂, W is the percentage of water in the ethanol co-solvent. Although the table is organized in this view, it was randomized before doing the experiments.

#	T (°C)	C (%)	W (%)
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0
16	0	0	0
17	0	0	0

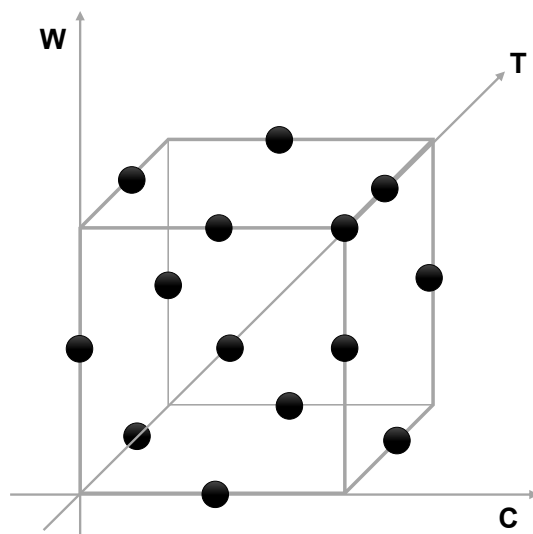
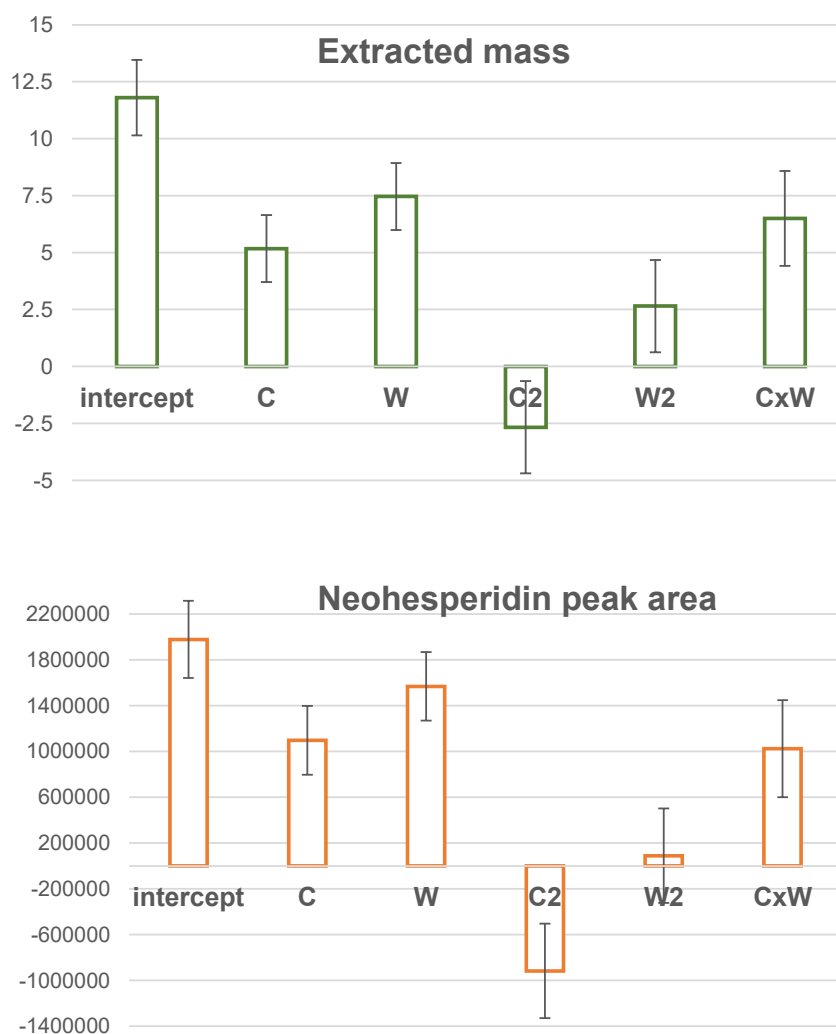
Figure IV.1.S1 - Representation of 13 experimental conditions tested in the Box-Behnken design. The central point was repeated 5 times.**Figure IV.1.S2** - Significant terms in the quadratic models established for the total extracted mass and the neohesperidin peak area, based on the 17 experiments in the Box-Behnken design.

Figure IV.1.S3 - Response curves observed for the total extracted mass and peak area of neohesperidin, based on the quadratic models in Figure IV.1.S2, and depending on the percentage of co-solvent introduced in pressurized CO₂ (C) and the percentage of water introduced in ethanol co-solvent (W).

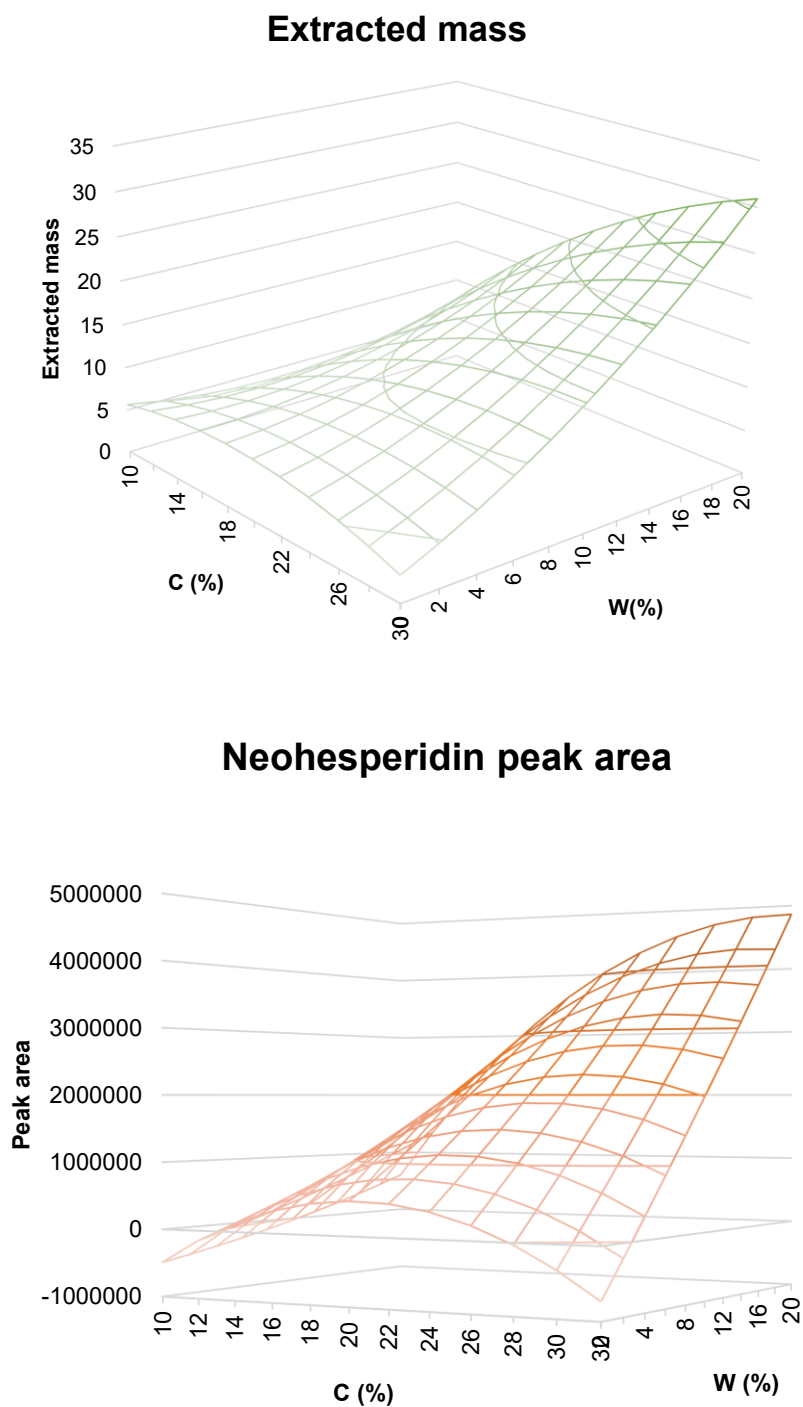


Table IV.1.S3 - Gradient profiles in the two dimensions of the SFC-SFC method.

Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)
a. First-dimension method			
0.0	20	150	1.7
0.5	20	150	1.7
8.0	100	110	0.6
10.0	100	110	0.6
10.5	20	110	0.6
12.0	20	150	1.7
12.5	20	150	1.7
b. Second-dimension method			
0.0	15	150	2.0
0.5	15		
17.0	50		
19.0	50		
19.5	15		
22.0	15		

Table IV.1.S4 - Peak areas and ratios obtained for neohesperidin (*m/z* 609) and brutieridin (*m/z* 753) with LC-MS analysis of two samples of bitter orange peel (Pe-DS) extracted with maceration or SFE.

Analysis conditions: SymmetryShield RP18 (150 × 3 mm, 3.5 µm). solvent A: H₂O + 0.1% FA, solvent B: ACN + 0.1 % FA, gradient: 5% to 100% of B in 5 min, 0.5 mL/min, ion source: ESI.

		Area (µV*sec)	
		Maceration	SFE
1	Neohesperidin (<i>m/z</i> 609)	211166108	192656524
2	Brutieridin (<i>m/z</i> 753)	179542576	177697634
	Ratio (1/2)	1.18	1.08

Table IV.1.S5 - Peak areas and ratios obtained for neohesperidin (*m/z* 609) and brutieridin (*m/z* 753) with three replicate SFC-SFC-MS analyses of a sample of bitter orange peel (Pe-DS) extracted with SFE

Analysis conditions for dim1: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm), co-solvent: MeOH + 0.1% MSA, 150-110 bar, 1.7-0.6 mL/min (gradient conditions in Table IV.1.S3, gradient a). Analysis conditions for dim2: CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 µm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table IV.1.S3, gradient b), 150 bar, 2.0 mL/min.

		Area (µV*sec)		
		Rep 1	Rep 2	Rep 3
1	Neohesperidin (<i>m/z</i> 609)	39865346	29219825	27316053
2	Brutieridin (<i>m/z</i> 753)	13125267	9459259	6875987
	Ratio (1/2)	3.04	3.09	3.97

Figure IV.1.S4 - 6-day stability study of the d.e. of neohesperidin (A) and naringin (B) in an orange peel sample (blue) and in standards (purple).

SFC-SFC analysis conditions are identical to Figure IV.1.2.

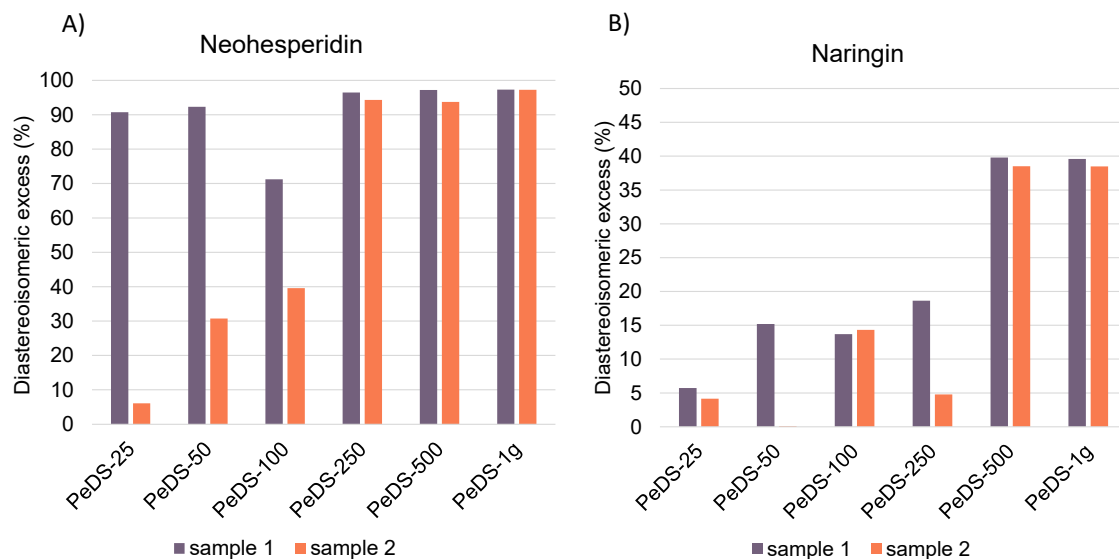
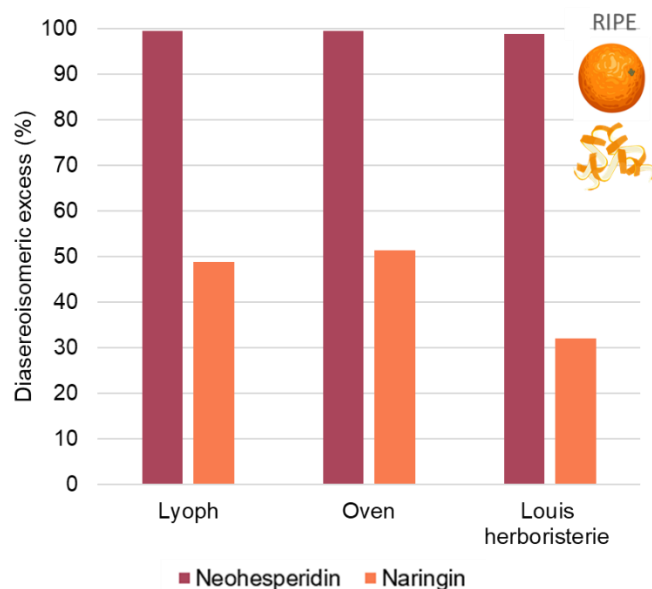


Figure IV.1.S5 - Comparison of the d.e. values of neohesperidin (purple) and naringin (orange) for samples of ripe orange peel with different drying methods (lyophilisation, oven and unknown drying conditions for the commercial sample from Louis Hesboristerie).

SFC-SFC analysis conditions are identical to Figure IV.1.2.



Partie 2 : Choix de colonne chirale et application au miel

Article 4: Stereoisomeric separation of flavonoids by two-dimensional supercritical fluid chromatography: identification of adequate chiral columns and application to honey analysis

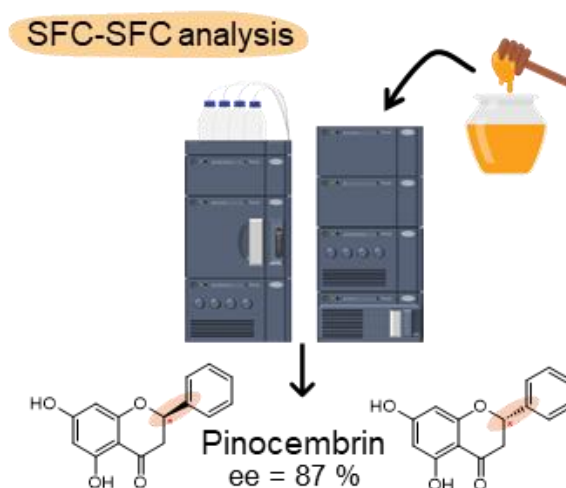
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Graphical abstract



Abstract

Chiral flavonoids are frequently encountered in natural products consumed as food or therapeutic products. However, the taste and other bioactivities of stereoisomers are known to differ. Hence, stereoseparation methods are necessary to resolve these isomers, whether to measure stereoisomeric ratios or to purify individual stereoisomers for activity testing. In this study, we looked for the best conditions to resolve different families of chiral flavonoids, including aurones, flavanones, aglycones, and glycosylated flavanones. Furthermore, we aimed to include this stereoseparation as the second-dimension method in a two-dimensional supercritical fluid chromatography system (SFC-SFC), to achieve the resolution of chiral flavonoids in complex samples. First, a set of 10 standards of chiral flavonoids was examined on 5 immobilized polysaccharide chiral stationary phases. With the help of Derringer desirability functions, the columns offering the best options for rapid resolution were identified. Then, a sample of honey was analyzed in an SFC-SFC system in heart-cutting mode. The first-dimension achiral separation

resolved the complex mixture of honey and isolated the fraction of pinocembrin enantiomers, which were re-analyzed in the second-dimension chiral separation to measure the enantiomeric excess, and resolve co-eluting isobaric species. This experiment demonstrates the relevance of this strategy in the resolution of flavonoid enantiomers from complex samples.

Keywords: flavonoids; honey; natural products; supercritical fluid chromatography; two-dimensional chromatography

1. Introduction

Chirality is a significant factor in the bioactivity of chiral flavonoids [1,2]. The latter are natural polyphenolic compounds frequently found in complex natural and food matrices such as honey [3,4]. The stereoisomers of a chiral flavonoid may have very different tastes [5], pharmacological properties, biological interactions, or bioavailability, making their separation and individual characterization essential to improve understanding of these effects [6]. Different chromatographic methods have been proposed for this purpose, mostly in the liquid phase (LC). While derivatization followed by achiral reversed-phase analysis was long employed, it has now been largely supplanted by direct resolution on a chiral stationary phase, where polysaccharide and cyclodextrin selectors are largely dominating the field [7]. In this context, supercritical fluid chromatography (SFC) is emerging as a promising environmentally friendly technique, combining high resolution with speed, particularly for chiral separations [7–9].

The success of an enantioselective SFC method largely depends on the judicious choice of the chiral stationary phase [10,11]. A wide range of chiral selectors is available to interact selectively with stereoisomers, based on bonded ligands [12,13], macrocycles [14] or polymers. Among those, the most used in chiral SFC are based on the natural polymers of cellulose or amylose derivatives, which were initially introduced by Okamoto and co-workers [15,16], and were firstly commercialized by Daicel Corporation and Chiral Technologies. The older phases mainly comprised non-halogenated aromatic groups, while more recently commercialized phases developed by Chankvetadze and co-workers [17] included chlorinated ligands to diversify the interactions between chiral analytes and chiral selectors, thereby offering alternate selectivity [18]. In addition, later developments proposed by Francotte and co-workers [19] improved column stability through the immobilization of the polysaccharides. Indeed, older versions of these stationary phases were simply coated on the modified silica surface, rendering them prone to column bleeding in strong solvents. On the contrary, the modern, immobilized versions are much

more stable, thus offering more possibilities for mobile phase optimization [20]. As a result, there is now a large diversity of such stationary phases available to increase the probability of achieving chiral resolution for any pair of stereoisomers. However useful these stationary phases may be, the predictability of a separation is still rather poor, making the screening of chiral columns an essential step in method development [21].

To rationalize the choice of a stationary phase to resolve multiple flavonoid stereoisomers in a timely fashion and with good chromatographic quality, a multi-criteria approach based on the Derringer and Suich method is appropriate [22]. This method allows several analytical responses to be weighted according to their significance, offering a versatile strategy that is adapted to the complexity of the chromatographic parameters involved. Such desirability functions can be used for column screening [23,24] or analysis conditions [25,26].

Moreover, flavonoids in natural products may be included in samples of high complexity. Consequently, the simple use of one chiral column would not necessarily be sufficient to resolve the targeted stereoisomers from all other compounds present in such samples. For this reason, it is useful to include the stereoseparation method in a two-dimensional chromatographic system, where the first dimension may be an achiral analysis, serving to isolate the target flavonoid stereoisomers, and the second dimension would serve to resolve the stereoisomers. Therefore, a heart-cut SFC-SFC instrument we had previously developed [27] was used in the present study to demonstrate the feasibility of this approach in the analysis of a complex honey sample.

2. Experimental

2.1. Chemicals and reagent

HPLC-grade methanol used as the mobile phase and sample diluent, was purchased from VWR (Fontenay-sous-Bois, France). CO₂ with a purity of 99.7% was delivered by Air Liquide (Paris, France). Ultra-pure water was supplied by a Milli-Q IQ 7000 system from Merck (Darmstadt, Allemagne). Methanesulfonic acid (MSA) was purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Hydrochloric acid was purchased from ThermoFisher Scientific (Illkirch-Graffenstaden, France)

The flavonoid standards were kindly provided by Extrasynthese (Genay, France): pinocembrin, naringenin, hesperetin, pinocembroside, naringenin-7-O-glucoside, naringin, neohesperidin, taxifolin, dihydromyricetin, and alphonosin. As will appear in the following, the stereoisomeric ratio was unknown at this stage.

The honey sample was produced in the territorial collectivity of French Guiana and harvested in the commune of Remire Montjoly in December 2023. It is classified as forest honey.

2.2. Sample preparation

Flavonoid standard solutions were prepared at a concentration of 1 mg/mL in MeOH.

Honey polyphenols were extracted by solid-phase extraction (SPE) following a procedure adapted from previous works [28] as follows: 3 g of honey was dissolved in 10 mL of Milli-Q water acidified with HCl to pH = 2. The sample was then stirred magnetically at room temperature for 10 min and filtered through a 0.45 µm filter. The SPE C18 cartridge (Starta C18-U, 55 µm, 70 Å, 500 mg/6 mL, Phenomenex) was preconditioned with 10 mL MeOH followed by 10 mL Milli-Q water acidified to pH = 2. The sample was then passed through the cartridge at a flow rate of 2 mL/min. The cartridge was then rinsed with 10 mL of Milli-Q water acidified to pH = 2 to remove undesirable compounds, then 10 mL of MeOH eluted the polyphenols. The extract was then evaporated to dryness and reconcentrated in 0.5 mL MeOH for analysis.

2.3. Supercritical fluid chromatography (SFC)

2.3.1. Instrumentation

The supercritical fluid chromatography system was a Waters Corporation (Milford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography (UPC²). It was equipped with an autosampler with a partial loop volume injection system, an automated back-pressure regulator (BPR), a column oven compatible with two 150-mm length columns, via column switching valves. The system also included a binary solvent delivery pump compatible with a maximum mobile phase flow rate of 4 mL/min and a maximum pressure of 414 bar. Two detectors were available: a diode-array detector (ACQUITY PDA) and a single-quadrupole mass spectrometer equipped with an electrospray ionization source (ACQUITY QDa). An isocratic solvent manager was used as a make-up pump and was positioned before a flow splitter, where one portion of the flow was directed to the mass detector and the other was sent to the back-pressure regulator. Empower 3 was used for chromatographic acquisition and for data treatment.

2.3.2. Analysis conditions

For the analysis of all the standards, different immobilized polysaccharide stationary phases were obtained from Chiral Technologies (Illkirch-Graffenstaden, France). CHIRALPAK IA-3, IB-3, IC-3, IG-3 and IJ-3 (150 × 4.6 mm, 3.0 μm) were used. Their structures are presented in Figure IV.2.1. The mobile phase used was CO₂ with MeOH. The injection volume was 2 μL. The screening conditions used for IG-3 (gradient 1.b.) and for the other columns (gradient 1.a.) are presented in Table IV.2.1.

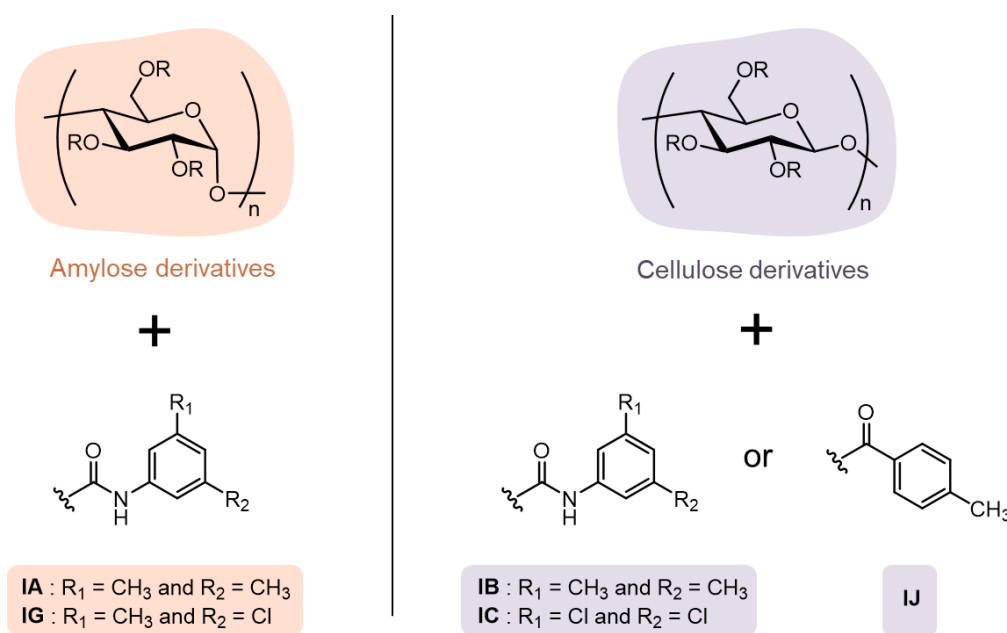


Figure IV.2.1 - Structures of the CHIRALPAK IA-3, IB-3, IC-3, IG-3 and IJ-3 compared in this paper.

For the analysis of the honey sample in SFC-SFC-DAD-MS, two different columns were used. The first-dimension (dim1) column was ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm) from Waters to isolate the target flavonoids (pinocembrin enantiomers) from the other compounds in the honey sample. The second-dimension (dim2) column was CHIRALPAK IG-3 (150 × 4.6 mm, 3.0 μm) from Chiral Technologies (Illkirch-Graffenstaden, France). This served to separate the enantiomers of pinocembrin. The mobile phase used was CO₂ with MeOH containing 0.1% MSA for dim1, and only MeOH for dim2. The injection volume was 5 μL. The gradient programs, including variation of solvent composition, back-pressure and flow rate, are presented in Table IV.2.1-Gradient 2.

Table IV.2.1 - Gradient profiles used at all stages for column screening or for SFC-SFC analysis of the honey sample

Gradient 1				Gradient 2			
Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)	Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)
a. Gradient used for IA, IB, IC, IJ columns				a. First-dimension method			
0	5	150	2.0	0.0	2	150	1.7
0.5	5			1.0	2	150	1.7
12	50			7.0	30	130	1.7
13	50			10.0	90	110	0.6
13.1	5			11.0	90	110	0.6
14	5			12.0	60	130	0.9
15	5			13.5	30	150	1.2
				15	2	150	1.5
				16	2	150	1.7
b. Gradient used for IG column				b. Second-dimension method			
0	5	150	2.0	0	5	150	2.0
0.5	5			0.5	5		
25	100			12	50		
27	100			13	50		
27.5	5			13.1	5		
28	5			14	5		
30	5			15	5		

The column oven was heated to 30°C and the sample compartment was kept at 10°C. All chromatograms were recorded in the 210–400 nm range, with 1.2 nm resolution. Scan rate was 20 Hz. Visualization and peak integration were done at 280 nm for standards and sample.

2.3.3. Mass spectrometry conditions

The previously optimized MS parameters were as follows [29]: make-up fluid was MeOH, 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 3.7 Hz. The mass range for total ion recording was set from m/z 100 to 1000. The honey sample was analyzed in dim2 with single-ion monitoring (SIM) mode to target pinocembrin at m/z 257.

3. Results and discussion

3.1. Column Screening

3.1.1. Set of compounds

A set of 10 flavonoid compounds was selected for the column screening. This set consisted of three major classes of chiral flavonoids: auronols, flavanonols, and flavanones (Figure IV.2.2).

The flavanones selected included a wide structural diversity with aglycon structures (hesperetin, naringenin, pinocembrin) and glycosylated structures comprising monosaccharide (naringenin-7-O-glucoside, pinocembroside) and disaccharide moieties (naringin, neohesperidin). While the

aglycon flavonoids in this selection comprise one or two chiral centers, the glycosylated ones contain many more chiral centers in the sugar moieties. For this reason, aglycon molecules would yield one or two pairs of enantiomers while the glycosylated molecules would yield a pair of diastereomers, only considering the chiral center on the genin root. Strictly speaking diastereoisomers could be resolved in an achiral environment. However, in our experience, the diastereoisomers of flavonoids are mostly unresolved on achiral columns.

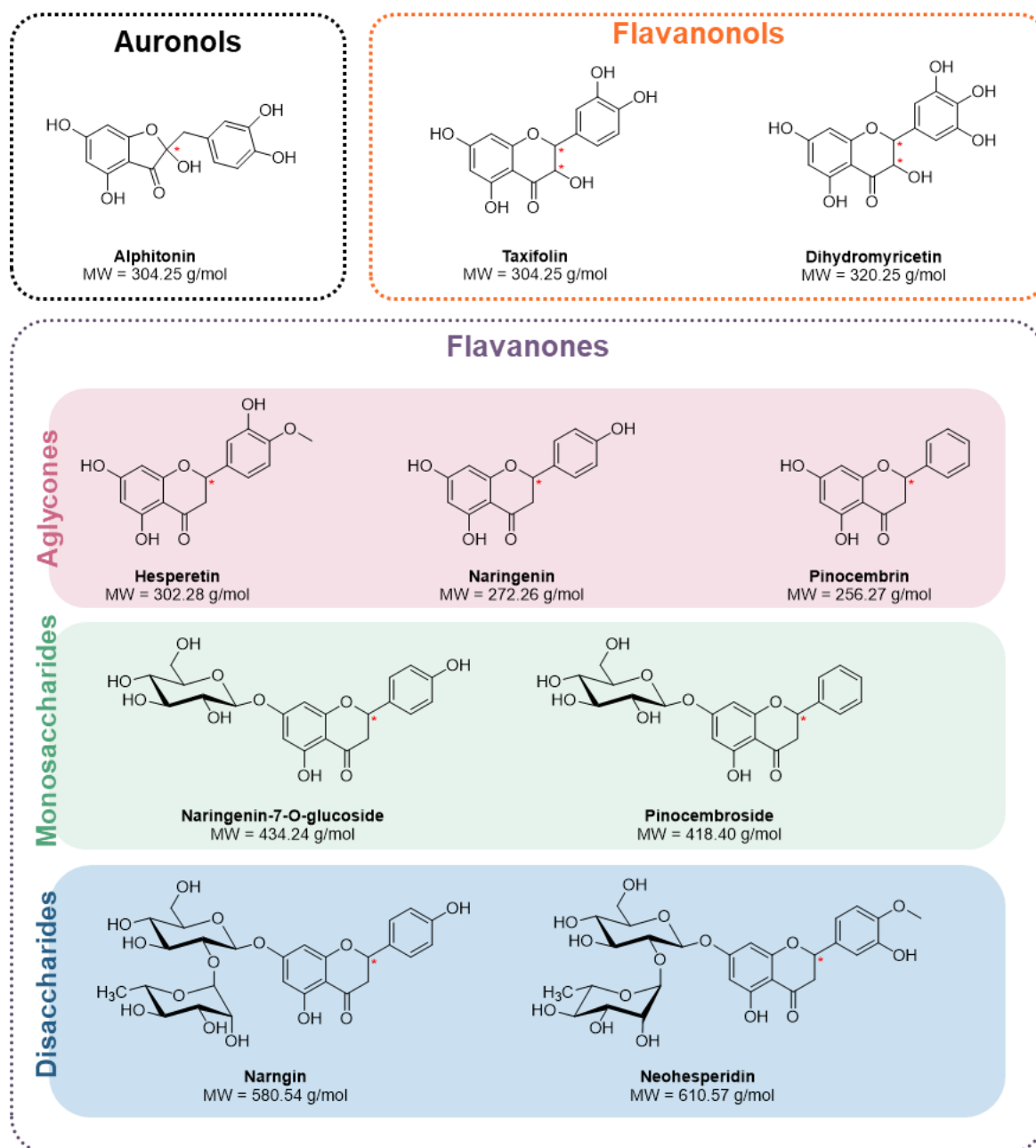


Figure IV.2.2 - Set of flavonoid standards

3.1.2. Definition of the desirability functions

As part of the development of a chiral separation method applied to flavonoids, a multi-criteria approach was employed using so-called Derringer desirability functions. The aim was to identify the columns that would perform well in separating flavonoid stereoisomers, ideally identifying one fit-for-all column, or possibly revealing different columns for different classes of chiral flavonoids. Derringer functions make it possible to transform several analytical responses, initially expressed in different units or different modalities, into a single standardized value enabling different chromatographic conditions to be compared. To achieve this, it is first necessary to define the comparison criteria and translate them into functions yielding corresponding di values.

Four criteria were selected: retention time and proportion of co-solvent at the moment of analyte elution for the second peak, resolution (calculated from the measured width at half-height $w_{50\%}$), and peak asymmetry (measured at 10% of peak height). Each of these criteria was modeled using a desirability function, ranging from 0 (unacceptable result) to 1 (ideal result). They are presented in Figure IV.2.3 and further described below.

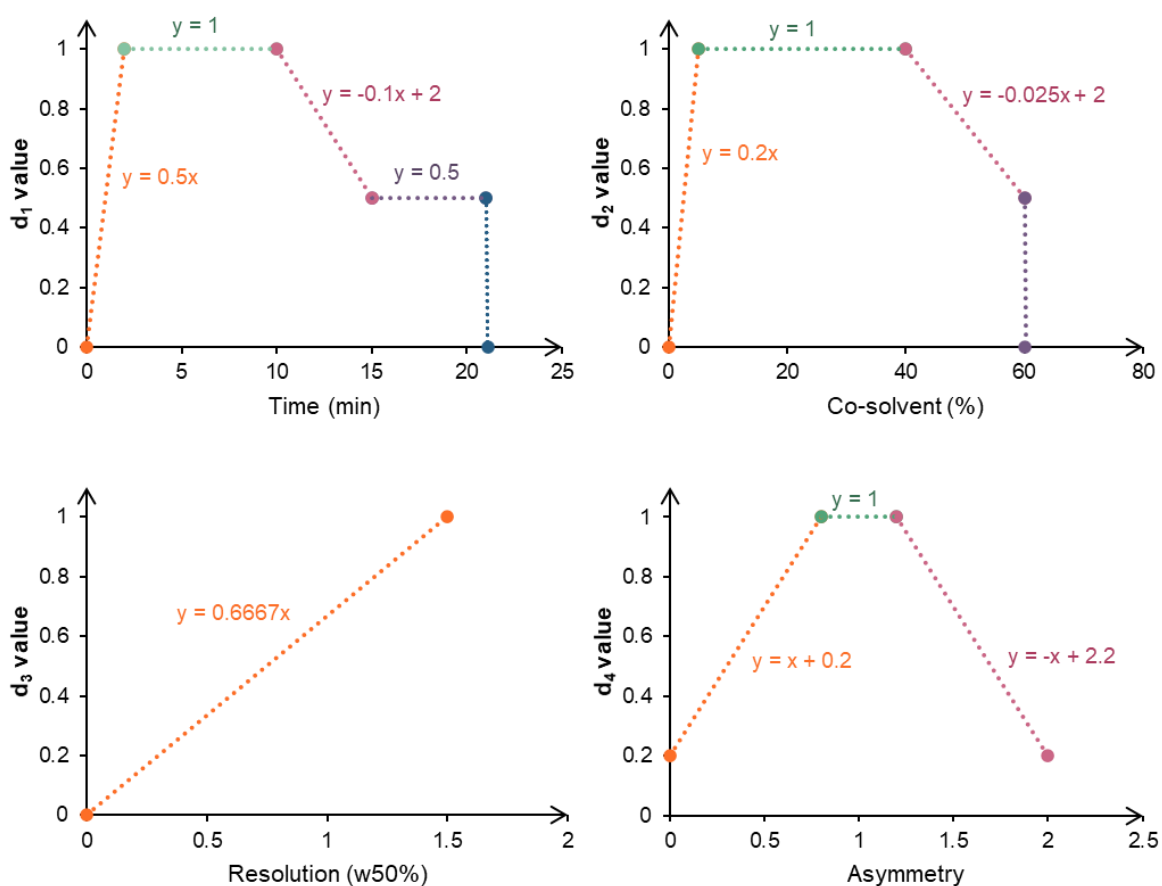


Figure IV.2.3 - Derringer functions based on: retention time (d₁), elution composition (d₂), resolution calculated with width at half-height ($w_{50\%}$) (d₃) and EP asymmetry at 10% peak height (d₄)

3.1.2.1. Retention time (d_1)

The first criterion was retention time, represented by the d_1 function. The retention time was considered optimal when comprised between 2 and 10 min. Below this range, the time was considered too short to guarantee effective separation, while above 10 min, a progressive penalty was applied to avoid lengthy analyses. Beyond 20 min, the d_1 value was set to zero as the analysis was considered too long.

3.1.2.2. Elution composition (d_2)

The second criterion was the composition of the co-solvent at the moment of analyte elution, represented by the d_2 function. This was also evaluated according to a three-zone function. Values between 10% and 40% were considered ideal, as they ensure a good balance between solubility (to favor good peak shapes), elution and interaction with the chiral selector. Below 10%, desirability increased progressively, while above 40% it decreased. A limit value was set at 60%, beyond which desirability would be zero. Note that such conditions could only be reached on the IG column, because its high retentivity made it necessary to further increase the co-solvent proportion.

In addition to analytical-scale considerations, when seeking stereoisomer purification, this composition would also ensure good solubility to favor productivity, while retaining an acceptable volume of solvent to use, evaporate and send to waste. Low solvent proportion would be unfavorable to high productivity in preparative separations, because solubility would be impaired. On the opposite, high solvent proportion would be undesirable for economic and ecological reasons.

3.1.2.3. Resolution (d_3)

The third criterion considered was resolution, represented by the d_3 function. The resolution between two peaks is of course the most important criterion in chiral separations. This criterion was thus heavily weighted in the overall desirability formula, as it directly determines the quality of the chiral separation. It was modeled by an increasing linear function, where the maximum d_3 value of 1 was set at the theoretical value of baseline resolution (1.5). Above 1.5, the d_3 value would remain equal to 1. This model reflects the idea that a gain in resolution beyond baseline resolution does not bring any additional benefit in the case of analytical-scale separations. An optimization function for preparative-scale separations may have adopted a different threshold,

since overloading conditions usually employed at preparative scale would always result in larger peaks, thereby requiring higher resolution to ensure good peak purity.

3.1.2.4. Asymmetry (d_4)

The final criterion was asymmetry, represented by function d_4 . Asymmetry was measured at 10% of the peak height. Ideal asymmetry is expected to be in the 0.8–1.2 range, reflecting a balanced peak shape with no excessive head or tail drag. Peaks that are too asymmetric may be linked to poor analyte solubility, adsorption phenomena, column overloading, or solvent/stationary phase incompatibility. The d_4 function was thus constructed to give maximum desirability (1) in the target range, between 0.8 and 1.2. Outside this range, the function tends towards zero. However, the minimum d_4 value was set at 0.2 instead of 0, so as not to penalize visible but distorted peaks too much.

3.1.2.5. Total desirability (D)

These four individual desirability functions were combined to calculate an overall score, called total desirability, denoted D . It was calculated using a weighted arithmetic mean, taking into account the relative importance of each criterion, with a much heavier weighting put on resolution (factor 6), in line with the importance it has in the outcome of a chiral separation.

Thus, total desirability was calculated using Equation (1) below:

$$D = \frac{d_1 + d_2 + d_3 \times 6 + d_4}{9} \quad (1)$$

This overall score provided an objective comparison of the chiral columns tested to separate flavonoid stereoisomers. Similarly, it could be used to compare different operating conditions to quickly identify the most promising ones, taking into account the trade-offs between analysis speed, solvent consumption, resolution and peak quality.

3.1.3. Columns ranking based on flavonoids families

The best columns, defined by the best average performance on the four identified criteria, will then be those with the highest D values. The results are shown in Figures IV.2.4 and IV.2.5. The detailed values of the four desirability functions (d_1 , d_2 , d_3 , and d_4) for each column and each compound, are detailed in Table IV.2.S1.

First, Figure IV.2.4 shows the sum of the total desirability (D) values obtained for each flavonoid compound analyzed on the five chiral columns tested (IA, IB, IC, IG, IJ). This cumulative representation reveals the relative easiness of resolving certain flavonoids compared to others. Indeed, some flavonoids had high desirability profiles spread over several columns, indicating good overall compatibility with different chiral phases. This was the case for naringenin and its glycosylated forms. Conversely, compounds such as taxifolin, dihydromyricetin, and pinocemboside had low overall values, indicating that enantioresolution was achieved only on one or two columns. Finally, alphononin, the only auronol in this test set, could never be resolved, raising questions about the composition of the standard, possibly comprising a single enantiomer, as it seems unlikely that none of the chiral selectors could have (even partly) resolved them.

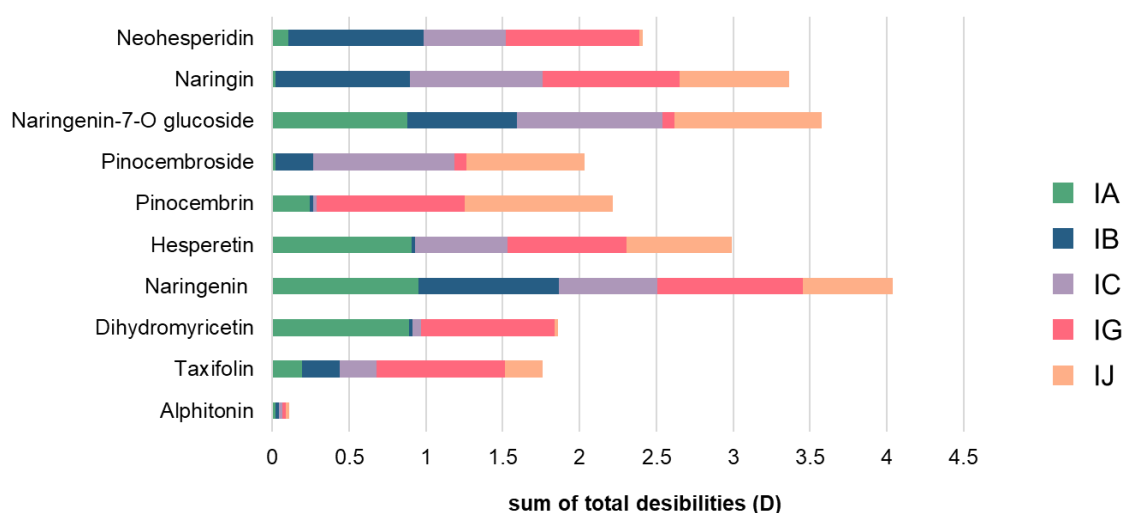


Figure IV.2.4 - Cumulated total desirability values measured for each compound on the 5 columns tested (IA, IB, IC, IG, IJ)

Secondly, another way of representing these results is shown in Figure IV.2.5, which is more focused on columns, to reveal those that were most successful for certain categories of compounds.

The IG column was the best performing overall, with high values of overall desirability for the majority of compounds. It was particularly effective for non-glycosylated flavonoids, both flavanones and flavanonols, with an overall mean desirability of 0.8. It was also suitable for flavanones with a disaccharide moiety (blue). However, and somewhat surprisingly, it was unsuccessful for flavanones glycosylated with monosaccharide groups (green), with less than 0.1 total desirability. The IG column therefore has a more favorable chiral interaction capacity for the flavonoid structures tested.

The IJ column showed an intermediate profile. It was effective for most of the flavanone family but not for flavanonols. It worked well for aglycone forms (purple and red) and flavanones with a monosaccharide group (green). However, it was less successful for flavanones with a disaccharide group (blue) as it resolved the diastereoisomers of naringin but not those of neohesperidin.

The IC column performed well for glycosylated flavanones (green and blue) with a total D value of around 0.9 for monosaccharides and 0.7 for disaccharides. But it remained limited for the other compounds.

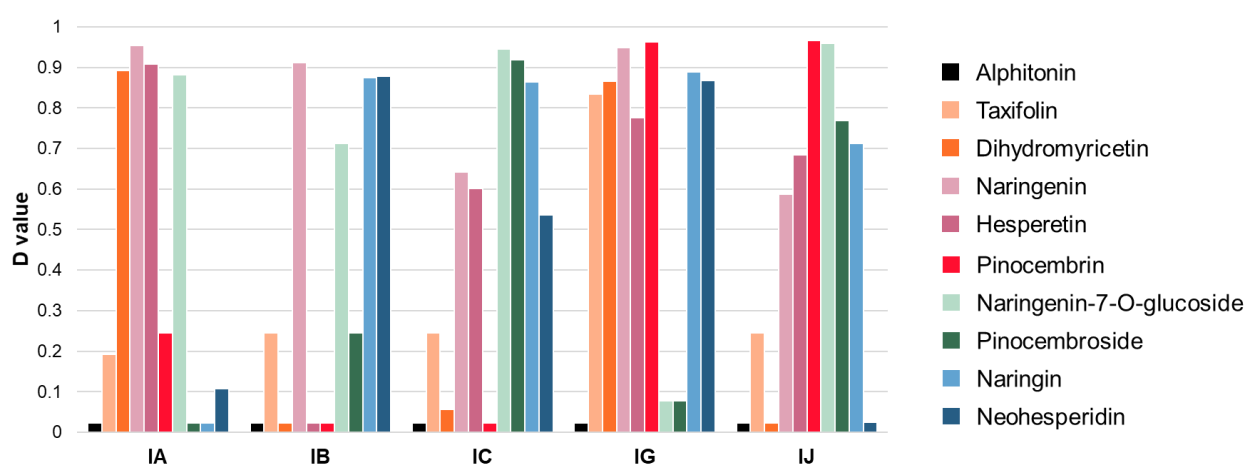


Figure IV.2.5 - Individual total desirability values measured for each test compound on all 5 columns (IA, IB, IC, IG, IJ)

Conversely, the IA and IB columns showed more erratic behavior. The IB column performed particularly well for flavanones with a disaccharide group ($D = 0.88$) (blue) and moderately well for compounds with a monosaccharide ($D = 0.47$) (green). However, it is ineffective for other compounds. The IA column was suitable for the aglycone forms of flavanones and flavanonols, but not for glycosylated structures, except for naringenin-7-O-glucoside.

Interestingly, reverse effects were observed between certain columns. For example, compounds resolved on the IA column were not well resolved on the IB column, and conversely, even though these two columns have the ligands but are based on different polymers (Figure IV.2.1). A similar observation can be made for the IC/IG column pair, which both carry chlorinated ligands, although slightly different. As was previously observed in many occasions, this suggests that the nature of the amylose or cellulose polymer constituting the stationary phase strongly influences the possibility to resolve stereoisomers. In this study, the amylose-based phases appeared generally more selective for aglycone forms, while those based on cellulose showed better selectivity for glycosylated forms.

Overall, this analysis highlights the benefits of using different columns within the same compound family. The total desirability approach is an objective tool for guiding the choice of columns, particularly during the analytical optimization phase or the development of robust chiral methods.

3.2. Honey sample application

Having identified the best columns in relation to flavonoid structure, we proceeded to examine possible applications. Complex natural samples were of particular interest as they contain many different species, and usually include many isomeric compounds. In such cases, even when flavonoids could be partly isolated through selective extraction or sample preparation procedures, they would still contain many compounds with high structural similarity. It is therefore unlikely that a single chiral column would be able to resolve the stereoisomers of a particular flavonoid from all other species. As a result, a two-dimensional strategy was considered, where the first dimension would comprise an achiral stationary phase to start resolving all species, isolate target flavonoids from other species, then the second dimension would comprise the chiral column to resolve the stereoisomers.

To demonstrate this idea, a sample of honey from French Guiana was analyzed. The sample was expected to contain a variety of flavonoids, including pinocembrin, an aglycone flavanone derived from propolis (Figure IV.2.2), which was often described in the phenolic composition of honey [30–33]. As appears in Figure IV.2.5, pinocembrin, shown in red, was well resolved on IG and IJ columns, where the total desirability values were close to 1. Although the IJ column was relevant, the IG column was selected for its broader applicability on different sorts of flavonoids. Full results of retention times and resolution measured on this column are provided in Table IV.2.S2. In the literature, pinocembrin enantiomers had been previously resolved in reversed-phase liquid chromatography (LC) on Chiralpak AD-H [34], which is the coated version of the IA column examined in the present paper, but also in normal-phase LC on Chiralpak AS-H and on Chiralcel OD-H, which is the coated equivalent of the IB column [35]. Bee pollen flavonoids were previously resolved in SFC-UV on the Chiralpak AD-H column, but pinocembrin was not identified in this study [8].

The two-dimensional SFC-SFC system with diode-array and mass spectrometric detection had been previously developed [27]. The first-dimension method was based on a previously developed method for flavonoid analysis [29]. The gradient programs are described in Table IV.2.1 (Gradient 2).

Although SPE was applied to extract polyphenols, the honey sample was still highly complex as appears with its rich chromatographic profile in Figure IV.2.6A. It would therefore be unrealistic to

determine the enantiomeric excess of flavonoids in a single dimension of separation. To determine the presence of pinocembrin, a standard was first analyzed in the first and second dimensions (dim1 and dim2) to determine the retention times in each method. As a result, the pinocembrin peak was detected in dim1 in UV at 280 nm on the diethylaminopropyl-bonded column (Figure IV.2.6A). However, this peak was clearly not well resolved from other species. To simplify reading the chromatogram, the SIM chromatogram recorded in positive ionization mode, tracking the $[M + H]^+$ ion of pinocembrin (m/z 257) is represented in Figure IV.2.6B. On this chromatogram, close to the retention time corresponding to pinocembrin, two peaks were visible. In order to ascertain which of them corresponded to pinocembrin, these two peaks were both collected in storage loops and re-injected in the second-dimension column.

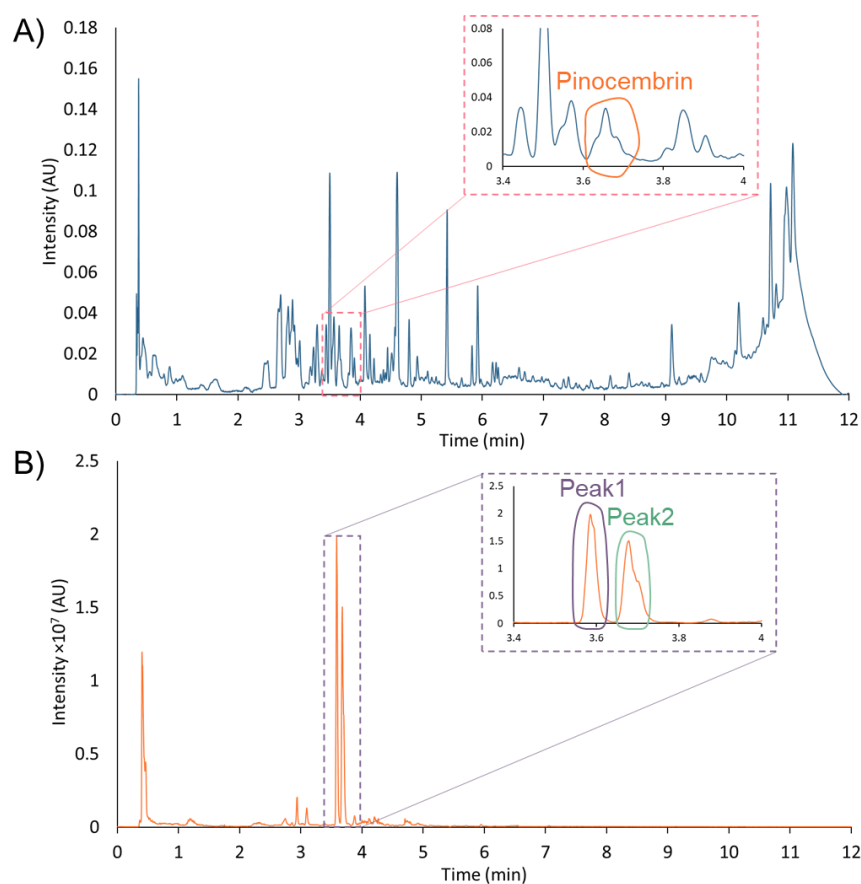


Figure IV.2.6 – 1D chromatograms of honey sample. A) SFC-UV analysis (280 nm). B) SFC-MS analysis (m/z 257). Analysis conditions (dim1): ACQUITY UPC² Torus DEA (100 $\times$ 3.0 mm, 1.7 μ m), co-solvent: MeOH + 0.1% MSA, 30 °C, 150-110 bar, 1.7-0.6 mL/min (gradient conditions in Table IV.2.1, gradient 2.a)

Injection of peak 1 in dim2 on the IG column enabled the pinocembrin enantiomers to be separated as the retention times corresponded to those of the pinocembrin standard (Figure IV.2.7). An enantiomeric excess of 87% was measured. When peak 2 was transferred from dim1 to dim2,

four peaks were visible. Two of them corresponded to the two enantiomers of pinocembrin, but in smaller quantities than in peak 1. This small presence nevertheless enabled the enantiomeric excess to be calculated at 85%. This suggests that the presence of pinocembrin in peak 2 probably corresponded to a remnant of peak 1, due to their proximity in the dim1 chromatogram. In addition, a second pair of peaks was detected in the dim2 chromatogram of peak 2. As the m/z value was identical, these peaks were probably originating from pinocembrin isomers.

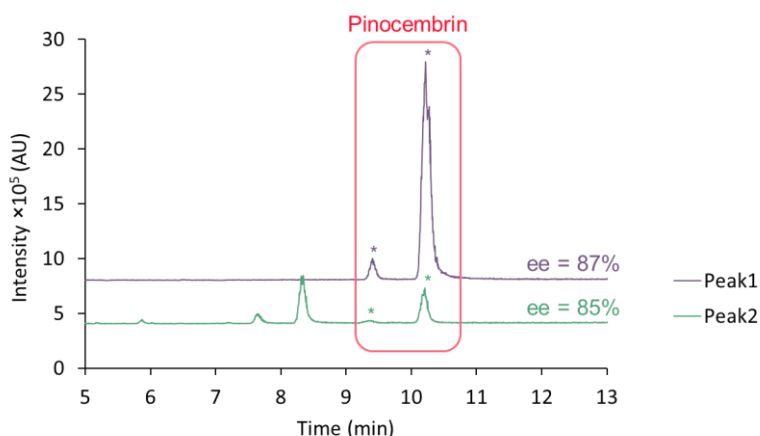


Figure IV.2.7 – SFC-MS (m/z 257) chromatograms of honey sample in dim2 corresponding to the peak of pinocembrin extracted from dim1. Analysis conditions (dim2): CHIRALPAK IG-3 (150 × 4.6 mm, 3.0 μ m), co-solvent: MeOH, 30 °C, 150 bar, 2.0 mL/min (gradient conditions in Table IV.2.1, gradient 2.b)

For instance, dihydrodaidzein (7,4'-dihydroxyisoflavanone) was previously described in honey [36]. Since it is an isoflavanone (B-ring in the C3 position) while pinocembrin is a flavanone (B-ring in the C2 position), it is reasonable to think that they could have been resolved both in the achiral column and in the chiral column. However, more confident identification would have required the analysis of a standard compound, or purification of the peaks to carry out NMR experiments, which was beyond the scope of the present study.

Finally, it is important to mention that scalemic mixtures analyzed in an achiral chromatographic system may cause self-disproportionation of enantiomers (SDE), as was first demonstrated by Cundy and Crooks [37]. To put it simply, intermolecular associations of the enantiomers in solution may yield enantiomerically enriched or enantiomerically depleted fractions in achiral chromatography. In other words, the enantiomeric excess may vary when the front, apex and tail of the peak are examined. While this does not appear to be significant in the present example (the ee values were measured as 85% and 87% in the two fractions), it further supports the interest of collecting the full peak in a single fraction to be transferred from the first dimension to the second, in order to obtain an accurate measurement of ee. This phenomenon should also be considered

to implement comprehensive methods of two-dimensional chromatography involving an achiral step and a chiral step, where the peak containing both enantiomers eluting from the achiral column would be divided into several fractions. Further information on SDE in achiral chromatography can be found in the literature [38,39].

4. Conclusion

In the above study, it was shown that certain polysaccharide chiral selectors appeared to be specific to particular flavonoid structures, including a marked difference between the amylose phases performing better for aglycone forms and the cellulose phases for glycosylated flavanones. Based on these observations, suitable columns were defined for different classes of flavonoids, using Derringer desirability functions. This made it possible to map the best-performing column/compound combinations for separating stereoisomers.

A complex sample of French Guiana honey containing a variety of flavonoids was analyzed with heart-cutting two-dimensional supercritical fluid chromatography (SFC-SFC-DAD-MS). The first-dimension method was used to isolate the enantiomers of pinocembrin, a bioactive aglycon flavanone naturally present in bee products; then the second-dimension method resolved the enantiomers, and further separated them from other isobaric compounds. Determining the enantiomeric excess provides a better understanding of the composition of the sample that would be helpful in bioactivity studies, or when biosynthetic pathways are examined.

Furthermore, these results illustrate the usefulness of a two-dimensional chromatographic system to characterize chiral molecules in complex natural matrices. This methodology opens the way to a better understanding of the chiral profile of flavonoids present in natural products such as honey, illustrating the full potential of SFC-SFC in advanced chiral analysis.

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Supplementary information

Table IV.2.S1 – Detailed values obtained from desirability functions for each standard compound: retention time (d₁), composition of the mobile phase at the moment of elution (d₂), resolution calculated with width at half-height (w_{50%}) (d₃), and asymmetry at 10% peak height (d₄).

		Auronols		Flavanonols				Flavanones					
		Alphitonin	Taxifolin	Dihydromyricetin	Naringenin	Hesperetin	Pinocembrin	Pinocembroside	Naringenin-7-O glucoside	Naringine	Neohesperidin		
IA	d1	0.50	0.82	0.97	1.00	0.76	1.00	0.00	0.88	0.50	0.44		
	d2	0.50	0.78	0.92	1.00	0.91	1.00	0.00	0.88	0.48	0.74		
	d3	0.00	0.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00		
	d4	0.20	0.20	0.20	0.58	0.54	0.20	0.20	0.42	0.20	0.32		
IB	D	0.02	0.19	0.89	0.95	0.91	0.24	0.02	0.88	0.02	0.11		
	d1	0.50	0.50	0.47	1.00	0.50	0.50	1.00	1.00	0.90	0.90		
	d2	0.50	0.50	0.44	1.00	0.50	0.50	1.00	1.00	0.84	0.84		
	d3	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.70	1.00	1.00		
IC	d4	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20		
	D	0.02	0.24	0.02	0.91	0.02	0.02	0.24	0.71	0.87	0.88		
	d1	0.50	1.00	0.50	1.00	1.00	0.50	1.00	0.97	0.79	0.68		
	d2	0.50	1.00	0.50	1.00	1.00	0.50	0.96	0.92	0.73	0.63		
IG	d3	0.00	0.00	0.00	0.60	0.53	0.00	1.00	1.00	1.00	0.56		
	d4	0.20	0.20	0.50	0.20	0.20	0.20	0.33	0.66	0.34	0.20		
	D	0.02	0.24	0.06	0.64	0.60	0.02	0.92	0.94	0.86	0.54		
	d1	0.50	0.53	0.79	0.88	0.27	0.99	0.50	0.50	0.96	0.80		
IJ	d2	0.50	0.26	0.75	0.83	0.25	0.97	0.00	0.00	0.91	0.75		
	d3	0.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.98		
	d4	0.21	1.00	0.48	0.88	0.99	0.75	0.20	0.20	0.20	0.51		
	D	0.02	0.83	0.87	0.95	0.78	0.96	0.08	0.08	0.89	0.87		
IJ	d1	0.50	0.50	0.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50		
	d2	0.50	0.50	0.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50		
	d3	0.00	0.00	0.00	0.51	0.66	1.00	0.73	1.00	0.70	0.00		
	d4	0.20	0.20	0.20	0.20	0.20	0.69	0.53	0.63	0.20	0.23		
	D	0.02	0.24	0.02	0.59	0.68	0.97	0.77	0.96	0.71	0.03		

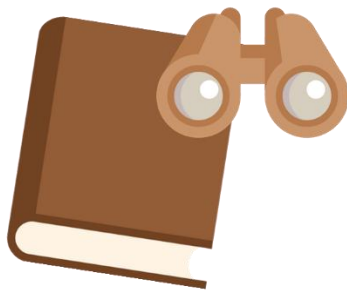
Table IV.2.S2 – Detailed values of retention times (Tr), asymmetry, elution composition (corresponding to the proportion of co-solvent at the moment of analyte elution) and resolution observed on the IG column.

Name		IG			
		Tr(min)	Asym	elution composition	R°(w50%)
Naringenin	1	10.91	1.35	45.52	3.32
	2	11.51	1.29	47.85	
Hesperetin	1	14.63	1.22	59.93	17.47
	2	20.61	1.19	83.15	
Pinocembrin	1	9.36	1.44	39.51	4.80
	2	10.18	1.46	42.69	
(±) Taxifolin	1	14.42	0.81	59.14	2.81
	2	15.36	1.06	62.76	
Dihydromyricetin	1	10.96	2.61	45.70	8.28
	2	13.23	1.44	54.52	
Alphitonin	1	6.47	1.99	28.30	-
	2			0.00	
(±) Taxifolin	1	15.36	1.08	62.77	-
	2			0.00	
Pinocembroside	1	16.12		65.73	0.00
	2	16.46		67.04	
Naringenin-7-O glucoside	1	15.68		64.00	0.00
	2	16.03		65.37	
Naringine	1	10.09		42.35	1.89
	2	10.77		44.99	
Neohesperidine	1	11.37	1.54	47.32	1.47
	2	12.67	1.84	52.35	

Conclusion chapitre

Ce chapitre a démontré l'efficacité du système SFC-SFC utilisé pour l'analyse stéréospécifique de flavonoïdes dans des matrices naturelles complexes. La détermination des excès diastéréoisomériques de la néohesperidine et de la naringine dans le *C. aurantium* a permis d'évaluer les différences de composition dans les différentes parties d'orange. Un criblage de colonnes chirales a permis d'identifier les colonnes les plus adaptés à chaque classe de flavonoïdes, avec une application concrète sur un échantillon de miel de Guyane. Ces résultats soulignent l'intérêt de la SFC-SFC pour la caractérisation des composés bioactifs dans les extraits naturels complexes.

Conclusion et perspectives



Conclusion

Dans ce manuscrit nous avons présenté la mise en place d'un système bidimensionnel en chromatographie en phase supercritique. L'efficacité de ce système a été démontrée pour des produits naturels complexes tels que le *C. aurantium* et le miel.

La première partie de ces travaux a été consacrée au développement instrumental d'un système SFC-SFC en mode multiple heart-cut. Pour cela l'ajout de trois vannes automatiques à un instrument de SFC unidimensionnelle a été effectué. Cette configuration a montré une excellente robustesse sans engendrer de problèmes de surpression, tout en conservant les performances chromatographiques d'un système unidimensionnel. L'optimisation du transfert des pics entre les dimensions a permis de récupérer jusqu'à 83 % du pic d'intérêt, confirmant l'efficacité du développement. Cette approche, facilement transposable, rend la 2D-SFC accessible à tout laboratoire déjà équipé d'un système SFC unidimensionnel.

Ensuite, l'évaluation des performances analytiques du système a permis d'identifier les paramètres critiques à maîtriser. L'impact des vannes, des tubings et des volumes de boucles sur les délais de gradient et la qualité chromatographique a été étudié. Si la première dimension n'a pas été affectée, une légère augmentation de la largeur des pics en seconde dimension a été observée, sans compromettre la résolution globale. Un effet d'élargissement lié au solvant de réinjection dans la deuxième dimension a été observé dans certains cas extrêmes, mais reste faible dans les conditions classiques d'analyse en SFC. Ces résultats montrent que le système 2D mis en place est stable, performant et facilement optimisable pour de nombreuses applications. De plus, ce travail ouvre la voie à la 2D-SFC en mode compréhensif, en mettant en évidence les zones de compatibilité entre les dimensions pour des gradients d'élution couvrant une large gamme de concentration de co-solvant.

Une deuxième partie de ces travaux a porté sur les applications de ce système, notamment l'analyse des flavonoïdes chiraux présents dans des matrices naturelles. L'extraction de ces composés par fluide supercritique s'est révélée reproductible en termes de rendement global, mais sensible à la variabilité de la représentation des échantillons, avec des fluctuations marquées dans les ratios stéréoisomériques. Une masse minimale de 500 mg s'est avérée nécessaire pour garantir des résultats fiables et répétables. L'étude des différents organes de *C. aurantium* a mis en évidence la stabilité de la néohespéridine, contrairement à la naringine, plus sensible à la lumière ou à la maturation.

Par ailleurs, un travail de criblage de phases chirales a mis en évidence la sélectivité des polysaccharides vis-à-vis de certaines structures, les phases amylose étant plus adaptées aux

flavonoïdes aglycones, et les phases cellulose aux flavanones glycosylées. Ces observations ont été faites grâce à des fonctions de désirabilité de Derringer, permettant de rationaliser le choix des colonnes selon la nature des flavonoïdes. L'analyse SFC-SFC d'un échantillon complexe de miel de Guyane française a permis de séparer efficacement les énantiomères de pinocembrine, et de déterminer leurs excès énantiomériques. Ces résultats illustrent la performance de la SFC-SFC pour l'analyse fine de la chiralité dans des matrices naturelles complexes.

Perspectives

Pour aller plus loin sur ces travaux, plusieurs pistes d'investigation peuvent être envisagées. Tout d'abord, l'étude des zones de compatibilité entre les dimensions pourrait être approfondie en intégrant des colonnes présentant des polarités intermédiaires. Une telle approche permettrait de déterminer si les tendances observées pour les colonnes polaires et apolaires se retrouvent également pour ces phases à polarités intermédiaires, et d'affiner ainsi la compréhension de la compatibilité des quantités de co-solvant.

Par ailleurs, bien que le système développé ait démontré son efficacité pour l'analyse de matrices naturelles complexes, son applicabilité pourrait être étendue à d'autres types d'échantillons, notamment des plantes non encore étudiées ou des produits pharmaceutiques. En effet, que ce soit avec de la chiralité ou encore avec simplement deux dimensions de séparation, le domaine pharmaceutique peut trouver son intérêt à l'utilisation d'un tel système afin de séparer des énantiomères de molécules thérapeutique ou encore pour la séparation d'impuretés.

Concernant les aspects instrumentaux, l'évaluation de boucles de plus grand volume en mode heart-cut permettrait de caractériser l'impact de l'injection sur la performance de la seconde dimension. De même, évaluer l'impact de la modification des dimensions de colonne en première et seconde dimension. L'utilisation de solvants différents entre les deux dimensions pourrait également être envisagée. Dans ces conditions, un mode de modulation de type *Active Solvent Modulation* (ASM) constituerait une stratégie prometteuse pour pallier d'éventuelles incompatibilités de phase et maximiser la qualité des séparations.

En parallèle, les travaux préliminaires menés sur le mode compréhensif en SFC ont montré la faisabilité d'une approche SFC×SFC, bien que celle-ci requière des contraintes instrumentales plus strictes qu'en mode multiple heart-cut. En effet cette nouvelle configuration nécessite l'ajout d'un deuxième système SFC ce qui complexifie sa mise en place. La mise en œuvre d'un système en ligne, impliquant une commutation rapide des vannes et une gestion synchronisée des boucles de stockage, représente ainsi un objectif ambitieux mais réaliste. Les résultats obtenus en mode *off-line* sur les furocoumarines témoignent du potentiel de cette stratégie pour l'analyse exhaustive d'échantillons complexes.

Enfin, dans une perspective industrielle, un système 2D-SFC, qu'il soit utilisé en mode heart-cut ou compréhensif, pourrait offrir de nombreux avantages. Ses capacités de séparation élevées et la possibilité de cibler sélectivement des composés d'intérêt en font un outil particulièrement adapté aux secteurs pharmaceutique, agroalimentaire et des produits naturels. Il pourrait notamment être mobilisé pour la purification analytique ou préparative d'énantiomères, le contrôle qualité de matières premières complexes, ou encore la détection d'impuretés difficiles à résoudre en chromatographie unidimensionnelle.

Développement d'outils chromatographiques multidimensionnels en phase supercritique pour l'analyse de produits naturels

Résumé :

Les produits naturels sont une source majeure de composés bioactifs avec des intérêts pharmaceutiques et cosmétiques. Leurs analyses restent complexes en raison de la diversité structurale et stéréochimique des molécules présentes. La chromatographie en phase supercritique (SFC), efficace et respectueuse de l'environnement, peut être couplée en deux dimensions (SFC-SFC) pour augmenter la puissance séparative face à ces mélanges complexes.

Cette thèse décrit le développement d'un système SFC-SFC en mode multiple heart-cut. En plus de la mise en place du système, différents paramètres ont été optimisés : volumes morts, boucles de transfert, pression et composition des phases mobiles, sur colonnes polaires et apolaires. Ces travaux ont permis de déterminer les combinaisons de co-solvant compatibles, permettant un transfert efficace et reproductible entre les deux dimensions, tout en préservant la forme des pics et la résolution.

Le système a été appliqué aux échantillons de *Citrus aurantium* L., plante riche en flavanones (néohesperidine, naringine). Différentes parties de la plante, dont celles du fruit à trois stades de maturité, leurs origines et leurs conditions de séchage ont été comparées. Une séparation achirale en première dimension a été développée pour séparer les flavonoïdes et une séparation stéréosélective (sur colonne chirale) en seconde dimension pour déterminer leurs excès diastéréoisomériques.

Afin d'appliquer la méthode à un nombre plus large de flavonoïdes, un criblage de colonnes chirales a été réalisé. Celui-ci a permis d'identifier des colonnes adaptées selon le degré de glycosylation des flavonoïdes.

Finalement, ces premiers développements instrumentaux ouvrent la perspective d'un couplage SFC×SFC en mode compréhensif pour une analyse plus exhaustive et rapide des matrices naturelles complexes.

Mots clés : SFC, chromatographie, multidimensionnelle, produits naturels, flavonoïdes, chiralité

Development of multidimensional chromatographic tools in the supercritical phase for the analysis of natural products

Summary:

Natural products are a major source of bioactive compounds of pharmaceutical and cosmetic interest. Their analysis remains complex due to the structural and stereochemical diversity of the molecules present. Supercritical phase chromatography (SFC), which is efficient and environmentally friendly, can be coupled in two dimensions (SFC-SFC) to increase the separative capacity for these complex mixtures.

This thesis describes the development of an SFC-SFC system in multiple heart-cut mode. In addition to setting up the system, various parameters were optimized: dead volumes, transfer loops, pressure and composition of the mobile phases, on polar and non-polar columns. This work enabled compatible co-solvent combinations to be determined, enabling efficient and reproducible transfer between the two dimensions, while preserving peak shape and resolution.

The system was applied to samples of *Citrus aurantium* L., a plant rich in flavanones (neohesperidin, naringin). Different parts of the plant, including the fruit at three stages of maturity, their origins and drying conditions were compared. A first-dimension achiral separation was developed to separate the target flavonoids and a second-dimension stereoselective separation (on a chiral column) was used to determine their diastereoisomeric excesses.

In order to apply the method to a larger number of flavonoids, a screening of chiral columns was carried out. This enabled us to identify columns adapted to the degree of glycosylation of the flavonoids.

Finally, these initial instrumental developments open up the prospect of coupling SFC×SFC in comprehensive mode for more exhaustive and rapid analysis of complex natural matrices.

Keywords: SFC, chromatography, multidimensional, natural products, flavonoids, chirality