



# Production d'extraits non colorés de plantes enrichis en composés bioactifs. Investigation des techniques d'extraction modernes (US, MO, SFE, PLE)

Thibault Lefebvre

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**HAL Id: tel-03166445**

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# UNIVERSITÉ D'ORLÉANS

**ÉCOLE DOCTORALE SANTE, SCIENCES BIOLOGIQUES ET CHIMIE DU  
VIVANT (SSBCV)**

**Institut De Chimie Organique Et Analytique - ICOA UMR7311**

## **THÈSE** présentée par : **Thibault LEFEBVRE**

soutenue le : **27 novembre 2020**

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

Discipline/ Spécialité : Chimie Analytique et Phytochimie

**Production d'extraits non colorés de  
plantes enrichis en composés bioactifs.  
Investigation des techniques d'extraction  
modernes (US, MO, SFE, PLE)**

**THÈSE dirigée par :**

**Pr. DESTANDAU Emilie**  
**Dr. LESELLIER Eric**

Professeur, Université d'Orléans  
Maitre de conférences, Université d'Orléans

**RAPPORTEURS :**

**Dr. SUBRA-PATERNAULT Pascale**  
**Pr. CHEMAT Farid**

Directeur de recherche, Université de Bordeaux  
Professeur, Université d'Avignon

---

**JURY :**

**Dr. SUBRA-PATERNAULT Pascale**  
**Pr. CHEMAT Farid**  
**Dr. BOUESOCQUE-DELAYE Leslie**  
**Dr. TORIBIO Alix**  
**Pr. DESTANDAU Emilie**  
**Dr. LESELLIER Eric**

Université de Bordeaux, Rapporteur  
Université d'Avignon, Rapporteur  
Université de Tours, Examineur  
Chanel Parfums Beauté , Examineur  
Université d'Orléans, Co-directeur de thèse  
Université d'Orléans, Co-directeur de thèse







# REMERCIEMENTS

Ces trois années de thèse furent pour moi épanouissantes, autant sur le plan intellectuel que personnel. Je tiens à remercier les personnes qui en sont responsables en espérant n'en oublier aucune.

Tout d'abord, je tiens à remercier les différents membres du jury qui ont accepté d'évaluer ces travaux de thèse. Merci à Pascale Subra-Paternault et Farid Chemat qui sont rapporteurs ainsi qu'à Leslie Boudesocque et Alix Toribio qui sont examinateurs.

En 2017, Emilie Destandau et Eric Lesellier m'ont sélectionné afin de travailler, à leurs côtés, sur ce projet de thèse. Je tiens à remercier mes directeurs de thèse pour la confiance qu'ils ont placée en moi en me donnant la possibilité de vivre, à leur côté, cette grande expérience qu'est le doctorat. C'est grâce à leurs enseignements, leur patience et leur sympathie que j'ai pu vivre trois belles années.

Deux directeurs, deux spécialités et deux personnalités

Emilie, je te remercie pour tous nos échanges qu'ils soient scientifiques ou non. Tu étais là pour répondre à mes questions ou pour me rassurer lorsqu'il y avait des baisses de moral. Tu m'as permis d'étendre mes connaissances en phytochimie et tu as confirmé mon souhait d'évoluer dans ce domaine.

Eric, tu m'as fait découvrir le monde (merveilleux) des fluides supercritiques avec leurs avantages leurs subtilités et leurs complexités. Je te remercie pour ta patience (car il en a fallu) pour revoir toutes les notions de chimie analytique. Tu as été un véritable professeur et j'ai énormément appris à tes cotés.

Je souhaite aussi remercier Pascal Bonnet, directeur de l'ICOA (Institut de Chimie Organique et Analytique), et Reine Nehmé, responsable de l'équipe SAAB (Stratégies Analytiques, Affinités et Bioactifs) dans laquelle cette thèse s'est déroulée. Je remercie tous les membres de l'équipe : Agnes, Bérengère, David, Laetitia, Reine et Caroline pour les différents échanges que l'on a pu avoir, au détour d'un couloir, d'une pause-café ou ailleurs. Un remerciement particulier pour Caroline avec qui j'ai eu le plaisir de collaborer pendant cette thèse. Je remercie aussi spécialement Reine, David et Bérengère avec qui j'ai partagé les joies de l'enseignement lors des séances de travaux pratique des deuxièmes années de l'IUT de Chimie d'Orléans.

Un grand merci à toutes les autres personnes de l'ICOA que j'ai pu côtoyer : Sophie ; Christophe, Alain-Michel, Stéphane, Cédric, Florian, Cyril, Laurent et Sandrine. Merci à Sophie pour sa patience pour les choses qui me tiennent le plus à cœur, à savoir, les démarches administratives. Merci à Alain-Michel et Stéphane pour leur aide dans le changement des bouteilles de CO<sub>2</sub> . Merci à Florian (et Madeol) pour son aide au niveau électronique dans l'amélioration et le développement de nouveaux systèmes (SFC et SFE/SFC). Cyril, merci pour toutes nos discussions et les essais réalisés en masse. Enfin, merci à Cédric pour son aide et sa disponibilité en cas de problèmes qu'ils soient d'ordre instrumental ou informatique.

Je tiens à remercier les non-permanents (docteurs, post-doc, ingénieurs, ATER, stagiaires) qui ont fait parti de cet épanouissement intellectuel mais surtout du personnel. Il y a BEAUCOUP de personnes que je souhaite remercier pour cela, je vais commencer par ceux qui ont déjà quitté l'ICOA : Farah, Nhi et Adrien. Merci Farah pour tous ce qu'on a pu partager au labo mais aussi en dehors. Merci petite herbe pour tous les moments passés ensemble que ce soit au bureau, lors de Wokasie ou lors d'enterrements de poissons. Adrien, je te remercie pour ce qu'on a pu vivre ensemble que ce soit en repas matures, en affaires, en D&D et j'en passe. Je remercie aussi toutes les stagiaires qui ont pu participer à ce projet : Arwa, Nesrine, Sirine et Carole.

Ensuite je souhaite remercier mes collègues de bureau qui m'ont supporté (même si faut avouer, que je n'y étais pas souvent). Merci à Rouba et Jeremy. Rouba ce fut un plaisir de faire les TP avec toi et de partager nos différentes anecdotes. Jeremy, je te remercie sincèrement pour tout ce que tu fais au labo qui a servi à toute la communauté. Le Wall of cake en fait partie mais attention de ne pas déraiper dans un régime totalitaire ! Je souhaite remercier maintenant mes collègues présents dans l'autre bureau (car j'ai passé beaucoup de temps dans votre bureau, en même temps c'était le plus proche de mes instruments et il y avait la bouilloire) : Merci Angéline pour ta sympathie et ton sens de l'organisation qui permet de réorganiser et ranger le laboratoire lorsque cela est nécessaire. Laurine, merci pour ta patience, en particulier lorsque tu étais la seule fille dans le bureau... Merci Ghassan pour tous les kebabs et notre amour commun pour l'hymne russe. Et enfin merci à toi, Quentin pour ta bonne humeur, ta motivation pour sortir en « afterwork » et ta générosité qui m'a permis de colorer le Shimadzu en vert (cette couleur lui va mieux je trouve). Je remercie les résidentes du labo plantes. Merci Gaëlle pour ta joie de vivre et ta gentillesse. Souhila, je te remercie pour tous les moments sympa que l'on a pu vivre pendant ces trois

années. Merci aussi d'avoir été présente et à l'écoute lorsque cela était nécessaire. Enfin, je vais terminer ce paragraphe en remerciant une personne tellement présente dans les labos d'analyse qu'elle est maintenant considérée comme faisant parti de l'équipe analytique. Merci beaucoup Mazarine (et Sebastien). On a commencé ce doctorat ensemble avec MT180 et on ne s'est jamais quitté. On était là l'un pour l'autre et ça nous a permis d'avancer même pendant les moments difficiles.

Je souhaite maintenant remercier mes amis, hors laboratoire, qui ont su me soutenir et m'encourager tout au long de la thèse : Clément, Pauline, Maxence, Grégoire, Yohan et Alexandre. J'espère que ces remerciements sont fins et bien dosés.

Je remercie Margaux pour son soutien pendant ma thèse. Tu as su m'écouter et m'encourager lorsque certaines périodes étaient compliquées. Tu es là, avec moi au quotidien et ça, ça ne doit pas être facile à vivre... Merci beaucoup pour tout. Je remercie aussi Berlioz qui est un bon compagnon de vie et de travail qui m'a beaucoup aidé, notamment pendant le confinement.

Pour finir, je suis et je serais toujours reconnaissant envers mes parents qui m'ont donné la possibilité d'être là où j'en suis aujourd'hui. Ils m'ont donné les moyens et la liberté de faire ce que je souhaitais et ils ont su me remettre sur le droit chemin lorsque j'avais tendance à m'égarer. Je vous dois cette thèse et je ne l'oublierai jamais.



# LISTES DES COMMUNICATIONS ECRITES ET ORALES

Cette thèse a été réalisée au sein de l'Institut de Chimie Organique et Analytique (Université d'Orléans). Elle a été financée par une bourse de la région Centre-Val de Loire dans le cadre d'un Appel à Projets de Recherche (APR-IR) du projet « Khloros » en collaboration avec Alban Muller. Cette thèse s'intitule « Production d'extraits de plantes non colorés enrichis en composés bioactifs. Investigation des techniques d'extractions modernes (US, MO, SFE, PLE) ». Elle a été supervisée par le Pr. Emilie Destandau et Dr. Eric Lesellier.

Dans le cadre de ce projet de recherche, différentes publications et communications orales ou par affiches ont pu être réalisées.

L'intégralité des publications acceptés et soumises sont dans ce manuscrit de thèse.

## Publications acceptées

- 1) Development of an analytical method for chlorophyll pigments separation by reversed-phase supercritical fluid chromatography  
*Thibault Lefebvre, Arwa Talbi, Sirine Atwi-Ghaddar, Emilie Destandau, Eric Lesellier*  
*Journal of Chromatography A, Volume 1612, 8 February 2020, 460643*
- 2) Evaluation of the extraction and stability of chlorophyll-rich extracts by supercritical fluid chromatography  
*Thibault Lefebvre, Emilie Destandau, Eric Lesellier*  
*Analytical and Bioanalytical Chemistry (2020)*
- 3) Supercritical Fluid Chromatography development of a predictive analytical tool to selectively extract bioactive compounds by Supercritical Fluid Extraction and Pressurised Liquid Extraction  
*Thibault Lefebvre, Emilie Destandau, Caroline West, Eric Lesellier*  
*Journal of Chromatography A Volume 1632 (2020) 461582*

- 4) Selectivity in plant extraction and the influence of modern extraction techniques (UAE, PLE, MAE and SFE): A Review  
*Thibault Lefebvre, Emilie Destandau, Eric Lesellier*  
*Journal of Chromatography A, Volume 1635 (2021) 461770*
- 5) Recent development for the analysis and the extraction of bioactive non-volatile compounds from *Rosemarinus officinalis* L.  
*Eric Lesellier, Thibault Lefebvre, Emilie Destandau*  
Accepté dans Trends in Analytical Chemistry le 16 Decembre 2020
- 6) Selective extraction of carnosic acid, rosmarinic acid and pigments (carotenoids and chlorophylls) from Rosemary by online SFE-SFC  
*Thibault Lefebvre, Emilie Destandau, Eric Lesellier*  
Accepté dans Journal of Chromatography A le 21 Novembre 2020

## Communications orales

- 1) Method development of Chlorophyll Pigments Separation by Supercritical Fluid Chromatography (SFC)  
T. Lefebvre, A. Talbi, E. Destandau, E. Lesellier  
ISCNP30, Athènes (Grèce), 28/11/2018
- 2) Mise au point d'une méthode de séparation des pigments chlorophylliens par chromatographie supercritique (SFC)  
T. Lefebvre, A. Talbi, E. Destandau, E. Lesellier  
SEP10, Paris (France), 26/02/2019

## Communication par affiche

Extraction method development for the creation of a bank of extracts

T. Lefebvre, S. Zubrzycki, M. Bunout, C. Catan, E. Lesellier, E. Destandau  
ISCNP30, Athènes (Grèce), 28/11/2018





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# LISTES DES ABREVIATIONS

ACN : acétonitrile

EtOH : éthanol

CLHP : chromatographie liquide haute performance

MeOH : méthanol

MeTHF : 2-méthyltétrahydrofurane

MO : extraction assistée par micro-ondes

PC : propylene carbonate

PLE : extraction par liquide pressurisé

QuEChERS : Quick, Easy, Cheap, Efficient, Rugged and Safe

SFC : chromatographie par fluide supercritique

SFE : extraction par fluide supercritique

US : extraction assistée par ultrasons

UV : ultraviolets



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## INTRODUCTION GENERALE



Depuis plusieurs années maintenant, une tendance vise à substituer des ingrédients issus de la pétrochimie par d'autres d'origine végétale, plus écologiques, plus verts. Par exemple, il est possible de raffiner à partir de biomasse du glucose et des acides gras qui pourront être utilisés pour produire des monomères et polymères qui sont classiquement issues de la pétrochimie. Le végétal s'impose alors comme une source prometteuse de nouvelles matières premières pour différents domaines.

L'extraction de végétaux permet de récupérer des substances naturelles afin de les utiliser dans différentes applications. Lors de l'optimisation de l'extraction des plantes, différents paramètres peuvent être étudiés pour en évaluer l'efficacité. Le paramètre principal est le rendement d'extraction (masse extraite vs masse de l'échantillon). Cependant, ce paramètre n'est pas spécifique des composés présentant une bioactivité éventuellement recherchée pour un ingrédient cosmétique. Dans certains cas, il est donc utile de prendre en compte d'autres paramètres afin d'étudier la sélectivité de l'extraction, dans le but d'obtenir des composés plus purs, un extrait enrichi en composés ciblés, ou pour éviter l'extraction de composés indésirables.

Ainsi, lors de l'extraction de composés d'intérêt, les pigments présents dans la plante peuvent aussi être extraits. Leur présence peut dans certains cas, perturber des analyses chimiques (par exemple : des analyses spectrophotométriques dans le cadre de l'évaluation d'activités). La couleur des extraits liée à ces pigments peut aussi être un frein à leur utilisation comme par exemple en cosmétique où les formulations blanches sont souvent recherchées.

L'objectif de cette thèse a donc été de développer une méthodologie afin de trouver des conditions d'extraction sélective pour récupérer séparément les composés bioactifs et les pigments.

Dans le premier chapitre, un état de l'art propose les différentes possibilités pour la production d'extraits non colorés. Il est possible d'utiliser des adsorbants afin de piéger certaines molécules après extraction. L'extrait peut aussi subir une étape de purification ou un fractionnement afin de récupérer un extrait riche en composés bioactifs et pauvre en pigments. Après avoir discuté des avantages et inconvénients des différentes possibilités, nous avons décidé d'orienter nos axes de recherches vers l'extraction sélective. L'objectif est d'extraire directement les composés cibles sélectivement sans procéder dans un second temps à une étape de purification ou de piégeage. Une review a été réalisée sur les

différents travaux présentant de la sélectivité dans des techniques d'extraction moderne : Extraction assistée par micro-ondes (MO), ultrasons (US), par fluide supercritique (SFE), et par liquide pressurisé (PLE). L'un des premiers paramètres étudiés est le solvant utilisé. Sa polarité va avoir une incidence directe sur les molécules extraites de la plante. Cette propriété peut être évaluée selon différents modèles qui sont aussi présentés et comparés. Les paramètres physiques induits par les différentes techniques (ex : puissance des ondes en microondes, température en ultrasons, etc...) sont ensuite présentés. La deuxième moitié de cette review se focalise sur la sélectivité en extraction par fluide supercritique (SFE). Cette technique est connue pour être sélective grâce au dioxyde de carbone qui est utilisé comme solvant d'extraction et dont les propriétés peuvent être modulées en fonction de la pression, de la température, ou par l'ajout de co-solvants appelés aussi modificateurs. De plus, le dioxyde de carbone présente des qualités intrinsèques qui en font un solvant vert. En effet, il n'est pas toxique, ce qui est un avantage pour une utilisation en cosmétique, c'est un sous-produit industriel peu cher, éventuellement recyclable dans les procédés à grande échelle, et qui permet de collecter des fractions concentrées puisqu'après utilisation sous pression, il s'évapore naturellement de l'extrait.

Il existe différents pigments, mais cette thèse s'est focalisée principalement sur une famille : les chlorophylles. Dans le deuxième chapitre, l'objectif est de produire un extrait riche en chlorophylle qui pourra être utilisé comme « standard » de chlorophylles. Devant la difficulté d'obtenir des standards de chlorophylles, la production d'extrait d'une plante riche en ces composés semble être la meilleure alternative. Cependant, ces composés se dégradent. Il a donc fallu étudier différentes conditions d'extraction (par MO, US, SFE et PLE) et de stockage (extrait non évaporé, évaporé avec différentes techniques comme à l'azote, évaporateur rotatif...) afin de préserver au maximum les composés natifs. Une fois que des conditions permettant de préserver au maximum les chlorophylles natives ont été identifiées, les chlorophylles et leurs produits de dégradations ont été étudiés par chromatographie en phase supercritique (SFC). Différentes conditions d'élution, notamment différents pourcentages de co-solvant, ont été appliquées et l'évolution de la rétention des chlorophylles a été étudiée. Le suivi de leur rétention a permis de trouver de bonnes conditions de séparation et ainsi de développer une méthode chromatographique d'analyse de ces composés et de leur produit de dégradation.

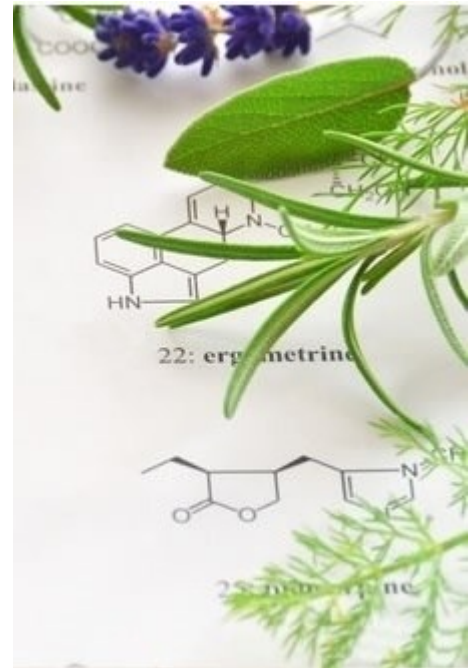
Pour la suite, le romarin, beaucoup utilisé en cosmétique pour ses molécules ayant des propriétés antioxydantes, a été sélectionné afin d'étudier la sélectivité de l'extraction par fluide supercritique et de produire des extraits enrichis en bioactifs. Ainsi, le troisième

chapitre décrit une première étape vers l'extraction sélective. Dans un premier temps, un état de l'art a été réalisé. Une présentation des principaux composés du romarin, de leurs différentes utilisations et des solutions permettant leur extraction et leur analyse sont présentées. La modélisation des comportements de rétention des chlorophylles par SFC a permis de définir des conditions favorables ou défavorables à leur extraction par SFE. Ainsi une composition de phase mobile donnant une faible rétention sera une composition qui pourra solubiliser et donc extraire facilement les chlorophylles (et réciproquement avec une forte rétention qui donnera des conditions défavorables d'extraction). Ce troisième chapitre présente donc le développement d'un modèle permettant d'extrapoler des conditions d'analyse en conditions d'extraction.

C'est dans le quatrième et dernier chapitre qu'une étude propose l'extraction sélective de composés bioactifs et de pigments chlorophylliens. Utilisant des conditions trouvées dans le chapitre précédent, un procédé d'extraction par fluide supercritique a permis la production d'extraits riches en composés bioactifs et d'autres riches en pigments. Cette étude s'est appuyée sur un couplage extraction/chromatographie supercritique en ligne afin de guider l'optimisation des paramètres en suivant les cinétiques d'extraction.



# CHAPITRE 1: La sélectivité en extraction





## 1. LES PIGMENTS DANS LES EXTRAITS VEGETAUX

Les végétaux sont utilisés depuis plusieurs milliers d'années pour une multitude d'applications que ce soit dans l'alimentation, la médecine (par exemple : utilisation du pavot comme somnifère [1]), la teinture de textiles, et d'autres encore. Cependant la compréhension de ces phénomènes, au niveau biologique et chimique, n'est apparue qu'à partir du XIX<sup>ème</sup> siècle. C'est en 1806 que Friedrich Wilhelm Sertürner a réussi à isoler la morphine du pavot [2]. C'est cet événement qui marque le début de l'étude de métabolites issues de plantes. [3].

Aujourd'hui, deux familles de métabolites sont dissociées : les métabolites primaires et secondaires, aussi appelés spécialisés. Les métabolites primaires sont impliqués dans les fonctions primaires, donc essentielles, de l'organisme. Ils vont participer au développement de la plante et à sa reproduction. Ils vont être formés pendant la phase de croissance de la plante. Les métabolites primaires peuvent être divisés en trois groupes et sont valorisés dans différentes applications : les lipides, les protéines et les glucides. Les métabolites secondaires, eux, ne sont pas essentiels au développement, à la croissance et à la survie de la plante. Ces métabolites sont synthétisés dans le but de résister aux différents stress pouvant être subits par la plante ou l'organisme [4]. Ils peuvent être aussi divisés en trois familles : les terpènes, les phénols et les alcaloïdes.

Les pigments appartiennent aux métabolites secondaires. Il en existe trois principaux groupes :

- **Les flavonoïdes** sont des molécules colorées allant du rouge à l'ultraviolet en passant par le jaune. Les anthocyanes qui sont un sous-groupes des flavonoïdes sont des pigments hydrosolubles caractéristiques d'une couleur de rouge à bleu. Toutes ces molécules sont constituées d'un squelette carboné avec un hétérocycle oxygéné (représenté figure 1), substitué une ou plusieurs fois par divers groupements tels que des hydroxyles, des alkyls, des éthers ou des motifs glycosides. Ces molécules sont synthétisées par la plante afin d'attirer certains insectes (exemple : la pollinisation des abeilles) ou repousser des espèces invasives. Leur absorption dans le visible et l'UV leur permet de servir d'agent protecteur lors de stress causés par divers rayonnements [5]–[7].

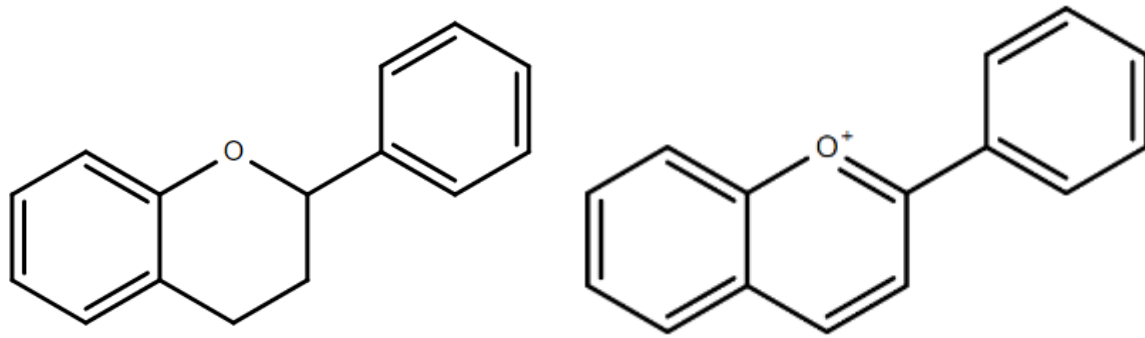


Figure 1: Squelette des flavonoïdes (à gauche) et des anthocyanes (à droite)

- **Les caroténoïdes** sont des pigments d'orange à jaune et sont également omniprésents dans la nature. Ils appartiennent à la famille des lipides. Ce sont donc des pigments liposolubles ayant des propriétés antioxydantes. Cela permet de capter des radicaux libres pouvant être formés lors du processus de photosynthèse. Les caroténoïdes peuvent se diviser en deux parties avec les xanthophylles qui peuvent être qualifiés de lipides polaires, et les carotènes qui sont plutôt non polaires (figure 2). Ces composés vont jouer un rôle de photoprotection qui permet de résister à certains stress, notamment pendant l'absence ou l'excès de lumière.-[8].

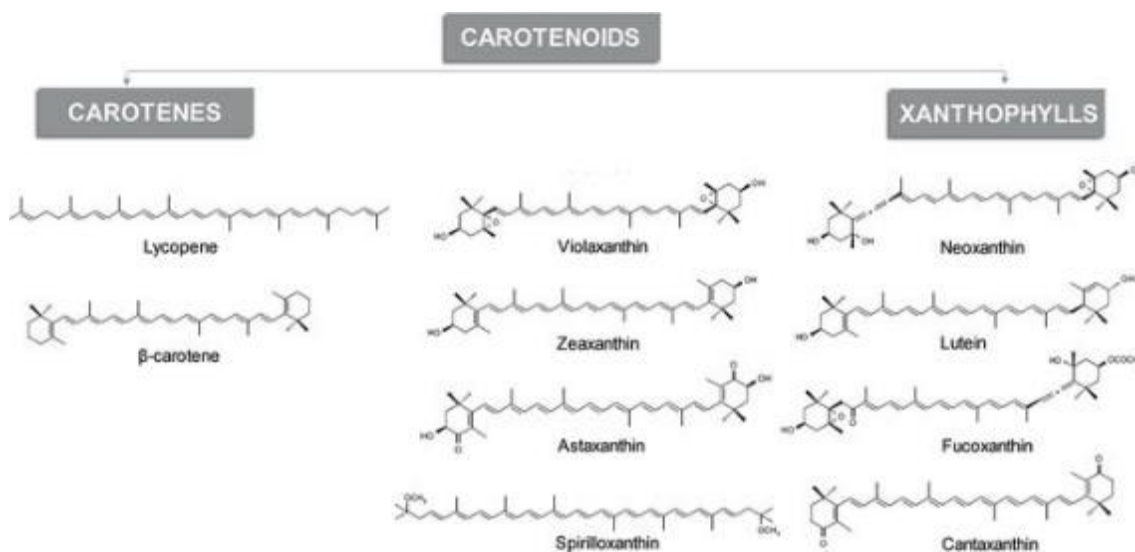


Figure 2: Exemples de carotènes et xanthophylles

- **Les chlorophylles** sont les pigments clés de toute espèce qui utilise la photosynthèse pour vivre. Leur rôle est de convertir cette énergie lumineuse en énergie chimique par la réaction de photosynthèse. Pour cette raison, les chlorophylles sont présentes dans des espèces diverses comme des plantes (partie aérienne), algues ou encore des bactéries. Comme le montre la figure 3, les chlorophylles ont un noyau porphyrinique (en vert) responsable de leur couleur et une chaîne phtyl (en jaune)

rendant ces pigments lipophiles. Ces molécules ont aussi la capacité d'émettre une fluorescence dans le rouge.

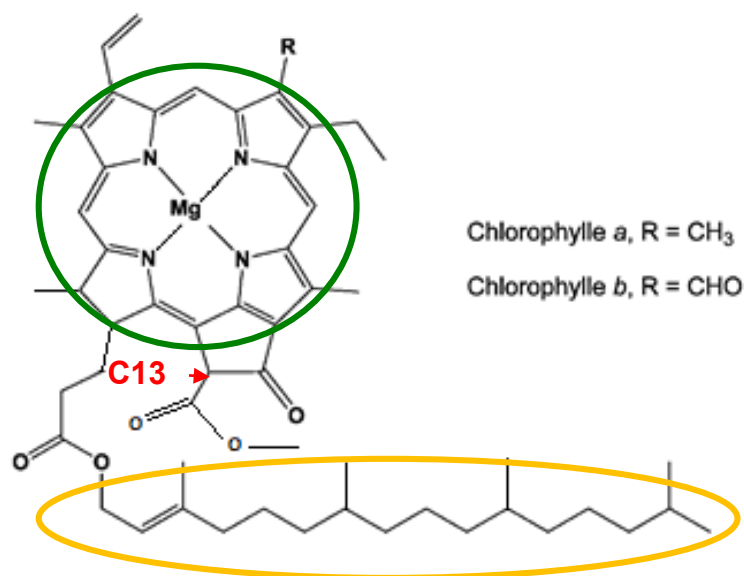


Figure 3: Formule semi développée de la chlorophylle a et b

La couleur d'un extrait peut venir des différents pigments. Les travaux de cette thèse se sont portés seulement sur les chlorophylles. La raison est qu'il s'agit d'une problématique qui intéresse le partenaire industriel de cette thèse : Alban Muller. La couleur verte intense de certains extraits est perçue comme un frein à leur incorporation dans une formule cosmétique. Les différentes problématiques liées à la couleur dans les ingrédients cosmétiques sont détaillées dans la partie suivante.

## 2. PROBLEMATIQUES LIEES AUX CHLOROPHYLLES

### 2.1. Formulation cosmétique

La couleur des extraits peut être un frein à leur utilisation. Lors de la formulation de produits cosmétiques, une couleur finale neutre, souvent blanche, est privilégiée. Des matières premières colorées comme des extraits peuvent donc être écartées car elles ne permettraient pas de répondre au cahier des charges. Si le produit final doit être coloré, le formulateur préférera ajouter un colorant stable, avec une quantité précise permettant d'avoir un résultat visuel répétable. Les pigments chlorophylliens ne sont pas stables et leur teneur dans les extraits peuvent fluctuer en fonction de l'échantillon de plante utilisé ou du procédé.

Malgré cela, formuler des extraits contenant des chlorophylles est tout de même faisable. Il est ainsi possible de masquer leur couleur en utilisant des poudres

blanchissantes comme de l'oxyde de titane (TiO<sub>2</sub>) ou autre .... Toutefois, lors de la formulation, les pigments peuvent subir des changements physiques (ex : température pendant une étape de chauffe) et/ou chimiques (ex : changement de pH), qui sont susceptibles d'altérer leur structure et de modifier la couleur des extraits.

Les pigments chlorophylliens se dégradent de différentes façons. Cette dégradation est présentée en détail dans le chapitre 2. Les différents produits de dégradations (ex : phéophytines) ont des spectres d'absorption différents de celui des chlorophylles natives (principalement chlorophylles *a* et *b*). Généralement, la couleur va tendre vers du gris/marron dû à l'apparition des phéophytines. Ce phénomène empêche donc le produit final de passer la phase d'étude de stabilité.

## 2.2. Perturbation des analyses dûes aux chlorophylles

Lors du développement d'extraits de plantes pour des actifs cosmétiques, les conditions opératoires sont optimisées en fonction d'une éventuelle bioactivité désirée, par exemple, vis-à-vis d'une problématique de vieillissement cellulaire des cellules de la peau.

Des essais biologiques in vitro permettent d'évaluer l'activité d'un extrait et donc d'optimiser l'extraction en fonction de la réponse souhaitée. Ces tests sont essentiellement basés sur des mesures d'absorbance ou de fluorescence dans le domaine UV-visible. Généralement, ces tests consistent en l'ajout d'un réactif à l'extrait qui va se colorer en fonction de l'activité et de l'intensité de celle-ci. Une lecture d'absorbance permet donc d'évaluer cette intensité. La présence dans un extrait de molécules colorées, comme des pigments, peuvent perturber ces analyses. Les pigments chlorophylliens ont des bandes d'absorbance entre 400 et 700nm (figure 4). Ces absorptions sont souvent dans la gamme de longueur d'onde où les réactifs colorés permettant l'évaluation d'activité absorbent. Ce chevauchement de longueur d'onde peut entraîner des résultats faussés.[9], [10] Par exemple, le test de la capacité antioxydante CUPRAC se base sur une absorbance à 450 nm [11]. La mesure de l'activité de la tyrosinase utilise une absorbance à 490 nm[12]. Ou encore, le dosage des flavonols totaux se base sur la lecture d'une absorbance à 430 nm[13].

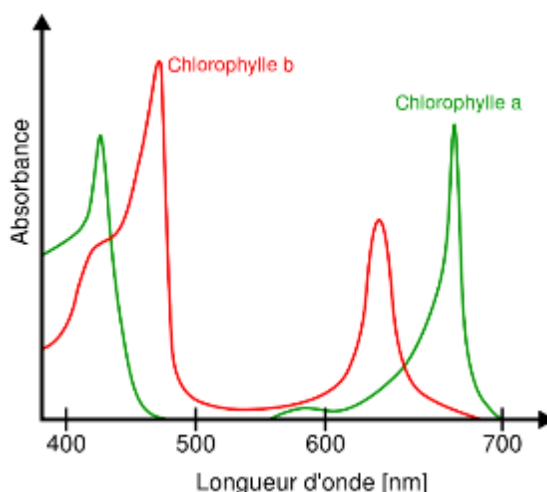


Figure 4: Spectres d'absorption UV-Visible des chlorophylles a et b

De plus, que ce soit les chlorophylles natives (a et b) ou leurs produits de dégradation (par exemple : phéophytines, phéophorbides, etc...), ces molécules sont naturellement fluorescentes. De ce fait, cette propriété peut gêner la lecture de test in vitro basé sur la fluorescence [14]–[18].

### 3. LES PROCEDES DE DECHLOROPHYLLATION

Il existe de nombreuses techniques de déchlorophyllation car elles permettent de prévenir différents problèmes qui peuvent survenir lors de la formulation (comme mentionné précédemment), ou lors de l'analyse d'activité d'extraits.

#### 3.1. Adsorption

Il est possible de retirer la couleur d'un mélange par adsorption en utilisant différentes phases solides.

Dans le cadre de la production et du raffinage des huiles végétales, une étape consiste en la décoloration de l'huile (composés colorés présents dans l'huile végétales comme des caroténoïdes ou même chlorophylles). Cette étape est réalisée en utilisant des terres naturelles décolorantes. Ce sont des argiles de type « Montmorillonite » qui peuvent être activée par un traitement acide afin d'en améliorer le pouvoir adsorbant. Il est possible d'utiliser ces adsorbants sur des huiles car la sélectivité des pigments vis-à-vis des autres molécules (comme les glycérides) n'est pas un critère important.

Le charbon actif est aussi utilisé dans l'extraction de pigments chlorophylliens [19]–[21]. Les chlorophylles contenant un noyau porphyrique plan s'adsorbent facilement sur le charbon actif qui présente une grande surface spécifique planaire. Les QuEChERS sont utilisés pour la préparation et la purification d'échantillons, notamment pour le dosage de pesticides dans les végétaux. Il existe différents adsorbants dont un à base de charbon actif permettant de retirer les pigments (chlorophylles et caroténoïdes). Il existe d'autres phases solides utilisées pour faire de l'extraction sur phase solide (SPE) des chlorophylles d'échantillons juste avant de les analyser. La phase Diaion HP-20 qui est une résine composée d'un copolymère de styrène-divinylbenzene est utilisée à cette fin [22]–[24].

Ces adsorbants permettent de séparer les pigments de l'extrait initial. Cependant, ils peuvent être trop peu sélectifs et adsorber aussi d'autres composés qui peuvent être d'intérêt [21], [25], [26]. La société UCT a développé un nouvel adsorbant capable de retirer les chlorophylles en limitant l'adsorption des analytes planaires [27]. Cependant aucun détail technique n'est donné sur ce gain de sélectivité.

Il est aussi possible d'améliorer la sélectivité de l'adsorbant par rapport aux chlorophylles en développant des polymères à empreinte moléculaire [25]. Cela consiste à synthétiser un polymère autour d'une molécule cible afin que celui-ci épouse la forme de la molécule créant ainsi des cavités sélectives de la molécule ciblée. Cependant, ce procédé est plus adapté pour la préparation d'échantillon à petite échelle afin de procéder à des analyses car il peut être long à mettre en place et coûteux.

L'utilisation d'un adsorbant pour retirer les chlorophylles d'un extrait est possible et est déjà mis en place pour certaines applications. Cependant, celui-ci reste peu sélectif, surtout à une grande échelle. De plus, une fois adsorbés, il restera difficile voire impossible de récupérer les pigments pour leur potentielle valorisation et un éventuel recyclage des adsorbants. Cette méthode n'a donc pas été sélectionnée afin de répondre à notre problématique.

### 3.2. Fractionnement

Afin de retirer les pigments dans un extrait coloré, il est aussi possible d'ajouter des étapes de purification.

L'une des plus anciennes approches est l'extraction liquide-liquide [28]. Il est possible d'extraire les pigments apolaires en utilisant des solvants non-polaires. De par la nature des chlorophylles, celles-ci vont migrer vers cette phase.

Dans les années 80, la chromatographie de partage centrifuge (CPC) a été développée. Une colonne est composée de disques placés horizontalement composés d'une série de cellules interconnectées par des capillaires (Figure 5). Dans chaque cellule, un mélange de deux phases non miscibles (avec une phase stationnaire et une phase mobile) est incorporé et stabilisé par un champ de force centrifuge. Il est alors possible de considérer la phase supérieure ou inférieure comme phase mobile ou stationnaire en fonction du mode choisi. Un mode ascendant va utiliser la phase avec la densité la plus faible comme phase mobile alors qu'un mode descendant utilisera l'autre phase. L'échantillon à purifier est alors injecté dans la colonne. Il va donc se fractionner en fonction de l'affinité des composés entre les deux phases. Cette technique est utilisée dans le fractionnement d'extraits de plantes et notamment dans des problématiques de séparation de chlorophylles [29], [30].

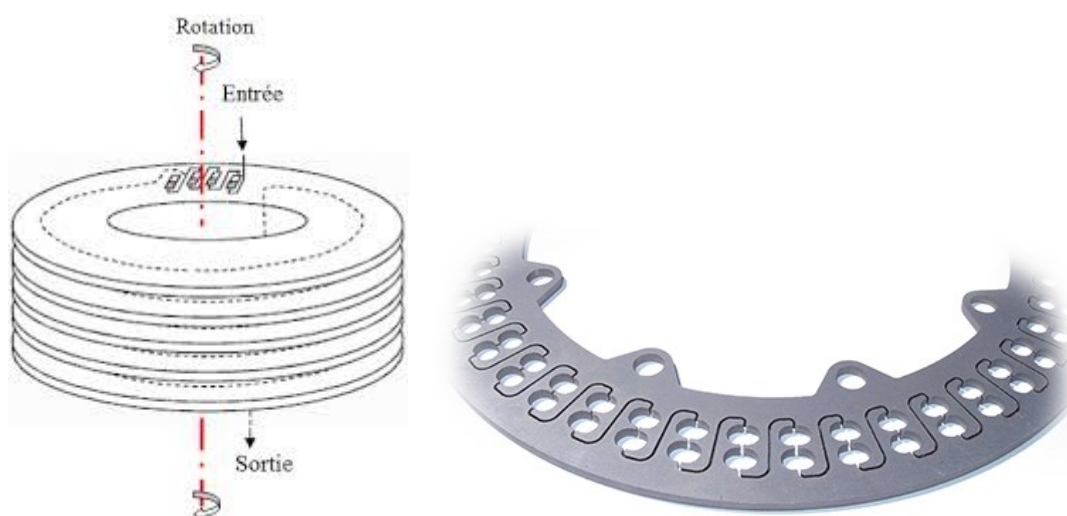


Figure 5: Schéma d'un appareil de Chromatographie de Partage Centrifuge (CPC)

Cette technique performante est très utilisée aujourd'hui à l'échelle semi préparative et préparative. Cependant, au niveau industriel il est très coûteux d'ajouter une étape de purification ou de fractionnement.

L'utilisation de techniques chromatographiques permet la séparation des pigments chlorophylliens des autres composés de l'extrait. De manière remarquable par rapport à notre sujet, une colonne contenant une phase solide est à l'origine de toute la chromatographie que nous connaissons aujourd'hui. En effet, en 1906, Mikhail

Semyonovich Tswett fut le premier scientifique à réaliser une séparation de pigments de plante [31]. Une phase stationnaire (comme du carbonate de calcium ou du sucre) et une phase mobile avec des solvants apolaires (comme de l'éther de pétrole, du disulfure de carbone ou du benzène) peuvent être utilisés pour la séparation des chlorophylles et d'autres pigments. La Figure 6 représente les différents systèmes chromatographiques utilisés par Mikhail Tswett en 1906.

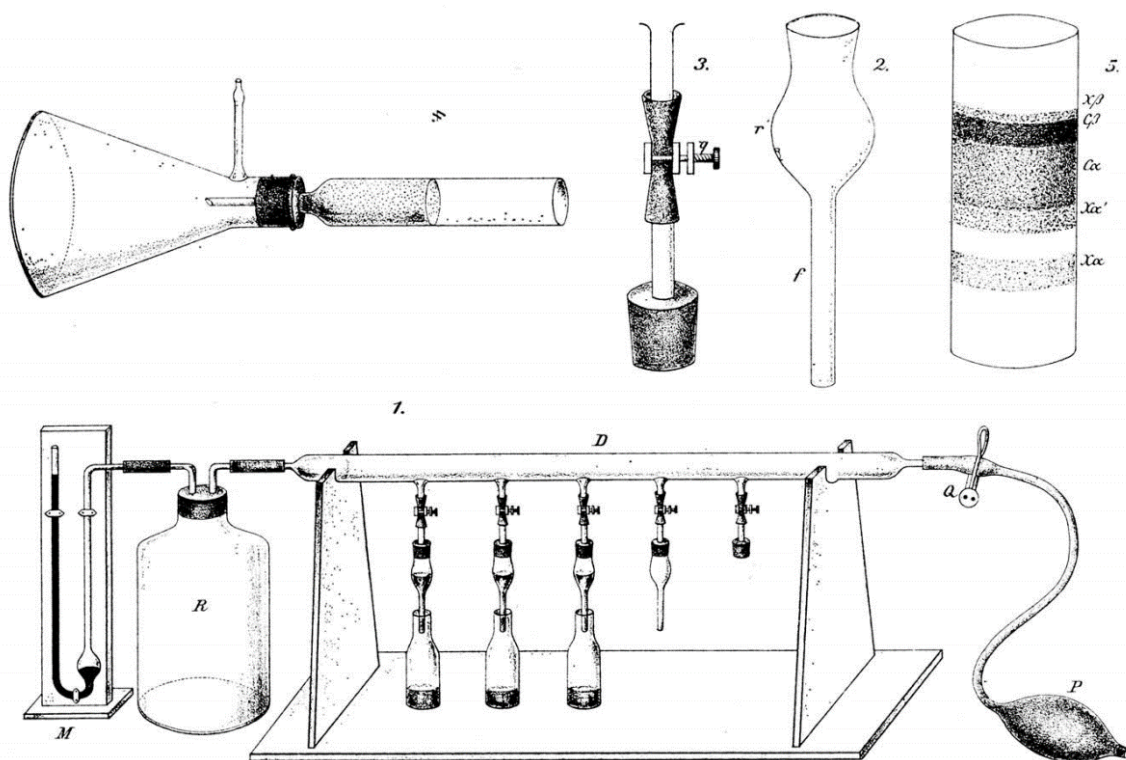


Figure 6: Système développé en 1909 par Mikhail Tswett avec de 1 à 3 des systèmes sous pression, 4 par sussions et 5 une séparation type

Sur le même principe, plusieurs méthodes existent afin de séparer sur colonne ouverte ces pigments. [32], [33] Cependant, la plupart des méthodes sont antérieures à 1970. Depuis, des méthodes plus efficaces en terme de séparation ont été utilisées et notamment la CPC.

Pour conclure, la possibilité d'un piégeage par adsorption ou la séparation des pigments chlorophylliens post extraction ont été écartées pour ce travail.

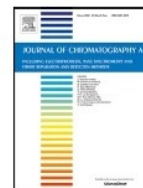
### 3.3. Extraction sélective

En sélectionnant judicieusement les conditions, il existe la possibilité d'extraire séparément des composés par rapport à d'autres. L'optimisation de ce procédé est alors focalisée sur la sélectivité. La review suivante présente un état de l'art de l'extraction sélective appliquée aux techniques d'extraction modernes (SFE, PLE, US et MO).



Contents lists available at ScienceDirect

Journal of Chromatography A

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## Review article

## Selective extraction of bioactive compounds from plants using recent extraction techniques: A review



Thibault Lefebvre, Emilie Destandau, Eric Lesellier\*

ICOA, UMR 7311, Université d'Orléans, rue de Chartres, BP 6759, 45067 Orléans, France

## ARTICLE INFO

## Article history:

Received 18 September 2020

Revised 25 November 2020

Accepted 26 November 2020

Available online 2 December 2020

## ABSTRACT

Plant extraction has existed for a long time and is still of interest. Due to technological improvements, it is now possible to obtain extracts with higher yields. While global yield is a major parameter because it assesses the extraction performance, it can be of interest to focus on the extraction of particular compounds (specific metabolites) to enrich the sample and to avoid the extraction of unwanted ones, for instance the primary metabolites (carbohydrates, triacylglycerols). The objective then is to improve extraction selectivity is then considered.

In solid-liquid extraction, which is often called maceration, the solvent has a major impact on selectivity. Its polarity has a direct influence on the solutes extracted, related to the chemical structure of the compounds, and modelling compound/solvent interactions by using various polarity or interaction scales is a great challenge to favor the choice of the appropriate extracting liquid.

Technical advances have allowed the development of recent, and sometimes green, extraction techniques, such as Microwave-Assisted Extraction (MAE), Ultrasound-Assisted Extraction (UAE), Pressurized Liquid Extraction (PLE) and Supercritical Fluid Extraction (SFE). This review focuses on the specificity of these recent techniques and the influence of their physical parameters (i.e. pressure, intensity, etc.). In addition to the solvent selection, which is of prime interest, the physical parameters applied by the different techniques influence the extraction results in different ways. Besides, SFE is a versatile and green technique suitable to achieve selectivity for some compounds. Due to its properties, SC-CO<sub>2</sub> allows tailoring conditions to improve the selectivity.

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## 1. Introduction

Primary and secondary metabolites can be extracted from plants by different techniques. For non-volatile compounds, the oldest technique is simply to bring the plant into contact with a solvent (water, alcohol, oil...). This technique is called solid-liquid extraction, or more commonly maceration. For several years now, so-called "modern" extraction techniques have been developed. The enhancement of performance comes from the use of "assisted" technology (i.e. ultrasonic waves, pressure, microwaves) which may reduce the duration of the process and induce energy and solvent saving through various intensification mechanisms. This physical treatment can also impact the extraction mechanism, possibly increasing extraction yields and causing different extraction selectivities compared to simple maceration.

To determine the best operating parameters, process optimization is frequently based on the global yield. However, in some cases, it is necessary to extract one family or one compound over another. Selectivity is then considered to find the best conditions. It can be used to enrich an extract with target compounds [1], or to avoid unwanted compounds such as pollutants [2–5]. In the latter case, purity is the optimized parameter.

The aim of this review is to investigate the different parameters that can induce extraction selectivity in numerous extraction methods.

First, the nature of the solvent is investigated as the right choice of solvent can by itself induce selectivity. In a second part, the review focuses on different techniques and their respective parameters to examine their role in selective extraction. Three extraction methods are described: Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE) and Pressurized Liquid Extraction (PLE). For example, the impact of frequency and power on selectivity is studied for UAE, pressure for PLE and so on. In the last part, the use of supercritical carbon dioxide (SC-CO<sub>2</sub>) in Supercritical Fluid Extraction (SFE) is detailed. This fluid is well known

\* Corresponding author.

E-mail address: [eric.lesellier@univ-orleans.fr](mailto:eric.lesellier@univ-orleans.fr) (E. Lesellier).

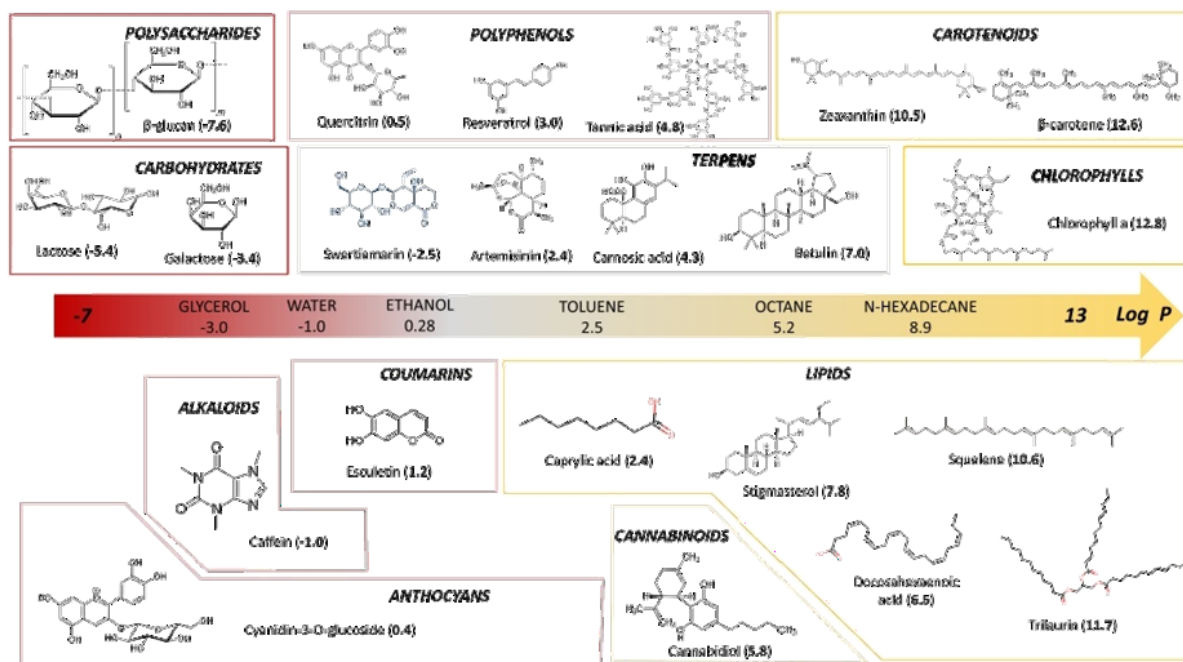


Fig. 1. Metabolite families and solvents organized as a function of log P and formulas of different metabolites.

for its selectivity properties. For this reason, this extraction method will be dealt with at greater length than the other three. Carbon dioxide modulation is studied and other parameters such as the use of separators or extraction vessel geometry are presented.

## 2. Extraction solvent

### 2.1. Solubility models

Whatever the extraction method used, the chemical nature of the extraction solvent is of primary importance to favor the compound solubility, i.e. the extraction recovery and the extraction selectivity.

The knowledge of these chemical properties, which are the basis of the interactions developed between the solvent and the compounds, guides the selection of an adequate solvent, to maximize the yield or purity of the target molecules. Various scales of solvent and compound chemical properties can help in this choice. For instance, the Rohrschneider polarity  $P'$  describes with one general value the ability of the molecules to dissolve in water or in alkanes. Other models such as the solvation parameter model, with the five Abraham descriptors (E, S, A, B, V), or the Cosmo-RS model, with five COSMOments (sig2, sig3, Hb don3, HB acc3, CSA), both detail five interaction types as reviewed elsewhere [6].

One polarity scale is commonly used to evaluate and compare polarities: log P. Also known as log  $K_{O/W}$ , this parameter is measured as the partition of the target analyte between two solvents (octanol and water). Similar to the Rohrschneider polarity parameter  $P'$ , one value describes the best solubility of the compound in the polar (aqueous) or non-polar (octanol) solvent. Concentrations in each phase are measured and log  $K_{O/W}$  is calculated using the following equation:

$$\text{Log } K_{O/W} = \text{Log} \left( \frac{C_{\text{octanol}}}{C_{\text{water}}} \right)$$

where  $C_{\text{octanol}}$  and  $C_{\text{water}}$  are the concentrations of the compound measured in each phase. *In silico* models also allow the direct calculation of this parameter computationally. In order to give an

idea of the polarity of different compounds, Fig. 1 locates different compound families as a function of their log P, and Tables S1-S15 summarize all the data for the molecules cited as examples in this review (chemical structures and log P). Log P values were calculated using the MolDesc platform designed by the Institute of Organic and Analytical Chemistry (ICOA) in Orleans – FRANCE (<http://moldesc.icoa.fr/>). This information can be used as a first-line value in order to estimate the polarity of the solvents and compounds discussed in this review.

The Hansen solubility parameter scale is another frequently used model. It is based on three parameters  $\delta_d$ ,  $\delta_p$  and  $\delta_h$ , which respectively describe dispersion, dipolar and hydrogen bond interactions. This scale may appear less accurate than the solvation parameter model (or linear solvation energy relationship - LSER) or the Cosmo-RS models, because of the smaller number of parameters (three against five). However, the possibility to calculate these three Hansen parameters simply from structural fragments of compounds makes it a popular scale. Numerous studies present its use both for the extraction of natural compounds, and to substitute classical and sometimes toxic solvents by green ones [7–20].

Among the various types of presentations used to assist in the choice of solvent such as a Hansen sphere or Teas triangle, the spider diagram is a useful figure to locate each solvent in relation to its three parameter values on a planar projection. Fig. 2 shows the location of classical solvents (blue bubbles) used for extraction, as further discussed below, namely water, methanol, ethanol, dimethylformamide (DMF), hexane, acetone and acetonitrile. On this diagram the bubble size is related to the total Hansen parameter. As expected, water is located at the top of the spider diagram because of the high value of the hydrogen bond  $\delta_h$  parameter; hexane is at the bottom right, having a high (and unique) dispersive contribution ( $\delta_d$ ); while acetonitrile is at the bottom left, because of its great dipole-dipole interactions ( $\delta_p$ ). The classical solvents presented on this diagram are well spread over the diagram space, showing the varied choices, which can be related to the diverse chemical structures of natural compounds. However, due to ecological and toxicity issues, these classical solvents are increas-

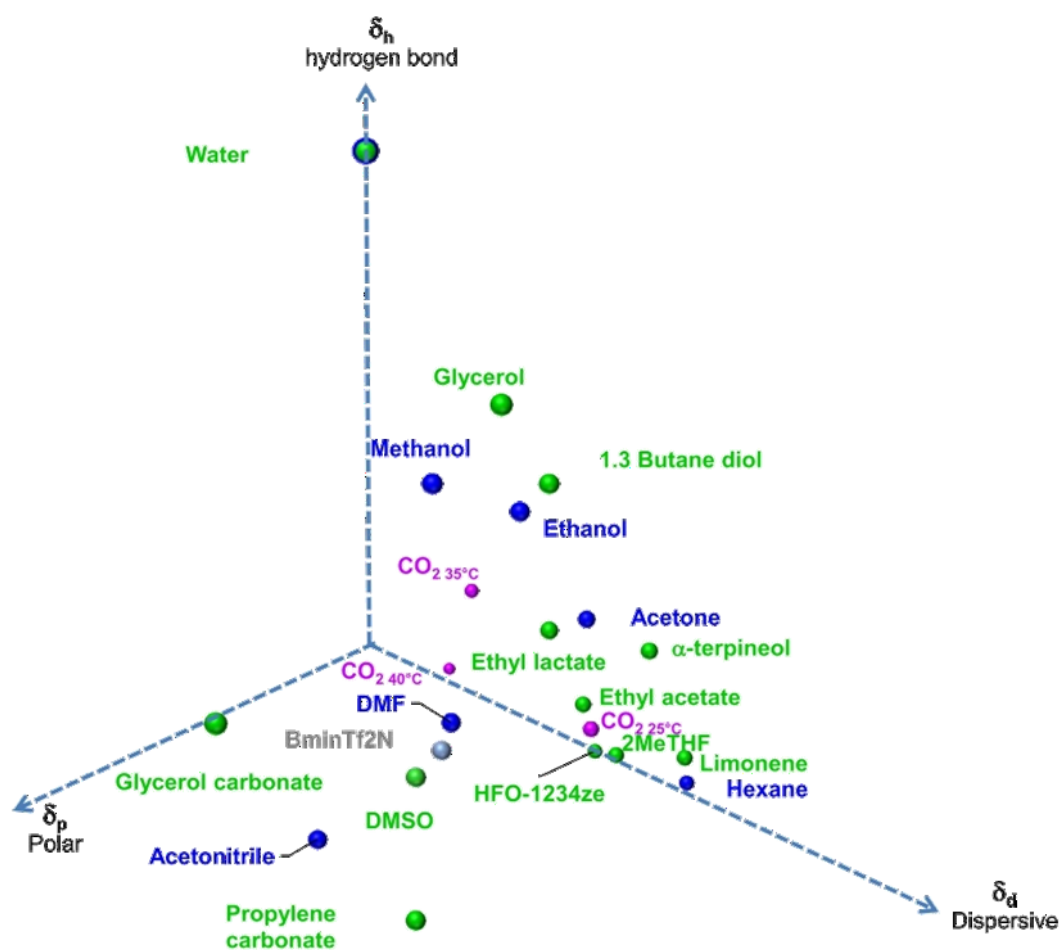


Fig. 2. Hansen solubility parameters of solvent (in blue: classical solvents, in green: green solvents, in purple: carbon dioxide). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ingly substituted by “green” ones. On the basis of previous work suggesting these replacements [7,8,20], one can see the location of green solvents (green bubbles) that can be used instead of classical ones (blue bubbles). Clearly, water is a green solvent, whereas glycerol and 1,3 butane-diol can replace methanol; limonene, methyl-tetrahydrofuran (MeTHF) or 1,3,3,3 trifluoropropene (HFO-1234ze) replace hexane, etc. Some ionic liquids are also used as green solvents, for instance B<sub>min</sub>Tf<sub>2</sub>N seems able to substitute DMF. Carbon dioxide is also often used for green extraction. As shown on Fig. 2, the location of carbon dioxide at 10 MPa varies according to the temperature (25–40°C) [10–18]. Because of the fluid compressibility, CO<sub>2</sub> density is in part related to temperature, which can modify the Hansen parameter values. Moreover, as further discussed, the calculation of these Hansen parameters depends on the equations and data used, which can lead to differences in the results, explaining some differences between the published values.

Fig. 3 shows the location of some bioactive natural compounds on the basis of their Hansen parameters. Four of these compounds (rutin, quercetin, oleuropein and chlorophyll) display a high value both for the polar ( $\delta_p$ ) and hydrogen bond ( $\delta_h$ ) values and are located on the left of the occupied space, whereas three other compounds (epicatechin, naringin, catechin) display a high  $\delta_h$  value and are located on the top and the right side of the spider diagram. The five compounds having a flavonoid skeleton with numerous hydroxyl groups that can scavenge free radicals in order to prevent damage on a cellular level (catechin, epicatechin, rutin, naringin, quercetin), appear to be best extracted with water or pro-

tic solvents, for instance ethanol, glycerol or 1,3 butanediol (Fig. 2). For naringin, a natural antioxidant which is used as a food additive to treat obesity and diabetes, its solubility appears to be higher in aqueous glycerol than in water, as the Hansen parameters of naringin are closest to that of glycerol [14]. However, the change from water to aqueous glycerol did not provide selective extraction of polyphenols from grape fruits (i.e. anthocyanins are more soluble in water than in aqueous glycerol). Several natural carotenoid pigments ( $\beta$ -carotene, astaxanthin, fucoxanthin, curcumin) are located in the same area, together with  $\alpha$ -tocopherol,  $\beta$ -sitosterol and resveratrol. All these compounds have biological activities, such as anti-inflammatory, anti-oxidant or anti-carcinogenic properties, whereas triterpene betulin acts as an antiviral agent [12].

Fig. 4 shows the location of some natural products and extraction solvents studied. First, and as discussed previously, depending on the calculation method, the values obtained can differ dramatically. For instance, the location of rosmarinic acid (RA) [18,21] can be close to ethanol or 1,3 butanediol when using the Stefanis method [22] whereas it is close to glycerol carbonate when using the Just method [23]. The differences between these calculation methods were recently discussed by Mathieu [24], who reported the superiority of the Stefanis model for polar compounds, as it takes into account local effects associated with conjugation.

Artemisin, a sesquiterpenoid known for its antimalarial activity, which is located lower on the diagram (Fig. 4), displays a very high solubility in dichloromethane (DCM) (1195 mg/mL), a rather high solubility in dimethylsulfoxide (DMSO) (103.7 mg/mL) and a

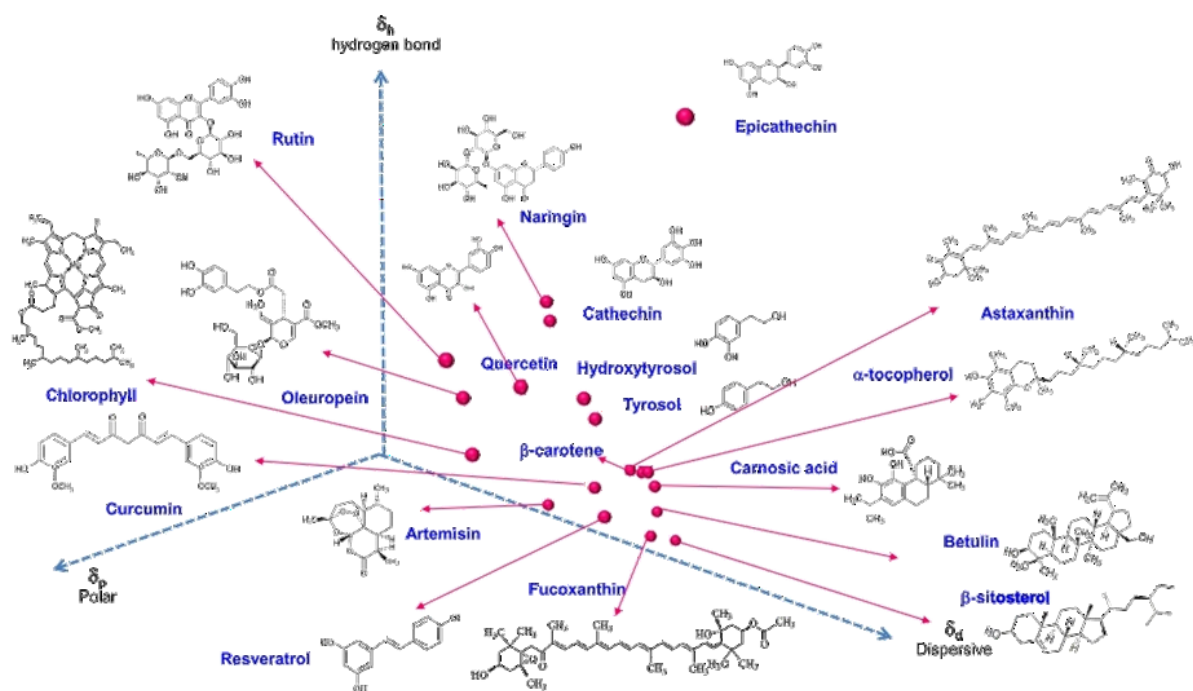


Fig. 3. Hansen solubility parameters of different metabolites and their formulas.



Fig. 4. Hansen solubility parameters (HSP) of different compounds (in yellow: different rosmarinic acid (RA) MSPs, in green: green solvents, in brown: organic solvents, in blue: polar natural products, in red and purple: mid and non-polar natural products). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

satisfactory one in ethyl acetate (39.4 mg/mL) [9]. However, the diagram shows that the location of artemisin toward these three solvents is not fully in accordance with the experimental solubility measurements. Similarly, the solubility of kahweol and cafestol toward varied solvents has been studied [12]. The normalized values range from 100% for the best extraction obtained in methyl-*t*-butylether (MTBE), to 0% for ethyl acetate, reaching 88% for DCM and 1.3% for hexane. Although these four solvents seem to be at equivalent distances from the two diterpenes, the experimental solubility values are quite different. Another study was done on the extraction of phenolic compounds (tyrosol, hydroxytyrosol) and phytosterol ( $\beta$ -sitosterol) from olive oil. As expected, the addition of a hydroxyl group to tyrosol improved the  $\delta h$  parameter of hydroxytyrosol, but despite these high  $\delta h$  values, both were only slightly soluble in water [16]. When looking at the respective solubility of these compounds towards green solvents such as ethanol, ethyl lactate, ethyl acetate and limonene, the use of ethanol allows the selective extraction of phenolic compounds, and the use of limonene the selective extraction of  $\beta$ -sitosterol. The other two solvents, ethyl lactate and acetate, could be used for the simultaneous extraction of interesting compounds [16]. The extraction recovery for fucoxanthin was studied in four green solvents, ethanol, ethyl acetate, ethyl lactate and limonene [20]. The best recovery was obtained with ethyl acetate, and the worst with limonene, despite its very close location to fucoxanthin on Fig. 4. The authors mentioned that the Hansen parameters were related to solubility, but not to the extraction kinetics, which depend on the matrices. Consequently, the use of Hansen parameters should be restricted to initial evaluation for the solvent selection. Nevertheless, when looking at the extraction selectivity between carotenoids and chlorophylls, the values were 0.52 for ethanol, 0.54 for ethyl lactate, 1.22 for ethyl acetate and 3.54 for limonene (at 40°C), showing the satisfactory relationship between the Hansen parameter location and this relative value.

All these models help to describe the polarities of different molecules based on different calculations. However, a common feature of all these models is that they evaluate a molecule without taking its environment into account. In the case of plant extraction, it is not clear that a solvent, which may solubilize a compound predicted by a previous model, will extract this same compound during plant extraction: other mechanisms can interfere during extraction such as co-extraction of other molecules, intra-particle diffusional resistance, etc.

## 2.2. Solvent selectivity

In solid-liquid extraction, solutes migrate from the solid (plant sample) to the liquid (extraction solvent) depending on the physicochemical properties of the solutes and solvent. It is therefore natural that the extraction solvent should play an important role during extraction and affect selectivity. Several studies have highlighted the selectivity of different solvents as a function of the targeted compounds. These solvents and their selectivity properties can be applied to any kind of extraction processes. This first part of the review focuses on solvent effects independently of other physicochemical effects induced by the extraction technique itself such as temperature, pressure, etc.

To illustrate the solvent effects, Santovo et al. [25] extracted antimicrobial molecules from freeze-dried *Haematococcus pluvialis* microalgae using PLE. Hexane and ethanol were used and compared. They confirmed that hexane (Log P = 2.6) favors the extraction of alkanes (i.e. octane Log P = 3.4) and methyl esters (i.e. Hexadecanoic acid ethyl ester Log P = 6.0) without carboxylic acids, while ethanol (Log P = 0.0) extracts carboxylic acids (i.e. butanoic acid Log P = 0.9) without any alkanes or methyl esters. Otero et al. [26] demonstrated that ethyl acetate is a better solvent to extract

fatty acids from freeze dried Galician Algae compared to less polar (hexane) and more polar (ethanol, and ethanol/water) solvents using PLE. The fatty acid content (expressed as mg per g of extract) was higher using ethyl acetate (693.20 mg/g) than hexane (426.12 mg/g) or ethanol (554.42 mg/g). However, it highlighted the fact that solubility is not a binary term (solutes are neither completely soluble nor not soluble at all).

Ethanol and hexane were also compared with respect to pigment content. Jaime et al. [27] studied the impact of these solvents through the extraction yield of different pigments such as chlorophylls and carotenoids from *Haematococcus pluvialis* microalgae by PLE. Both pigment families were found in ethanol and hexane extracts. This shows that even with a high log P gap, some compounds are soluble in any solvent. Differences were observed with xanthophylls such as lutein, which was more abundant in the ethanol extract than in the hexane extract (61.0% normalized area in ethanol vs 38.2% in hexane). The contrary was observed with carotenes such as  $\beta$ -carotene (5.4% in ethanol vs 8.4% in hexane). This can be explained by the fact that xanthophyll compounds are more polar than carotenes due to the presence of oxygenated functions (ketone, epoxy, hydroxyl). Saha et al. [28] studied different solvent compositions (using acetone LogP = -0.24, hexane and ethanol) on the extraction of lutein and  $\beta$ -carotene from orange carrot using PLE. Acetone mixtures led to extracts with a lower content of both carotenoids. However, ethanol mixed with hexane demonstrated a higher recovery of lutein and  $\beta$ -carotene. Macías-Sánchez et al. [29] extracted the same pigments by UAE from *Dunaliella salina* micro-algae using two other solvents, methanol and DMF (LogP = -0.83). The carotenoid/chlorophyll ratio was measured. Between these two solvents, the ratios were respectively around 5 and 8, demonstrating a higher carotenoid selectivity with DMF, which is less polar than methanol. This selectivity was also investigated by Sánchez-Camargo et al. [10] on *Phaeodactylum tricornutum* diatom by PLE. Among varied solvents, some considered as greener were tested such as d-limonene or ethyl lactate. They demonstrated a higher carotenoid/chlorophyll ratio, showing higher selectivity. The minimum ratio was obtained using ethanol (0.52) while the maximum was obtained with d-limonene at (1.73).

Selectivity can also be observed for more polar molecules. Belumori et al. [30] extracted rosemary leaves with MAE and UAE and different solvents. They showed that hexane was more selective for terpenoids (mainly carnosic acid), while selectivity for phenolic compounds such as rosmarinic acid was improved using ethanol. This was confirmed by Oliveira et al. [31] using different extraction solvents from water to acetone with maceration. Water favored the extraction of rosmarinic acid, while acetone favored carnosic acid extraction. Zachová et al. [32] examined the extraction of stilbenes (i.e. resveratrol, ...) from grape cane with methanol, acetone and ethanol. They demonstrated that ethanol was more selective than methanol by extracting similar amounts of stilbenes with a lower global yield. With an 18% lower yield compared to ethanol, acetone was the most selective solvent, extracting 2.4 times more concentrated stilbene. Boukhris et al. [33] developed a two-step PLE to extract germacranolides and phenolic compounds from *Anvillea radiata*. A first extraction with chloroform extracted germacranolides and a second step with methanol extracted phenolic compounds. To conclude, the above studies confirmed that selectivity can be obtained using non-polar and polar solvents, or by mixing them to obtain other extraction solvents with intermediate global polarity.

In the last decades, new kinds of solvent have been developed. Ionic liquids (IL) were some of the first. They were discovered at the end of the XIX<sup>th</sup> century, but found application only from the 1970s on (first used as electrolytes in batteries). They consist in blending two ionic compounds with opposite charges.

This blend creates a mixture with different physicochemical properties from those of the compounds used. For example, this difference can be visibly seen by mixing two solids and obtaining a liquid mixture. This kind of solvent has been used in plant extraction and shows interesting selectivity. Liu et al. [34] studied the use of an ionic liquid ( $[\text{HO}_3\text{S}(\text{CH}_2)_4\text{mim}][\text{HSO}_4]$ ) at different concentrations in water: from 0.2 to 1.6 M for the extraction of phenolic acids from *Eucalyptus globulus* leaves by MAE. The ionic solvent  $[\text{HO}_3\text{S}(\text{CH}_2)_4\text{mim}][\text{HSO}_4]$  at 1.0 M in water improved extraction by playing a catalytic role in the formation of ellagic and gallic acids from hydrolysable tannins such as tellimagrandin II. Selectivity was observed by changing the concentration of the ionic solvent, inducing different catalysis mechanisms that resulted in different ellagic and gallic acid compositions. Similar results were obtained by Sukor et al. [35] who used BmimTf2N for the extraction of gallic and tannic acids from oak galls during UAE. Different concentrations from 0.05 to 0.20 M led to different acid contents. Compared to other classical solvents, for example methanol, ionic liquids doubled the extraction yield of these phenolic compounds. These studies confirm the interest in these IL compared to more classical solvents such as methanol. This catalytic agent allows the extraction of compounds that are not extractable with other selective solvents.

Another family of solvents was developed historically from IL. Deep Eutectic Solvents (DES). DES are more recent, as they appeared at the beginning of the 2000s. The principle is close to that of IL. However, the compounds are not ionic and the interactions result from hydrogen bonding. To create a DES, one hydrogen-bonding donor and one hydrogen-acceptor are required. This results in a eutectic mixture with a lower melting point. Bubalo et al. [36] developed different DES for the extraction of phenols from grape skin using MAE and UAE. Choline chloride was used as hydrogen acceptor while different hydrogen donors were tested (i.e. glycerol, oxalic acid, malic acid, sorbose). Results showed that the selectivity could be tailored as a function of the different DES used. This was especially the case for the recovery of p-coumaroylmonoglucosides (i.e. malvidin-3-O-(6-p-coumaroyl)glucoside) using a choline chloride:glycerol 1:2 (molar ratio) mixture compared to other compounds such as anthocyanin-3-O-monoglucosides (i.e. petunidin-3-O-glucosides). Shang et al. [37] studied the extraction of *Cyclocarya paliurus* (Batal.) Ijinskaja leaves by UAE with respect to different flavonoid yields. They showed that alcohol- and amine-based DES are better for the extraction of these flavonoids than sugar- and acid-based DES. This can be explained by the fact that these compounds are better extracted by an alkaline solvent. It is then possible to selectively extract these flavonoids (kaempferol, kaempferol-7-O- $\alpha$ -L-rhamnoside, quercetin, quercetin-3-O- $\beta$ -D-glucuronide, and kaempferol-3-O- $\beta$ -D-glucuronide) from other compounds, which are better extracted in acidic solvents (i.e. anthocyanins, which are described next). Another name can be used for this kind of solvent: NaDES when only "natural" compounds are used such as carbohydrates (glucose, sorbitol...), acids (citric acid, amino acids, ...), etc. Guo et al. [38] worked on the extraction of anthocyanins (i.e. cyanidin-3-O-glucoside) from mulberry using NaDES. These compounds are sensitive to pH changes, and by using different kinds of solvents (acidic and alkaline), the authors demonstrated that anthocyanins are better extracted using acidic mixtures. The impact of pH itself on selectivity is further detailed later in this paper.

In order to change the extraction solvent viscosity, or to increase its polarity, water can be added to other solvents. Pagano et al. [39] confirmed the use of a water mixture with ethyl lactate and ethanol on *Ocimum basilicum* L by PLE to recover polyphenolic acids (rosmarinic and caffeic acids). Ethyl lactate mixtures seemed inadequate to extract these compounds. However, adding water had a positive impact for acids. The highest content expressed in

mg per g of extract was obtained using 25% of water in ethanol for rosmarinic acid (17.78 mg/g) while the caffeic acid content was higher with pure ethanol (2.42 mg/g). The caffeic acid/rosmarinic acid ratio was calculated and was higher with pure ethanol, showing that pure ethanol is the most selective solvent to extract caffeic acid over rosmarinic acid.

The impact of water was also investigated on pigments. Rudke et al. [40] showed a negative influence of water on carotenoid extraction from buriti (*Mauritia flexuosa* L.) using ethanol-water mixtures in PLE. In addition, carotenoids were little impacted by temperature changes. However, the phenolic content seemed less affected by solvent change than by temperature. Based on these results, it is then possible to increase selectivity for the extraction of carotenoids over polyphenols and vice versa using temperature (this parameter is discussed further in this review). Regarding more polar pigments such as tannins, Bosso et al. [41] showed a positive impact of an ethanol:water mixture for the extraction of condensed tannins and flavonoids by UAE. But when using pure solvent (water or ethanol), the tannin content decreased drastically and reached a minimum of 8.0 mg/g dry sample weight with EtOH:Water 50/50 v/v, and around 0.5 mg/g with EtOH and water. A maximum flavonoid yield was also reached using ethanol-water mixtures. But using pure ethanol, the decrease was not as significant as that observed for tannins. Using pure ethanol was then a more selective way to extract flavonoids over condensed tannins.

Carrero-Carralero et al. [42] extracted bioactive carbohydrates (i.e. inositols) from mung bean (*Vigna radiata*) and studied the impact of different extraction solvent mixtures between ethanol, methanol and water by MAE. Water extracted more carbohydrates and other non-bioactive compounds. However, despite the higher yield, water was less selective than alcohol mixtures as it also extracted non-bioactive carbohydrates, thereby producing bioactive extracts of lower purity.

As shown previously, pH affects extraction yields due to different forms of ionizable analytes. Machado et al. [43] compared different extracts obtained with an ethanol:water mixture to a mixture with an acidic pH (pH=2). The acidic solvent improved the global yield, but it did not improve the yield of phenolic and anthocyanin content. As a result, selectivity towards these compounds was lower. pH can also impact the plant matrix, as acidic conditions improve the breakdown of different cell walls. Qu et al. [44] studied the impact of pH on the extraction of seed oil from yellow horn. Reducing pH seemed to improve the oil release by changing the stability of oil bodies in the cell walls, thereby helping the release of lipids. However, for more acidic conditions (from pH=4 to 3), the extraction yield was no longer improved and this is possibly explained by the proteins that coalesced, which stopped oil release by blocking it in cells. This highlights the fact that pH can affect selectivity by changing the plant cell organization, thereby modifying the release of compounds (in the present case, lipids).

In addition to water, additives can also tune extraction selectivity. It is possible to use aqueous two-phase systems (ATPS) for plant extraction. ATPS are generally composed of alcohol and water with inorganic salts. Depending on their charge density and hydration capacity, salts can change solvent properties. For instance, water and ethanol are miscible. By adding salts, their hydration by water will reduce miscibility between ethanol and water. Ethanol is then excluded from the upper part of the mixture. For example, Guo et al. [45] used different salts ( $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{CaCl}_2$ ,  $\text{NaCl}$  and sodium citrate) to extract lignans (schizandrin, schisantherin A and deoxyschizandrin) from *Schisandra chinensis* (turcs.) Baill seeds by UAE. The ATPS excluded ethanol in the upper part but also other water-free molecules, and in the present case, lignans. Excluding lignans during the process enhanced extraction by maintaining a maximum concentration gradient between plant and

solvent, thereby favoring their solubilization. Although yields were similar to classic ethanol extraction, purities increased: the purity of the three target compounds doubled with UAE-ATPS compared to classic UAE-ethanol extraction. This technique was also used by Cheng et al. [46] to extract polysaccharides from *Gentiana scabra* Bunge by MAE. This two-phase system allowed the selective recovery of polysaccharides in the upper part, enhancing the purity, and hence the selectivity. Polysaccharides that are soluble in ethanol (homopolysaccharides made of glucose for example) were better extracted in the upper phase while those soluble in water (heteropolysaccharides made of arabinose, galacturonic acid for example) remained in the bottom phase. This is a selective way of extracting different groups of polysaccharides.

The solvent plays a major role in solid-liquid extraction. Its polarity directly influences the compounds extracted, from non-polar ones with the extraction of alkanes using hexane [25] to the extraction of carbohydrates using water [42]. In addition, green solvents such as NaDES proved to be effective for the extraction of anthocyanins [38]. However, in the last few decades, new modern extraction techniques have been developed in order to improve extraction both in terms of efficacy and in the way they are obtained: greener, safer and more cost-effectively. Different physical effects are put to advantage to intensify the extraction process and four techniques will be detailed in this review.

### 2.3. Selectivity in modern liquid extraction techniques

Ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and pressurized fluid extraction (PLE) can provide selectivity either during a pre-processing step, or during an extraction process, or both. In this review, selectivity during extraction steps is examined for different techniques.

### 2.4. Ultrasound assisted extraction (UAE)

Ultrasonics were discovered at the end of the XIX<sup>th</sup> century. These acoustic waves have been applied to food processing as a new green technology, but also in the pharmaceutical, nutraceutical and cosmetic fields.

The use of ultrasound enhances the extraction mechanism. These waves, at certain frequencies and amplitudes, create cavitation bubbles which, when they reach a non-stable point, release high temperature and high pressure by imploding. This phenomenon can break cell walls and favor the release of metabolites. Several parameters can modulate ultrasonic waves. The two main ones are the frequency (Hz) and the amplitude (MPa). Power (W) is the amplitude over time and intensity is power over surface area (W/m). These parameters change ultrasonic waves and can interact differently with plant samples.

The frequency of ultrasound waves is an important factor. However, no article was found demonstrating significant extraction selectivity by modifying the frequency. Chen et al. [47] studied the impact of different factors such as solvent, temperature but also ultrasonic frequency (from 2 to 8 kHz) on the extraction of betulin from white birch bark. However, frequency seemed to have a less significant effect than the solvent on betulin purity.

Ultrasound power, on the contrary, has shown a significant effect on selectivity. Alvez-Filho et al. [48] focused on the extraction of chlorogenic acids from sweet potato peels. Different power densities from 0 to 50 W/L (equivalent to 500 W in this case) were applied during the extraction and revealed that some compound yields such as 4,5-dicaffeoylquinic, 3-caffeoyl-4-feruloylquinic, and 3-caffeoylquinic acids improved with intensity. These compounds

result from hydrolysis reactions on other molecules such as tri-caffeoylquinic acid. This reaction is due to the formation of OH radicals, caused by ultrasonic waves, which produce H<sub>2</sub>O<sub>2</sub>. Hydrogen peroxide and hydroxyl radicals can induce a hydrolysis reaction. This article highlighted the fact that ultrasound-assisted extraction can be used to extract or modify solutes by adjusting the power, resulting in a selective process. Sousa et al. [49] observed similar results in an earlier study on gallic acid formation from other polyphenols extracted from *Phyllanthus amarus* using of different intensity range (99 to 301 W/cm<sup>3</sup>). They demonstrated that a higher gallic acid content was obtained with the highest intensity during the longest extraction time tested (9 min). Hydrolysis reactions were also observed on polysaccharide extraction from tea flower by Wei et al. [50]. Ultrasonic waves seem to be able to cut polysaccharide chains into small pieces, affecting the selectivity of the process. The same effect on polysaccharide was studied by Zhu et al. [51] on pomegranate peel, varying the power from 100 to 200 W.

Differences can also be seen with different instruments. Jacotet-Navarro et al. [52] compared three ultrasonic devices (bath, reactor and probe) by extracting rosmarinic, carnosic and ursolic acid from rosemary leaves. Differences were observed regarding the yield of these compounds and their purities. These differences were due to the different ultrasonic power provided by each device.

Independently of ultrasound waves' characteristics, it is also possible to use temperature in order to optimize the extraction process. Ramić et al. [53] studied the impact of different parameters such as temperature (30 to 70°C) and ultrasonic power (72 to 216 W) on the extraction of polyphenolic compounds from *Aronia melanocarpa* by-products. Optimized conditions were found for different groups: Total Phenolic (TP), Total Flavonoid (TF), Monomeric Anthocyanins (MA) and Total Proanthocyanidin content (TPA). The optimal temperature for all families was 70°C while the best ultrasonic power was around 200 W. However, the effect of ultrasonic power did not appear to be very significant compared to temperature. MA content decreased with high temperature (70°C) and long extraction time (90 min), suggesting degradation effects. Other compound families (TP and TF) showed an increasing extraction yield with temperature, indicating good thermal stability. It is therefore possible to selectively extract TP, TF and TPA from MA by using high and optimized temperatures with a long extraction time (90 min).

Chemat et al. [54] compared the impact of temperature on the yield and purity of artemisinin from *Artemisia annua* L. between conventional extraction and UAE. They showed that UAE with high temperature increased the extraction yield, but that the purity of artemisinin decreased due to the enhanced extraction of other compounds. Similar results were obtained by Lavoie et al. [55] with the extraction of betulinic acid from *Betula alleghaniensis* and *B. papyrifera*. The concentration of this compound increased with temperature. However, the extraction was more selective at low temperature, yielding purer extracts that were not contaminated with squalene.

To conclude, ultrasonic waves enhance plant extraction by facilitating cell wall breakdown and compound release. However, other mechanisms induced by different intensities [48] (chlorogenic acids from sweet potato peels), temperatures [53] (polyphenolic compounds from *Aronia melanocarpa* by-products) or devices [52] (by the comparison of bath, reactor and probe for the extraction of rosmarinic, carnosic and ursolic acid from rosemary leaves) can affect extraction and even selectivity. For example, thermal degradation and hydrolysis can chemically modify the solutes, making it possible to selectively obtain solutes. All the above-mentioned articles demonstrate how ultrasonic waves can change the extraction process, resulting in different selectivities.

## 2.5. Microwave assisted extraction (MAE)

Microwave-Assisted Extraction (MAE) uses microwave technology in order to heat a solid and liquid mixture. Heating is induced by microwave irradiation absorbed by molecules depending on their dielectric constant. Polar molecules with a high dielectric constant (i.e. water with a dielectric constant of 78.5 at 25°C, ethanol with 24.3 at 25°C) are able to absorb this energy and re-emit it, resulting in heating the system, whereas solvents with a low dielectric constant such as hexane (1.87 at 25°C) are insensitive to microwaves.

The main parameter for microwave-assisted extraction is temperature. It is possible to change the temperature by changing the irradiation duration and power. In practice, the temperature can be set by regulating the irradiation power in order to maintain the right temperature. Conversely, it is also possible to use power intensity directly.

Power must be considered a key parameter in order to optimize extraction when using MAE. Chen et al. [56] extracted trans-resveratrol from tree peony seed oil-extracted residues. Changing the microwave irradiation power impacted the yield of resveratrol: increasing the power from 120 to 385 W increased the yield (from 1.2 µmol/g to 2.9 µmol/g) with a similar purity level (between 80 and 90%). This can be due to increased diffusivity of the solvent, induced by the irradiation energy coming from the plant matrix to the solvent [57]. However, from 385 to 700 W, the yield decreased. Excessive irradiation can cause internal over-heating, leading to carbonization and then other reactions such as isomerization and/or degradation of the product. Kaur et al. [58] observed a similar behavior of swertiamarin, amarogentin and mangiferin content as a function of irradiation power. The best yield was observed around 500 W for the first two compounds, or 550 W for the third one.

As in UAE, temperature itself can selectively impact the extraction of compounds. Ruiz-Aceituno et al. [59] selectively extracted different sugars (inositols and inulin) from artichoke external bracts using temperature and MAE. The highest inositol yields were obtained at low temperature (50°C) while inulin was better extracted at 120°C. Moreira et al. [60] studied the impact of solvent using different alcohol-water mixtures on polyphenol extraction from apple tree wood. They showed that, at 100°C, mixtures increased the total phenolic content compared to pure methanol, ethanol or water. This can be attributed to the high dielectric constant of water, which increases energy absorption by the solvent mixture and thus, by increasing the temperature inside the sample, leads to an easier release of compounds. This variation of power with different ethanol-water compositions was studied by Koyu et al. [61]. Water is a good solvent for the absorption of microwaves due to its high dielectric constant. It was observed that mixtures containing high amounts of water (e.g. 65%) required higher microwave irradiation power (500 W) than mixtures with lower amounts of water (30%, 300 W) in order to extract the same anthocyanin content. This can be explained by the fact that solvents that absorb fewer microwaves can induce more waves in the sample and ultimately improve extraction.

It is also possible to irradiate the plant samples directly and use residual water as the extraction solvent. Michel et al. [62] evaluated this technique for the extraction of antioxidants from sea buckthorn (*Hippophaë rhamnoides* L.) berries. This technique was considered as a good alternative for the extraction of polar antioxidants (i.e. quercetin and isorhamnetin) which are usually not extracted with other techniques such as aqueous maceration or simple pressing. In addition, this technology can be used for other processes such as hydrodistillation (HD). Using only residual water, Lucchesi et al. [63] extracted essential oil from *Eleteria cardamomum* L. They observed that, compared to classical hydrodistillation,

oxygenated compounds were better extracted using solvent-free microwave extraction. The absence of solvent likely reduces thermal and hydrolysis reactions that may degrade oxygenated compounds.

To conclude, MAE can induce selectivity through irradiation power, which can play a role as seen for the extraction of trans-resveratrol from tree peony seed oil-extracted residues [56]. Temperature (induced by irradiation) also influences selectivity for the extraction of different sugars (inositols and inulin) from artichoke external bracts [59]. All the results obtained may come from selective heating due to solvent composition or plant matrix.

## 2.6. Pressurized liquid extraction (PLE)

In many studies, this technique has been used to extract pollutants from natural matrices such as soils, but others have focused on the extraction of metabolites from plants. This technique applies pressure in order to heat the extraction solvent above its boiling point. Thus, it enhances extraction efficiency by reaching higher temperatures than conventional extraction (maceration, soxhlet, etc.). Higher temperatures lead to a higher solvent solubility capacity, to a lower viscosity improving penetration of the solvent into plant cells, and to a reduction in solute-matrix interactions. All these effects lead to an improvement in the extraction yield, and may then lead to a decrease in selectivity.

The role of temperature (from 80 to 125°C) on extraction selectivity was reported by Dunford et al. [64] when extracting policosanols (a long-chain alcohol) from wheat straw, germ and bran. A higher temperature resulted in no significant change in policosanols extraction but in a higher global yield, resulting in a decrease in selectivity. An increase in the extraction yield of p-coumaric acid, trans-ferulic acid, rutin and hesperidin related to a temperature increase (62 and 90°C) was also observed by Gómez-Mejía et al. [65] when extracting polyphenols from citrus peel waste. Hossain et al. [66] observed different trends as a function of temperature (66 to 129°C) with antioxidants from rosemary. Some compounds were positively affected by temperature (caffeic acid, gallic acids) while others were negatively affected (rosmarinic acid, luteolin-7-O-glucoside, apigenin-7-O-glucoside, carnosic acid and carnosol). This study confirms that temperature does not always increase extraction yield for polyphenols. Rudke et al. [40] studied the impact of different parameters on the extraction of phenolic compounds and carotenoids from buriti (*Mauritia flexuosa* L.) shell. The impact of temperature (30 to 70°C) differed between these two compound families. Total polyphenol yield was improved at higher temperatures while carotenoids were not strongly impacted by this parameter. However, because carotenoids are sensitive to high temperature, the yield increase resulting from temperature was maybe hidden by a yield decrease due to degradation, finally resulting in no change observed. Alvarez-Casas et al. [67] observed similar degradation results on different polyphenols through the extraction from white grape marc. Some compounds such as gallic acid and catechin were negatively impacted by high temperature (above 100°C) while total phenolic and flavonoid contents were increased, even at the highest temperature. Similar effects were observed by Dobíáš et al. [68] for the extraction of esculetin, rutin, scopoletin, 7-hydroxy-coumarin and quercetin from different plant samples. While compound yields improved as a function of temperature, quercetin content decreased from 80° to 100°C.

Benito-Román et al. [69] optimized the extraction of  $\beta$ -glucans and phenols at high temperature (from 135 to 175°C) from waxy barley. Temperature had a negative impact on glucan yield and a positive one on total phenolic yield. However, high temperature can lead to a fragmentation of this polysaccharide, explaining the observed decrease in glucan recovery. Ruiz-Aceituno et al. [70] extracted different sugars from pine nuts. They highlighted the fact

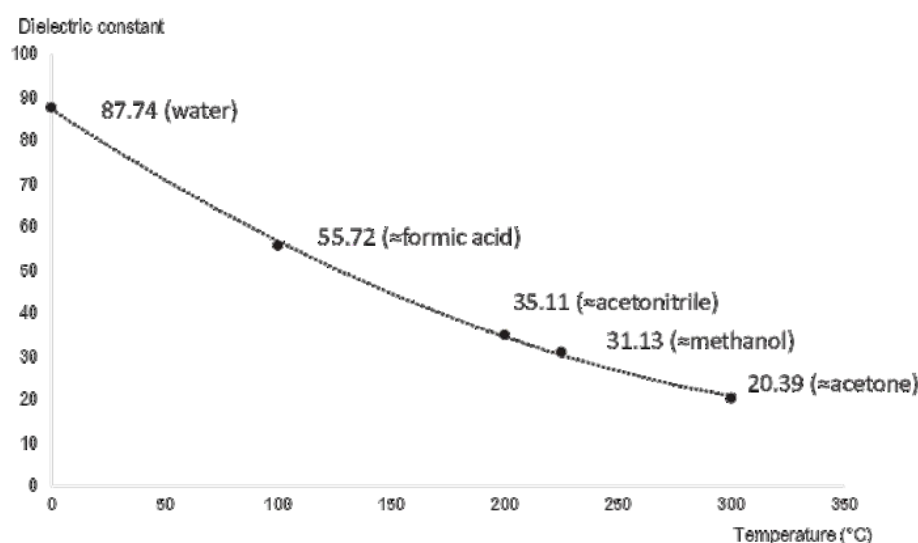


Fig. 5. Water dielectric constant variation as a function of temperature.

that between different sugars (i.e. inositols, pinitol, ...), different behaviors regarding temperature were observed, with some being positively affected, while others were negatively affected. All the above studies showed that it is not easy to predict the impact of temperature, although temperature can improve selectivity.

Pressurized hot water extraction (PHWE) or subcritical water extraction (SWE), which are considered as new “green” extraction techniques, are based on the dramatic change in the dielectric constant of water at high temperature (Fig. 5 reproduced from [71]). High temperature favors the movement of water molecules that reduces intermolecular hydrogen bonding, and induces a lower polarity. Selectivity behavior of heated water was reported by Ko et al. [72] for the extraction of flavonoids from different plant samples using SWE. The maximum extraction temperature for quercetin was 170°C, while the extraction of quercitrin, which contains more hydroxyl groups due to one glycoside substituent, was optimal at 110°C, indicating that it was more soluble when the polarity of water was higher (at the moderately high temperature of 110°C). However, another study by Kumar et al. [73] focusing on the same compounds did not show such a clear effect of temperature. This might be due to a shorter extraction time in the latter case. Time is also an important factor, especially at high temperature because it can increase degradation reactions which may occur directly in the extraction cell. Kwon et al. [74] observed this degradation effect at high temperature on curcuminoids from *Curcuma long L* using ethanol-water mixtures. An increase in temperature (from 110 to 135°C) improved curcuminoid content, but an extraction time above 7 min led to a decrease.

Pressurized extraction (PLE and PHWE) applies temperature and pressure but in a system that is mainly static for technological reasons. This mode keeps the extraction solvent in contact with the matrix for a certain time, and then a portion of solvent is refreshed (“flush” volume expressed in percentage). Zaghdoudi et al. [75] studied the impact of extraction cycles (1 and 5) on carotenoid extraction from Tunisian kaki, peach and apricot at 40°C and with an extraction solvent composed of methanol:methyl-tetrahydrofuran 20:80 v/v. The study demonstrated that different carotenoids are not affected in the same way when the number of cycles changes. Some compounds, such as lutein, were not affected, whereas others (carotene, zeaxanthin, cryptoxanthin) showed a yield decrease with a higher number of cycles. Before each new cycle, part of the solvent is renewed. This induces changes in the compounds, which prefer to migrate to a fresh solvent than to a

solvent already containing solutes. This difference can lead to selectivity.

In conclusion, temperature can be considered as the main optimizing parameter for PLE. Temperature may impact the selective extraction of the compounds positively or negatively, depending on their chemical structure, as observed in many of the papers discussed in this part. In addition, even if pressure allows the use of temperatures higher than the solvent boiling point, it is not always necessary to apply excess heat. What is more, the role of the number of cycles, which induces a pseudo-dynamic extraction, on the selectivity of extraction has been little studied.

This part has described three techniques (UAE, MAE and PLE) based on selectivity results. Each of them has advantages due to their technical parameters such as: power of ultrasonic waves, power intensity for microwaves and temperature for pressurised extraction. They all also have advantages over classical extraction methods, beyond the apparatus required, based on their lower solvent consumption, speed of extraction and energy consumption. Nevertheless, the main parameter influencing selectivity is not the physical parameters but rather the chemical nature of the extraction solvent. Consequently, and because of this major effect of the solvent, it is difficult to favor one technique over another in terms of selectivity. The easiness of the scale-up of these techniques for industrial processes should also be taken into account.

### 3. Supercritical fluid extraction (SFE)

Supercritical fluid extraction has been widely studied and is usually considered a green extraction technique [76,77] owing to the use of a green and renewable solvent: carbon dioxide. While other fluids can be used in plant extraction (such as propane), CO<sub>2</sub> has more advantages such as non-toxicity and thermodynamic parameters, which facilitate its use in the supercritical state (above 31.1°C and 7.4 MPa). This review will thus focus on supercritical extraction using carbon dioxide.

Fig. 6 presents a scheme of a supercritical fluid extractor. In green, the parameters that can lead to selectivity changes are highlighted. All these selective points are discussed in this review.

A first part focuses on supercritical carbon dioxide (Sc-CO<sub>2</sub>) interactions, i.e. on solubility. Parameters such as pressure, temperature and modifier can change these Sc-CO<sub>2</sub> properties. The extraction of non-polar molecules (lipids, terpenes, etc.) is examined first, followed by successively more polar molecules.

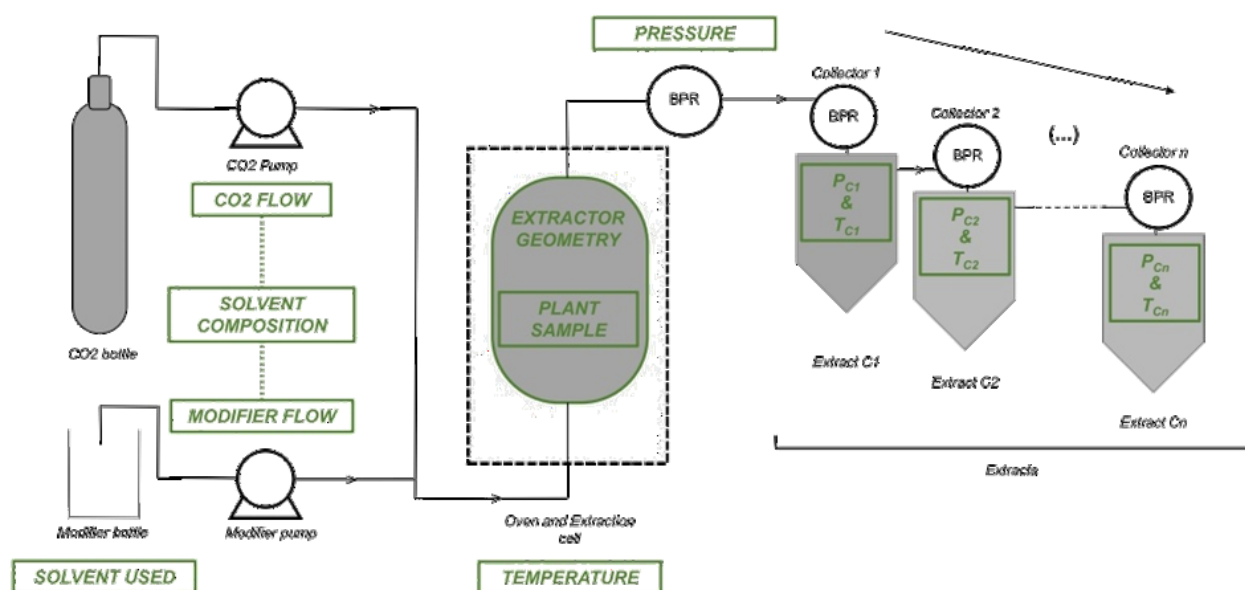


Fig. 6. Classical Supercritical Fluid Extractor (SFE) scheme highlighting selectivity key points (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A second part describes more technical aspects, such as the different extraction modes (static, dynamic), the vessel geometry and sequential extraction.

### 3.1. Solvent selectivity in SFE

Supercritical carbon dioxide is a particular extraction solvent. Due to its compressibility, it is sensitive to pressure and temperature changes. Density can change with pressure (an increase in pressure leads to an increase in density) and with temperature (an increase in temperature leads to a decrease in density). Density change may modify interactions with the compounds, and thus their extraction. In addition, a co-solvent, often called modifier, can be added in order to modulate the polarity of Sc-CO<sub>2</sub>. For instance, adding an alcoholic modifier (such as ethanol) to carbon dioxide increases the polarity of the extraction fluid.

#### 3.1.1. Lipophilic compounds

Due to the low polarity of carbon dioxide, supercritical fluid extraction has been widely applied to oils and more particularly lipids. Lipids are a large family with different subcategories (for example glycerides, phospholipids, waxes...). Sc-CO<sub>2</sub> SFE has been involved in oil removal processes in order to replace the classical process using n-hexane. Markom et al. [78] optimized process parameters to recover crude palm oil. After varying temperature (40°C to 60°C) and pressure (11 to 20 MPa), they studied the extraction kinetics and monitored fatty acid (FA) chain lengths (C12 to C20, saturated and unsaturated) in the glycerides extracted. For any conditions and any related density, light fatty acids such as C12:0 and C14:0 were extracted at the beginning of the process while heavier chains were extracted later. The FA chain length was the most influential parameter for selectivity compared to unsaturation number. It was then possible to collect lighter or heavier fractions of glycerides based on their extraction kinetic differences. Moreover, using a slower extraction kinetic and the right CO<sub>2</sub> density can induce a longer extraction process, making it easier to collect separate fractions (with light or heavy compounds).

In the field of oils and fats, Free Fatty Acid (FFA) content is an important property of oils. It can affect flavor perceived as a metallic taste. FFA come from a lipolysis reaction directly in oil/fat sam-

ples. The content of FFA was studied by King et al. [79] as a function of different pressures and temperatures. In conditions where the Sc-CO<sub>2</sub> density was the lowest (40°C and 13.8 MPa), larger amounts of FFA were extracted. Then, FFA content in the extracted oil decreased when the temperature increased (up to 100°C). Pressure and temperature had a selective impact on FFA content. To conclude, FFA can be considered as light lipids and thus, require low-density Sc-CO<sub>2</sub> to be extracted. They are similar to short chain glycerides.

Dos Santos Garcia et al. [80] worked on the extraction of *Mucuna* seed oil in order to produce defatted meal. Their study also focused on the extraction of L-DOPA (3,4-dihydroxyphenylalanine), an amino acid with antioxidant properties. The aim was to obtain an alternative to other conservatives, such as butylated-hydroxytoluene (BHT), butylated-hydroxy-anisole (BHA), or tocopherols, which are usually added to avoid oxidation. *Mucuna* seeds are known to be a high-yielding source of L-DOPA. Variation in the pressure (15 to 25 MPa) and temperature (40 to 60°C) showed that both L-DOPA and oil yield increased with CO<sub>2</sub> density. However, temperature had no visible impact on the chemical distribution of fatty acids. In a different paper, Salvador et al. [81] focused on the extraction of oil from pecan nut cake. After carrying out different extractions with various pressure and temperature settings, it appeared that a lower pressure (10 MPa), and therefore a lower density of Sc-CO<sub>2</sub>, may advantage the extraction of saturated fatty acids. No significant change in the ratio between saturated and unsaturated fatty acids was observed with pressure changes (from 15 to 30 MPa). From a certain pressure (especially 15 MPa), pressure did not seem to have much impact on the fatty acid profile of extracted oils. However, the use of a modifier showed greater differences. Couto et al. [82] compared the oils extracted with and without modifier (ethanol at 6.5 wt%). The ratio of saturated (S) and unsaturated (U) fatty acids was calculated with experiments using the same pressure and temperature (20 MPa and 50°C). Results showed that using a modifier improved the recovery of saturated acids (such as lauric, myristic or arachidonic acids) over unsaturated ones. The S/U ratio was around 0.9 without modifier and below 0.5 with 6.5% of ethanol.

Docosahexaenoic acid (DHA) is an omega-3 fatty acid present in different oils (from plant and fish). It is known for its benefi-

cial properties especially for health (brain functions, pregnancy ...). Tang et al. [83] focused on the selectivity of DHA applied to oil-rich microalgae. Different modifiers such as petroleum ether, acetone, methanol and ethanol were tested. Using ethanol or methanol as modifier increased the content of unsaturated lipids extracted (from 30 to 100%) and thus the selectivity vs. saturated lipids. Increased pressure (up to 35 MPa) resulted in a higher extraction efficacy but too high a pressure (above 35 MPa) caused the extraction of impurities. In a second step, temperature was also optimized. Temperature increase improved mass transfer by increasing the vapor pressure of solutes. However, from 40°C, increasing temperature decreased the Sc-CO<sub>2</sub> density and hence the solubility of compounds. This indicates that pressure, temperature and modifier can all modify Sc-CO<sub>2</sub> behavior in order to selectively extract DHA, but it also reveals the complexity of the influence of these three parameters.

Phospholipids are part of the polar lipid family. Montanari et al. [84] investigated selectivity applied to phospholipids in soybeans using Sc-CO<sub>2</sub>. Increasing the pressure (up to 68.9 MPa) increased the total phospholipid content. However, when a lower pressure (16.6 MPa) was applied, differences between the phospholipid families (phosphatidylcholine, phosphatidylethanolamine, a-phosphatidylinositol) were observed. The percentage of phosphatidylcholine decreased from above 70% of total phospholipids down to 20%. Conversely, the proportions of phosphatidylethanolamine and a-phosphatidylinositol increased. No significant change was observed when temperature (60°C to 80°C) was tested. In addition, it was necessary to add a modifier (here 10% ethanol) to Sc-CO<sub>2</sub> in order to extract phospholipids.

In oil refining, the non-saponifiable fraction has interesting uses especially in cosmetics. It is composed of fatty esters (waxes), sterols, squalene, triterpenes, etc. Felföldi-Gáza et al. [85] studied the influence of modifier (ethanol at 0, 5 and 10%), pressure (30 to 45 MPa) and temperature (40 to 60°C). It was observed that the non-saponifiable content can be decreased by increasing the temperature or increasing the modifier percentage. As expected, increasing the polarity of the extraction solvent by adding ethanol to Sc-CO<sub>2</sub> favored the extraction of polar lipids against less polar compounds. Martins et al. [86] optimized SFE parameters in order to extract sterols from *Eichhornia crassipes* biomass, which is classified as an invasive plant. They focused on 4 sterols: stigmasterol,  $\beta$ -sitosterol and methylcholesterol. The modifier (ethanol) had more impact on sterol yields than on the total yield, whereas pressure and temperature did not seem to be selectively significant. However, the  $\beta$ -sitosterol content had a different behavior and was better extracted at high pressure (30 MPa). In this case, it was the most abundant compound, so these conditions were selected. Sovova et al. [87] studied the extraction of 20-hydroxyecdysone from *Leuzea carthamoides* DC. They observed that this steroid was preferably extracted at the highest pressure tested (27–28 MPa at 60°C), confirming that this kind of molecule requires high pressure, and therefore a high Sc-CO<sub>2</sub> density, to be extracted. de Melo et al. [88] studied the recovery of stigmasterol from water hyacinth using Sc-CO<sub>2</sub>. The influence of temperature was observed at constant pressure (20 MPa). Between 40°C and 60°C, the lower temperature provided the best extraction yield due to a higher Sc-CO<sub>2</sub> density. Besides, selectivity was calculated from stigmasterol content and total extract content as a function of extraction time. In this case, a selectivity value above 1 indicated a favorable extraction of this compound. As expected due to the lower yield, selectivity at 40°C was better than at 60°C. Moreover, at this temperature of 40°C, the extraction selectivity varied with time (for instance between 1 h and 5 h extraction). This means that the first fractions obtained during the first hour were not selective. These results indicate that the extraction kinetic plays a key role in selectivity.

Dias et al. [89] studied the selective extraction of spilanthol (a fatty acid amide) from *Spilanthus acnella* flowers. Different temperatures (50 to 70°C) and pressure values (15 to 49 MPa) were tested. The selectivity was calculated as the ratio of spilanthol content over total content. The best selectivity was obtained at the highest temperature tested (70°C), while the best pressure was the intermediate point tested (32 MPa). This demonstrates the complexity of plant extraction. Notwithstanding this difficulty, selectivity (spilanthol/yield ratio) and spilanthol yield were both multiplied by 5 in the optimized conditions compared to initial temperature (50°C).

Supercritical fluid extraction using Sc-CO<sub>2</sub> is a suitable technique for lipid extraction. This review has highlighted some selective ways to extract specific lipid families from non-polar ones (i.e. waxes) to more polar ones (i.e. phospholipids). This selectivity comes from Sc-CO<sub>2</sub> density changes, and sometimes also from the addition of a modifier. Non-polar compounds (i.e. waxes) are extracted at high density while more polar compounds (i.e. FFA, short chain glycerides) are better extracted using low density. More polar compounds (i.e. phospholipids) also require a polar modifier in order to be extracted.

Also known as vitamin K, tocopherols are fat-soluble compounds, which can be extracted from oils by SFE. Montañés et al. [90] studied the extraction of apple seed oil and its tocopherol content, between 43 and 63°C, and 300 to 130 MPa (a very high pressure). Increasing the temperature (from 53 to 63°C) led to a decrease in tocopherol content, except for  $\delta$ -tocopherol, due to compound degradation. Nevertheless, the high pressure improved extraction yield both for oil and tocopherol [90].

SFE using Sc-CO<sub>2</sub> can also be used to selectively extract other metabolites such as terpenes. These metabolites are composed of isoprene units (C<sub>5</sub>H<sub>8</sub>). Terpenes are rather non-polar molecules. Isoprene units are organized in a linear or cyclic way, either oxygenated or not, and compose different families ranging from monoterpenes such as limonene, to tetra-terpenes such as carotenes, and even to natural rubber.

Pavlic et al. [91] studied the influence of pressure and temperature on monoterpene content from sage herbal dust. Monoterpene content increased with pressure (from 10 to 20 MPa at 50°C), related to a density increase. But this content decreased for pressures above 20 MPa up to 30 MPa, due to a loss of selectivity toward monoterpenes. Regarding temperature, selectivity decreased when the temperature increased (from 40°C to 50°C), due to a decrease in density. However, selectivity increased again from 50°C to 60°C due to the increase in the vapor pressure of the analytes. Reverchon et al. [92] investigated the SFE of jasmine concrete, a product used in fragrances and necessary for the production of various other fragrances. This product undergoes fractionation steps to remove unwanted compounds (fatty acids, waxes, etc.) in order to obtain a pure product called an absolute. The study showed that under different pressure settings, different ranges of compounds were extracted. The lowest pressure applied was 8 MPa (at 40°C) and it extracted low molecular weight compounds (i.e. linalool, benzyl acetate). A second step using Sc-CO<sub>2</sub> at 8.5 MPa (and the same temperature) was used. This difference may seem insignificant but it has a significant impact on Sc-CO<sub>2</sub> density (25% increase). The second set of conditions extracted heavier compounds such as diterpenes (i.e. phytol), but also undesired compounds such as fatty acid esters (i.e. methyl oleate and ethyl eicosanoate). Then, a last step with Sc-CO<sub>2</sub> at 20 MPa was used in order to extract lipophilic compounds such as waxes (i.e. nonacosane). This work is a perfect illustration of how the selectivity of Sc-CO<sub>2</sub> can be tailored by modulating pressure, and hence its density. Sonsuzer et al. [93] also studied the selectivity between different terpene families: monoterpenes, sesquiterpenes and oxygenated monoterpenes, extracted from *Thymbra spicata* oil. They

demonstrated that increasing the pressure (from 8 to 12 MPa) decreased the selectivity of monoterpenes. This is explained by the better extraction of the less volatile compounds (sesquiterpenes and oxygenated monoterpene) when the fluid density is higher. Similar results were also observed by Barjaktarovic et al. [94] in their study of SFE on *Juniperus communis* L. fruits. Different compound families (i.e. monoterpenes, sesquiterpenes, oxygenated monoterpenes, oxygenated sesquiterpenes and other heavier compounds such as waxes) were studied. Results showed that a lower pressure (8 MPa compared to 10 MPa) gave the best selectivity for the extraction of oxygenated vs. non-oxygenated and waxes.

Trabelsi et al. [95] extracted terpenes from *Citrus aurantium amara* peels. At a constant temperature and constant percentage of modifier (3 wt% of ethanol), limonene oxide (oxygenated monoterpene), 1,5-heptadiene,3,6-dimethyl,  $\alpha$ -caryophyllene (sesquiterpene), 3,9-dodecadien and bicyclo[10.1.0]tridec-1-ene were extracted with a pressure above 17 MPa and static time greater than 50 min. They were extracted separately from sabinene, ocimenol (monoterpenes) and  $\alpha$ -farnesene (sesquiterpene) which were extracted only at low pressure with a short static time (30 min). Pourmortazavi et al. [96] reported similar results regarding the impact of pressure and temperature. However, they investigated the impact of the modifier (methanol, ethanol, dichloromethane and hexane). Hexane (at 5%) was found to be a selective modifier for 1,8-cineole, limonene and  $\delta$ -3-carene (monoterpenes). While their content was similar to the one obtained with other modifiers at 5%, the number of compounds extracted changed from 22 with other modifiers to 7 with hexane. This demonstrates its good selectivity toward monoterpenes.

Allawzi et al. [97] optimized the extraction of Jordanian rosemary essential oil by SFE. The paper also confirmed that a pressure increase (from 7.6 to 9 MPa at 35°C) improved the extraction of monoterpenes ( $\alpha$ -pinene and eucalyptol), while methyl esters (i.e. palmitic acid methyl ester, hexadecenoic acid methyl ester) were extracted only at 7.6 MPa. The selectivity between oxygenated terpenes and other terpenes was investigated by Mira et al. [98]. They carried out SFE on orange peel and showed that the ratios of oxygenated compounds (mainly linalool) over non-oxygenated terpenes was high at the lowest pressure (8 MPa at 20°C) and then decreased when the pressure increased (up to 15 MPa). Then, from a certain pressure (i.e. 15 MPa at 20°C), it increased again but more slightly. It demonstrated that the selectivity of oxygenated compounds over non-oxygenated ones was better when using Sc-CO<sub>2</sub> at low density. The work by Van Opstaele et al. [99] confirmed the same behavior. In order to develop hop aromas by SFE, the fraction responsible for floral scent was more significant at low Sc-CO<sub>2</sub> density. One molecule responsible for this floral scent is, among others, linalool. Jokic et al. [100] worked on *Salvia officinalis* L. leaves and reported identical conclusions regarding oxygenated monoterpenes.

Glis et al. [101] focused on diterpenes from sage (*Salvia officinalis* L.) and studied 3 different diterpene (D) selectivities: one regarding the selectivity of monoterpenes and oxygenated monoterpenes ( $S_{D(M+OM)}$ ), the second with sesquiterpenes and oxygenated sesquiterpenes ( $S_{D(S+OS)}$ ) and the third with triterpenes, esters and waxes ( $S_{D(T+E+W)}$ ). These selectivities were plotted as a function of pressure. Increasing the pressure had a different impact on terpenes. For monoterpenes and oxygenated monoterpenes ( $S_{D(M+OM)}$ ), a pressure increase from 7 to 15 MPa decreased selectivity due to the higher solubility of monoterpenes in low-density Sc-CO<sub>2</sub>. Above 15 MPa, this selectivity increased slightly due to an increase in diterpene solubility. In comparison with the other two selectivities (sesquiterpenes  $S_{D(S+OS)}$  and triterpenes  $S_{D(T+E+W)}$ ), the increase in pressure increased both selectivities, meaning that the solubility of diterpenes increased without increasing that of other heavy compounds. This study confirmed the correlation be-

tween the volatility of extracted compounds with Sc-CO<sub>2</sub> density: low-density Sc-CO<sub>2</sub> extracts light terpenes better, while high-density CO<sub>2</sub> improves the recovery of heavier compounds.

Bogdanovic et al. [102] optimized SFE in order to obtain diosgenin (sapogenin family) rich extract from fenugreek (*Trigonella foenum-graecum* L.) seeds. Thanks to a response surface methodology, they optimized conditions (24.6 MPa and 43.5°C) in order to increase diosgenin yield with a decrease in total yield, showing the improvement of purity.

Klein et al. [103] worked on triterpenoid extraction from uvaia leaves (*Eugenia pyriformis* Cambess.) and particularly on  $\alpha/\beta$ -amyrin. They obtained the purest  $\alpha/\beta$ -amyrin extract using the lowest pressure tested (10 MPa) and the highest temperature (60°C). Higher densities increased yield, but decreased purity. Oliveira et al. [104] studied the extraction of *Piper divaricatum* essential oil, focusing on another family of volatile compounds: phenylpropanes. After varying the pressure and temperature parameters, they succeeded in selectively extracting high amounts of eugenyl acetate and  $\beta$ -elemene with low amounts of eugenol and methyl eugenol (at 35°C and 10 MPa), showing again the selectivity of low density Sc-CO<sub>2</sub>.

Morsy et al. [105] produced thymol-rich extracts from ajwain (*Carum copticum* L.) and thyme (*Thymus vulgaris* L.). Thymol is a phenol but is also a volatile compound. Different pressure conditions (10.4 and 16.7 MPa at 40°C) were tested and the use of higher pressure improved thymol yield. However, with higher pressures, it was possible to avoid the extraction of unwanted compounds such as fatty acids (i.e. 9-octadecenoic acid). Because these acids were better extracted at lower pressure (10.4 MPa), thymol selectivity toward fatty acids was improved.

Like lipid extraction, SFE is suitable for terpene extraction, from volatile monoterpenes to non-volatile triterpenes. Low-density Sc-CO<sub>2</sub> favors the selectivity of volatile compounds, whatever their polarity.

### 3.1.2. Pigments

Pigments are responsible for the color of plants. Carotenoids and chlorophylls are respectively responsible for yellow or green colors. These pigments are important because they are necessary for photosynthesis reactions in plant cells. However, it may be necessary to remove them from plant extracts because of color and stability issues during extraction processes, inducing color changes [106]. Carotenoids often display antioxidant and provitaminic activities that explain the interest in their selective extraction from varied matrices. The studies presented below demonstrate how SFE can selectively extract these compounds.

Carotenoids are divided into two families: carotenes and xanthophylls. The latter group is differentiated by the presence of oxygen atoms, increasing the compound polarity, which may cause them to interact differently with Sc-CO<sub>2</sub>. Filho et al. [107] studied the impact of pressure (10 to 40 MPa) and temperature (40 and 60°C) of pure Sc-CO<sub>2</sub> on different compounds from the carotene and xanthophyll families. It was shown that oxygen-containing molecules were not, or only weakly, extracted at any setoff pressure and temperature. However, one xanthophyll behaved differently: rubixanthin. This can be explained by the fact that it contains only one hydroxyl group against two for other xanthophylls (i.e. cryptoxanthin). Regarding the behavior of carotenes, higher pressure and/or temperature values increased carotene yields. Gomez-Prieto et al. [108] confirmed this behavior using *Mentha spicata* L. In addition, they suggested a sequential extraction of  $\beta$ -carotene and lutein, using first Sc-CO<sub>2</sub> at 40°C and 11.3 MPa for carotene, and then increasing the temperature to 60°C to extract lutein. Contrary to what Filho et al. [107] observed, lutein was affected by temperature compared to the other xanthophylls they studied. Chronopoulou et al. [109] selectively extracted

menaquinon-7 from other carotenoids at low Sc-CO<sub>2</sub> density (10 MPa and 40°C) using *Tetrademus Obliquus* microalgae. Gomez-Prieto et al. [110] extracted trans-lycopene from tomato. At 40°C, changing the density of Sc-CO<sub>2</sub> through pressure increases (from 7.7 to 28.1 MPa) changed the extraction of the lycopene isomer ratio between *cis* and *trans* forms. At lower density, the extraction of *cis*-lycopene over the *trans* form was favored. For densities above 0.65 g/mL (approximately 13.5 MPa), the contrary was observed, showing extraction selectivity between two isomers obtained only with pressure changes.

Wrona et al. [111] studied the impact of pressure and temperature on lipids, phenolic and chlorophyll content by carrying out SFE on *Solidago gigantea* Ait. They confirmed that lipidic and phenolic contents increased when pressure increased (up to 80 MPa). But contents decreased when the temperature increased (up to 70°C). However, chlorophyll content was improved using high pressure and temperature. Because pressure had a positive impact on every yield, temperature appeared to be a good factor for the selective extraction of chlorophyll pigments over lipids and phenolic compounds.

Garcia et al. [112] studied the influence of temperature (40 to 60°C) and pressure (25 to 35 MPa) on carotenoid and chlorophyll contents. Both families were influenced in the same way: the contents increased with pressure and decreased with temperature. In addition, ethanol was used as modifier at 1 wt% and 5 wt%. The contents of chlorophylls and carotenoids improved with the percentage of modifier. Abrahamsson et al. [113] studied the impact of experimental conditions for chlorophyll *a* and carotenoids and confirmed previous observations, especially the impact of modifier (ethanol). However, selectivity can still be improved for these two pigment families. Guedes et al. [114] examined the ratio of carotenoids to chlorophyll *a* on *Scenedesmus obliquus* extraction. The maximum ratio was obtained using 25 MPa pressure and 60°C temperature. Even if the chlorophyll content increased as a function of temperature and pressure, carotenoids increased even more, which resulted in a better selectivity. However, this ratio decreased when ethanol was used as modifier due to a great increase in carotenoids and chlorophylls.

Even if SFE using carbon dioxide is an extraction technique of interest for non-polar molecules, it is also possible to selectively extract polar metabolites from plants when modulating Sc-CO<sub>2</sub> properties.

### 3.1.3. Alkaloids and cannabinoids

Cannabinoids and alkaloids are known for their pharmaceutical properties [115,116]. Pimentel et al. [117] extracted amides (piperovatine and pipercollosidine) from *Piper piscatorium* (Trel Yunc). With pure CO<sub>2</sub>, temperature (40 and 70°C at 40 MPa) had a different impact on these two compounds. While selectivity (expressed in g/100g of extract) increased for pipercollosidine at higher temperature (from 0.004 to 0.031 g/100g), the selectivity of piperovatine did not change significantly (from 0.26 to 0.30 g/100g). This can be explained by autooxidation which can occur for piperovatine at higher temperature, which balances selectivity changes. However, the biggest selectivity changes for these compounds were obtained with a polar modifier (especially ethanol at 10%). At 40°C, the selectivity obtained for pipercollosidine was 0.24 g/100g and for piperovatine it was 0.93. Selectivity was lower at 70°C but this can be explained by the extraction of other compounds due to an increase in solubility in the extraction solvent. Liu et al. [118] observed that the modifier can reduce selectivity. They studied the extraction of two alkaloids: evodiamine and rutaecarpine from *Evodia Rutaecarpa*. Different modifiers were tested (at 0.4 mL/min for 2 L/min gaseous CO<sub>2</sub>): no modifier, pure methanol and methanol with water at 50, 30, and 5%. Water decreased the yield of evo-

diamine and rutaecarpine, but also their purity, whereas methanol increased their yield with a similar purity.

Gallo-Molina et al. [119] optimized the extraction of tetrahydrocannabinol (THC) from *Cannabis sativa* L. and observed different behaviors. They obtained the highest levels of THC using 2 wt% of modifier (ethanol) at the highest (34 MPa and 60°C) and lowest (15 MPa and 40°C) sets of pressure and temperature. This demonstrates that ethanol can significantly impact Sc-CO<sub>2</sub> behavior even when used in low amounts (2 wt%). Attard et al. [120] studied the influence of pressure and temperature for the extraction of waxes and cannabidiol (CBD) from hemp wastes. They observed that at low Sc-CO<sub>2</sub> densities (8 MPa, 35°C), selectivity was high for lipophilic compounds (alkanes and fatty acids) with a low extracted content for other molecular families. At higher density (i.e. 35 MPa and 50°C), other groups of compounds (such as CBD) were better extracted, reducing selectivity for the previous compounds.

### 3.1.4. Polyphenols

Polyphenols are also part of secondary metabolites and are considered as quite polar plant components. They are of interest due to their antioxidant properties [121].

Antunes-Ricardo et al. [122] worked on the extraction of isorhamnetin from *Opuntia ficus-india* L. A higher recovery of this compound was observed with high pressure and intermediate temperature (30 MPa and 50°C). At the highest temperature (65°C), isorhamnetin content decreased while global yield increased. As described previously, an increase in density increases yields, and thus can result in a decrease in selectivity. Another parameter can influence extraction selectivity: the flow rate. A maximum amount of isorhamnetin was obtained at the maximum flow rate (100 g/min) while the maximum global yield was obtained at an intermediate flow rate (80 g/min). When the Sc-CO<sub>2</sub> flow rate increases, it may reduce mass transfer resistance between compounds and extraction solvent, resulting in an improvement of the extraction kinetic. However, increasing the flow rate may prevent the solvent from entering sample pores, thereby decreasing the extraction of other compounds.

Conde et al. [123] extracted flavonoids from *Pinus pinaster* wood. They also observed that the extraction yield of polar compounds was higher at high pressure and temperature (25 MPa and 50°C). However, the use of 10 wt% ethanol as modifier also significantly improved the extraction of flavonoids against other compounds. Yilmaz et al. [124] confirmed the interest of using ethanol as modifier for the extraction of polar molecules. They investigated the influence of pressure, temperature and modifier percentage on the extraction of catechins (EGC, CT, ECT, EGCG and ECG). Setting the conditions at 30 MPa, 50°C and 20% of ethanol extracted a maximum of GA, EGC and EGCG, while 30 MPa, 30°C and the same modifier were optimal conditions for CT and ECT. The best conditions for ECG were 25 MPa, 30°C and 15% of modifier. These extractions highlight the fact that using high temperature (i.e. 50°C) may degrade compounds, resulting in a lower yield (i.e., 50°C with CT, ECT and ECG).

Nagavekar et al. [125] extracted oleoresin from *Curcuma longa* and *Curcuma amada*. They studied the influence of previously tested parameters: pressure, temperature and modifier percentage. They confirmed the need to use high pressure (35 MPa), high temperature (65°C) and polar modifier (ethanol) at 30 wt% in order to extract curcuminoids. These conditions improved both the yield and selectivity of these interesting polar compounds. Konar et al. [126] extracted caffeic acid derivatives (echinacoside, chlorogenic acid, cynarin and caftaric acid) from *Echinacea purpurea* aerial parts. They also observed similar results for these kinds of compounds with high pressure (30 MPa), high temperature (60°C) and modifier (10% ethanol). Gourguillon et al. [127] evaluated different extraction methods (MAE, PLE and SFE) to extract dicaffeoylquinic

acids from a halophyte plant. They demonstrated the need to use a hydro-alcoholic modifier (EtOH:water 80/20 v/v) at different percentages (5 to 20%) in order to extract these compounds with SFE. Chromatographic analysis also showed the selectivity achieved with this polar modifier regarding polar compounds against less polar compounds. Polyphenols can also be extracted under their glycosylated forms. However, this requires the use of highly polar modifiers (i.e. a high percentage of water) which may be incompatible with supercritical CO<sub>2</sub>.

### 3.1.5. Carbohydrates

Carbohydrates are important molecules from plants, but no studies were found on their extraction using Sc-CO<sub>2</sub>. This can be explained by the same reason stated at the end of the previous part. Montañez et al. [128–130] developed SFE methods on home-made and commercial carbohydrate samples. Different conditions such as temperature, pressure and modifier were compared for the extraction of carbohydrate compounds (lactose, lactulose, galactose, tagatose, etc.). Different alcohols from n-butanol to ethanol and different mixtures of ethanol and water were tested. Results highlighted the need for a high-polarity modifier, confirming the necessity to introduce water in the modifier. These studies confirmed that at high temperature (80°C) high pressure (30 MPa) and high co-solvent flow rate, the addition of 5% of water in ethanol selectively modified the carbohydrate extraction [130]. As expected, all the previous studies show the need to use both high-density Sc-CO<sub>2</sub> and polar modifier in order to extract polar metabolites.

### 3.1.6. Selectivity loss

As described previously, Sc-CO<sub>2</sub> behavior can be modulated using different parameters, but these changes are not always selective towards the target compounds. This was the case in the study by Naziri et al. [131] who worked on the recovery of squalene from wine lees. As it contains only carbon and hydrogen atoms, squalene is one of the least polar compounds in plants. Thus high-density Sc-CO<sub>2</sub> is required, implying high pressure. However, high density will increase the total yield but reduce squalene selectivity. As pointed out in the previous section, a modifier, also known as co-solvent, is often used to increase the extraction capacity of Sc-CO<sub>2</sub> by increasing its polarity. But in some cases, this “improvement” can lead to a decrease in selectivity. This was observed by Valente et al. [132]. They worked on the extraction of antioxidant molecules from *Botryosphaeria dothidea*. They observed that antioxidant capacity was reduced using 10 wt% modifier (ethanol) compared to pure Sc-CO<sub>2</sub>. Even if a modifier helps to extract more polar compounds, it shows that these new compounds have a lower antioxidant capacity than the less polar ones obtained without a modifier. Pereira et al. [133] also showed a selectivity decrease using a modifier for the extraction of lignans from *Phyllanthus amarus* Schum and Thonn.

Modulating Sc-CO<sub>2</sub> properties through temperature, pressure and modifier represents an important key point to achieve extraction selectivity. In addition, selectivity can be obtained for a wide range of compounds, from non-polar (squalene)[85] to very polar (carbohydrates)[128–130]. However, certain technical parameters can also affect selectivity.

## 3.2. Technical parameters

### 3.2.1. Extraction modes

Beyond the complexity of Sc-CO<sub>2</sub>, other parameters, related to the technology itself, can provide differences in selectivity. This is the case of the extraction modes: static or dynamic. The static mode can be limited by the lower diffusivity of compounds within plant cells reducing their migration into the extraction solvent,

whereas the dynamic mode favors compound diffusion by maintaining at the optimum the concentration gradient between the plant and the solvent since the solvent is continuously renewed. Due to these different effects, the extraction of some solutes can be favored. Bermejo et al. [134] compared the two modes, using static and dynamic extraction with different modifiers (ethyl lactate, ethyl acetate and ethanol) on caffeine extraction from green tea. A slight change is that a modifier was introduced in the extraction cell with the plant, and then Sc-CO<sub>2</sub> was pumped. It showed that, depending on the modifier used, the static or dynamic mode showed a higher yield for caffeine. For example, with ethyl lactate, a higher concentration of caffeine in the extract (expressed in g of caffeine per g of extract) was obtained with the dynamic mode (14.3% in static mode and 18.2% in dynamic mode). On the contrary, with ethyl acetate, the dynamic mode seemed to have a negative impact compared to the static mode for the same compound (8.1% in static mode vs. 5.5% in dynamic mode). This highlighted the fact that the extraction mode affects extraction yield differently depending on the solvent used. Massias et al. [135] confirmed the previous study by showing that some compounds such as flavonoids or phenolic acids could be affected by this mode. Using CO<sub>2</sub>:EtOH:H<sub>2</sub>O 75:22:3 %mol as solvent, phloridzin was better extracted in static mode while chlorogenic acid was better extracted in dynamic mode. However, no explanations were found to correlate these modes to the kind of compounds affected.

### 3.2.2. Sequential extraction

Supercritical fluid extraction systems allow the use of successive extraction conditions. It is possible to start extracting the sample at a certain temperature, pressure and percentage of modifier, then use other parameters for a second step, change from static to dynamic mode and so on. Vardanega et al. [136] carried out a two-step method in order to extract different terpenes from annatto seeds. They carried out a first extraction step using low density fluid (60°C and 10 MPa) in order to extract lighter molecules (i.e. geranylgeraniol) and a second step at higher density (40°C and 20 MPa) to extract heavier compounds (i.e. tocotrienols). Bogdanovic et al. [137] developed a 4-step method in order to extract different compounds from Lemon balm (*Melissa officinalis* L., *Lamiaceae*) at different temperature and pressure settings (respectively 10 MPa/40°C, 30 MPa/25°C, 30 MPa/40°C and 30 MPa/100°C). The contents of the extracts for different families (monoterpenes, sesquiterpenes, diterpenes, fatty acids, methyl esters and alkanes) were evaluated by monitoring the content in specific molecules (i.e. geraniol for monoterpenes, palmitic acid for fatty acids ...). The results demonstrated that some compounds such as monoterpenes and sesquiterpenes were mostly extracted during the first and second steps, while other compounds such as alkanes and methyl esters were extracted during the third and last steps. A similar process was developed by Vigano et al. [138] with a 3-step extraction (60°C/17 MPa, 50°C/17 MPa and 60°C/26 MPa) in order to extract respectively tocopherols (i.e.  $\gamma$ -tocopherols,  $\gamma$ -tocotrienols), fatty acids (i.e. oleic and linoleic acids) and carotenoids (evaluated with total carotenoid content).

It is also possible to carry out sequential extraction with a modifier. Sökkmen et al. [5] extracted caffeine and catechin separately (from green tea) in a 2-step process using respectively pure Sc-CO<sub>2</sub> (25 MPa and 60°C) and Sc-CO<sub>2</sub> modified with ethanol (same pressure and temperature, with 0.5 mL/min of ethanol in 10 g/min CO<sub>2</sub> flow). A similar study was carried out by Domingues et al., [139] in order to extract fatty acids, long-chain aliphatic alcohols and triterpenoids from *Eucalyptus globulus* bark. Likewise, Serra et al. [140], demonstrated that a first step using pure Sc-CO<sub>2</sub> (25 MPa and 50°C) was helpful to recover anthocyanin compounds in the second step with modified Sc-CO<sub>2</sub> (with 10 wt% ethanol). This can be explained by the fact that the first step removed lipophilic

and non-polar compounds, which makes anthocyanins more available during the second extraction step. Finally, it is possible to go beyond modified Sc-CO<sub>2</sub> and to use only modifier as the extraction solvent. Bitencourt et al. [141] carried out a 4-step extraction starting from a pure Sc-CO<sub>2</sub> step (50°C, 30 MPa), adding modifier (30% wt% ethanol) in the second step, using only modifier (ethanol) in the third step, and finishing with a mixture of ethanol and water. During the first step, unidentified non-polar compounds were extracted. The second step extracted  $\beta$ -ecdysone and less polar saponins. The third, pure ethanol step extracted  $\beta$ -ecdysone with more and less polar saponins. Finally, hydro-alcoholic extraction showed more polar saponins. This extraction process can selectively extract different saponins based on their polarity. However, using only ethanol or hydro alcoholic solution during extraction is moving away from the realm of supercritical fluid extraction.

### 3.2.3. Extractor geometry

Another technical aspect that can affect selectivity is the geometry of the extraction vessel. Zabet et al. [142] worked on two different extractor geometries which led to two different effects. The first one was on temperature diffusion. Different shapes of the vessel may cause different temperature profiles. As we saw previously, temperature changes Sc-CO<sub>2</sub> behavior, which may in turn change solubility. The second change is due to carbon dioxide velocity changes. This change can impact the extraction kinetic, and then result in a faster extraction process. In other words, an equivalent extraction time would require less solvent in a more efficient extractor. The same authors applied these changes to rosemary extraction [143]. Results confirmed that different components from volatile (monoterpenes) to non-volatile (terpenic acids ...) compound families can be extracted differently depending on the extractor geometry.

### 3.2.4. Separators

The separator, which is located after the extraction cell, controls precipitation using the appropriate pressure and temperature. The controlled release of pressure can selectively precipitate particular compounds over others. Vasquez et al. [144] used this feature in order to purify squalene from vegetable oil. High pressure was needed to extract this compound. However, it has been shown previously [131] that high pressure was unable to improve selectivity towards squalene extraction. Varying the collector pressure from high to low (23 to 15 MPa) made compounds precipitate according to their thermodynamic properties. Thus, it was possible to improve the purity of this compound using separators. A similar study was carried out by Reverchon et al. [145]. Likewise, Baldino et al. [146,147] used these separators in order to separate cuticular waxes from other active compounds using different pressure settings (25 and 20 MPa). They also used the same process but with a low temperature (-7°C) in the collector, in order to remove paraffinic compounds from *Artemisia annua* L. extract. Rovetto et al. [148] used a 3-separator system in order to extract different fractions of *Cannabis sativa* L. extract. Three conditions were used: 13 MPa/ 55°C; 9 MPa / 55°C; 6 MPa / 35°C. The results demonstrated that cannabinoids were mainly collected in the first separator (with cuticular waxes).

As previously presented, Schukla et al. [149] extracted volatile and non-volatile compounds using high-pressure Sc-CO<sub>2</sub>. Then two separators were used in order to separate these two families. The first separator was set at 17.5 MPa and the second one at 4 MPa. These pressures resulted in the separation of non-volatile compounds in the first separator and then the collection of volatile compounds in the second one. Cavero et al. [150] carried out a similar study in order to selectively precipitate antioxidant compounds in a first separator with Sc-CO<sub>2</sub> at high density (10 MPa

and 40°C), and recover volatile compounds (essential oil) in a second separator at a lower density (2 MPa and 20°C). In the same vein, Fornari et al. [151] separated volatile (i.e. thymol) from less and non-volatile compounds (i.e. carnosic acid) using two separators (at 10 MPa and 1 atm). Separation using separators can be more selective when pressures are closer. Costa et al. [152] carried out the extraction of volatile compounds from *Thymus lotocephalus* using separators at 18 MPa and 12 MPa. The first separator extract was composed of sesquiterpenes such as globulol (a caryophyllene oxide), while the second separator extract was composed of lighter compounds such as linalool or 1,8-cineole. Fuentes-Gandara et al. [153] carried out extraction using pure Sc-CO<sub>2</sub> and modified CO<sub>2</sub> with different percentages of ethanol and water (50% modifier with pure ethanol, 50/50 ethanol/water, and pure water) in order to extract allelopathic compounds from *Helianthus annuus* L. leaves. The extracts were then fractionated using three collectors at different pressure and temperature settings (20 MPa/45°C; 9 MPa/40°C; 1 atm/30°C). This combination of conditions allowed the selective recovery of different bioactive compounds. For example, using CO<sub>2</sub>:water 50/50 extraction conditions allowed the collection of the free chlorophyll fraction in the third collector. This fraction was also rich in bioactive compounds.

Supercritical fluid extraction provides high selectivity due to the numerous parameters that can affect it. This high selectivity may come from pure Sc-CO<sub>2</sub> density differences, from the addition of various modifiers, and also from technical parameters (vessel geometry, separators), or even from the kind of plant (terrestrial plant, algae...). A large range of compounds can be addressed by this selectivity, from volatile terpenes to non-polar waxes and polar carbohydrates. This last part of the review has described the different possible approaches providing selectivity, in relation to the extraction objective. However, the same parameters can also lead to a decrease in selectivity.

## 4. Conclusion

This review has surveyed the key points that need to be considered in order to modify selectivity during the extraction of bioactive compounds from plants. In particular, it has highlighted the importance of the chemical nature of the solvent, and of the physicochemical properties of the different techniques: wave modulation using UAE, microwave irradiation power using MAE, pressure with PLE and finally pressure, temperature and modifier addition for supercritical extraction using carbon dioxide.

The aim of this review was to provide an overview of the different possibilities as a function of technique and molecular family. For techniques using liquid solvents (MAE, UAE and PLE), it highlighted the strong impact of the extraction solvent in improving selectivity. Moreover, physical parameters can be more suitable for some compounds and matrix couples. For example, microwaves favor the extraction of polar compounds in fresh plants thanks to their dipole moment.

SFE is a major technique for selective extraction. Due to Sc-CO<sub>2</sub> behavior, it is possible to modulate its density with pressure and temperature and thus to favor or disfavor the extraction of target compounds. Low density (with pressure below 15 MPa and temperature below 40°C) will help recover volatile compounds (i.e. monoterpenes, ...) while higher densities (with pressures above 25 MPa and temperatures below 40°C) will extract non-volatile and non-polar compounds (i.e. tocopherols, non-saponifiable lipids) more efficiently. For more polar compounds (i.e. phenols), the introduction of a polar solvent as modifier improves their yields. Other parameters can affect selectivity such as vessel geometry, separators, and so on.

Considering all these different effects, it is also possible to develop new systems by coupling many parameters that was de-

scribed before. This represents a good perspective for selective extraction.

### Declaration of Competing Interest

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

### CRediT authorship contribution statement

**Thibault Lefebvre:** Writing - original draft. **Emilie Destandau:** Supervision, Writing - review & editing. **Eric Lesellier:** Supervision, Funding acquisition, Writing - review & editing.

### Acknowledgements

This work was supported by the Centre-Val de Loire region, which provided the funding of the "Khlors" APR-IR (2017).

### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461770.

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Cette review sur l'extraction sélective a permis de présenter les différents paramètres affectant la sélectivité.

L'un des paramètres majeurs est le choix du solvant. Sa polarité va avoir une incidence directe sur les molécules extraites. Il existe différents modèles permettant d'évaluer la polarité d'une molécule. Le coefficient de partage LogP est utilisé couramment de par sa simplicité apparente. Il permet d'évaluer, en une valeur, si la molécule est plus hydrophile ou hydrophobe. Cependant, ce paramètre unique ne permet pas de prendre en compte les différentes interactions qui peuvent rentrer en jeu lors de l'extraction et la solubilisation de composés. Le modèle de solubilité d'Hansen lui, prend en compte trois paramètres (forces de dispersions, intermoléculaire et liaisons hydrogènes). Il est alors possible de placer dans un repère en trois dimensions, différentes molécules et de voir en fonction de leur distance, si elles sont solubles entre elles ou non. Alors que le LogP est initialement une valeur expérimentale, les valeurs du modèle de Hansen sont théoriques. Il est alors possible d'utiliser ces données afin de trouver des conditions de bonne solubilisation. Cependant, ces modèles ont certaines limites car ils se basent sur la molécule seule. L'environnement est aussi un facteur à prendre en compte, particulièrement dans des procédés d'extraction car d'autres mécanismes peuvent interférer comme la co-extraction d'autres molécules, la diffusion dans les cellules de la plante, etc....

Par ailleurs, pour chacune des différentes techniques d'extractions (MO, US, SFE et PLE), les différents paramètres (physique et chimiques) affectant la sélectivité ont été présentés.

L'extraction assistée par ultrasons améliore l'extraction en provoquant une rupture des membranes de la plante ce qui favorise la diffusion des solutés dans le solvant d'extraction. Il a été démontré que l'intensité des ondes appliquées au mélange plante/solvant peut avoir un impact sur la sélectivité, surtout pour l'extraction de molécules polaires. Les ondes peuvent aussi être modulées dans l'extraction assistée par microondes. La puissance a aussi un impact sur la sélectivité. Celle-ci peut augmenter le rendement d'extraction de certains composés par rapport à d'autre. Cependant, une trop haute puissance peut aussi avoir l'effet inverse c'est-à-dire, qui entraîne la diminution du rendement pour ces mêmes composés. L'extraction par liquide pressurisé utilise ce paramètre avec celui de la pression qui permet de chauffer

au-delà de la température d'ébullition du solvant d'extraction. Cependant, il n'a pas été observé que la pression seule ait un impact sur la sélectivité.

Pour toutes les différentes techniques d'extraction, la température reste le paramètre qui impacte le plus la sélectivité. Elle peut favoriser la solubilisation de certains composés par rapport à d'autres, peut améliorer la diffusion du solvant dans les cellules ou les tissus de la plante ou encore elle peut réduire les interactions matrice/soluté et donc faciliter la récupération du composé hors de la plante. Mais une température trop élevée peut aussi avoir des effets négatifs de dégradation sur certaines familles de composés (par exemple : anthocyanes) ou de co-extraction qui diminuent la sélectivité.

L'une des quatre techniques sélectionnées a montré un plus gros potentiel en terme de sélectivité : l'Extraction par Fluide Supercritique (SFE). Les propriétés solvatantes du CO<sub>2</sub> supercritique peuvent être modulées en fonction de la pression et de la température appliquées et du co-solvant utilisé. Certains composés plus volatils, par exemple les constituants des huiles essentielles, vont nécessiter une faible densité alors que de plus grosses molécules vont avoir besoin d'une forte densité de CO<sub>2</sub> pour être extraits (par exemple des cires). Il est alors possible de jouer sur la densité au cours de l'extraction pour gagner en sélectivité, mais aussi pendant la phase de collecte (en utilisant des séparateurs) en faisant précipiter sélectivement certaines fractions.

Cette technique étant prometteuse pour notre problématique, elle a été sélectionnée afin de produire des extraits sélectifs non-colorés (chapitre 3 et 4).





## CHAPITRE 2: Etude des chlorophylles et de leurs produits de dégradations





## 1. INTRODUCTION

Avant de déterminer les conditions d'extraction sélective des chlorophylles, il a fallu trouver un moyen de produire un échantillon contenant des chlorophylles. Cet échantillon de pigments sera principalement utilisé pour les différents développements analytiques, notamment pour étudier leur comportement chromatographique et la séparation des différents pigments natifs et de leurs produits de dégradation en fonction de la composition de la phase mobile, mais aussi lors du développement du modèle de prédiction des conditions d'extraction sélective présenté dans le chapitre 3.

La première partie de ce chapitre se focalise sur la production de cet extrait présentant l'étude des conditions d'extraction et de stockage limitant la formation de produits de dégradation. Une fois des conditions trouvées, cet extrait a été utilisé pour développer une méthode de séparation chromatographique par SFC des chlorophylles et de leurs dérivés.

## 2. ETUDE DE LA DEGRADATION D'EXTRAITS RICHES EN CHLOROPHYLLES

Le prix d'un standard de chlorophylle *a* est élevé (plus de 150€ le milligramme – prix Sigma-Aldrich en 2020). De plus, à cause de la faible stabilité de ces composés, celui-ci se conserve difficilement très longtemps. L'utilisation d'un extrait de plante riche en chlorophylles est apparue alors comme une solution économiquement plus intéressante. De plus, celui-ci peut être refait en fonction des besoins afin de garantir une bonne quantité de chlorophylles natives.

Il existe différentes plantes riches en chlorophylles (épinard, basilic ...) mais l'une d'entre elle était plus accessible que les autres : le lierre (*Hedera helix* L.), une espèce dont la prolifération naturelle en tout milieux est bien connue. Cette plante a donc été sélectionnée pour la fabrication d'un extrait riche en chlorophylles.

Les chlorophylles sont une famille de pigments très complexes de par leurs diversités et leur stabilité. Leur capacité à capter l'énergie solaire pour alimenter la cellule végétale par la photosynthèse les rendent sensibles aux rayons UV. Ces composés sont stables lorsque la cellule vit. Cependant, une fois qu'un déséquilibre apparaît, pendant la senescence, un stress subit par l'organisme ou une

transformation du milieu (par exemple : une extraction), ces composés peuvent se dégrader de façon plus ou moins spontanée. Il existe différentes réactions qui peuvent donner de nombreux produits de dégradation. De plus, certaines de ces réactions sont plus au moins difficile à éviter (par exemple avec l'oxygène de l'air).

L'objectif n'était pas d'éviter tout type de dégradation, mais de déterminer des conditions d'extraction limitant la formation de produits de dégradation. De plus, pour un stockage dans des conditions classiques (réfrigérateur à 4°C), qui est souvent nécessaire lors de l'analyse de nombreux échantillons, il pouvait être important d'étudier comment évoluait la composition en chlorophylles en fonction des jours.

La première étude de ce chapitre porte sur la détermination des meilleures conditions pour l'extraction et le stockage de cet extrait de lierre riche en chlorophylles.



# Evaluation of the extraction and stability of chlorophyll-rich extracts by supercritical fluid chromatography

Thibault Lefebvre<sup>1</sup> · Emilie Destandau<sup>1</sup> · Eric Lesellier<sup>1</sup>Received: 2 June 2020 / Revised: 17 July 2020 / Accepted: 3 August 2020  
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## Abstract

In this study, a rapid (less than 10 min) analytical method by reverse-phase supercritical fluid chromatography was developed with an isocratic mobile phase, enabling the separation of 11 compounds, chlorophyll *a* and *b*, pheophytin *a* and numerous allomers or epimers. This method was used to examine the stability of chlorophyll pigments of plant extracts obtained with various extraction methods including microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and ultrasound-assisted extraction (UAE), with ethanol as solvent or modifier. The effect of storage was studied for both liquid and dried extracts. Irrespective of the extraction method, the evaporation of the extracts induced partial degradation of the chlorophyll pigments. It was found that liquid extracts could be stored at 4 °C for 3 weeks without a dramatic change in allomer forms of chlorophylls. However, during this storage period, epimerization appears to be important, leading to a significant decrease in the chlorophyll *b* native form.

**Keywords** Chlorophyll · Supercritical fluid chromatography · Plant extraction · Sample stability

## Introduction

Chlorophyll pigments are responsible for the green color of external parts of plants, which is a quality attribute of vegetable products. Post-harvest treatments can be responsible for pigment degradation leading to color changes. Many studies have investigated the effects of various methods, including (i) plant drying, done to improve the duration of plant storage [1–3]; (ii) blanching, applied to inactivate enzymes [4] during specific maturation treatments, for instance maturation of tea leaves [5, 6]; (iii) the ripening of grape berries [7, 8], fruits [9] and olives [10]; and (iv) during many other manufacturing processes such as canning [11], freeze-drying [12], pasteurization [13] or microwave cooking [14].

Beyond the many parameters acting on pigment stability during these processes, the increase of temperature is often reported to play a major role in the chemical change of native chlorophylls *a* and *b*, either by replacing the magnesium cation of the porphyrin group by two hydrogen atoms, leading to

pheophytin forms, or by inducing epimerization on carbon 13<sup>2</sup>, leading to *a'* and *b'* forms, both for chlorophylls and pheophytins. High thermal treatment also yields the loss of carboxy groups (decarboxymethoxylation) on carbon 13<sup>2</sup>, leading to pyropheophytin derivatives [14].

Endogenous enzymatic activity can also be promoted by processing conditions leading to dephytylated chlorophyll derivatives (chlorophyllides, pheophorbides) that are more soluble in water [4, 5, 12, 15]. Other oxidative enzymatic activity was reported in olive fruits to provide 13<sup>2</sup> hydroxy- or methoxy-pheophytin forms or 15<sup>1</sup> lactone-pheophytin [16–18] or in herbs *Gynostemma pentaphyllum* Makino (19) favored by heat treatments. The presence of 15-OH-lactone chlorophyll has also been reported in *Rhinacanthus nasutus* (L.) after a hot-air drying process (60 °C, 4 h) [2].

Other studies described attempts to control and stabilize these compounds [19, 20]. From a more practical view, different industrial processes exist to avoid degradation. Salts of Cu<sup>2+</sup> or Zn<sup>2+</sup> serve to stabilize chlorophyll by changing the magnesium complex and obtain a brighter green for a longer time [21]. Heat treatment can also be used, which will inactivate chlorophyllase enzyme and then reduce the pheophytination reaction.

Extraction processes are also employed in order to obtain extracts containing bioactive, mainly antioxidant, compounds.

✉ Eric Lesellier  
eric.lesellier@univ-orleans.fr

<sup>1</sup> ICOA, CNRS UMR 7311, Université d'Orléans, rue de Chartres, BP 6759, 45067 Orléans Cedex 2, France

These extracts are introduced in body care or nutraceutical products. Among extraction processes, pressurized liquid extraction (PLE) makes use of high extraction temperature, above the boiling point of the solvent used, by applying back-pressure after the extraction cell which is generally equal to or greater than 10 MPa. PLE studies have reported the effect of extraction temperature or a comparison of PLE to maceration (MAC) (typically 6 h at room temperature), Soxhlet extraction (2 h) and ultrasound-assisted extraction (UAE) (2 h), for instance, with a solvent mixture of ethanol and water 90/10 (v/v) [22, 23]. The ratio between pheophytin *a*, produced by the removal of the magnesium cation from the porphyrin group, and chlorophyll *a* was in the same range for MAC, UAE and PLE at 160 °C. This ratio was strongly depleted for Soxhlet extraction, showing the probable degradation of chlorophyll *a* due to both the temperature and the duration (6 h) of the Soxhlet process. UAE and Soxhlet extraction both seemed to favor an increase in pheophorbide *a* due to an enzymatic reaction. Also, the use of high temperature (higher than 130 °C) for PLE favored the increase in pyropheophytin derivatives.

Supercritical fluid extraction (SFE) was also performed to extract plant pigments, mainly from microalgae, which contain both carotenoids and chlorophylls [24–28]. Most of these papers described the effects of changes in back-pressure or temperature, with pure carbon dioxide as extracting fluid, on the carotenoid/chlorophyll ratio, and comparison with pure liquid solvents like methanol or dimethylformamide (DMF). However, these ratios are calculated from spectroscopic measurements, which does not allow the study of individual pigment degradation during the extraction process. Indeed, it was shown that because the absorption spectrum of chlorophyll *a'* and *b'* was identical to that of chlorophyll *a* and *b*, cooking processes had no effect on the total chlorophyll concentration, i.e. on the visual color, whereas the concentration of epimer forms was strongly improved [9].

Beyond the effects of various parameters during the extraction processes (MAC, UAE, Soxhlet, PLE, SFE), the stability of chlorophylls in extraction solvents is also of interest.

Several solvents have been suggested for the extraction and analysis of plant pigments, including acetone or aqueous acetone [1, 5, 6, 9], ethanol/acetone [3], ethanol/hexane [13], DMF [15–17, 29], methanol [4, 14] or even hexane/acetone/ethanol/toluene [2, 30]. The solvent can be evaporated under nitrogen stream at room temperature [6] or under vacuum at temperature varying from ambient to 40 °C [2, 3, 13, 15, 17, 30]. Liquid chromatography (LC) analysis can be performed immediately by dissolving the dry residue [2, 13, 20], or after storage at –80 °C in 2-propanol until analysis [4] or at –18 °C in acetone for 18 h [17]. Some papers reported the degradation of chlorophylls in solvents. No loss in absorbance was observed for DMF extract after 21 days at 4 °C [31], whereas a decrease in the green color of ethanol/water mixtures was

reported to follow first-order kinetics [32]. Spontaneous allomerization (the replacement of a hydrogen atom at the C-10 position with an oxygen-containing moiety) of chlorophylls in methanol exposed to atmospheric oxygen was often observed, which produced 13<sup>2</sup> hydroxy-, 13<sup>2</sup> methoxy- or 15<sup>1</sup> hydroxy-lactone derivatives [12, 13, 33] and could be completed after 6 h in ambient light [33] or 3 days in the dark [34]. Another paper reported that when extraction was performed at 60 °C, because oxygen is insoluble in organic solvents, no allomerization of chlorophylls was observed [35].

To summarize, the study of the effect of processes on chlorophylls first requires extraction for identification and quantification. Obviously, degradation of chlorophylls can occur during extraction processes (MAC, UAE, PLE, SFE), but also during storage of liquid extracts or dry residues before analysis. Unfortunately, in the case of the analysis of numerous samples, storage of extracts is necessary because of the limited number of analyses that can be performed in a laboratory.

The aim of this paper is to study by supercritical fluid chromatography the changes in the chlorophyll content of ivy (*Hedera canariensis* Wild) extracts obtained by various extraction methods and stored under different conditions for several days. This plant was selected as a model because of its high chlorophyll content and also because of its pharmacological and therapeutic properties [36].

## Material and methods

### Chemicals and plant material

The solvents used were methanol and ethanol, gradient grade (VWR International, Fontenay-sous-Bois, France). Carbon dioxide (>99.5%) was from Air Liquide (Fleury-les-Aubrais, France).

Wild ivy was collected in Orleans (France). The leaves were dried using a Memmert 500 dryer (Mettler GmbH, Schwabach, Germany) at 50 °C for 24 h. The dried leaves were then ground using a Kinematica Polymix PX-MFC 90 D Kinematica (Lucerne, Switzerland). This device grinds the plant continuously, which means that grinding duration depends on the quantity to be ground. However, the speed employed was the maximum possible on this device. The same batch of plant samples was used for all extraction processes.

For PLE and SFE, the dispersant used was diatomaceous earth (DE) of general purpose grade (Fisher Scientific, Waltham, MA, USA).

### Extraction and solvent evaporation

The general treatment of the dried leaves of ivy (sample) is summarized in Fig. 1. Four extraction techniques were used:

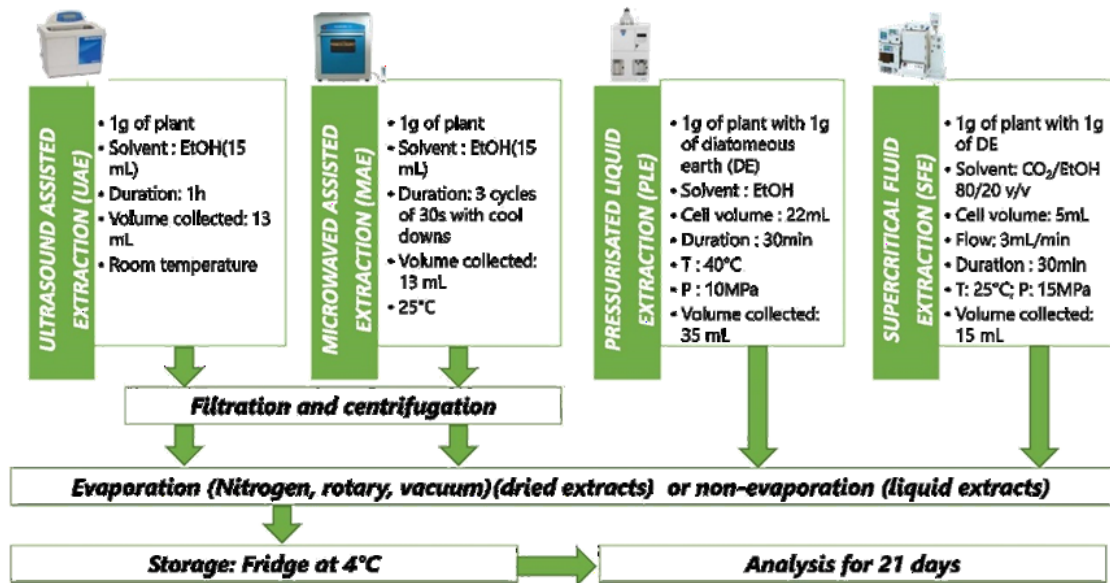


Fig. 1 Experimental scheme of methods and protocols used for chlorophyll extraction from dried ivy leaves

- Ultrasound-assisted extraction (UAE) was performed with an ultrasonic bath using a Branson 3510 device (Vlierberg, Netherlands) at room temperature. One gram of sample was extracted in 15 mL of ethanol for 1 h. The final collected volume was 13 mL after filtration and centrifugation.
- Microwave-assisted extraction (MAE) was carried out with a MicroSYNTH device from Milestone Srl (Soriso, Italy). Extraction was carried out in a 50 mL reactor (QRS1550). One gram of sample was extracted in 15 mL of ethanol during three cycles of 30 s at 500 W. A cooling step was done after each extraction cycle to reduce the temperature of the extract to around 25 °C. The final collected volume was 13 mL after filtration and centrifugation.
- Pressurized liquid extraction (PLE) was performed with a Thermo Scientific Dionex ASE 150 system (Thermo Scientific, Waltham, MA, USA). One gram of the sample mixed with 1 g of dispersant (DE) reagent was extracted in a 22 mL extraction cell. One extraction cycle of 30 min with ethanol at 40 °C was used. The final collected volume was 35 mL.
- Supercritical fluid extraction (SFE) was carried out with a Waters MV-10 system (Waters Corporation, Milford, MA). In order to monitor the extraction kinetics, a Gilson 151 UV/Vis multi-wavelength detector (Gilson Company, Lewis Center, OH, USA) was added between the oven and the back-pressure regulation (BPR) module, allowing an extraction duration of 30 min to be selected. One gram of sample mixed with 1 g of dispersant (DE) was extracted in a 5 mL extraction cell. The remaining cell space was filled with cotton. Extraction was done with a mixture of CO<sub>2</sub>/EtOH 80/20 v/v at a flow of

3 mL/min and back-pressure of 15 MPa at 25 °C for 30 min. The final collected volume was 15 mL.

The collection system was modified from the original version. Usually, vials of 100 mL are used and connected to the outlet of the extraction cell with capillaries of 2 mm internal diameter (ID). This large internal diameter allows the CO<sub>2</sub> to return to the gaseous state without inducing high pressure into the capillary. In that case, to avoid the adsorption of extracted compounds onto the internal surface of the connecting capillary that would result from the strong decrease in the compound solubility, a make-up pump located after the back-pressure regulator adds a make-up solvent to favor the full transfer of the compounds into the collection vial. However, we wanted to avoid the use of the make-up solvent, to reduce the final collected volume and the duration of an evaporation step, but also to favor the collection of concentrated extracts, allowing an easier direct quantitative analysis. We then replaced the 2 mm ID connection capillaries with polyether ether ketone (PEEK) tubing of 1 mm ID. When using 20% of ethanol in the extraction solvent, we can assume that with this lower internal diameter, the ethanol flow will be able to flush the compounds out of the capillary despite no addition of make-up solvent. Moreover, we tested two types of collection tubes with this new capillary of smaller internal diameter. First, the collection was done in an open tube of 15 mm × 18 cm, maintaining the capillary at the bottom of the tube, in order to avoid loss of the aerosol droplets during the CO<sub>2</sub> depressurization. The relative standard deviation measured on the peak area of chlorophyll *a* was equal to 7.1%, which was rather high in comparison to the other extraction methods tested. Therefore, we changed to collection tubes of 28 mm ×

14 cm covered with a septum through which the collection capillary was passed, and the RSD was decreased to 4.2%, which was in the same range as those measured with the other extraction methods.

In order to prepare samples for analysis, some of the extracts containing solid plant residues needed to be filtered with filter paper (UAE and MAE extracts). The extracts were then centrifuged (9000 rpm, 10 min) using a Jouan BR4i multi-function centrifuge (Thermo Electron, Waltham, MA, USA). Supernatants were then collected for analysis.

Different conditions were selected to study possible chemical changes during storage. Each liquid extract, provided by the four extraction methods, was first divided into four fractions. The first one was kept in the liquid form, and the other three were dried using three different solvent evaporation methods:

- Vacuum rotary evaporation (50 mbar, room temperature; 20 min) was implemented using a BUCHI device (BUCHI Corporation, New Castle, DE, USA) with different modules (B-480, R-114, I-100, V-100 and F-308).
- Nitrogen evaporation was carried out using a Techne sample concentrator (Techne, Staffordshire, United Kingdom) (ambient temperature; 6 h).
- Vacuum concentration was performed with a Savant SpeedVac™ SPD 2010 instrument (Thermo Fisher Scientific, Waltham, MA, USA) (25 °C, pressure 13 mbar; for 4 h).

The four extracts, three dried and one liquid, were then stored at 4 °C and monitored for 21 days to follow the evolution of chlorophyll compounds (Fig. 1).

## Analysis of chlorophylls

Supercritical fluid chromatography (SFC) was implemented using the same JASCO instrument as the SFE system, only replacing the extraction cell with a chromatographic column. This device was equipped with a JASCO MD40-10 PDA detector (and an LC-NETII/ADC interface unit). In order to quantify the chlorophyll compounds, the samples were monitored at 650 nm. Chromatograms were recorded using ChromNAV software (JASCO, Tokyo, Japan). The chromatographic column was an octadecyl-bonded silica Cortecs C18 (4.6 × 150 mm, 2.7 µm) (Waters, Milford, MA, USA). This column was selected for the type of silica particle (core-shell), which provides high chromatographic efficiency. The mobile phase was composed of CO<sub>2</sub>/MeOH 80/20 v/v in isocratic mode. The flow rate used was 1.5 mL/min, and the column oven temperature was equal to 25 °C. Back-pressure was set at 10 MPa.

Molecular characterization of chlorophylls in ivy extract was carried out with a Nexera UC SFC system with a

single-quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), using the chromatographic conditions described above.

The following parameters were used for the characterization: electrospray ionization (ESI) in positive mode; interface voltage, 4.5 kV; interface temperature 350 °C; nebulizing gas flow, 1.5 L/min; heat block, 200 °C. Make-up solvent was also used to enhance the ionization of the molecules. Methanol at 0.100 mL/min with 20 mM ammonium formate was added after the column. The compounds were identified based on UV-visible absorption spectra and comparison of the  $m/z$   $[M + H]^+$  values with data from the literature [4, 6, 12, 30, 37].

Before SFC analysis, the dried samples (5 mg) were dissolved in 500 µL of methylene chloride. The injection volume was 5 µL.

## Results and discussion

### Influence of the extract drying method on the chlorophyll degradation

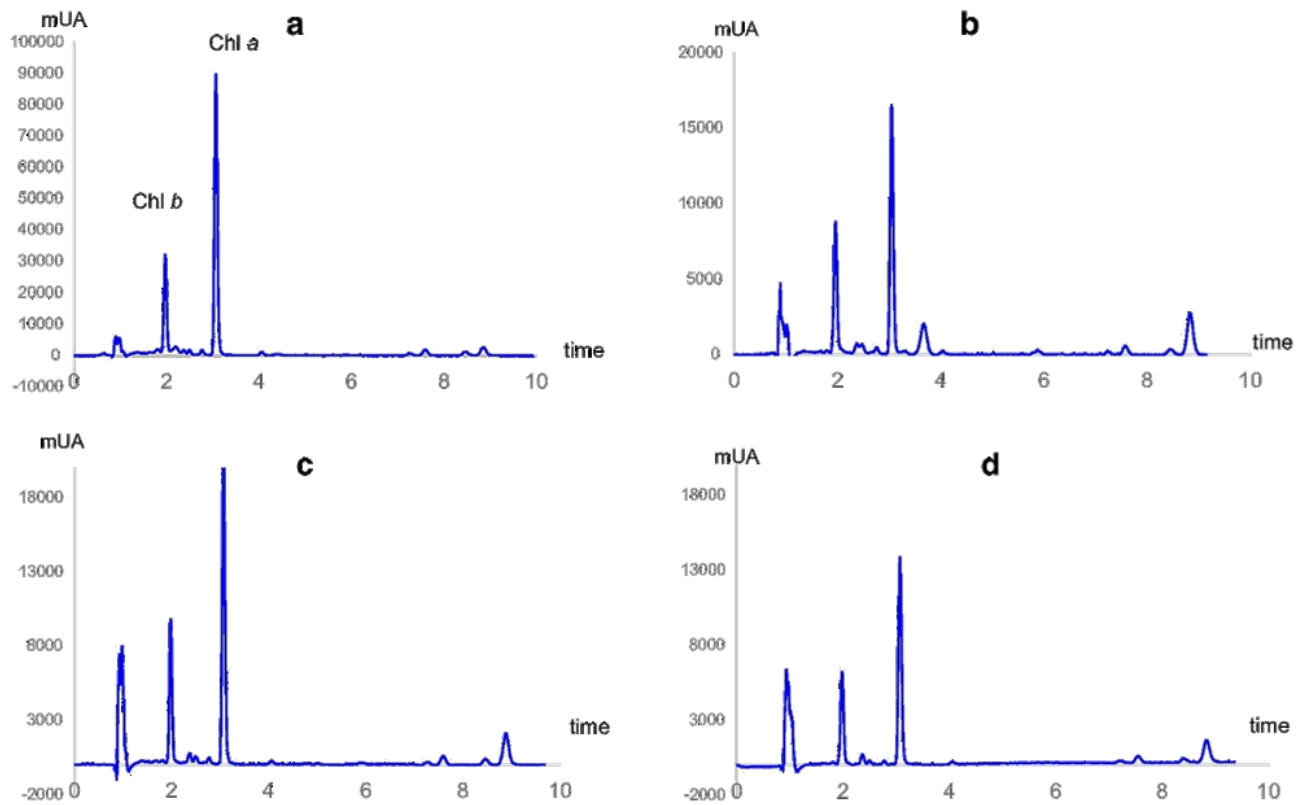
As reported in Experimental section, different solvent evaporation techniques, including rotary evaporation, nitrogen flow and vacuum concentrator (SpeedVac), were used to dry the extracts for sample conservation (conditions described in Experimental section).

Figure 2 presents the chromatograms obtained for the UAE extracts for the liquid extract (without drying) or after solvent evaporation using the three evaporation methods.

First, we can observe that satisfactory separation of the compounds was achieved with a C18-bonded phase with 20% of methanol in the mobile phase. In a previous publication, which was devoted to the separation of pheophytins and pheophytin derivatives produced under strong degradation conditions, we recommended a higher modifier percentage of 70% to provide both good separation and elution [38]. With these new conditions, 20% of methanol was sufficient to ensure the study of the observed compounds, with a short analysis time.

All chromatograms show two main peaks around 2 and 3 min for chlorophyll *b* and chlorophyll *a*, respectively, the native pigments in the studied plant. From Fig. 2a, corresponding to the liquid extract of dried plant, we can suppose that the drying process at 50 °C for 24 h required for plant conservation does not induce significant degradation of the two chlorophylls. Numerous small peaks appear between the two chlorophylls, with four minor peaks with longer retention time, around 7 and 9 min. The identification of these additional (degradation) peaks was performed by SFC-MS on a UAE extract stored in liquid form for 20 days at 4 °C (Fig. 3). As shown in Fig. 3 and Table 1, which presents the values of both the maximum absorption wavelength and the  $m/z$  value of the  $[M + H]^+$  protonated molecular ion, some of these compounds

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**Fig. 2** Supercritical fluid chromatography chromatograms of chlorophyll extracts obtained by UAE (details in Experimental section). **a** Liquid extract without evaporation stage; **b** sample obtained after nitrogen

evaporation; **c** sample obtained after rotary evaporation; **d** sample obtained after SpeedVac evaporation

are epimers of the main form of chlorophyll *a* and *b* (peaks 7 and 2), and others are 13-hydroxychlorophyll forms: peaks 4 and 5 from chlorophyll *a* and *a'*, and peaks 9 and 10 from pheophytin *a* and *a'*. Peak 11 corresponds to pheophytin *a*, which is produced from chlorophyll *a* by the loss of the magnesium cation, whereas peak 8 could be a 15-lactone form of pheophytin *a*. The presence of pheophytin allomers in teas or in Chinese herbs has been previously reported [6, 30], and the

retention order observed for all compounds in our study with an ODS stationary phase with supercritical fluids is identical to that observed in HPLC. We can also note the absence of pyropheophytin derivatives that are generally produced in plant processes at high temperatures [12, 38]. In addition, no presence of pheophytin *b* or *b'* was observed, which should be eluted between peaks 7 and 8, or of 13-hydroxy-chlorophyll *b* and *b'*, which should be eluted between peaks 6 and 7 [6] or

**Table 1** UV-visible and MS identification of pigments observed in the extracts

Peak number	Lambda 1 (nm)	Lambda 2 (nm)	[M+H] <sup>+</sup> m/z	Compound name
1	461	646	907.5	Chlorophyll <i>b</i>
2	455	654	907.5	Chlorophyll <i>b</i>
3	462	658	908.0	Chlorophyll derivative
4	418	654	909.5	13-Hydroxychlorophyll <i>a</i>
5	418	654	909.5	13-Hydroxychlorophyll <i>a'</i>
6	430	663	893.5	Chlorophyll <i>a</i>
7	431	658	893.5	Chlorophyll <i>a</i>
8	399	665	903.5	15-Hydroxy-lactone pheophytin <i>a</i>
9	405	666	887.5	13-Hydroxypheophytin <i>a</i>
10	402	664	887.5	13-Hydroxypheophytin <i>a'</i>
11	406	662	871.5	Pheophytin <i>a</i>

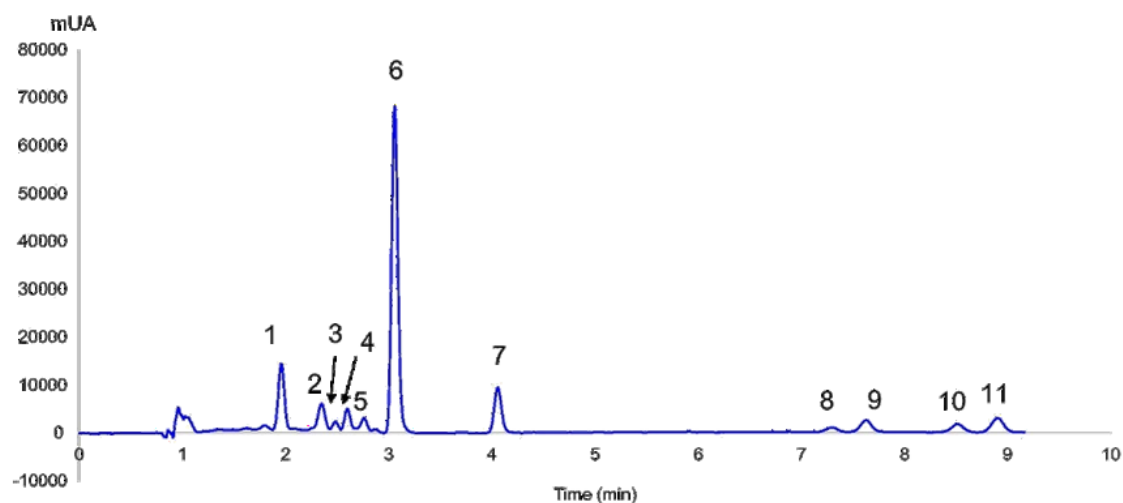


Fig. 3 Chromatogram of a UAE liquid extract stored for 20 days at 4 °C. For peak identification, see Table 1

between peaks 7 and 8 [30]. Other studies on thermal treatment of parsley products report identical conclusions on the absence of pheophytin *b* [4].

Numerous papers report the allomerization of chlorophyll *a* exposed to atmospheric oxygen under ambient light [4, 12], or the oxidation of chlorophyll pigments by free radicals at room

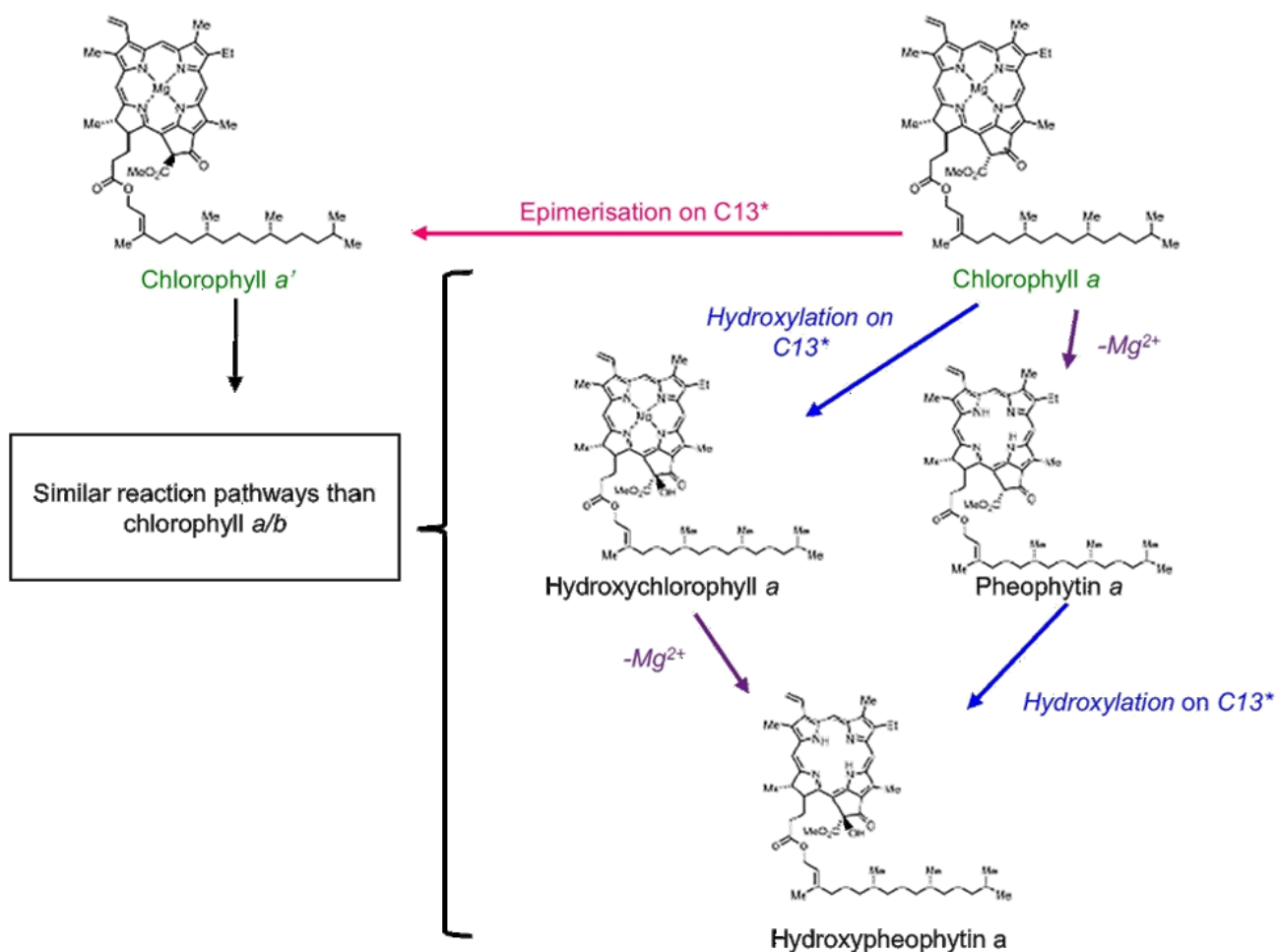
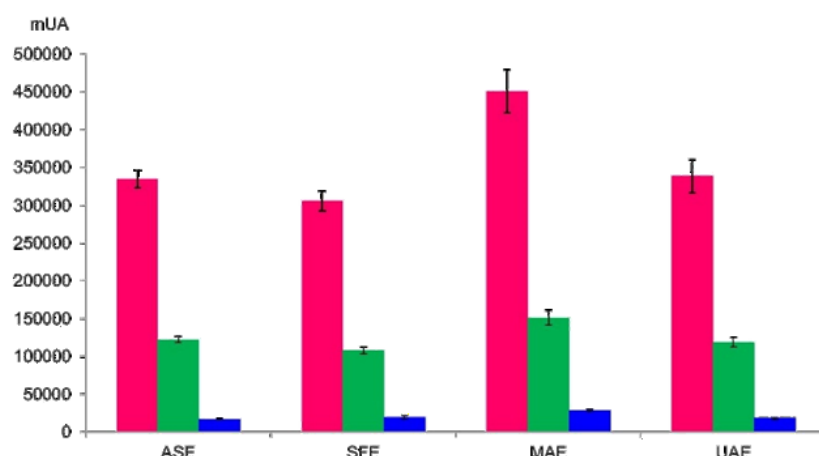


Fig. 4 Degradation pathways of chlorophyll *a* and compound structures

**Fig. 5** Peak area and relative standard deviation for triplicate extractions of chlorophyll *a* (pink), chlorophyll *b* (green) and pheophytin *a* (blue) using various methods (PLE, SFE, MAE, UAE)



temperature leading to 13-hydroxy derivatives [34]. Moreover, all the derivatives found in our extracts result from the degradation of chlorophyll *a*, which is often reported as the most sensitive pigment compared to chlorophyll *b* [4, 13, 39]. Figure 4 presents different chlorophyll degradation pathways. This scheme focusses on chlorophyll *a*, but these reactions are identical with chlorophyll *b*.

The concentration of pigments in the liquid extract (direct injection from the extract) and the three dried samples is different (Fig. 2), explaining the higher peak intensity for the liquid extract (30,000 mAU for chlorophyll *b* and 100,000 mAU for chlorophyll *a*) with respect to the three dried samples (10,000 mAU for chlorophyll *b* and 20,000 mAU for chlorophyll *a*). These chromatograms also show a change in the ratio between the peak areas of the two native chlorophyll pigments, from 3 in the liquid extract to 2 after evaporation. This indicates that the drying step causes more degradation of chlorophyll *a* than chlorophyll *b*. Moreover, the relative intensity of the pheophytin *a* peak (around 9 min), in comparison to the chlorophyll peaks, varies depending on the evaporation method. Lower intensities for pheophytin are observed for the liquid extract (Fig. 2a), without any evaporation. For rotary (Fig. 2c) and SpeedVac (Fig. 2d) evaporation, the increase in the relative intensities for the pheophytin peak are

similar, whereas a higher peak increase of pheophytin is observed for nitrogen evaporation (Fig. 2b). Beyond the type of solvent evaporation, the duration of evaporation is dramatically longer with nitrogen than with the rotary or SpeedVac method (6 h vs. less than 1 h). This chemical modification is mainly observed for the chlorophyll *a* molecule, which seems to be more sensitive than chlorophyll *b* to the difference in evaporation treatments. Such difference in the behavior of the two chemical forms of chlorophyll has often been reported [1, 4, 13, 14, 39].

### Influence of the extraction method on the chlorophyll degradation

To study the recovery from the various extraction methods (UAE, PLE, MAE and SFE), as well as their relative standard deviation, three independent extractions were carried out for each method, using ethanol as solvent (conditions described in Experimental section). The quantitative results from the liquid extracts are compared after normalization relative to the final volume of the extracts.

Figure 5 shows the peak areas for chlorophyll *a*, chlorophyll *b* and pheophytin *a* obtained from the four extraction methods. The amounts of chlorophyll *a* and *b* extracted by PLE and UAE, obtained with pure ethanol as extraction solvent, and obtained by SFE, obtained with a mixture of 20% ethanol in carbon dioxide, are in the same range. Lower recovery in chlorophyll content is found when using SFE (CO<sub>2</sub>/ethanol 95/5) in comparison to conventional extraction with methanol or dimethylformamide from microalgae [26]. Greater quantities are obtained with MAE with pure ethanol, despite the much shorter extraction time. The higher temperature reached during the extraction cycles with microwaves (above 100 °C) could be responsible for the better chlorophyll solubility or accessibility toward ethanol, possibly because more chloroplasts are broken open during the process, which would also explain the more significant formation of

**Table 2** Relative standard deviation (%) (calculated from triplicate extractions) of chlorophyll *a* peak area and peak area ratio for varied extracted compounds of liquid extracts using different extraction methods (C is chlorophyll, P is pheophytin)

	RSD Ca	Ratio Ca/Cb	Ratio Ca/Pe	Ratio Ca/Ca'
	%			
ASE	3.5	2.7	19.9	49.6
SFE	4.2	2.8	15.7	67.4
MAE	6.3	2.9	15.7	11.8
UAE	6.5	3	18.5	76.0

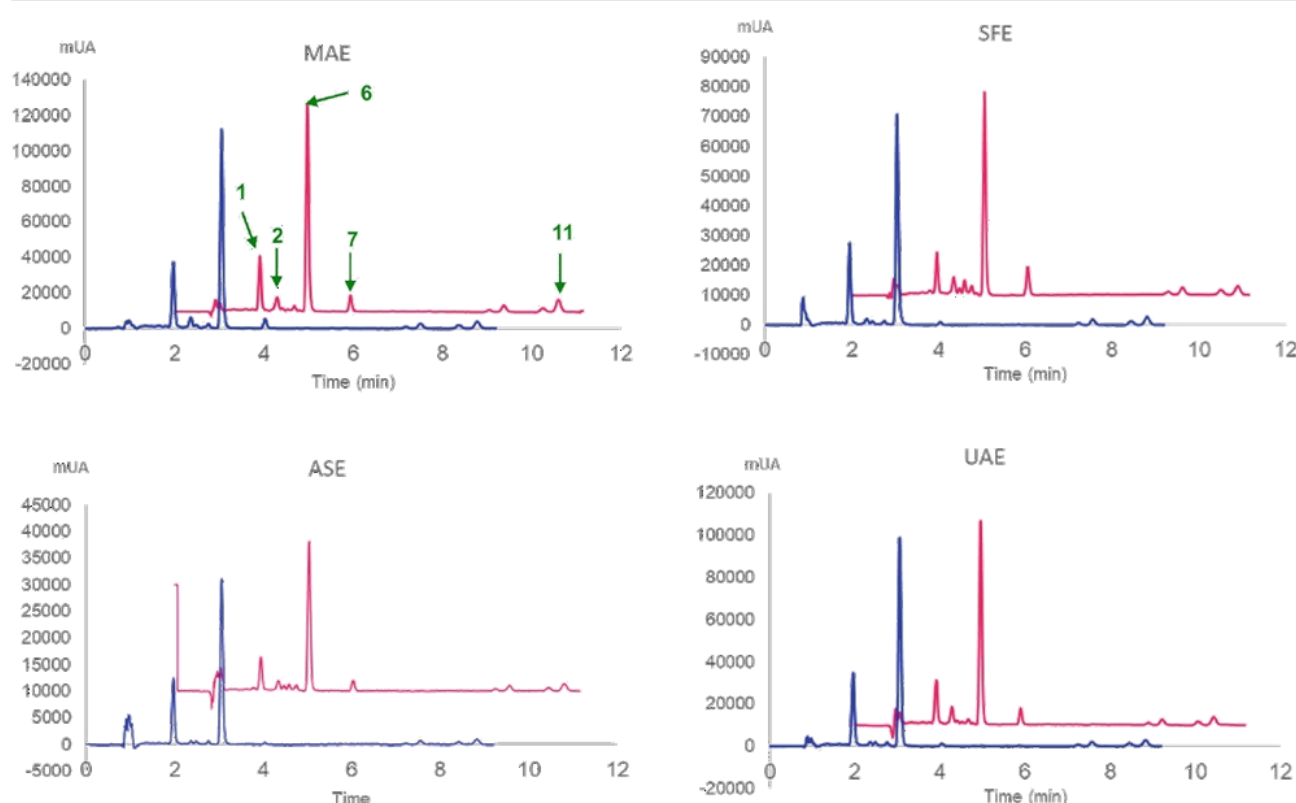


Fig. 6 Chromatograms at day 0 (blue) and day 21 (pink) for the four liquid extracts obtained by MAE, SFE, PLE and UAE

chlorophyll derivatives with the use of microwaves in the extraction process [14].

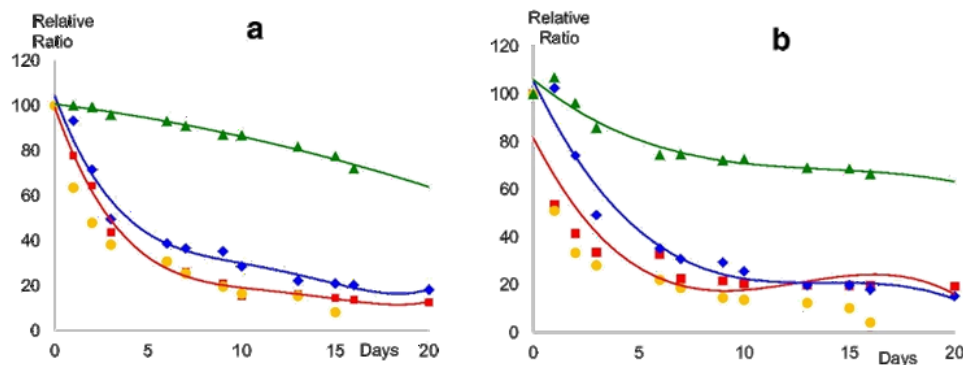
The ratio of peak area for chlorophyll *a* and *b* is also similar (from 2.7 to 3; Table 2) regardless of the extraction method used, and this ratio is typical for plants.

The lowest relative standard deviation is obtained for PLE (3.5%) and SFE (4.2%), probably because of the better control of the volumes used and collected (Table 2). For SFE, to avoid the loss of compounds during the depressurization stage, the collection system provided by the MV-10 device was modified (see Experimental section). The relative deviation standard (RSD) was 7% with open-vial collection but only 4.2% with closed-vial collection, showing the importance of the compound loss in droplets of the aerosol formed at the

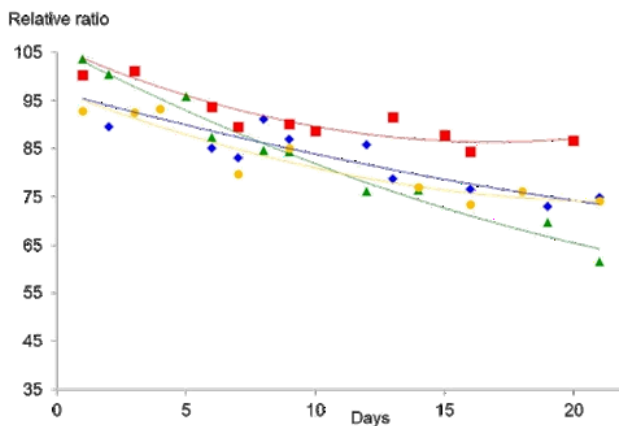
capillary extremity with an open collection system. For UAE (6.3%) and MAE (6.5%), the RSD was higher, which could be due either to additional treatments, filtration and centrifugation of the sample, or to the extraction method itself (for example, due to less accurate control of temperature).

Figure 5 and Table 2 also show the ratio of the chlorophyll *a* to pheophytin *a* peak area. This ratio ranged from 15.7 to 19.9. The pheophytization, meaning the replacement of the magnesium cation of the porphyrin ring in the native chlorophyll by two hydrogen atoms, is in part related to the presence of acidic compounds in the extract. MAE could favor a greater break of the vacuoles containing these acidic compounds in the plant [4]. Another ratio was measured between the two epimers of chlorophyll *a*. These values are very high for both

Fig. 7 Relative ratio percentage of chlorophyll *a/a'* (a) and *b/b'* (b) for the liquid extract obtained by SFE (red squares), PLE (blue diamonds), UAE (yellow circles) and MAE (green triangles)



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**Fig. 8** Relative ratio percentage of chlorophyll *a*/pheophytin *a* peak area during storage for liquid extracts obtained by SFE (red squares), PLE (blue diamonds), UAE (yellow circles) and MAE (green triangles)

PLE and UAE (around 70), slightly lower for SFE (around 50) and very low for MAE (around 12).

Finally, the MAE with higher extraction temperature and ability to favor compound release provides the highest chlorophyll amount, with the highest relative standard deviation and also the highest pheophytin amount, showing both the efficiency of this extraction method and its degradation capability.

### Chlorophyll stability in liquid or dried extracts

All the extracts from the four extraction methods, evaporated and non-evaporated, were analyzed over a period of several weeks, and the content of the various pigments was monitored. However, the content of chlorophyll *a* and *b* in dried extracts decreased dramatically from the first day of conservation and with the drying step. Consequently, no results are presented for the dried extracts.

Figure 6 shows the chromatograms of the extracts just after extraction (day 0) and after 20 days of storage at 4 °C. First, the decrease in chlorophyll *b* (peak 1) seems to be greater than that of chlorophyll *a* (peak 6). Figure 7 shows the change in the value of the relative ratio (expressed from the value obtained at day 0, which is equal to 100%, meaning the greatest possible value) of the epimeric forms for the two chlorophylls. The epimeric forms *a'* (Fig. 7a) and *b'* (Fig. 7b) dramatically increase (the relative ratio decreases) with the storage duration, except for the MAE extract. However, for this MAE sample, as mentioned above, the ratio between the chlorophyll *a* and *b* and their epimeric forms was very small (Table 2), meaning that the great amount of the epimeric forms was mainly due to the extraction process, and not to the extract storage. For the other three extraction methods, this decrease is similar, with the main decrease of the ratio during the five storage days.

Figure 8 shows the variation in the ratio of chlorophyll *a*/pheophytin *a* peak area for the liquid extracts. The results are also expressed in percentage calculated from the ratio measured just after the extraction (day 0). For all liquid extracts, this ratio decreases, indicating a regular increase in the pheophytin *a* content vs. the chlorophyll *a* content. This decrease seems more significant for the MAE extract, whereas the final values for PLE and UAE are close to 75%, and only 85% for SFE, indicating limited formation of pheophytin *a* during the storage of the SFE liquid extract. The main increase in pheophytin *a* (peak 11) is observed for the MAE extract, whereas peaks 8, 9 and 10, corresponding to allomers of pheophytin, are rather unchanged, regardless of the extraction technique used (Fig. 6).

Finally, Fig. 6 also shows the increase of the 13-hydroxychlorophyll *a* and *a'* (peaks 4 and 5) in all extracts. This increase seems to be greater for the SFE extract, with regard to other extraction methods. Because SFE is well recognized to reduce compound oxidation during the extraction process, this oxidation could occur in the collection liquid, maybe due to the lack of protective compounds (polyphenol types). Generally, polyphenols are extracted with the chlorophylls when utilizing classical methods (PLE, UAE, MAE), allowing for better protection against oxidative degradation. These compounds, especially phenolic acids, are also largely found in ivy extract when using polar extraction solvent [40]. However, a previous study showed that in SFE, the addition of 20% of water to ethanol used as modifier was required to extract dicafeoylquinic acids from a halophytic plant [41], whereas the use of pure ethanol mixed with carbon dioxide was unable to recover these antioxidant compounds.

To conclude on the liquid extract stability, the epimeric forms of chlorophyll *a* and *b* increase during the storage of the liquid extracts, whereas the allomer forms of pheophytin *a* are rather unchanged during this storage.

Other parameters could have been evaluated (energy or solvent consumption, waste production) in order to study the ecological impact of the varied used techniques. However, due to the scale of the extraction process (1 g of sample), these points were not addressed during the development. In addition, the advantages and drawbacks in the use of these four extraction methods, especially for industrial extract production, have been summarized elsewhere [42].

### Conclusion

Rapid and accurate separation of chlorophyll pigments (native and derivatives) was achieved by supercritical fluid chromatography, using a modern C18-bonded phase together with an isocratic mobile phase. This method was used to study both the effect of the extraction method applied to plants and the storage conditions of extracts.

From the experiments carried out in this study, the use of microwave-assisted extraction seems to favor the appearance of degradation products from chlorophyll pigments. Moreover, for this extract, the increase in pheophytin *a* is greater during the storage of liquid extracts. Numerous allomeric forms are also observed in extracts obtained from the four extraction methods, but these compounds seem rather stable during storage (for liquid extracts).

The evaporation of liquid extracts induces a strong increase in pheophytin *a* and sometimes in chlorophyll *a'*. In addition, the conservation of these dried extracts leads to a dramatic decrease in the native form of chlorophylls. A practical conclusion is that, if analysis of the extract cannot be done immediately after extraction, the extracts should be stored in a liquid form prior to analysis. For these liquid extracts, after 21 days of storage at 4 °C, and depending on the extraction method used, the increase in the pheophytin *a* amount is limited, whereas the epimerization of chlorophyll *a* and *b* or of 13-hydroxychlorophyll *a* is the main degradation observed.

## Compliance with ethical standards

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

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L'objectif de cette étude était de trouver les conditions d'extraction et de stockage permettant de garder le plus longtemps possibles les chlorophylles natives des plantes supérieures (*a* et *b*).

Différentes techniques d'extraction ont été utilisées (US, MO, SFE et PLE) ainsi que différentes techniques d'évaporation des extraits (azote, speedvac et évaporateur rotatif). Afin d'analyser la teneur en différents dérivés chlorophylliens, une méthode a été développée en SFC en utilisant une colonne greffée C18 avec un mode d'élution isocratique à 20% de méthanol (à 1,5 mL/min, 25°C et 10 MPa)

Cette étude a permis d'identifier des conditions facilitant la formation de produits de dégradation. L'extraction assistée par micro-ondes semble favoriser l'apparition rapide et en grande quantité de produits de dégradation. De plus, lors du stockage, cet extrait est celui qui est le moins stable parmi ceux obtenus avec les différentes techniques.

L'évaporation des extraits induit également l'apparition de nombreux produits de dégradation, quelle que soit la technique d'évaporation utilisée, par rapport aux extraits conservés à l'état liquide. Le stockage d'extrait liquide avant analyse a donc été privilégié dans ce travail.

Lors des travaux présentés par la suite, l'extrait de lierre a été réalisé en utilisant des ultrasons avec de l'éthanol pendant soixante minutes. Les prochaines parties utilisent donc ce protocole d'extraction afin d'avoir un échantillon riche en chlorophylles.

### **3. ETUDE CHROMATOGRAPHIQUE DES CHLOROPHYLLES**

Comme vu précédemment, un échantillon contenant des chlorophylles peut vite donner lieu à de nombreux produits de dégradations. Il était donc intéressant d'étudier le comportement des chlorophylles natives mais aussi les différents produits de dégradation en chromatographie par fluide supercritique (SFC). Ce qui est présenté dans l'article suivant, a permis de voir le comportement de ces composés sous différentes conditions chromatographiques.



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Journal of Chromatography A

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## Development of an analytical method for chlorophyll pigments separation by reversed-phase supercritical fluid chromatography

Thibault Lefebvre, Arwa Talbi, Sirine Atwi-Ghaddar, Emilie Destandau, Eric Lesellier\*

ICOA, CNRS UMR 7311, Université d'Orléans, rue de Chartres, BP 6759, 45067 Orléans Cedex 2, France

### ARTICLE INFO

#### Article history:

Received 1 September 2019

Revised 17 October 2019

Accepted 19 October 2019

Available online 21 October 2019

#### Keywords:

Reverse phase supercritical fluid chromatography

Retention behaviour

Phytolated chlorophyll derivatives

Modifier percentage and nature

Method development

Backpressure modulation with flow rate gradient

### ABSTRACT

Chlorophyll pigments give the green colour to plants, which is a quality attribute of food and vegetables. However, the chemical structure of native chlorophyll can change during varied processes (drying, freezing, extraction) applied to plants, which produce degradation compounds that could have a brown and unwanted colour.

Systematic experiments have been conducted in supercritical fluid chromatography with a C18 stationary phase to understand and model the chromatographic behaviour of the compounds with respect to the nature of the modifier (MeOH, ACN, and MeOH/ACN 50/50) and its percentage, from 10% to 100%. Specific retention changes were observed, which provide numerous analytical conditions to achieve compound separation. The chromatographic profile of the extract containing native chlorophyll a, b and numerous phytolated chlorophyll derivatives (pheophytin a, a', b, b'; hydroxypheophytin a, a', b, b'; pyropheophytin and lactone derivatives) is strongly impacted by the nature of the modifier and, because of the complexity of the extract, the optimal conditions obtained are unusual for supercritical fluid chromatography. An original method development using an optimization criterion was discussed for the analyses of samples, leading to a fast analytical method with a very low backpressure and a flow rate gradient, but a simplest and rapid method is also suggested for samples displaying fewer derivatives.

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### 1. Introduction

Chlorophyll compounds are involved in photosynthetic chemical reaction of plants. They are divided into two main classes, the non-polar phytol esterified (chlorophylls *a* and *b*), and the non-esterified and polar ones (chlorophylls *c1*, *c2*, *c3*). The non-polar ones, chlorophyll *a* and chlorophyll *b*, are found in the leaf chloroplasts of higher plants, while the polar ones are found in marine phytoplankton. These pigments are responsible for the bright green colour of vegetables, or vegetable extracts, and their degradation can be damageable for the quality of the products. The chlorophyll *a* and *b* pigments are composed of a porphyrin-type skeleton, consisting of four pyrrole groups linked by methyne bridges (Fig. 1). These porphyrin rings chelate a magnesium ion in the centre, and are esterified by a terpenoid alcohol, called the phytol chain, on carbon 17. A vinylic group is located on carbon 3, and a methyl ester on carbon 13<sup>2</sup>. The *a* and *b* forms differ by the nature of the substituent on carbon 7, which is either a methyl group for chlorophyll *a* or an aldehydic one for chlorophyll *b*.

Under normal maturing or postharvest storage, natural degradation of pigments can be observed in vegetables leading to changes in colour, which could depreciate the quality of the products [1,2]. During the ripening of olive fruit, the decrease in chlorophyll content associated to the carotenoid esterification is responsible for the colour evolution, from green to black [3]. For instance, an enzymatic reaction due to chlorophyllase induces the loss of the phytol chain to give acidic forms of pigments called chlorophyllides (S1).

Other colour changes, related to chlorophyll structure modification, are reported during plant processes, such as dehydration [4–7] or freezing [8]. Most often, the increase in temperature during drying, the extraction with protic solvents or long storage all promote the demetallation of magnesium, leading to pheophytins *a* and *b*, or the epimerisation on carbon 13<sup>2</sup>, leading to *a'* and *b'* compounds, either for chlorophylls or pheophytins [9] (S1). Additional decarbomethoxylation (on carbon 13<sup>2</sup>) can also be favoured by heating, leading to pyro-pheophytin from pheophytin [10]. Besides, the presence of hydroxylated chlorophyll compounds is also reported, either related to the vegetable maturity [11] or to the conditions of the extraction process [12].

The chromatographic separation of these compounds is very useful to study the effects of the varied processes applied to vegetables: dry- or moist-heating, freeze-drying [13], rehydration,

\* Corresponding author.

E-mail address: [eric.lesellier@univ-orleans.fr](mailto:eric.lesellier@univ-orleans.fr) (E. Lesellier).

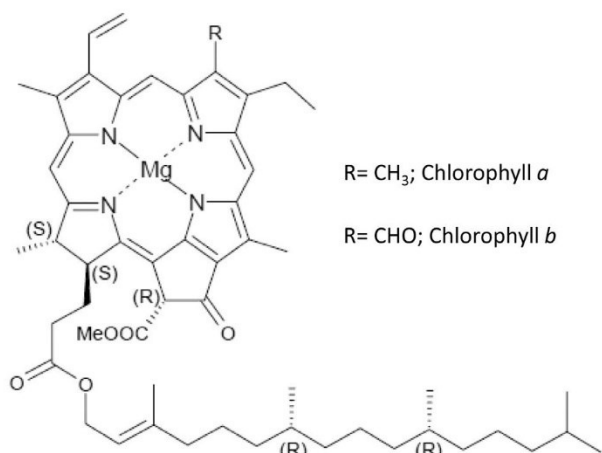


Fig. 1. Chemical structure of chlorophyll a and b.

canning or extraction, to check the adulteration due to the addition of food colorant (copper complexes of chlorophyll) ([9] or the re-greening process in the presence of zinc [13]. Moreover, a chromatographic method can also follow the eventual degradation of chlorophyll during plant extraction processes when using modern extraction methods such as pressurised fluid extraction [14] or supercritical fluid extraction [15].

The presence of the phytyl chain, which contains 20 carbons, makes chlorophylls and some of their derivatives strongly non-polar (Log  $P > 8$ ).

One paper reported the separation of chlorophylls and chlorophyll derivatives with normal-phase liquid chromatography on a silica stationary phase with a dioxane-hexane elution gradient [16]. Thanks to the good quality of new generation-silica, the stabilization time between the analyses was lower than 10 min.

Apart from this example, most of the separations reported in the literature were achieved in non-aqueous reversed-phase liquid chromatography (NARP-LC), i.e. without any water in the mobile phase [6,7,11,17], or with a constant small amount of water of 4% [5,10]. To observe both the derivatives that have lost their phytyl chain, meaning the more polar compounds (chlorophyllides and pheophorbide), water was used in the first part of the gradient. To achieve an optimal separation of these acidic derivatives, an ion-pairing agent was required. Ammonium acetate was selected in most cases [1,4,9,13,15,18,19], whereas tetrabutylammonium acetate [3,12] or varied amines (pyridine, piperidine, pentylamine) were also added to the mobile phase [20–22].

Whatever the nature of the derivatives, elution gradients were largely used, with varied organic solvents: methanol, acetonitrile, acetone, methyl-*t*-butyl-ether, isopropanol, ethyl acetate or tetrahydrofuran. These organic solvents were used alone or in binary or ternary mixtures. The number of steps in the gradients generally varied from 4 to 8, including the stationary phase equilibria.

The use of C8-bonded phase was reported [19,22] but most of the stationary phases employed were C18-bonded ones, with fully porous particles. For these C18 phases, some studies reported retention changes related to the type of bonding, monomeric or polymeric [19,20]. The use of polymeric C18 phases, for instance Vydac 201 TP 54, provided different separations for mono-vinyl and di-vinyl chlorophyll c3, or for chlorophyll c1 and c2. These two pairs of compounds differ by the nature of substituents on carbon 8, either an ethylene or an ethane group. The specific behaviour of these polymeric C18-bonded phases for shape-constrained solutes was described elsewhere [23].

A C30 ligand was also selected for the separation of chlorophylls [5,14] for its ability to separate compounds with rigid well-

defined molecular shape [23]. Recently, this C30 phase was used for an SFC x LC two-dimensional separation (in the second LC dimension) [24], to be applied to the separation of both chlorophylls and carotenoid pigments of bell pepper (*Capsicum annuum* L.).

In order to improve the speed of chlorophyll compounds analysis, monolithic C18-bonded silica phases, which have a high porosity, have been used with a flow rate of 4 ml/min allowing the separation of the two chlorophylls a and b and the two pheophytins a and b in less than 5 min [19]. Recently, the use of core-shell particles was reported to achieve the separation of 24 pigments in a 28 min-analysis [25], including the epimer forms and the pyroforms of chlorophyllide, pheophorbide, chlorophyll and pheophytin.

Reversed-phase supercritical fluid chromatography (RP-SFC), which can be achieved with a non-polar C18-bonded phase, is an interesting alternative for the separation of this type of non-polar compounds. With regards to the organic solvents used in NARP-LC, carbon dioxide has many advantages: ecological, economic and physico-chemical. Moreover, it can be mixed with many other solvent (often called modifier) to tune the polarity of the mobile phase, inducing large changes in retention and sometimes great improvement in the separation performances.

The first use of supercritical fluid chromatography (SFC) for the separation of chlorophyll pigments was reported twenty years ago. It was achieved with a modifier gradient of methanol from 6 to 25% on a C18-bonded phase [26], but no separations between hydroxylated and epimeric pheophytins were studied.

The goal of the present paper was to study the effects of a large composition range of varied modifiers (MeOH, ACN, MeOH/ACN) on the retention behaviour and on the separation of chlorophyll pigments, and to describe the analytical development of a separation method by RP-SFC based on an combined optimisation criterion.

## 2. Material and methods

### 2.1. Instruments

Chromatographic separations were carried out with the following instruments: the carbon dioxide pump was JASCO PU-2080-CO<sub>2</sub>, the modifier pump was JASCO PU-2080, the mixing unit was GILSON Dynamic mixer model 811, the column oven was a column thermostat JETSTREAM 2 PLUS (Sigma-Aldrich, l'Île d'Abeau, France), the back-pressure regulator was BP-2080 Plus. This device was equipped with a PDA detector JASCO MD40-10 (and an interface unit LC-NETII/ADC). Chromatograms were recorded using CHROMNAV software (Jasco, Lisses, France). The chromatographic column was an octadecylsiloxane-bonded superficially porous silica CORTECS C18+ (4.6 × 150 mm, 2.7 μm) provided by Waters (Dublin, Ireland). This column was selected for the type of particles (core-shell) providing high efficiencies, and for the positive charge surface modification. Retention factors ( $k$ ) were calculated using the following equation.

$$k = \frac{t_r - t_0}{t_0}$$

with retention times ( $t_r$ ) and dead time ( $t_0$ ). The dead time was measured at each analytical condition, because it varies in function of the compressibility of the carbon dioxide that can change following the inlet pressure of the column, which is related, at constant flow rates, to the mobile phase viscosity, i.e. to the modifier percentage [27,28].

Characterisation of an ivy extract was carried out with a Nexera UC SFC system coupled with a mass spectrometer, equipped with a dual ionization source and a simple-quadrupole analyser from SHIMADZU (Kyoto, Japan). The following parameters were used for the characterization: ionization in positive mode; interface voltage, 4.5

kV; interface temperature 350 °C; Nebulizing gas flow, 1.5 L/min; heat block, 200 °C. A make-up solvent was also used to enhance the ionization of the molecules: 0.100 ml/min of methanol with 20 mM ammonium formate were introduced after the column.

The plant dryer was a Memmert model 500. The sample was dried at 50 °C for 48 h. Plant extraction was carried out with Ultrasonic bath BRANSON 3510. The centrifugation device was BR4i multifunction centrifuge (Thermo-electron, Nantes, France).

The microwave extractor was a Microsynth from Milestone (Soriso, Italy). Plant extraction was carried out in a 50 ml-reactor (QRS1550). Evaporation was made with a BUCHI rotary evaporator using the different modules (B-480, R-114, I-100, V-100 and F-308).

## 2.2. Chemicals and plant material

The solvents used for extraction and chromatographic purpose were methanol and ethanol, gradient grade (VWR International, Fontenay-sous-Bois, France), and acetonitrile, gradient grade (Carlo Erba, Val de Reuil, France). Carbon dioxide (>99.5%) was from AIR LIQUIDE (Fleury-les-Aubrais, France).

The plant used was wild ivy (*Hedera canariensis* Willd) collected in Orleans (France).

## 2.3. Extraction methods

Two extracts were used. The first one called in the next pages "fresh extract" was made using 1 g of the dried plant material and 15 mL of ethanol. The mixture was then sonicated for 60 min. Another extract called "degraded extract" was obtained with microwave assisted extraction with three heating cycles at 500 W for 30 s, and cooldown to 25 °C between two consecutive cycles. Both extracts were filtrated and centrifugated at 7000 rpm and at 20 °C for 10 min. The Fresh extract was stored in the fridge in tainted bottle during analyses, whereas the Degraded extract was evaporated with a rotary evaporator. It was then solubilised in methanol for analysis.

## 2.4. Analytical methods

Different composition of mobile phase comprising carbon dioxide and methanol and/or acetonitrile were employed as further detailed in the results section. The flow rate was 1.5 mL/min, outlet pressure 10 MPa, oven temperature 25 °C, and UV detection was monitored at 650 nm.

## 3. Results and discussion

### 3.1. Fresh extract

A first extract was analysed. As shown in the following chromatogram (Fig. 2), this extract mainly contains two compounds absorbing visible light at 650 nm. After characterisation using mass spectrometry and UV spectroscopy, chlorophyll *a* and *b* were identified. As expected, when using a C18-bonded stationary phase, chlorophyll *b* having an aldehyde function thus being slightly more polar is eluted before chlorophyll *a* bearing a methyl group. In addition, the relative peak heights are in accordance with other reported studies on plants, with chlorophyll *a* being more abundant than chlorophyll *b*.

In order to better understand the chromatographic behaviour of these two compounds and develop a separation method, different mobile phase compositions were tested. Then, for all these conditions, retention times of the two compounds were monitored at 650 nm.

From methanol 10% to 100%, retention factors were plotted for both chlorophylls (Fig. 3). For all the studied conditions,

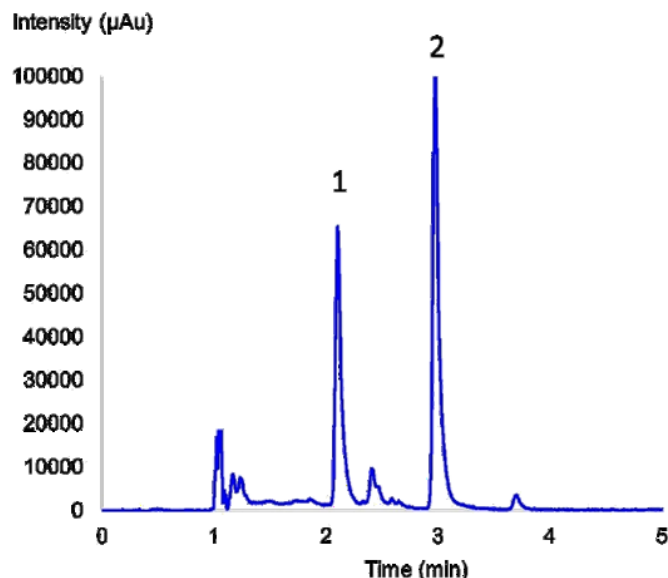


Fig. 2. Chromatogram of fresh ivy extract in SFC. Column: Cortecs C18+; flow rate 1.5 mL/min,  $T = 25^{\circ}\text{C}$ ,  $P_{\text{out}} = 10\text{ MPa}$ , mobile phase:  $\text{CO}_2$ -MeOH (60/40, v/v) with (1) chlorophyll *b* and (2) chlorophyll *a*.

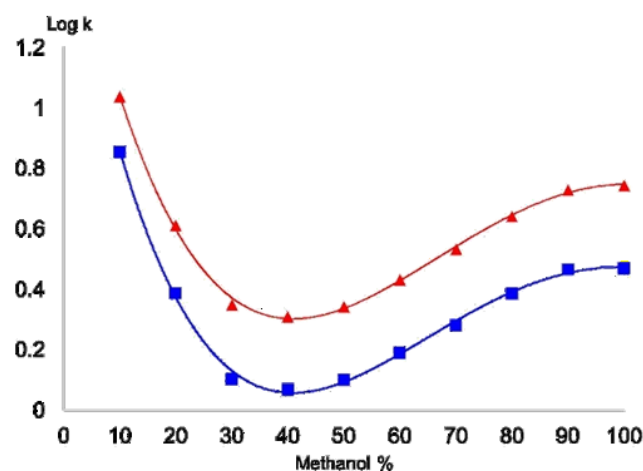


Fig. 3. Variation of  $\log k$  vs the percentage of MeOH. Blue squares: chlorophyll *b*; red triangles: chlorophyll *a*.

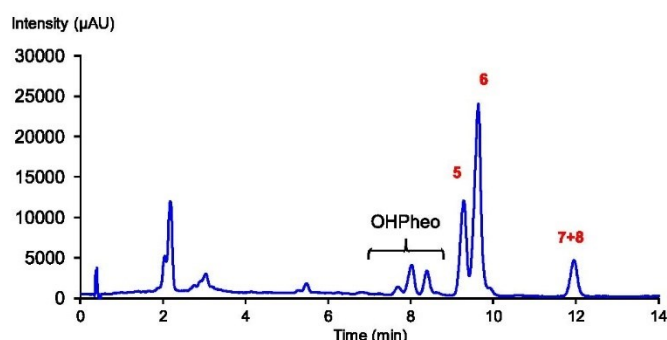
both chlorophylls *a* and *b* were separated. Moreover, the two chlorophylls had similar retention behaviours. From 10% to 40% methanol, there is a decrease of the retention factor. This decrease is explained by the increase of the polarity of the mobile phase which favours the solubility of compounds in the mobile phase. However, from 40% methanol, the polarity of the mobile phase continues to increase that decreases the solubility of the compounds. This phenomenon was already observed with other non-polar compounds (carotenoids [29,30], UV absorbers [27] or triglycerides) with octadecyl-bonded silica stationary phase in SFC [31,32].

### 3.2. Degraded extract

To develop a separation method that would also be suited to chlorophyll derivatives formed during varied processes applied to plants, a degraded extract was examined. As shown on Fig. 4, it contains more peaks than was observed in the fresh extract, and they are eluted later (between 4 and 12 min) than the native chlorophylls eluted in the first 4 min. Because the stationary phase

**Table 1**  
Identification of glycytylated derivatives of chlorophylls by UV/visible and Mass-spectrometry. (peak number refer to Fig. 10).

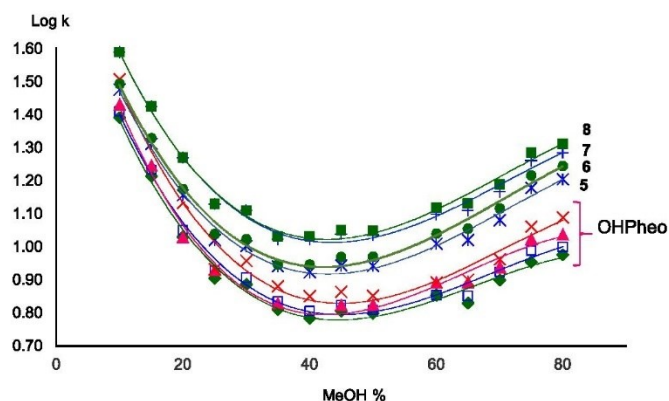
Peak	Identification	RT with method Fig. 10 (min)	UV-vis max, nm	[M+H] <sup>+</sup> m/z calculated	[M+H] <sup>+</sup> m/z detected	Fragments	Ref.
1	13 <sup>2</sup> -Hydroxypheophytin a	3,88	666/407	888	887	869/610	[9]
2	13 <sup>2</sup> -Hydroxypheophytin a'	4,26	664/408	888	887		
i	15 <sup>1</sup> -Hydroxy-lactone-pheophytin a	4,45	668/400	904	904	565	[9]
3	13 <sup>2</sup> -Hydroxypheophytin b	4,59	652/435	902	902	623/605	[9]
4	13 <sup>2</sup> -Hydroxypheophytin b'	4,79	656/436	902	902		
5	Pheophytin a	6,11	665/408	872	872	593	[9]
6	Pheophytin b	6,57	665/408	886	886	607	[13]
7	Pheophytin a'	7,37	665/408	872	872	593	[9]
8	Pheophytin b'	7,73	665/408	886	886	607	[13]
9	Pyro-pheophytin a	10,95	665/408	814	814		[33]



**Fig. 4.** Chromatogram of degraded Ivy extract in SFC. Mobile phase: CO<sub>2</sub>/MeOH 90/10; other conditions as in Fig. 3. Identification of peaks in Table 1 and Fig. 5.

is not polar, this increase of the retention time for these new compounds indicates that they are rather less polar than the chlorophylls. It means that these compounds are not chlorophyllides, which are obtained after the loss of the non-polar phytyl chain. The molecular weight of the main compounds were obtained from SFC-MS analysis (Table 1). From these mass values and from the relative retention of the compounds, an identification attempt was done. With the MS parameters described previously, [M+H]<sup>+</sup> ions could be detected and correlated with expected degradation products. In addition, the ionisation parameters allowed to confirm the main compounds with fragments found in MS/MS methods [9,13,33]. However, due to the weak ionisation conditions and the use of a simple-quadrupole MS, the fragments observed in literature were detected with a much lower abundance.

The four compounds eluted first on Fig. 5 are hydroxy-pheophytin (OHPheo), whereas compounds 5 and 6 are pheophytin



**Fig. 5.** Variation of log *k* vs the percentage of MeOH. 5 = pheophytin a; 6/ pheophytin b; 7/ pheophytin a'; 8/ pheophytin b'.

*a* and *b*, and compounds 7 and 8 are pheophytin *a'* and *b'*. In this work, the retention order of pheophytin compounds is reversed compared to the one observed in other publications in SFC or NARP-LC. Whereas the pheophytin *b* is generally less retained than pheophytin *a*, due the presence of the aldehydic group on pheophytin *b* with the C18 columns used in literature, our experiments show that the pheophytins with an aldehydic group (*b* and *b'*), were more retained than the ones with a methyl group (*a* and *a'*). This unexpected chromatographic behaviour can be explained by the positive charge surface modification of the selected C18-bonded phase, which favours the retention of compounds having an aldehydic group. However, this unexpected behaviour did not occur for the chlorophylls, indicating that this behaviour may be related to the presence of the magnesium group in the porphyrin ring.

Fig. 5 shows that the chromatographic behaviour of these phytylated chlorophyll derivatives vs methanol percentage is similar to the one of chlorophylls. The separation behaviour of the two pairs of pheophytin (*a/b* and *a'/b'*) follows the same pattern, i.e. increasing separation when increasing the methanol percentage. It also appears very clearly on Fig. 6, showing the chromatograms obtained from 60% to 75% methanol in CO<sub>2</sub>. Using a temperature of 25 °C and a back-pressure of 10 MPa, we can consider that the method is not supercritical or subcritical fluid chromatography anymore, but rather non-aqueous enhanced-fluidity liquid chromatography (EFLC). When comparing the separation quality of the chromatograms plotted in Fig. 6, for the peaks that were eluted before pheophytins 5 to 8 (see chromatogram at 70%), it appears that the best separation was achieved for 70% methanol. In this condition, the four hydroxy-pheophytins *a*, *b*, *a'* and *b'* (compounds 1, 2, 3 and 4) were well resolved, as well as one additional minor impurity (*i*) which co-elutes either with compound 2 or 3 for other methanol percentages. From its retention time with regards to pheophytin compounds, this impurity could be a 15-lactone form of pheophytin *a*, which is eluted before [9,12,34,35], or a pyro form of hydroxy-pheophytin which is eluted after [6].

However, increasing the methanol percentage increases the fluid viscosity and the inlet pressure. Because we did not wish reach 40 MPa inlet pressure with regards to the pumping system, flow rate was reduced at the highest methanol proportions (superior to 50%), thus pressure drop was maintained stable (around 30 MPa).

Consequently, the study began, for 10% of modifier, at 3 mL/min, and for the optimal conditions (70% methanol added to CO<sub>2</sub>), the flow rate was reduced down to 1.8 mL/min. To overcome this flow rate decrease, and to shorten the analysis duration, the back-pressure was decreased to the lowest possible value. At the optimal conditions, i.e. 70% methanol, it was possible to decrease the back-pressure value down to 4 MPa, without causing any phase separation between methanol and CO<sub>2</sub>. Supplementary information (Figure S2) shows the separation variation of the chlorophyll

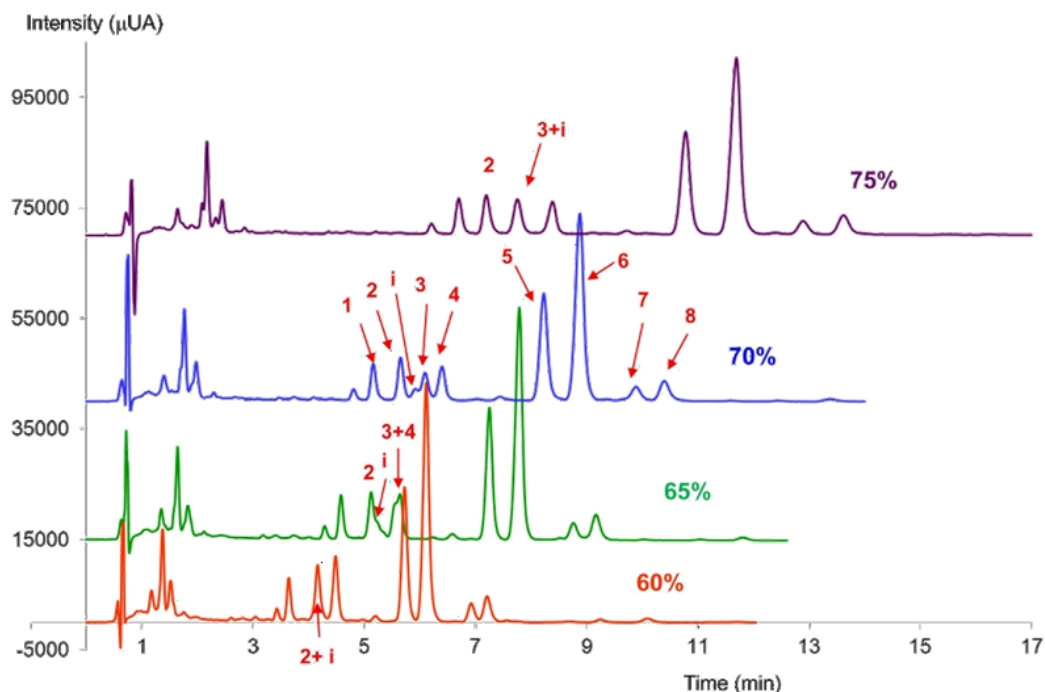


Fig. 6. Chromatograms of chlorophyll derivatives at varied methanol percentages from 60% to 75%.  $P_{out} = 10$  MPa.

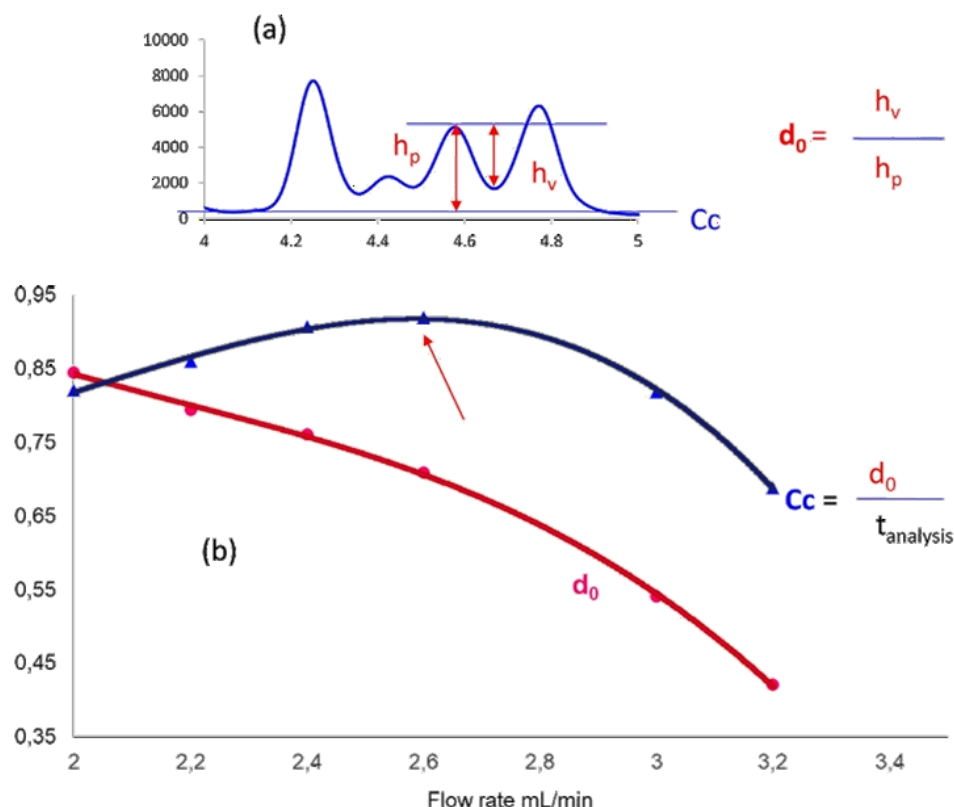


Fig. 7. Variation of the discrimination factor  $d_0$  and of the combined criterion ( $Cc$ ) for the pair of hydroxypheophytin 3 and 4 vs the flow rate with a back pressure of 4 MPa.

derivatives at this low back-pressure for varied flow rates, from 1.8 to 3 mL/min. At 2 mL/min and 4 MPa, the chromatogram shows an almost identical separation quality, with a little smaller retention, with regards to the analysis achieved at 10 MPa and 1.8 mL/min. This retention decrease was not as significant as could be expected from an increase of 0.2 mL/min of the flow rate. However, as the

back-pressure was decreased from 10 to 4 MPa, the average pressure, i.e. the average density of the fluid was also lower, thereby reducing the elution strength of the mobile phase. At higher flow rates, from 2.2 to 3 mL/min maintaining constant back-pressure (4 MPa), the analysis duration was shortened. However, for most pairs of compounds, 1/2, 3/4, 5/6 and 7/8, a reduction of the

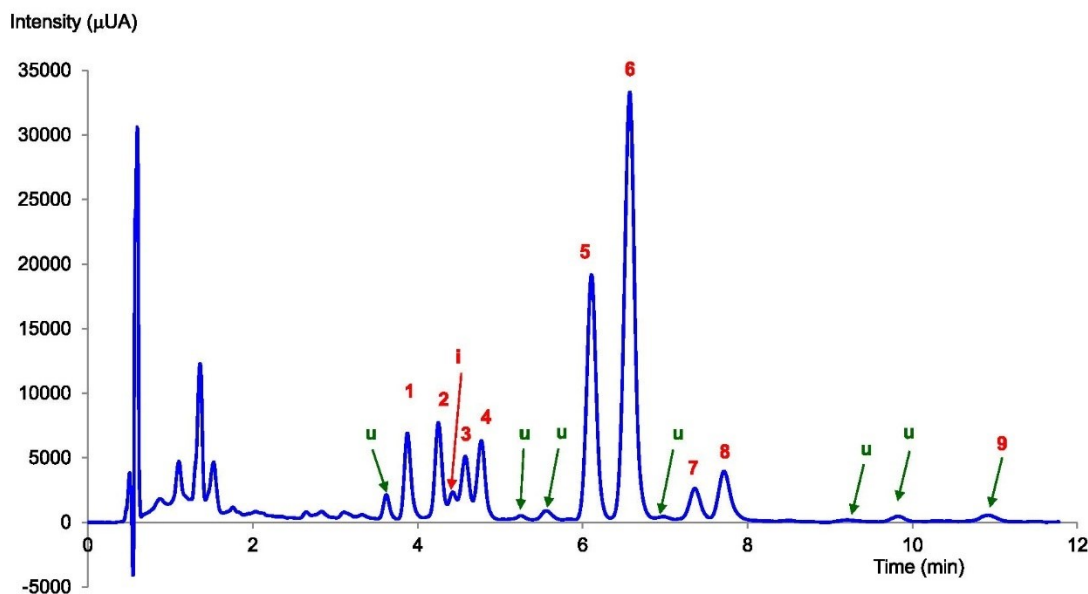


Fig. 8. Chromatogram of the degraded Ivy extract at 2.6 mL/min.  $P_{out} = 4$  MPa;  $T = 25$  °C, mobile phase  $CO_2/MeOH$  30/70.

resolution was observed. In general, the change in resolution is due either to separation factor modifications, *i.e.* to interactions variations between the compounds and the mobile phase, or to the efficiency, related to diffusion or mass transfer coefficients of the compounds. When modifying the flow rate, no change in the mobile phase composition occurs, except for compressible fluids, because of density changes. However, with 70% methanol added to  $CO_2$ , no significant density variation should occur with flow rate increase. This observed behaviour of the regular decrease in resolution for the four pairs of compounds due to the flow rate increase from 2 to 3 mL/min can mostly be related to kinetic phenomena, and is in accordance with the one observed elsewhere for C18-bonded superficially porous particles [32]. From a practical point of view, this decrease in resolution was measured by the discrimination factor  $d_0$  [36] for the 3/4 pair of compounds, which displayed the worst resolution. As can be seen in Fig. 7a, this criterion is well suited to examine the changes in separation quality without measuring peak width as it is required for the resolution factor. The discrimination factor can vary from zero for co-eluted peaks to one for fully separated peaks (meaning with a baseline resolution). As shown in Fig. 7b, for the 3/4 pair, this factor varied from 0.85 at 2 mL/min, to 0.4 at 3.2 mL/min. It underlines that the gain in analysis duration, from 10.5 min to 7 min (S2), was counterbalanced by the loss in separation quality.

In order to manage these opposite effects a combined criterion ( $C_c$ ) was calculated as the ratio between the discrimination factor and the analysis duration ( $t_a$ ). Fig. 7b shows that this combined criterion first increased when flow rate increased from 2 to 2.6 mL/min, due to the greater effect of the analysis time reduction, then decreased when flow rate further increased from 2.6 to 3.2, due to the greater loss in separation quality. As a consequence, the value of 2.6 mL/min can be selected as a satisfying compromise between the two chromatographic changes. The chromatogram achieved at this flow rate and a back pressure of 4 MPa (Fig. 8) displays the eight chlorophyll derivatives pheophytins and hydroxy-pheophytins, the impurity (i) and at least seven unknown minor compounds (u) that also absorb visible light at 650 nm, indicating that they must all have a porphyrin structure. The retention time of the last unknown compound is 11 min. Finally, to reduce the analysis duration, a flow rate gradient was applied from 2.6 to 3.2 mL/min (S3). The first gradient (S3a) was a step one at 5 min, allowing to maintain good resolution for hydroxy-pheophytins that

were eluted before 5 min. The chromatographic profile was satisfactory both for hydroxy-pheophytins eluted at 2.6 mL/min and for pheophytins eluted at 3.2 mL/min. As expected, this gradient allowed to reduce the analysis duration because the last unknown compound was then eluted at 9.5 min instead of 11. Three other gradient profiles (S3b,c,d) were attempted, by beginning earlier the increase in the flow rate (4.5 min, 4 min, 3.5 min) with a more or less smooth gradient. The use of a smoother slope could provide a more progressive change in average pressure in the column, *i.e.* a less dramatic variation of the fluid properties (density, viscosity, diffusion coefficients). However, no significant change of the chromatographic profile was noticed for these different gradient profiles, indicating once again that, with this high methanol proportion, the fluid behaves more as an incompressible liquid than a compressible supercritical fluid.

Additional studies were carried out replacing methanol by acetonitrile or by a 50/50 mixture of acetonitrile and methanol. The proportions in carbon dioxide varied from 10% to 80%. In Fig. 9, the chromatographic behaviour of pheophytin *a* and *b* are represented to exemplify the effects observed, because they are the main compounds produced by physico-chemical treatments on plants or plant extracts. As appears in Fig. 9, the chromatographic behaviour of the compounds followed the same pattern as the

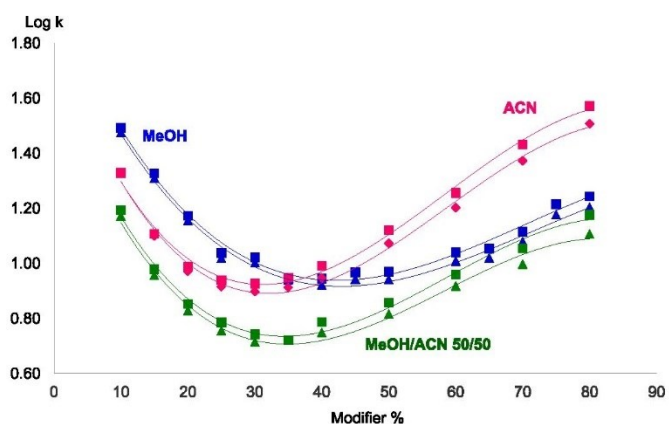
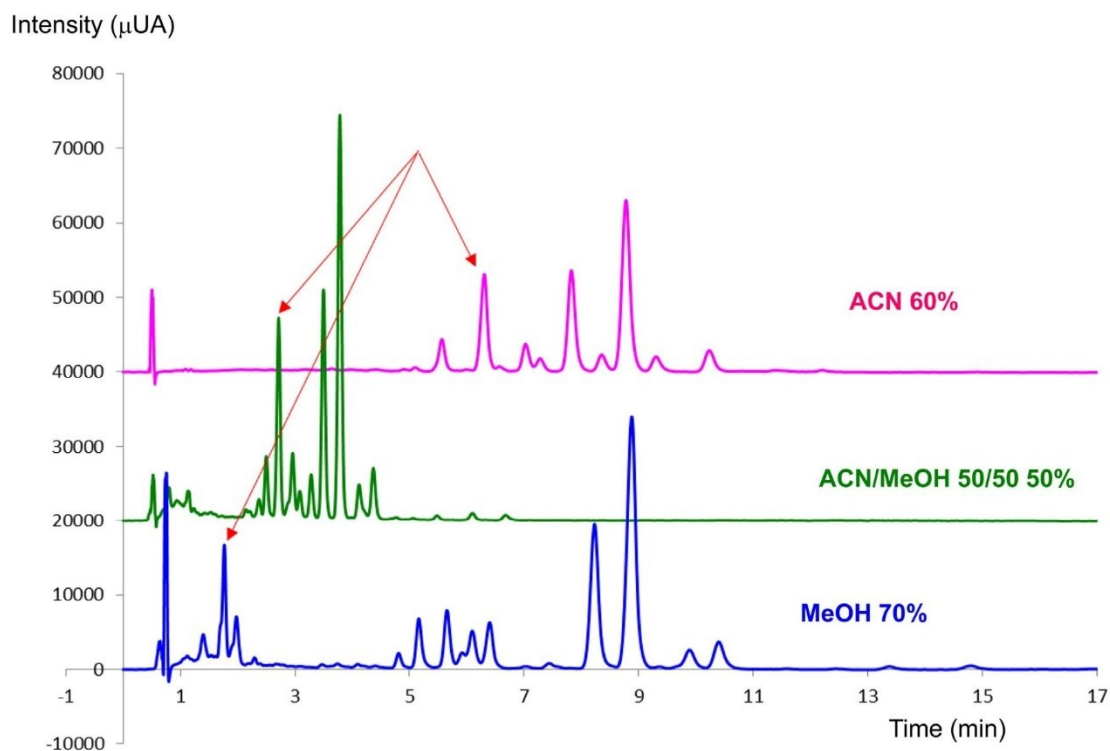


Fig. 9. Variation of  $\log k$  of pheophytin *a* (triangles) and *b* (squares) with different modifiers (MeOH, ACN, ACN/MeOH). Other analytical conditions as in Fig. 4.



**Fig. 10.** Chromatograms of the degraded Ivy extract at the optimum of the separation for three modifiers. Lower chromatogram: methanol 70%; medium: methanol/acetonitrile (50/50) 50%; upper: acetonitrile 60%.

one observed when methanol alone was used as modifier. The percentage of acetonitrile providing minimum retention (25–30%) was lower than the one of methanol (40–50%). For the MeOH/ACN mixture, this percentage was intermediate (30–40%). Moreover, the separation factor ( $\alpha$ ) for these two pheophytins increased continuously with the modifier percentage (S4). For high modifier percentages, the use of acetonitrile or methanol/acetonitrile 50/50 favoured the separation of these two compounds. Fig. 10 displays the chromatograms obtained with the three different modifiers at the percentages providing the best separation for the degradation compounds of chlorophylls. Unfortunately, one impurity is located at the beginning of the chromatogram when using methanol (see arrows). When using the two other modifiers (acetonitrile or methanol/acetonitrile mixture), this impurity is shifted later in the chromatogram and is co-eluted with some of the target degradation compounds. The presence of hydrophilic compounds eluted in the early part of the chromatogram when using C18-bonded phase was also reported elsewhere [14].

Finally, when studying the effects of the plants drying or storage, the extraction conditions with solvents or the conditions of evaporation of these solvents, in most cases, few degradation products from chlorophylls are observed, and the separation method can be simplified, by using only 20% of modifier (S5). The main chlorophyll derivatives observed are the pheophytins, but in comparison to the abundant native chlorophylls, their concentrations are very low. This chromatogram was obtained with only 20% methanol in carbon dioxide, which was sufficient to analyse samples which do not contain too many chlorophyll derivatives.

#### 4. Conclusion

This study displays the chromatographic behaviour of chlorophylls and chlorophyll derivatives versus the nature and the percentage of modifier for different modifiers. The variations of retention factor on a C18-bonded phase is rather unusual, although

it was observed before for carotenoid pigments, triglycerides or UV absorbers. This change is related to the non-uniform solubility variation of the non-polar compounds towards the mobile phase. These observations could also be used in the future to improve the modelling of extraction conditions by using supercritical fluids.

This paper also describes an original method development to achieve the separation of phytolated chlorophyll derivatives, varying the modifier nature and percentage, but also other parameters impacting the fluid density and viscosity. For instance, the modifier addition changes the interactions between the compounds and the mobile phase, as expected, but also impacts the fluid viscosity, that changes the inlet pressure. With regards to the pressure limit of the pumping system, this side-effect can be managed by reducing the back-pressure in the goal to increase the flow rate to reduce analysis duration. Besides, when using high modifier percentages, this study also underlines that the changes in the gradient slope when modifier proportion is high, keeping constant the medium time between the initial and the final compositions, does not affect the chromatogram, because of the low compressibility of the mobile phase, which is rather in a liquid state.

Finally, the separation obtained at optimal conditions allows the separation of epimers of pheophytin and hydroxy-pheophitin, *a* and *b*, with a reduced analytical retention time compared to classical HPLC methods.

#### Declaration of Competing Interest

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

#### Acknowledgements

This work was supported by the Centre-Val de Loire region, which provided the funding of the “Khloros” APR-IR (2017).

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2019.460643.

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Dans cet article, une méthodologie a été développée afin de proposer des conditions d'analyses permettant la séparation de chlorophylles et de leurs produits de dégradation. Ce développement analytique a été réalisé par SFC en utilisant une phase greffée C18.

La méthodologie suivie consistait en l'étude de la rétention des composés en faisant varier le pourcentage de co-solvant utilisé lors d'analyses en conditions isocratiques. En comparant les différences de rétention entre chacun des composés, il est possible d'identifier des conditions optimales de séparation. Ainsi, une phase mobile composée à 70% de méthanol a été identifiée comme la condition permettant d'obtenir la meilleure séparation de l'ensemble des composés d'un extrait volontairement dégradé. Toutefois, en utilisant 1,05 mL/min de méthanol comme co-solvant (70% de 1,5 mL/min), cette méthode a une consommation de solvant élevée.

Cette étude a été réalisée sur un échantillon contenant beaucoup de produits de dégradation (extrait et stocké dans les pires conditions). Rappelons que dans un autre contexte (par exemple celui de la partie précédente sur l'étude de la dégradation d'extrait riche en chlorophylles), ou moins de produits de dégradation ont été identifiés, une méthode d'analyse en SFC isocratique avec 20% de méthanol donnait une séparation suffisante pour étudier la dégradation d'un extrait de lierre riche en chlorophylles au cours du temps.

## **4. CONCLUSION**

Dans ce chapitre, des conditions d'extraction de chlorophylles à partir de lierre ont été trouvées afin de pouvoir avoir un échantillon riche en chlorophylles natives et suffisamment stable pour pouvoir l'utiliser plusieurs jours. De plus, les chlorophylles ont pu être étudiées chromatographiquement dans le but de développer une méthode analytique par SFC.

Ce comportement chromatographique peut être utilisé pour le développement de méthode analytique comme décrit précédemment, ou pour la détermination de conditions d'extraction sélective. C'est ce deuxième point qui sera étudié dans le chapitre suivant.





# CHAPITRE 3: Etude et modélisation des conditions d'extraction sélective





## 1. INTRODUCTION

L'objectif de cette thèse est de développer une méthodologie afin de trouver des conditions d'extraction sélective de composés bioactifs non-colorés de plantes. Pour cela, il a fallu choisir une plante sur lesquelles produire des extraits sélectifs.

Le romarin (*Rosemarinus officinalis* L.) est utilisé en cosmétique pour ces molécules qui ont des propriétés antioxydantes. Cependant, des conditions favorables pour l'extraction de ces molécules, sont aussi favorables pour les chlorophylles (ex : extraction par macération éthanolique).

Le partenaire industriel de cette thèse (Alban Muller), commercialise un extrait de romarin pour la cosmétique. Il s'agit d'un extrait réalisé avec un solvant composé d'eau et d'éthanol. Ce solvant permet donc l'extraction de composés polaires (par exemple : acide rosmarinique) en limitant l'extraction des chlorophylles. Cependant, ces conditions d'extraction ne permettent pas de récupérer d'autres composés intéressants comme l'acide carnosique. Une extraction éthanolique pourrait solubiliser ces composés mais il s'agit aussi de condition favorable pour l'extraction de chlorophylles.

Dans un premier temps une review sur la famille des *Lamaceae* (incluant le romarin) présente un état de l'art de la composition en métabolites des plantes de cette famille, des différentes méthodes analytiques développées afin d'analyser ses constituants, ainsi que des méthodes d'extraction ou de purification mise en œuvre vis-à-vis de ces molécules cibles. Dans un deuxième temps, un modèle de prédiction de conditions d'extraction sélective est développé.

## 2. LE ROMARIN

Avant de développer un modèle permettant de trouver des conditions d'extraction sélective de bioactifs, une review sur le romarin, allant de l'extraction à l'analyse a été réalisée.

**Recent development for the analysis and the extraction of bioactive compounds in *Rosemarinus officinalis* and in medicinal plants from the *Lamacieae* family.**

Eric Lesellier, Thibault Lefebvre, E. Destandau

ICOA, UMR 7311, Université d'Orléans, Rue de Chartres, 45000 Orléans

Accepté dans Trends in Analytical Chemistry le 16 Decembre 2020

**Highlight**

- Chromatographic behaviors (expected and unusual) of natural compounds in reverse phase chromatography
- Description and comparison of varied extraction methods for *Rosemarinus officinalis* L.
- Focus on fractionation of extracts for improving their purity (Prep-SFC, coupling approaches). f

**ABSTRACT**

Numerous studies are carried out to identify the chemical structure of bioactive compounds from plants, including chromatographic and spectrometric approaches, or to select the extraction method and conditions enabling the higher recovery of these compounds, and the better activity of the extracts.

In Lamacieae, these targeted compounds are mainly polyphenols, flavonoids, di- or triterpenes and their separation have been mainly achieved by reversed phase liquid chromatography using C18 bonded phases. Gradients with acetonitrile are generally used, in association with UV or mass spectrometry detection. The main issues of these separations, that are discussed in this review, are the great number of compounds in extract, that requires high efficiencies of the chromatographic system, the peak tailing of the acidic compounds, which depends both on the addition of acidic additives in the mobile phase and on the type of C18 stationary phase, the separation of epimers frequently encountered for natural compounds which do have rings in their skeleton, and the elution out of the column of the more hydrophobic compounds, for instance the triterpenoid acids.

Varied extraction approaches have been applied to rosemary leaves; maceration, ultrasonic (UAE) and microwave (MAE) assisted extractions, pressurized liquid (PLE)

and supercritical fluid extraction (SFE). Numerous organic solvents, and water, display varied extraction efficiency, related both to their chemical structure and the one of compounds. The stability in solvents of some major compounds were studied, as well as their degradation pathway.

This review reports the range of conditions used for each extraction methods, and comparison for also innovative ones, dealing with coupling between extraction and separation, both for analytical and preparative. The different ways of extract fractionation are also detailed, mainly with supercritical fluids, either by applying successive conditions during extraction, or during collection. Some more unusual processes are also described, coupling PLE and SFE or adsorption/SFE/Trapping.

## **1. INTRODUCTION**

Lamacieae plants, Rosemary (*Rosemarinus officinalis*), Sage (*Salvia officinalis*), Thyle (*Thyus vulgaris*), Lemon balm (*Melissa officnalis*), wild Marjoram (*Origanum vulgare*) are a rich source of antioxidant compounds, in which the main molecules are rosmarinic acid, carnosic acid and carnosol [34], [35]. Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid [36], [37], founded in more than 240 plant species [37], whereas carnosic acic is a phenolic diterpene (fig. 1). Of course, many other phenolic compounds are present in Lamacieae, including hydroxynnamic acid and hydroxybenzoic acid derivatives, flavonoids and phenolic terpenes [34]–[38].

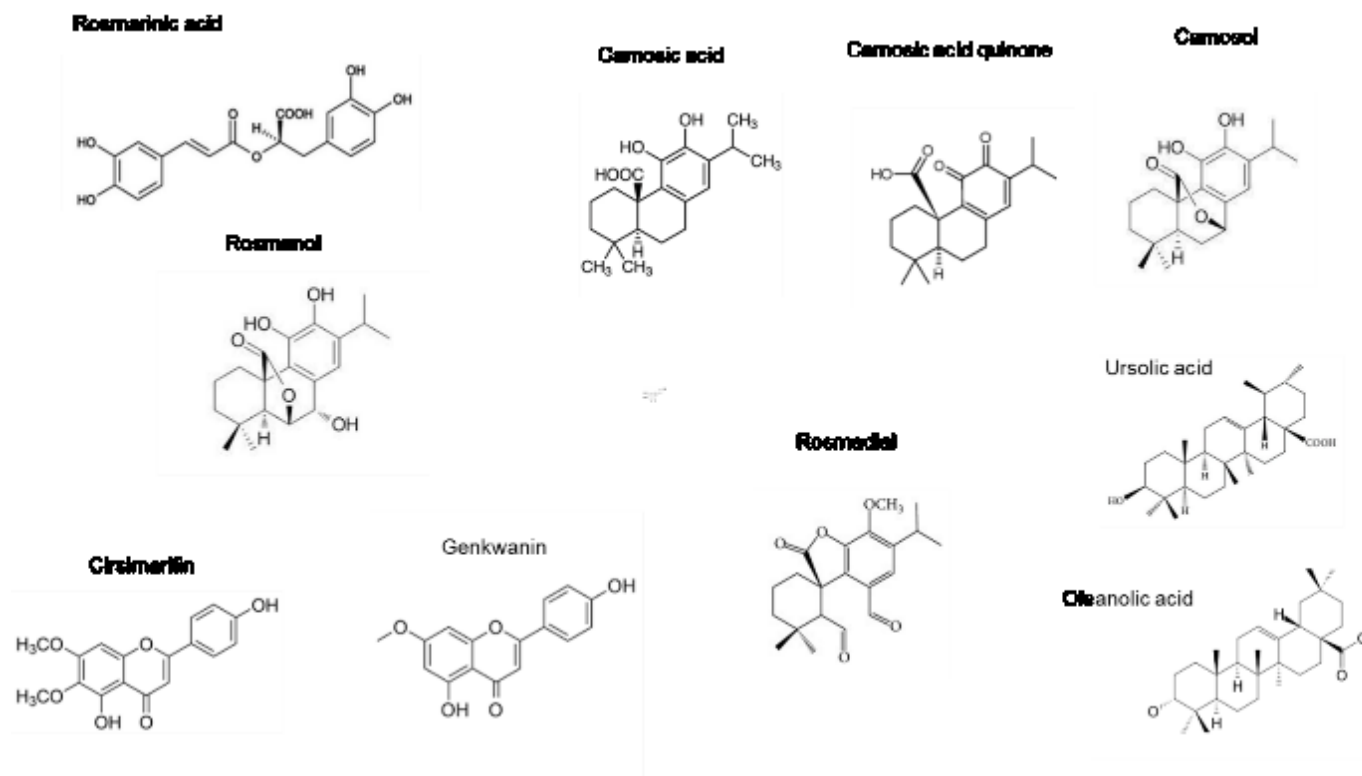


Figure 1. Chemical structures of varied bioactives compounds of rosemary.

A multitude of biological activities have been described for these phenolic compounds, astringent, antioxidative, antiinflammatory, antibacterial, antiviral [36], whereas they also display anti-diabetes property [39], and antiproliferative effects against various human cancer cell lines [40], both *in vitro* and *in vivo* [41].

This review presents the state of the art of the last 20 years about separation and extraction of nonvolatile compounds mainly from *rosmarinus* leaves, especially focused on their suitability for rosmarinic acid, carnosol and carnosic acid.

## 2. SEPARATION

### 2.1 LIQUID CHROMATOGRAPHY

Numerous high-performance liquid chromatography (HPLC) analytical methods were developed for the diterpene compounds, i.e. for carnosol and carnosic acid [42]–[44], or for studying the degradation products from carnosic acid in methanol [45], [46], by using octadecyl bonded stationary phases. Both isocratic [43], [44], [46] and gradient [42], [45] mobile phases were used, with mixtures of water plus acetic or trifluoroacetic acids, acetonitrile [42]–[44], [46] and/or acetonitrile and methanol [45]. Depending on the UV detection wavelength, the relative abundance of triterpene varies, the response of carnosic acid is greater around 230 nm, whereas the one of carnosol is favored at 280 nm [43]. Strangely, carnosol always elutes before carnosic acid whereas the acid part of carnosic acid is esterified in an intramolecular bond for carnosol. It could mean that the acidic group is linked to an hydroxyl group in an intramolecular interaction for carnosic acid, that would reduce its polarity toward the one of carnosol. Or that the intramolecular bond for carnosol, which is located crossing a benzene structure, decreases planearity of carnosol, i.e. the surface contact between this compound and the C18 bonded chains, or even reduces the penetration of carnosol into the bonded phase due to the increase in the length/breadth ratio [47], [48]. Moreover, the use of some ODS phases provide symmetrical peak for carnosic acid (Zorbax SB-C18 [10], Lichhrosphere 100 RP 100 [44]). When using a mobile phase gradient, using a 50/50 MeOH/ACN v/v as organic solvent mixed to water, instead of pure acetonitrile for all the other studies, carnosic acid is eluted with 100 % of organic solvents in the last plate of the gradient, that leads to a peak displaying a tailing. This could be due to a lack of solubility in MeOH of carnosic acid, which explain that most of separation method dealing with diterpene compounds favor the use of acetonitrile as organic solvent.

The analysis of rosmarinic acid from plants of the Lamiaceae family was recently reviewed [49]. Reverse phase methods were often selected, by using acidic mobile phase to prevent peak tailing [39]. The separation between rosmarinic and caffeic acids was easily achieved with a gradient water/methanol [50], whereas acetonitrile is also used for studying the phenolic composition of Lamiaceae herbs [51]. A validated HPLC method was proposed for studying the rosmarinic acid content in sage (*Salvia officinalis*) with the use of a methanol/water 0.1% phosphoric acid gradient, showing

the stability of sample extracts for 3 days when stored and the small influence of matrix effects on accuracy [52]. A study on the column performance of varied C18 bonded phases showed that the polar endcapping of the stationary phase reduces the peak tailing for rosmarinic acid, from Symmetry to Symmetry Shield phases [53].

By using a Lichrosphere 100 C18 bonded phase, two different mobile phases were reported for studying an aqueous extract of leaves, with a water/acetonitrile gradient for the analysis of rosmarinic acid, and for a DMSO extract, with a water/methanol one for the analysis of carnosol and carnosic acid [54]. Both analyses were achieved with acetic acid in water, with a higher percentage for the aqueous extract containing rosmarinic acid (2.5% vs 1%), allowing to provide a very good peak symmetry for both studied acids.

Most of analysis of rosmarinic acid, carnosic acid and carnosol were achieved by RP-LC using octadecyl bonded phases [34], [35], [38], [55]–[61]. Octadecyl bonded phases was used for these studies, with either binary water/ACN [55]–[58], [60], [61] or water/MeOH gradient [59], or ternary water/MeOH/ACN gradient [35], [38]. As expected, the retention of rosmarinic acid was lower than the one of the two diterpene compounds. The use of Ultrabase C18 [56], or Atlantis T3 C18 [38] columns, which are both recognize as fully endcapped, i.e. able to avoid silanophilic interactions, seems favor the separation performances especially for rosmarinic acid, which is well resolved and appears as a symmetrical and sharp peak.

The use of mass spectrometry [34], [38], [46], [56]–[58], [60]–[63] strongly helps for compound identification, due to some partial coelutions, for instance between carnosol and carnosic acid quinone [60], which are the first degradation compounds of carnosic acid, and which do have the same  $m/z$  value. Identification of some compounds also having the same  $m/z$  value is not always easy, for instance between 12-O-met, and oxydated carnosol derivatives [62]. Moreover, the relative retention of some compounds seems unexpected, maybe depending on the C18 bonded phase used. For instance, cirsimaritin with one more methoxy group with regard to genkwanin is eluted before [34], [56], [57], [62], [63], and sometimes before or after rosmarinol, whereas one study reports that methyl-carnosate is eluted before carnosic acid, or rosmadial before carnosol [38]. These surprising chromatographic behaviors reinforce the importance of the use of mass spectrometry for identification of complex

mixtures of natural products. Most of the mass analysis (ESI-MS) were achieved in negative polarity producing  $[M-H]^-$  ions for deprotonated molecule. Whatever, the separation of rosmanol, epirosmanol and epiisorosmanol with both cirsimarin and genkwanin remains a great challenge [34], [56].

One can remark (fig.2), that the use of small particle size (1.8  $\mu\text{m}$ ), with is a suited ODS phase for avoiding peak tailing for acidic compounds (Zorbax Eclipse C18 plus) with 0.1% formic acid in the mobile phase, and a probable performing LC-MS interface in term of chromatographic efficiency, which used a split ratio 1:3 to reduce the flow rate from 0.8 to 0.2 mL/min, gives a great separation quality of around 60 compounds in 40 minutes [62].

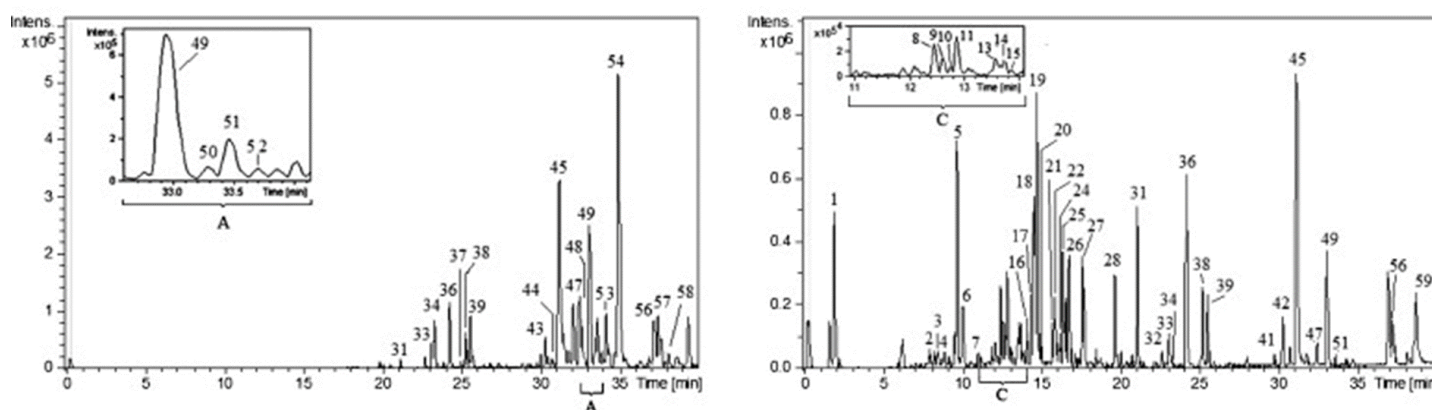


Figure 2. Chromatograms obtained in negative ionization mode by TOF-MS, for SFE (left) and PLE (right) extracts of rosemary (from ref. [62])

Finally, a cyclobond stationary phase, bonded with cyclodextrine, was also used, with a water (0.1% formic acid) acetonitrile mobile phase [63]. The retention order of the three main compounds was identical to the one observed for C18 bonded phases, whereas a coelution between rosmarinic acid and epirosmanol was reported, that was not the case with the two C18 phases previously discussed.

## 2.2 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis with UV detection was also used for the separation of phenolic compounds from rosemary extracts [64], [65]. Silica capillary was around 50 cm length, the pH ranged between 9 and 10, to favor the anionic phenolic compounds to migrate towards the anode, but the separation was carried out from the anode to the cathode. The analysis duration was very short, around 3.5 min, with a retention order carnosol, carnosic acid and rosmarinic acid [65]. A coupling between CE and mass

spectrometry has been developed by using ammonium acetate buffer (40 mM, pH=9), instead of sodium borate or tetraborate which are not compatible with ESI-MS [66].

### **3. EXTRACTION**

#### **3.1 LIQUID/SOLID EXTRACTION**

Varied pure solvents were used for maceration extraction (MeOH, EtOH, DCM, acetone, petroleum ether, chloroform, DMSO, butanone, ethyl acetate) [35], [38], [52], [54], [56], [58], [59], [65], [67], and sometimes with water/methanol, water/ethanol and water/acetone solvent mixtures [38], [52], [59], [63], which are able to extract all the target compounds as well as pure methanol or pure ethanol [56], [59]. A central composite design was achieved to find optimum conditions for the simultaneous extraction of rosmarinic acid, carnosol and carnosic acid from rosemary, leading to the following conditions: 55 minutes for the extraction duration, a ratio liquid (extraction solvent) to solid (rosemary power) equal to 5 mL/g and a mixture ethanol/water 70/30 v/v [59]. Authors claim that the obtained extract do have an antioxidant content comparable to those provided by supercritical fluids or pressurized and heated liquids.

When comparing petroleum ether, DCM and ethanol, the last one provides the highest amount of both rosmarinic and carnosic acid [35], whereas chloroform did not extract these two acids [65]. Besides, the extraction efficiency is ethanol<ethyl acetate<butanone for carnosic acid from dried leaves after 60 minutes from 25 to 50°C [67]. As expected, rosmarinic acid was extracted by water when carnosol and carnosic acid were obtained with DMSO [43], [54], or acetone [54], [59], in this last case with a sequential extraction achieved on the same raw material (rosemary leaves) [54]. However, in the case of extract concentration, DMSO is not easily to remove. CO<sub>2</sub>

The degradation pathway of carnosic acid in methanol or ethanol after extraction of plant was studied [46], [63]. Both reported the oxidation of carnosic acid into carnosic acid quinone, then producing carnosol by reduction, whereas rosmarinic acid seems stable in ethanol solution [63]. The stability of carnosic acid was strongly improved when diluted in fish oil [63], whereas the addition of ascorbic acid and EDTA reduced the rosmarinic oxidation during extraction [52].

#### **3.2 ULTRASONIC AND MICROWAVE EXTRACTION**

Sonication of samples during extraction generally favor the recovery of compounds, and it was largely applied to rosemary [50], [55], [57], [67]–[72]. The

frequency of the ultrasonic bath varied from 20 KHz to 43 KHz and the sonication duration from 5 to 120 minutes, with temperature from 25 to 50°C. The use of sonication improves the recovery of carnosic acid mainly with ethanol as extraction solvent, with regards to ethyl acetate or butanone, and strongly reduces the extraction duration with regards to shaking [67]. When performing extraction with methanol and ethanol, the use of sonication and stirring favors the recovery of rosmarinic and carnosic acid for both solvents [68]. Besides, the use of ultrasonic assisted extraction during 10 minutes at 40°C did not induce any improvement in the extraction of phenolic compounds from rosemary leaves dried 12 hours in a tunnel microwave dryer at 70°C, both with water or water/ethanol 50/50 [68]. Strangely, the TOF-LC-MS-MS analysis of aqueous extracts, which provided the greater extraction recovery, did not show the presence of rosmarinic acid in the extract. On an experimental point, one can mention the coupled sonicated extraction of Lamiaceae herbs with liquid chromatography [69].

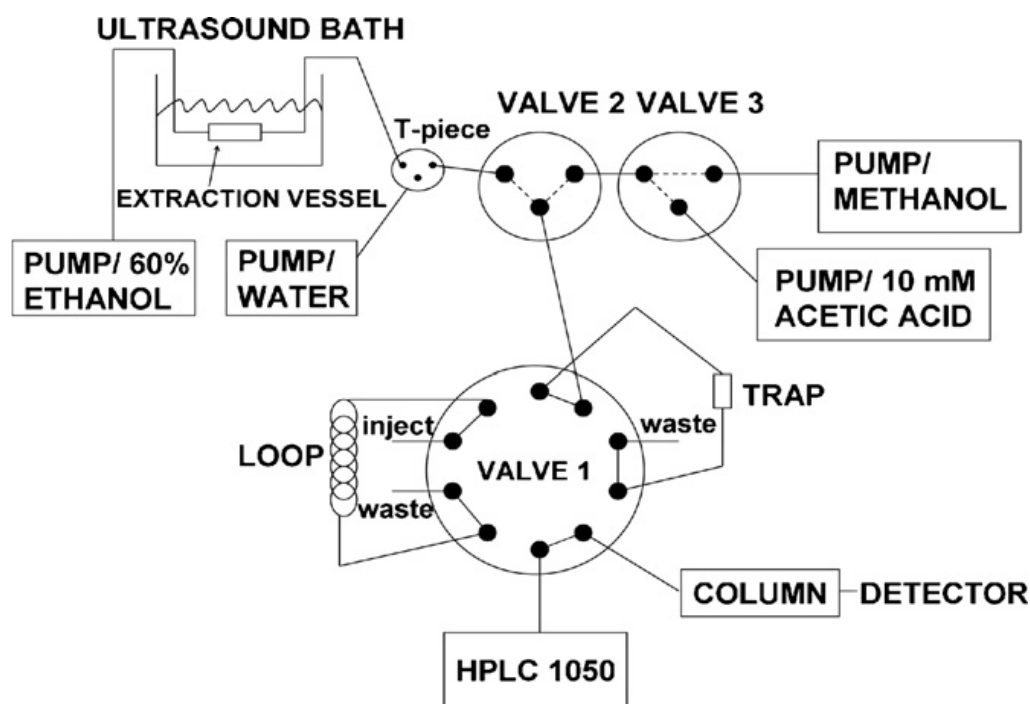


Figure 3. Scheme of the dynamic sonication assisted extraction liquid chromatography (DSAE-LC) system (from ref. [69])

The extraction cell (fig 3) is immersed in a ultrasound bath, the dynamic extraction was achieved by a mixture Ethanol/water 60/40 v/v, then water (1/1) is added to the extract to reduce the compound solubility and favor the trapping in a small column containing an SAX adsorbent (anion exchanger). After the trapping step, 500 microliters of an acidic solution filled the trap for analytes desorption, then directed in the analytical column. The comparison of chromatograms obtained by coupling or by

direct injection shows a limited loss of the chromatographic resolution due to the coupling, that does not hinder the study of acidic compounds from these herbs [69]. However, rosmarinic acid was not studied due to its too high concentration which would require a separate analysis with regards to the other studied acidic compounds. Solvent free microwave extraction (SFME), applied to fresh rosemary, provided higher recovery for both rosmarinic and carnosic acid with regards to steam or hydrodistillation [72].

Comparison between ultrasonic assisted (UAE) and microwave assisted extractions (MAE) have been done [73], [74]. The amount of rosmarinic and carnosic acids extracted by using discontinuous processes, by using 14 cycles of 30 s at the same energy input for MAE and USE, were identical, whatever the solvent, water and ethanol (as usually the rosmarinic acid was better extracted in water and carnosic acid in ethanol) [73].



*Figure 4. Varied equipment for the assisted ultrasound and microwave extraction (from ref.[74])*

From 10 varied extraction methods (fig. 4), heat reflux extraction (HRE; 78°C with or without grinding), maceration (40°C), ultrasound bath (40°C), ultrasound reactor (40°C), ultrasound probe (40°C) and microwave assisted (70, 125 and 150°C), by using an ethanol/water 90/10 v/v solvent, and 30 minutes of duration, except for HRE (30 minutes and 5 hours), and the US probe display an higher extraction for carnosic

acid, whereas, the increase in temperature favors the rosmarinic acid recovery, both for HRE and MAE [74]. The purity (in term of relative standard deviation; RSD) of the carnosic acid in extracts, the energy consumption and the carbon emission were also studied, showing the poor RSD values for ultrasonic methods with regards to the microwave ones, but the great advantages of US bath and probes in terms of low energy and CO<sub>2</sub> emission. Besides, the recovery of carnosol and carnosic acid from rosemary residues by maceration with ethanol was favored by using first solvent free microwave extraction (SFME) applied on rosemary fresh leaves [72]. A very interesting point from that paper is the recovery of ursolic acid whatever the extraction method used. This triterpenoid is the main compound observed in *Rosmarinus* leaves [58], [74] or callus [61] extracts, whereas, in a lot of paper reporting extraction with identical extraction solvents, the presence of this compound is not observed. A close look on chromatographic conditions applied for the analysis of *Rosmarinus* extract shows that the elution of ursolic acid from the C 18 column requires a mobile phase containing at least 90% of acetonitrile [58], [60], [61], [74]. Moreover, because of the close chemical structures between ursolic, oleanolic and, in a lower range, betulinic acids, these three compounds are not easily separated with classical liquid chromatographic systems. On the basis of fundamental works [75], the addition of 7.5 mM HP- $\gamma$ -CD (a modified cyclodextrin) in the acetonitrile phosphate-buffer pH 3.5 was reported to strongly improves this separation for rosemary extracts [58], but the use of this additives provides a dramatically increase in the retention time, by twice. Besides, due to the lack of chromophoric groups in these compounds, the detection of the triterpenoidic acids (ursolic, oleanolic and betulinic) should be done with very low wavelength in UV (210 nm) [74] or mass spectrometry [58], [60], [61].

### **3.3 PRESSURIZED LIQUID EXTRACTION**

Pressurized liquid extraction has also been used for the extraction of rosemary, either with water [66] or methanol/water [76] or pure methanol and ethanol [61]. The pressurization of extraction solvent allows to improve the extraction temperature above the boiling point of the solvent. The increase in temperature from 60 to 100°C with water extracts carnosic acid and improves the recovery of rosmarinic acid [66], whereas, a response surface methodology, applied to temperature and composition of the extraction mixture methanol/water, leads to select a mixture 59/41 methanol/water v/v and a temperature of 129°C which provide the highest antioxidant activity [76].

When comparing methanol and ethanol PLE extraction from rosemary leaves, working at 10 MPa, 150°C during 20 min, as expected, methanol favored the recovery of rosmarinic acid whereas the use of ethanol favored the one of carnosic acid and carnosol [61]. Strangely, the extraction using maceration (24 hours), provided higher recovery for carnosic acid and carnosol with regards to PLE, whereas, PLE by using ethanol was giving better results for rosmarinic acid.

### **3.4 SUPERCRITICAL FLUID EXTRACTION**

Supercritical fluid extraction of bioactive compounds of rosemary has been extensively studied [42], [77]–[81]. The solubility of carnosic acid was measured for different temperature (from 40°C to 60°C) and modifier (ethanol) percentage (from 2 to 10%) [77]. At 40°C, the increase in back pressure (with 6% of modifier) improved the carnosic acid solubility, whereas at 33.5 MPa, the increase in the modifier percentage do have an identical effect [77]. Another study showed that the increase in backpressure from 10 to 30 MPa favored the recovery of rosmarinic acid, but decreased the one of carnosic acid, both at 30 and 40°C [78]. However, the recovery of both compounds was lower by using pure CO<sub>2</sub> vs an extraction with ethanol under reflux for 3h. At 34.5 MPa, with 10% of ethanol and with a static extraction step (1 hour) followed by a dynamic one (1 hour), the increase in temperature favored the rosmarinic acid extraction, but reduces the one of carnosic and carnosol [79]. When comparing the amount from SFE extracts to the one from ethanolic or acetonic extracts (5 minutes of extraction in an ultrasonic bath), better recoveries were achieved for SFE extract at 80°C for the three antioxidant compounds.

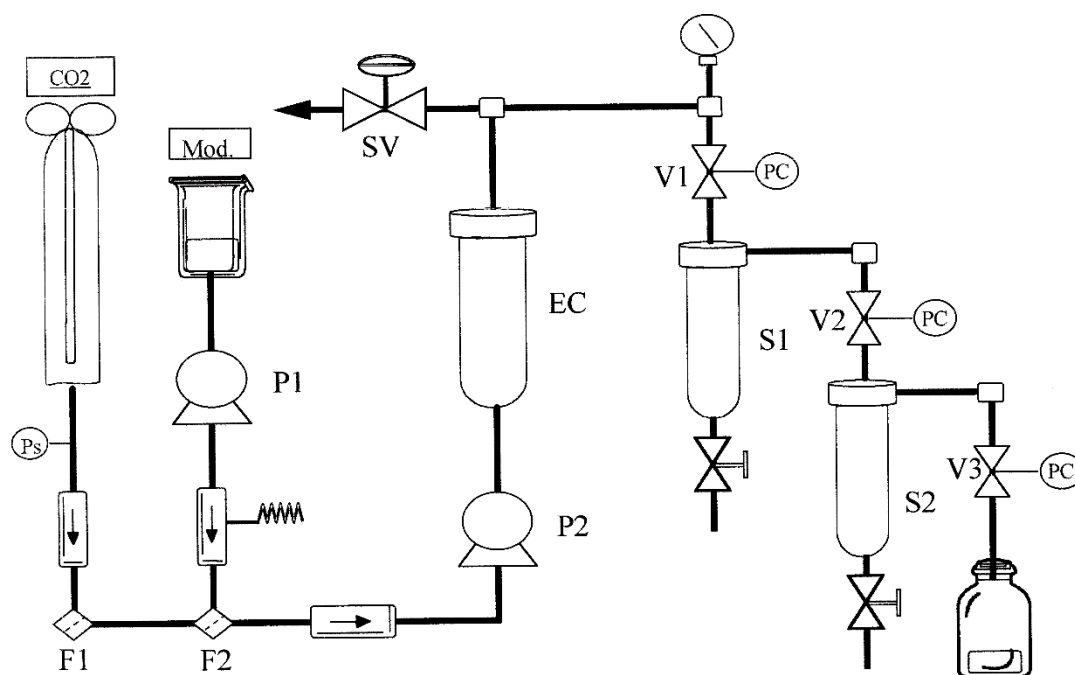


Figure 5. Scheme of a supercritical extractor equipped with two separators (S1,S2), three micrometering valves (V1,V2,V3), two pumps (P1,P2), one extraction cell (EC)(from ref [42]).

Attempt to induce selectivity in the composition of extract can be achieved with pure carbon dioxide by the use of two separators after the extraction step (fig. 5). The extracts collected at different temperatures and backpressure (S1: 40°C, 15MPa; S2: 25°C, 5.5 MPa) did not have a significant differences in the relative amount of carnosic acid, carnosol and carnosol isomer [42], whereas the best extraction condition to get antioxidant activity was achieved with 2 % of ethanol in carbon dioxide. These studies have been extended with higher ethanol percentages (up to 7%) [80].

#### 4. EXTRACT FRACTIONATION OU EXTRACT ENRICHMENT

Supercritical antisolvent fractionation (fig.6) is also used for selective recovery of carnosic acid, carnosol and rosmarinic acid [81].

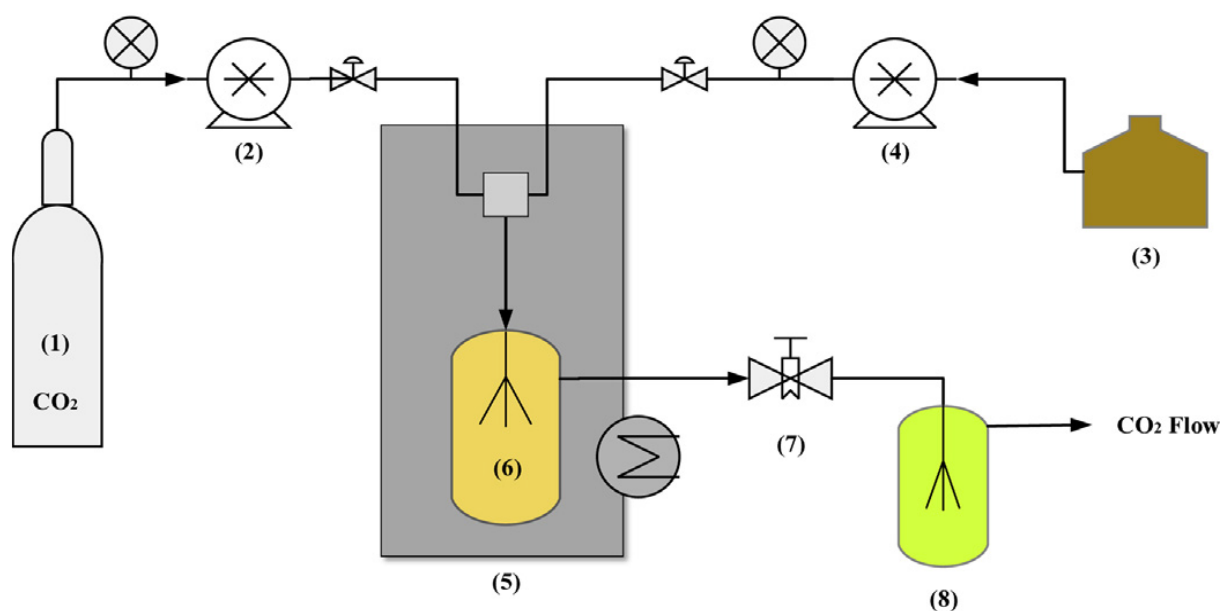


Figure 6. Scheme of an antisolvent fractionation equipment. (1) CO<sub>2</sub> cylinder; (2) CO<sub>2</sub> pump; (3) PLE extract reservoir flask; (4) high-pressure liquid pump; (5) oven temperature; (6) separator cell for raffinate; (7) back-pressure regulator; (8) separator cell for extract (from ref. [81])

After performing an extraction by pressurized fluid with varied ethanol/water mixtures at 150°C and 10MPa, the PLE extract (called feed) and CO<sub>2</sub> are concurrently mixed into a separator, which collect the non-soluble fraction called the raffinate, whereas the soluble one (the extract) goes through a BPR and is collected in a second separator. The water percentage in ethanol for the PLE was ranged from 20 to 50%, and the ratio between the PLE extract (feed) and CO<sub>2</sub> varied from 0.025 to 0.1, with pressure in the first separator between 10 and 30MPa. As expected, the rich water PLE extract favored the recovery of rosmarinic acid and the reverse for both carnosic acid and carnosol. Then, whatever the ratio feed/CO<sub>2</sub>, the rosmarinic acid was fully found in the raffinate, and greater amount of carnosic acid and carnosol were found in the extract. Finally, by taking into account the both steps, PLE and antisolvent precipitation, the better conditions for having a high recovery during extraction, and a high selectivity during the precipitation were a mixture water/ethanol 50/50 for PLE, a ratio feed/CO<sub>2</sub> equal to 0.025 (meaning 5% of CO<sub>2</sub> added to the PLE extract), a pressure of 10 MPa [81].

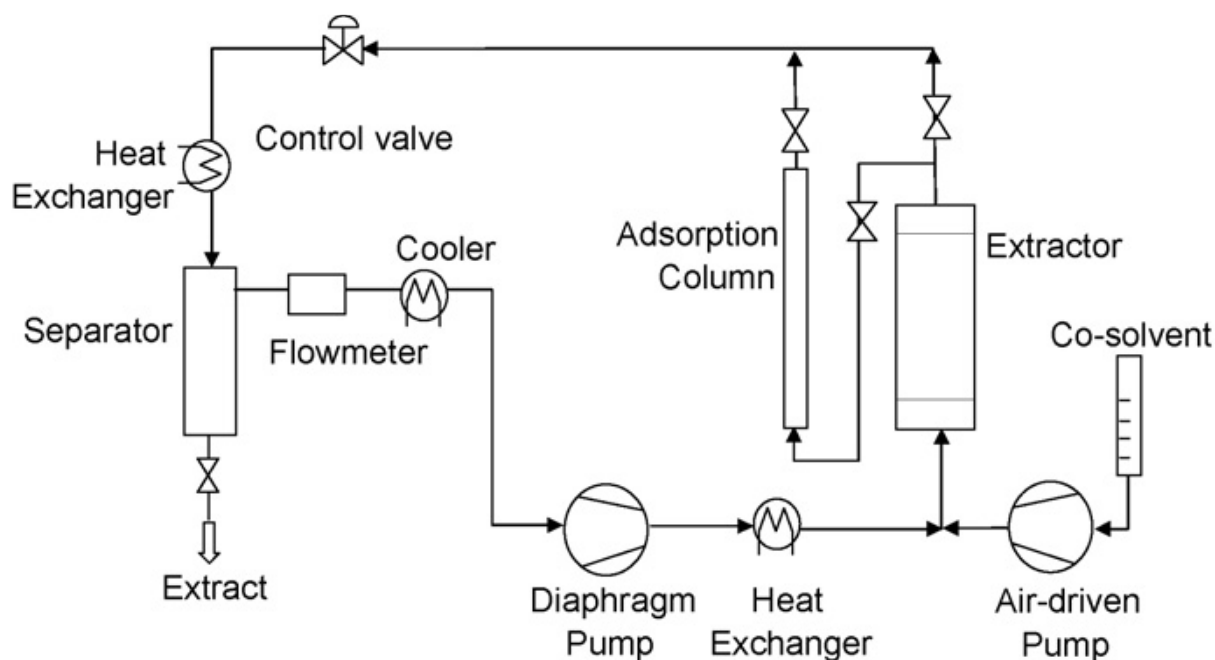


Figure 7. Scheme of the adsorption-extraction equipment (from ref. [82])

A mixed method (fig.7) was reported by using several steps [82]. First the oleoresin of rosemary was obtained by liquid/solid extraction with acetone, which do have a low boiling point. After mixing this oleoresin with glass particle, the solvent was eliminated by rotary evaporator, and the glass particle were located in a bed into an extractor. Pure carbon dioxide at 10 MPa and 40°C was fed to flush the volatile compounds. Then the pressure was increased to 25 MPa, to extract back the antioxydant compounds from the glass particle, i.e. rosmarinic acid, carnosic acid and carnosol. During this extraction, the fluid was directed through a trapping column filled with varied adsorbents (magnesium silicate, silica gel, activated carbon), to concentrate the solubilized compounds. Finally, the addition of ethanol (1-2%) allowed to retrieve the adsorbed compounds. This extract was odorless and light yellow, whereas the adsorbent was light green. The extract contained high amounts of carnosic acid and carnosol with regards to the crude oleoresin, but few rosmarinic acid was present in these extracts. Moreover, the recovery of compounds by comparison with the crude oleoresin was ranged from 5.3 to 15.7%, showing the lack of efficiency of the adsorption step.

Preparative (Prep) SFC was also applied for fractionation of SFE extracts [83]–[85]. The SFE was achieved by using pure CO<sub>2</sub> and a homemade prep-column with a SE-54 (5% phenyl-95% methylsilicone) coating stationary phase [83] or with CO<sub>2</sub>/Ethanol 93/7 v/v and a diol column [84]. The mobile phases were CO<sub>2</sub>/ethanol

with 5 to 10% of ethanol. Each time, two fractions were collected in different cyclones, depending on the analysis duration, one in the beginning of the analysis, and the second one at the end. For each study, carnosic acid is eluted at the end of the analysis, whereas the more volatile compounds are eluted in the first fraction. From varied extraction condition by using SFE, pure CO<sub>2</sub>, CO<sub>2</sub>/Ethanol 90:10 w/w, it was reported that the extraction of carnosic acid was improved by the ethanol addition, whereas, with pure CO<sub>2</sub>, the main amount was extracted in the first 60 minutes [86]. Whatever, several stationary phases was studied for a use in prep-SFC, silicas and 2-ethyl-pyridine. As usually seen in SFC [86]–[89], the carnosic acid was more retained and the carnosol is eluted first with the basic stationary phases (2-EtPy), whereas the classical retention order carnosol/carnosic acid was observed with the silicas. Because of the higher resolution, the 2-EtPy was selected for the prep-SFC with 20% of ethanol as cosolvent, allowing a concentration factor equal to 4.5, against 2 for the silica phases [85]. The specific retention of 2-EtPy column towards acidic compounds was also described for triterpenoic compounds, and more largely, and the retention behavior of polar stationary phases in supercritical conditions has been largely reported [86]–[89].

Extraction selectivity can also be achieved by using successive conditions [90]–[94]. By increasing the backpressure from 14(F1) to 35(F2) MPa at 50°C, the first condition extracted the rosemary essential oil, whereas carnosic acid was extracted at higher backpressure [90]. Identical pressure and temperature changes provide same fractionation between essential oil and nonvolatile compounds by using higher temperature (100°C) for the second conditions [92]. Another study showed that the use of 10% of ethanol to carbon dioxide dramatically improves the recovery of carnosic acid when a first extraction with pure carbon dioxide where applied at least for 60 minutes [91]. Besides, after the extraction of essential oil of rosemary by supercritical fluid at 8 or 15 MPa, the extraction of rosmarinic acid from the plant waste was achieved by a batch extraction (15 min) in a static bath, with an ethanolic solution (0,30,50,70,90%), at different temperature (30,60°C) and with or without ultrasound assistance (25kHz) [93]. The backpressure of the first extraction by SFE did not have any effect on the rosmarinic acid recovery from plant waste, as well as the temperature or the use of ultrasound, whereas, the use of 30% of ethanol provided the highest recovery and was finally selected. A two steps process has also been proposed with a

first extraction with neat CO<sub>2</sub> (60 min) either at 15 or 30 MPa, followed by an extraction with 7% of ethanol (120 min) [94]. The essential oil was recovered from the first step, whereas the use of ethanol in the second step provided enriched carnosol and carnosic acid concentration. Due to higher concentration in bioactive compounds, these enriched extracts displayed a higher anti-proliferative activity against colon cancer cells, with regards to a classical one step extraction using 7% of ethanol during 300 minutes.

Finally, a comparison was done (fig.2) between PLE with water (P=20Mpa) and SFE with 6.6% of ethanol as cosolvent (P=15MPa), static extraction during 20 min for both [62]. Rosmarinic acid and a lot of flavonoids and glycosylated derivatives were obtained with water (PLE), carnosol with both method, whereas carnosic acid was founded only in SFE extract.

To conclude, one can mention the use of subcritical water (6 MPa) for the extraction of dried rosemary leaves [95]. The increase in temperature from 100 to 200° favors the extraction of carnosic acid but reduces the one of carnosol. Selectivity of extraction can be achieved by changing the temperature from one to another extraction applied on the same sample. In both case, one can ask what is the usefulness of a water extraction vs a carbon dioxide one, in term of concentration or formulation for cosmetic product. One again, we can see in this paper that the retention order between cirsimarín and genkwanin is reversed with regards to the one described previously. This chromatographic behavior was also mentioned elsewhere [42], and can be related to the use of "old generation" C18 bonded phases (Zorbax C18, Nova-pak C18).

## **5. CONCLUSION**

The extraction of bioactive compounds from rosemary is well addressed in literature, on an analytical point of view, i.e. for studying the content in varied compounds. The scale up of these extraction methods for an industrial use would depend on economical and ecological issues. Some studies provide interesting ways to follow for increasing the extract purity or the recovery of targeted compounds, for instance with preparative SFC or by some unusual coupling between extraction and adsorption steps.

Due to the great number of compounds, their large range in polarity, their little structural differences, their lack of UV response, and for some their acidic character, the chromatographic method should take into account many parameters. Reverse phase liquid chromatography is actually the suited approach, but the column specificity, the additives included in the mobile phase, the performance of interface with detection systems are of a great importance to ensure satisfying analyses.

Les différentes méthodes permettant l'analyse des composés du romarin ont donc été présentées. L'analyse la plus courante est en chromatographie liquide en phase inverse. De par le nombre de composés différents dans le romarin, le développement analytique peut être complexe. De plus, les phénomènes d'élargissement de pics peuvent survenir pour les composés acides, comme les composés majoritaires que sont les acides carnosique et rosmarinique, alors que si les gradients d'élution ne sont pas prolongés, des triterpènes peuvent ne pas être élués de la colonne.

Par ailleurs, un état de l'art a été fait sur l'extraction de ces molécules par le biais de différentes techniques. Le solvant d'extraction va avoir une incidence sur les molécules extraites. L'eau va favoriser l'extraction de l'acide rosmarinique et l'éthanol celle de l'acide carnosique.

### **3. MODELE DE PREDICTION DE CONDITIONS SELECTIVES**

La précédente étude sur le comportement chromatographique présenté chapitre 2 a permis d'observer l'évolution des chlorophylles et de leurs produits de dégradation en fonction de différentes conditions analytiques. Malgré quelques différences dans le suivi de rétention liées à leur structure chimique respective, l'ensemble des composés chlorophylliens (contenant une chaîne phytyle) suivent une même tendance : une diminution suivie d'une augmentation de la rétention en fonction du pourcentage de modificateur (pour un même co-solvant). Ce comportement peut être transposé en condition d'extraction.

Le prochain article détaille le développement d'un modèle de prédiction de sélectivité en extraction sur la base du comportement chromatographique.



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# Supercritical Fluid Chromatography development of a predictive analytical tool to selectively extract bioactive compounds by supercritical fluid extraction and pressurised liquid extraction

Thibault Lefebvre, Emilie Destandau, Caroline West, Eric Lesellier\*

ICOA, CNRS UMR 7311, Université d'Orléans, rue de Chartres, BP 6759, 45067 Orléans Cedex 2, France

## ARTICLE INFO

### Article history:

Received 30 July 2020

Revised 21 September 2020

Accepted 22 September 2020

Available online 25 September 2020

### Keywords:

Supercritical Fluid Chromatography and extraction

Pressurised fluid extraction

Rosemary

Bioactive compounds and chlorophylls

Relationships retention vs extraction

Hansen solubility parameters

## ABSTRACT

Selective extraction is a great concern in the field of natural products. The interest is to apply specific conditions favouring the solubility of targeted secondary metabolites and avoiding the simultaneous extraction of unwanted ones. Different ways exist to reach selective extractions with suited conditions. These conditions can be determined from experimental studies through experimental design, but a full experimental design takes time, energy, and uses plant samples. Prediction from varied solubility models can also be applied allowing a better understanding of the final selected conditions and eventually less experiments.

The aim of this work was to develop and use a chromatographic model to determine optimal extraction conditions without the need for numerous extraction experiments. This model would be applied on the selective extraction of the desired antioxidant compounds in rosemary leaves (rosmarinic and carnosic acids) vs chlorophyll pigments to limit the green colour in extracts. This model was achieved with Supercritical Fluid Chromatography (SFC) and then applied to Supercritical Fluid Extraction (SFE) and Pressurised Liquid Extraction (PLE) assays.

SFC models predicted low solubility of chlorophylls for low (5%) and high (100%) percentage of solvent in carbon dioxide. Also, low solubility was predicted with acetonitrile solvent compared to methanol or ethanol. This was confirmed with different extractions performed using SFE with different percentages of solvent (5, 30, and 70%) and with the three solvents used in the SFC models (acetonitrile, methanol and ethanol). Also extractions using PLE were carried out using the same neat solvents in order to confirm the SFC models obtained for 100% of solvent. Globally, extractions validated the SFC models. Only some differences were observed between ethanol and methanol showing the complexity of plant extraction due to matrix effect. For all these extracts, the content of carnosic acid and rosmarinic acid was also monitored and selective extraction conditions of bioactive compounds could be determined.

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## 1. Introduction

The need for selectivity during plant extraction has different reasons. One of them, maybe the simplest and most logical one, is to enrich the final extract in particular bioactive metabolites, without the need for additional purification steps by preparative chromatography or counter-current chromatography. Numerous articles were published about enrichment of extracts. For example, it has been studied in order to extract more bioactive compounds such as hydroxytyrosol from olive waste [1], or chlorophyll pigments extraction in order to valorise and use them as dye [2]. On the con-

trary, another interest can be the necessity to avoid particular compounds. Some compounds considered as interferences can be toxic compounds coming from the plant or from the soil or the water used during the plant growth [3–6].

Among many extraction approaches, supercritical fluid extraction with carbon dioxide as extracting fluid has shown interesting selectivity properties, which result from the carbon dioxide itself, and from the possibility to extract sequentially different family of compounds when applying a dynamic and continuous method [7,8]. When the fluid is in the supercritical state, different parameters such as pressure, temperature and the addition of co-solvents can modify carbon dioxide behaviour. This change of parameters can then be used to selectively extract the target compounds, due to their varied solubilities depending on the fluid properties.

\* Corresponding author.

E-mail address: [ericlesellier@univ-orleans.fr](mailto:ericlesellier@univ-orleans.fr) (E. Lesellier).

The classical approach to determine extraction conditions is based on the selection of some parameters and their variation in a defined range. All the extractions are carried out, to determine the best. A more efficient way to obtain optimal conditions with a minimum of experiments exists with design of experiments (DoE).

Different parameters are screened simultaneously then, based on a pre-defined response (concentration of target analytes, yield...), optimum conditions are determined [9–11]. This strategy also helps understanding the influence of the different parameters tested and their interactions. Even if a DoE reduces the number of experiments, it still remains long. Applied to plant extraction, the number of extracts increases exponentially with the number of parameters to be tested.

The aim of this study was to develop a model which would allow identifying the best extraction conditions without the need for a DoE with numerous extractions. This work was focused on chlorophyll pigments. On the one hand, there is an interest for the extraction of chlorophyll pigments to use these compounds as natural dyes. On another hand, there is also an interest for not extracting chlorophyll pigments. For instance, for cosmetic raw materials, plant extracts must contain the lowest possible amount of chlorophyll pigments (with the highest possible amount of bioactive compounds), to obtain white final products. Also, chlorophyll compounds are very sensitive and transform themselves into degradation products such as pheophytin which change the colour from green to gray [12] inducing possible colour change of the final products. Rosemary (*Rosmarinus officinalis*) is a well known plant widely used in cosmetic or food applications. Rosemary extracts can be used as conservative agents for example for milk and dairy products [13]. The aerial part of this plant from the *Lamiaceae* family, contains chlorophyll but also interesting bioactive molecules such as carnosic or rosmarinic acids known for their antioxidant or their antiproliferative properties [14,15].

In this paper, the behaviour of chlorophyll compounds is first studied by Supercritical Fluid Chromatography and Liquid Chromatography with pure organic solvents as mobile phases. The retention observed in function of the different conditions is then correlated to real extraction processes, and then to SFE or pressurised liquid extraction (PLE). Finally, the different conditions favoring the extraction or non-extraction of chlorophyll will also be studied regarding the extraction of the main bioactive compounds of rosemary. Thus the behaviour of carnosic acid and rosmarinic acid will be also investigated in this work because they have different polarities ( $\log P_{\text{Carnosic acid}} = 5.14$ ,  $\log P_{\text{Rosmarinic acid}} = 1.82$ ) [16].

## 2. Material and methods

### 2.1. Solvents and chemicals

For all this study, the solvents used were methanol, ethanol and 2-methyltetrahydrofuran, gradient grade (VWR International, Fontenay-sous-Bois, France), and acetonitrile, gradient grade (Carlo Erba, Val-de-Reuil, France). Propylene carbonate used was for synthesis (Merck KGaA, Darmstadt, Germany). Carbon dioxide (>99.5%) was from Air Liquide (Fleury-les-Aubrais, France). Different additives were tested: water was supplied by an Elga Purelab Flex station from Veolia (Antony, France), and citric acid was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). In order to develop the analytical method, different standards were used. Carnosic and rosmarinic acid standards were supplied by Extrasynthese (Genay, France). Chlorophyll *a* and *b* were obtained from ivy extracts (protocol described in extraction method part). The dispersant was diatomaceous earth (DE) from Fisher Scientific (Illkirch- France), general purpose grade.

### 2.2. Analytical SFC method development

The chromatographic system used for the retention model or the quantification of extract was a Jasco instrument (see details in [12]).

During the analytical method development in order to analyse rosemary extracts, different columns were tested: HSS C18 (5  $\mu\text{m}$ , 4.6 x 150 mm), Torus DEA (1.7  $\mu\text{m}$ , 3.0 x 100 mm) (diethylamine group), Torus 2-Pic (1.7  $\mu\text{m}$  3.0 x 100 mm) (picolylamine group), Torus DIOL (1.7  $\mu\text{m}$  3.0 x 100 mm), all from Waters (Saint Quentin en Yvelines). Two elution gradients were used. "Gradient 1": 5% of methanol during 1 min, then from 5 to 50% until 15 min. The following conditions with "Gradient 1" were used for the initial column screening: flow rate: 1.0 mL/min, column oven temperature: 35 °C, injection volume: 5  $\mu\text{L}$ . The columns packed with small diameter particles and the elution gradient up to 50% methanol both induce a high pressure drop along the column. As a result, the back-pressure was set at 10 MPa, to avoid reaching the upper pressure limit of the pumping system (40 MPa).

Once the stationary phase was selected, and depending on the retention times observed for the target compounds, the gradient was optimised and shortened into "Gradient 2": 5% methanol during 1 min, then increase to 50% in 4 min and maintain at 50% during 4 min, to finally come back to initial condition during 3 min at 5%.

The final SFC method developed used coupled columns: HSS C18 coupled with Torus DIOL.

### 2.3. Plants and extraction methods

Two plants were used in this study. Ivy leaves (*Hedera canariensis* Willd) used for the production of chlorophyll-rich extract were collected in Orleans (France). Rosemary leaves (*Rosmarinus officinalis*) for the study of selective extraction of carnosic acid, rosmarinic acid and chlorophyll *a* were provided by one of our industrial partners, Alban Muller (Fontenay-sur-Eure, France) specialised in plant extraction.

Ivy was harvested and then dried using a Memmert model 500 dryer, at 50 °C for 24 h. Then the dried leaves were grinded using Polymix PX-MFC 90 D Kinematica from Cosserson (Coueron, France).

Plant extraction for "standard" extracts used in the two first parts was carried out with Ultrasonic bath Branson 3510. Ivy and Rosemary extracts were prepared from 1 g of plant material and 15 mL ethanol. The mixture was then sonicated for 60 min. The extracts were centrifuged at 7000 rpm at 20 °C for 10 min using BR4i Multifunction Centrifuge (Thermo-electron, Nantes, France). The extracts were used for no longer than one week, in order to maintain low amounts of chlorophyll degradation products.

A Waters MV-10 ASFE was used for supercritical fluid extraction. In addition, a UV detector was added between the oven and back-pressure regulator (BPR) modules in order to monitor extraction kinetics. The detector was a Gilson 151 UV / VIS Multiwavelength Detector (GILSON, Villiers-le-Bel, France). The 5-mL extraction cell was prepared using 1 g of plant material mixed with 1 g of dispersant. Cotton served to fill the cell completely and was placed at the top and bottom of the cell. Extraction parameters for all rosemary extracts were using the percentage of modifier with a total flow of 3.0 mL/min for 30 min at 25 °C, 10 MPa. For 5% modifier, a make-up solvent (ethanol) was used at 0.15 mL/min introduced before the BPR to avoid compound precipitation in the transfer capillary. Final volume obtained was 24 mL when using 5% modifier (plus make-up solvent) and 23 mL when using 30% modifier.

Pressurised liquid extraction (PLE) was performed with a Dionex ASE 150 from Thermo (Thermo, Villebon sur Yvette,

France): 1 g of plant material mixed with 1 g of dispersant (DE) were extracted in a 22-mL extraction cell.

All extractions were carried out at ambient temperature (Temperature regulation off), 10 MPa, and 3 cycles of 10 min static extraction with 60% flush. Final volume was 32 mL.

Different fluid compositions were selected from varied points of retention values and were applied in extraction by supercritical fluid, with ACN, MeOH, EtOH at 5, 30, 70% in CO<sub>2</sub> (SFE), and 100% liquid (PLE). All extractions of Rosemary leaves were carried out three times in order to evaluate repeatability. The latter is then expressed as standard deviation on the different figures.

Peak areas were measured with the SFC method developed in section 3.1.3. The results are expressed in terms of the area of the peak of chlorophyll *a* normalised by taking into account the final collected volume.

The volumes collected at the end of each extraction being different, it was necessary to find a way to be able to compare these areas with each other. A simple way is to evaporate the sample dissolve it again to a specific volume. With the mass of dry extract and the volume, it is possible to use the concentration. However, since chlorophylls are very sensitive to evaporation (possible degradation), another way was implemented. The final volume of each extract was measured, and then normalization by volumes was carried out. For example, if a normalised volume of 15 mL is selected, the area of an extract of 60 mL is then multiplied by 60 (volume of the extract) and divided by 15 (reference volume).

#### 2.4. Retention model

To establish the model  $\log k = f(\text{solvent percentage})$ , a HSS C18 column (5  $\mu\text{m}$ , 4.6 x 150 mm) was used (Waters, Guyancourt, France). The flow rate was 1.5 mL/min, the outlet pressure set at 10 MPa, the oven temperature at 25 °C, and UV detection monitored at 650 nm.

Retention factors ( $k$ ) were calculated using the following equation.

$$k = \frac{t_r - t_0}{t_0}$$

with retention times ( $t_r$ ) and dead time ( $t_0$ ). The dead time was measured at each analytical condition, because it could vary with the compressibility of carbon dioxide.

The latter can change depending on the inlet pressure of the column, which is related, at constant flow rate, to the mobile phase viscosity, i.e. to the modifier (co-solvent) percentage and nature [17,18].

An Ivy extract was used to examine the retention variation of chlorophyll *a* and *b*. The characterisation of this extract was carried out with a Nexera UC On-line SFE-SFC System with Mass-spectrometer in a previous study [12].

#### 2.5. Correlation with Hansen solubility model

In order to correlate our retention model with Hansen solubility parameters, other experiments were done with an HPLC instrument LC20-Series (two LC-20AD pumps; autosampler SIL-20AC; degassing unit DGU-20A; Column oven CTO-20AC; Diode array detector SPD-M20A) from Shimadzu (Kyoto, Japan) with the same flow rate, temperature and detection conditions as the SFC experiments.

XLSTAT 2018.1.1 software (Addinsoft, Paris, France) was used for data analysis.

### 3. Results and discussion

In this study, two different analytical SFC methods were used. Chlorophyll retention to serve for extraction models was moni-

tored using conditions summarised in Section 2.2. For this purpose, an isocratic SFC method using a C18 stationary phase allowed the analysis of chlorophylls from Ivy extracts. However, for other compounds with different polarities, this method was limited. Another method was needed to analyse chlorophylls together with carnosic and rosmarinic acid in rosemary extracts (details in Section 2.4). The following part presents the development of this second method.

#### 3.1. Analytical method development for quantification

The objective was to develop an analytical method to separate the phenolic acids of interest in rosemary: carnosic and rosmarinic acid, as well as chlorophylls *a* and *b*. During the development of an analytical method, many parameters influence the separation quality. The use of carbon dioxide in sub- or supercritical conditions makes the development of such a method even more complex. However, even if this can be seen as an additional difficulty, it also allows us to have more parameters to play on in order to separate the compounds and thus refine the method [19]. A diode array UV-visible detector was used for selective detection, and different wavelengths were selected depending on the absorbance of the compounds analysed: carnosic acid: 221 nm, rosmarinic acid: 332 nm, chlorophylls: 650 nm.

One of the major parameters influencing SFC separations is the stationary phase. In order to find the most suitable, a so-called "initial" analysis method was used (described in Section 2.2). Unlike RPLC or NPLC, chromatography in supercritical phase allows a wide choice of columns to be used, in particular with different polarity. Different columns were selected (Section 2.2).

From the first column screening, the polar Torus DEA column was rejected because chlorophylls eluted in the dead volume. On another hand, the Torus 2-PI-C column was unable to retain the two acidic compounds (rosmarinic and carnosic acids), which may be surprising for a basic ligand. As expected, the HSS C18 column was not able to retain rosmarinic acid but provided a satisfactory separation between carnosic acid and chlorophylls. Finally, the Torus DIOL column was selected because all compounds were sufficiently retained. In order to save time, the method was shortened by programming the elution with Gradient 2 (described in Section 2.2). It was then possible to analyse all target compounds within 10 min.

Despite this change in the mobile phase gradient, the chromatogram obtained with the Torus DIOL column showed broad peak shape for rosmarinic acid. The mobile phase composition was varied to improve peak efficiency. Different additives such as water or acid were added to the modifier in order to improve the peak shape of rosmarinic acid. Finally, citric acid was selected, a poly-acid which was reported in other SFC methods [20].

The first noticeable thing is the change in retention time between the different modifiers (Figure S1a). Rosmarinic acid is eluted faster using methanol with water than methanol alone, and even faster using methanol with acidified water. The addition of 5% of water in the modifier slightly decreases the retention of rosmarinic acid.

Adding citric acid to water induced both a greater decrease in retention time of the acidic compound and a strong peak width decrease. The modification of the ionization state of rosmarinic acid (from anionic to neutral), and by consequence, its change in solubility into the mobile phase can explain this change of retention. Besides, as the column ligands carry hydroxyl groups, the addition of an acid in the mobile phase could also change the apparent chemical nature of stationary phase [21–23]. Citric acid may adsorb onto the polar groups, which will affect the nature of the interactions between the analyte and the stationary phase as in HILIC mode [24–26]. The adsorption of citric acid may repulse

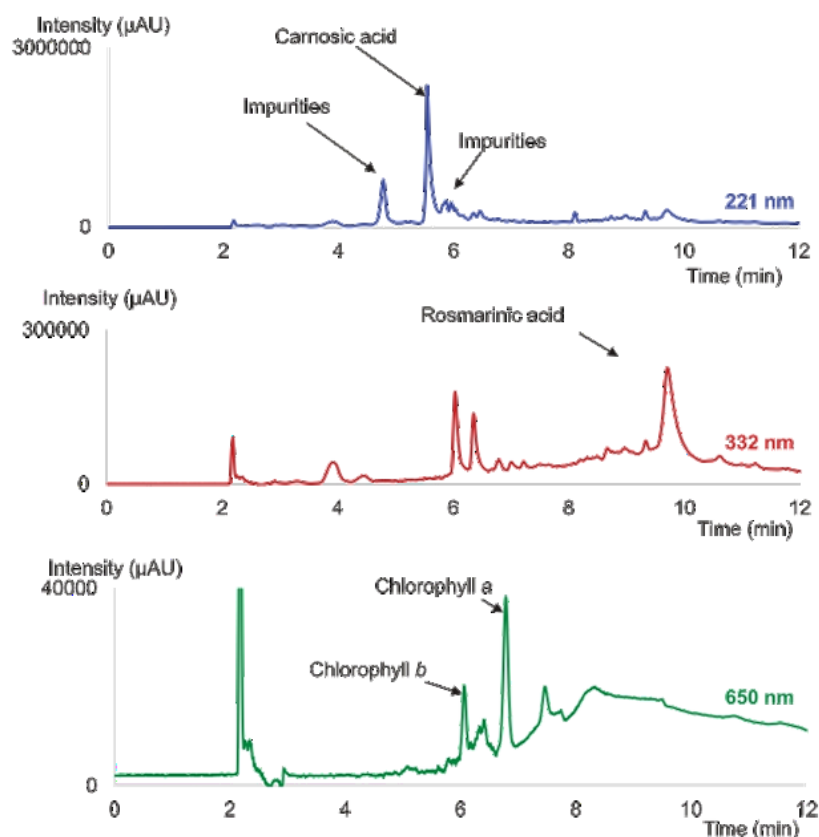


Fig. 1. SFC chromatograms of carnosic acid, rosmarinic acid and chlorophylls with final gradient conditions (gradient 2) and the coupled column system (HSS C18 and Torus DIOL).

other acidic compounds from the stationary phase and thus, reduce interactions between analytes and stationary phase.

Despite the best improvement of peak shape of rosmarinic acid obtained with citric acid, the use of this acid leads to visible degradation of the chlorophylls which are therefore no longer detected (Figure S1b). Chlorophyll compounds are sensitive to pH changes and degrade into other forms such as pheophytin (magnesium-free chlorophyll).

Consequently, as water also refined the rosmarinic acid peak, the modifier methanol /water 95/5 v/v was selected for the rest of the development. Thanks to previous optimization steps, a method allowing the analysis of carnosic, rosmarinic acid and chlorophyll *a* and *b* in less than 10 min was developed on the Torus DIOL stationary phase. Figure S2 shows chromatograms obtained with the new optimised conditions.

However, chlorophylls also absorb at 221 nm, the wavelength which is used to study carnosic acid. Because it elutes close to the chlorophylls, the peak integration could be biased. For this purpose, another approach with coupled columns was developed.

From the previous column screening, the HSS C18 column seemed to be suitable for carnosic acid and chlorophyll separation. However, a reversal of elution order between chlorophyll *a* and *b* occurred, compared to the one obtained with the Torus DIOL stationary phase. Chlorophyll *b* being more polar than chlorophyll *a*, it was more retained on the polar diol stationary phase and less retained on the non-polar C18 stationary phase. Whatever this drawback, the HSS C18 column was coupled with the Torus DIOL column in order to combine the different effects. Besides, due to the low viscosity of the mobile phase used in SFC, the column coupling does not induce a great pressure drop increase, around 3 MPa more.

However, when coupling column with an elution gradient, placing the most retentive column first is not advised, because the compounds leave the first column and enter the second one with a high modifier amount. Therefore, the most retained analytes in the first column will have very few interactions with the stationary phase of the second column.

For these reasons, the first column was HSS C18 and the second was Torus DIOL. As expected, this coupling made it possible to increase the difference in retention times between chlorophylls and carnosic acid of about one minute (Fig. 1). Moreover, the separation of carnosic acid from impurities was enhanced. The chlorophylls were well separated, with chlorophyll *b* eluting first, showing for these two pigments the predominance of HSS C18 phase on their selectivity. To conclude, the coupled columns are used with the elution conditions developed previously in order to quantify carnosic acid, rosmarinic acid and chlorophylls *a* and *b* from extracts obtained with SFE or PFE.

### 3.2. Solubility predictive model

To achieve a solubility predictive model, we monitored the retention of chlorophyll *a* in different analytical SFC and LC conditions. Plotting the logarithm of the retention factor according to the nature and the percentage of modifier is usually used to improve the understanding of the retention of one compound compared to another in order to develop an analytical method [12,27,28]. Another interest, related to extraction field, is to study the solubility of an analyte in a fluid that is either a mobile phase or an extraction phase. Consequently, specific extraction conditions, extrapolated from the retention curves, may be applied to extraction techniques such as supercritical fluid extraction (SFE) for

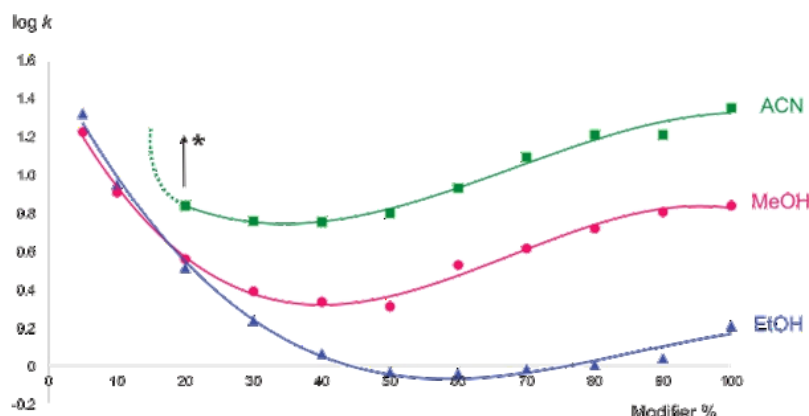


Fig. 2. Logarithm of retention factor of chlorophyll *a* vs modifier percentage. Column: HSS C18, Flow rate: 1.5 mL/min;  $T = 25^{\circ}\text{C}$ ;  $P = 10\text{ MPa}$ .

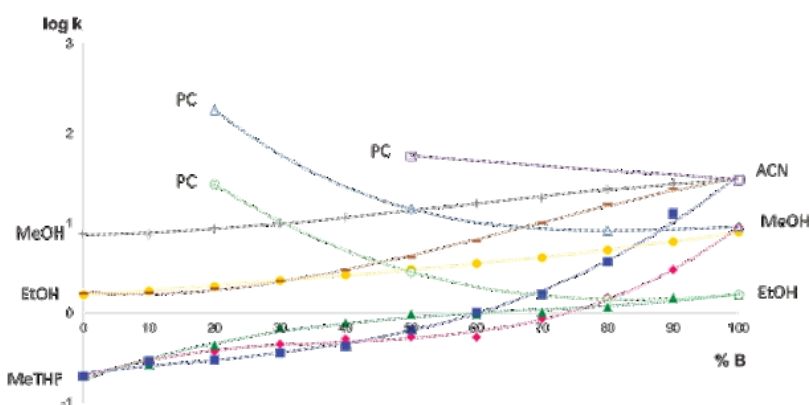


Fig. 3. Logarithm of retention factor of chlorophyll *a* vs different percentage of solvent B for different mixtures noted A – B with the solvents identified on the figure. Column: HSS C18, Flow rate: 1.5 mL/min;  $T = 25^{\circ}\text{C}$ .

$\text{CO}_2$ /modifier eluents, or pressurised fluid extraction (PFE) for neat solvent eluents.

Chlorophyll retention curves were obtained in SFC and LC, on a C18-bonded phase. Chlorophylls have high log *P* values (superior to 8) [29]. Thus a non-polar C18 stationary phase is able to provide some retention for such compounds. In addition, only limited changes in the stationary phase properties due to adsorption of the mobile phase components are expected. On the opposite, a polar stationary phase would not provide satisfactory retention of the non-polar chlorophylls, and the strong mobile phase adsorption on the polar stationary phase surface when changing the modifier percentage would induce some retention modification that would not be solely related to changes in the analyte solubility.

Three solvents were selected in this study: acetonitrile, ethanol, and methanol. Acetonitrile and methanol are quite different. Their log *P* may look similar (log  $P_{\text{acetonitrile}} = -0.34$  [30] Log  $P_{\text{methanol}} = -0.77$  [31]), but acetonitrile is a non-protic solvent, which can interact with the analytes through dipole-dipole interactions, whereas methanol is protic and interacts through hydrogen bonding interactions, which leads to different chromatographic behaviours. Ethanol, parent of methanol, is quite close in terms of its polarity (Log  $P_{\text{ethanol}} = -0.31$  [32]) but has an additional methylene group. In the field of plant extraction, this solvent is widely used due to its polarity, its origin (from agricultural resources), its biodegradability, its low toxicity, its moderate cost, and favourable regulations allowing its use in regulated fields such as cosmetics.

An ivy extract used as chlorophyll standard (preparation method described in Section 2.3) was therefore injected on the HSS

C18 column in SFC and LC with different percentages of solvent in  $\text{CO}_2$ , ranging from 5 to 100%. The other parameters such as temperature, flow-rate and back-pressure are described in Section 2.2. The results are presented in Fig. 2. Only the plots for chlorophyll *a* are presented because the plots for chlorophyll *b* had a very similar shape [12]. For acetonitrile, between 5 and 20%, retention was too high and was thus not measured.

The first noticeable point is the curve shape. Conventionally in RP-HPLC, when using C18-bonded stationary phase, the retention is expressed with the logarithm of the retention factor in order to have a straight line with a negative slope as a function of the percentage of organic solvent. In Fig. 3, two areas are identifiable. The first one is a decrease in retention until about 40% for acetonitrile and methanol, and 50% for ethanol. Because carbon dioxide is considered as a nonpolar solvent similar to pentane, this decrease was fairly predictable. The more modifier there is, the more the polarity of the mobile phase increases, approaching the polarity of chlorophyll *a*, which favours the analyte solubility in the mobile phase. However, beyond these percentages, the retention increases showing that the polarity of the mobile phase continues to increase above the polarity of the analyte. This behaviour was described before with carotenoids [33] and triacylglycerols [19] on C18 stationary phase in SFC.

The shape of this curve also has other consequences. It shows that it is possible to have equal log *k* values at different percentages of modifier. Identical log *k* indicates conditions of isosolubility. For example, similar log *k* values are measured for the methanol curve around 20–30% and 50–60%. A similar trend is observed

with acetonitrile. For ethanol, we observe equal log  $k$  values between 40 and 50% and 80%. These conditions applied to extraction could then yield similar results taking into account this property of isosolubility.

From these curves, it is possible to identify different zones which may be useful in the extraction or non-extraction of chlorophyll, on the basis of the compound solubility expected from the retention models. At low percentage of modifier, chlorophyll *a* has a high retention which means low solubility in the mobile phase. For example, it is the case with CO<sub>2</sub>/EtOH 95/5 v/v. This fluid composition can therefore be used in SFE in order to obtain extracts with low chlorophyll content.

Conversely, the minimum retention represents a high solubility in the mobile phase. Lowest retention was obtained between 30 and 50% MeOH or 50–80% EtOH. Similar compositions (i.e. 30% and 70% modifier) can be applied in SFE in order to obtain chlorophyll-rich extracts. The retention curve obtained with acetonitrile is interpretable in the same manner, but because this curve is located higher than the methanol and ethanol ones, it indicates lower solubility of chlorophylls in these CO<sub>2</sub>/acetonitrile mobile phases.

A final area is observed with 100% modifier, thus in near-HPLC conditions. The only difference is the back-pressure of 10 MPa applied to the SFC system. As a result, this specific point in the curve can be related to pressurised liquid extraction (PLE).

We can see that at 100% modifier, acetonitrile is not a suited solvent for the extraction of chlorophylls, whereas pure methanol is more efficient, and pure ethanol should solubilise chlorophylls well.

In order to validate the expected compound solubility from this chromatographic model, different extractions were done with these three solvents. But first, we attempted to correlate this retention models with the Hansen solubility prediction model.

### 3.3. Correlation with Hansen solubility prediction model

Varied solubility models exist in order to theoretically determine if compounds are soluble in a given environment, based on chemical interactions. From these varied models [34], the Hansen solubility parameters (HSP), derived from the Hildebrand solubility parameters, are widely used in plant extraction [35]. This model uses three different parameters to describe the different interactions that can take place between the compounds and the extraction solvent:  $\delta_D$  for dispersion (van der Waals forces),  $\delta_P$  for polarity (related to dipole moment) and  $\delta_H$  for hydrogen bonding. Based on these parameters it is possible to organize all chemicals into a 3-dimensions space ( $\delta_D$ ;  $\delta_P$ ;  $\delta_H$ ). Mixtures of liquid solvents can also have their own HSP. It is simply calculated using the HSP values of the different components, and then pondered according to composition. Eq. (1) was used to calculate HSP values for different solvent compositions:

$$\delta_{ab} = \frac{(\delta_a \cdot \%a + \delta_b \cdot \%b)}{\%tot} \quad (1)$$

With  $\delta_{ab}$  the HSP of the mixture,  $\delta_a$  and  $\delta_b$  the HSP values of solvent *a* and solvent *b* respectively,  $\%a$  and  $\%b$  the percentages of solvent *a* and solvent *b*.

From these parameters, solubility can be determined by calculating a distance ( $R_a$ ) between a solvent (neat or mixed) and the compound to solubilise (here chlorophyll *a*). This distance is calculated using Eq. (2):

$$R_a = \sqrt{4(\delta_{D2} - \delta_{D1})^2 + (\delta_{P2} - \delta_{P1})^2 + (\delta_{H2} - \delta_{H1})^2} \quad (2)$$

With  $\delta_D$ ,  $\delta_P$  and  $\delta_H$  the HSP for component number 1 (solvent) and 2 (analyte). Considering that the lowest distance should reflect the highest compatibility between the solvent and analyte,

and vice versa, it is then possible to predict the compound solubility.

In this study, we carried out a statistical study in order to correlate the solubility parameter of solvents (for the three solvents previously used as modifier in SFC model ACN, MeOH and EtOH and their mixtures at different compositions) to the log  $k$  values of chlorophyll *a* measured in SFC and LC conditions. Moreover, to improve the relevance of this study, two more solvents (2-methyltetrahydrofuran and propylene carbonate) were added in the study. These solvents, recognised as green solvents, were chosen to extend the HSP range of tested solvents (i.e. lower  $\delta_P$  with 2-methyltetrahydrofuran and higher  $\delta_D$  with propylene carbonate). By including these solvents, the population of three Hansen parameters is well scattered, improving the applicability domain of the model. The values of log  $k_{\text{chlorophyll } a}$  for different solvent compositions were measured by HPLC and retention curves are presented in Fig. 3. For the three solvents previously studied (MeOH, EtOH and ACN), the retention with pure solvent is close to the ones obtained with 100% modifier in SFC. The curves for the mixtures between these solvents evolve quite linearly. For the two other solvents (Propylene carbonate - PC and 2-methyltetrahydrofuran - MeTHF), mixtures with PC show high retention with high PC content. It is even impossible to elute chlorophyll *a* with PC percentage above 80%. For MeTHF, the contrary is observed as this solvent yields the lowest retention of all the solvents tested.

In addition, different mixtures of methanol and acetonitrile (5/95; 10/90; 20/80 and 50/50) as modifier were used in SFC to extend HSP range.

Population distribution is plotted in supplementary figures S3 and S4 (respectively for liquid-liquid and liquid-CO<sub>2</sub> mixtures) with  $\delta_P$  in function of  $\delta_H$ .  $\delta_D$  was not plotted because this parameter was less dispersed than the others, varying only between 15.1 and 19.1, while  $\delta_P$  varied between 4.8 and 18, and  $\delta_H$  varied between 4.6 and 22.3. Due to very high retention, some curves are not complete, mainly when using propylene carbonate (PC). All the HSP for the different composition are summarised in supplementary data. Data of calculated solubility parameters for liquid mixtures are summarised in Tables S5–S11 and for liquid-CO<sub>2</sub> mixtures in Tables S12–S18.

A multilinear regression was computed using  $\delta_P$ ,  $\delta_D$ ,  $\delta_H$  as independent variables and log  $k$  values as dependent variable.

In the first place, only data using liquid mixtures are studied.

The regression on liquid mixtures yielded Eq. (3) with  $R^2 = 0.905$ :

$$\log k_{\text{chlorophyll } a} = -4.02 + 0.13 \cdot \delta_D + 0.17 \cdot \delta_P + 0.03 \cdot \delta_H \quad (3)$$

The high regression coefficient demonstrates that log  $k$  values obtained chromatographically can be correlated with theoretical solubility parameters, in this case, HSP. This result first shows that the calculation of the solubility parameters of liquid mixtures in proportion of the volume fraction seems relevant, and secondly, that the retention of chlorophyll *a* should be predictable from the HSP values. In our point of view, this validates the use of chromatographic retention to study the solubility changes of this compound in liquids. A linear regression between experimental and calculated values of log  $k$  is represented in supplementary material Figure S19.

Besides, from the coefficient values of the three solubility parameters, it is difficult to deeply understand which interactions, dispersion, dipole-dipole or hydrogen bonding play a preponderant role in the retention changes, i.e. in the solubility changes. However, the dispersion and dipole-dipole interactions, which have the highest coefficient values in Eq. (3), seem mainly involved in the retention changes of chlorophyll *a*.

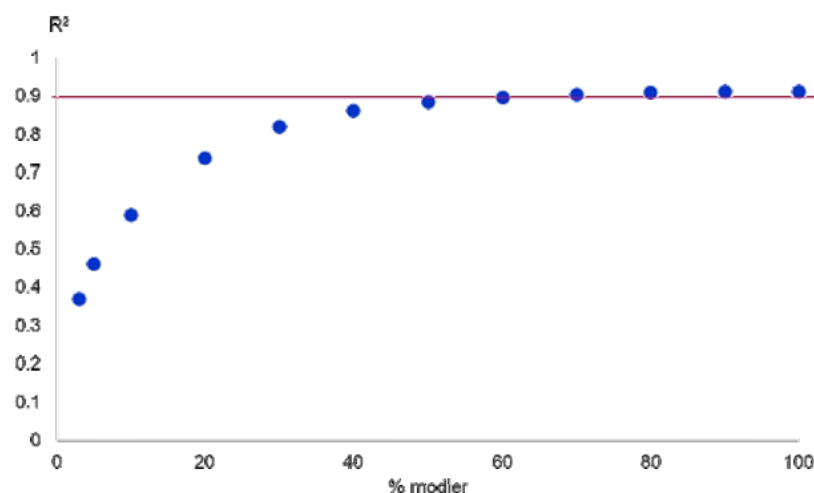


Fig. 4. Variation of the regression coefficient  $R^2$  from cumulative retention values of chlorophyll *a* measured either with pure mobile phases (100%) or added with values from mixed  $\text{CO}_2$ /liquid mobile phases.

Based on the chromatographic retention model in Eq. (3), solvents with high  $\delta P$  and  $\delta D$  would induce a poor solubility of chlorophyll *a*. It is the case for PC ( $\delta P = 18$ ,  $\delta D = 20$ ) which shows poor solubility, so high retention in chromatographic model.

The contrary is observed with MeTHF ( $\delta P = 4.8$ ;  $\delta D = 16.8$ ): low retention indicating high solubility.

The same data processing has been applied on  $\text{CO}_2$ -solvent data (log  $k$  values measured in SFC conditions). However, these parameters depend on the fluid density, they can change in function of pressure and temperature [36]. Due to the complexity of the determination of its HSP,  $\text{CO}_2$  HSP were selected for the closest condition possible (40 °C and 100 bar) [37].

Fig. 4 shows the change in the  $R^2$  regression coefficient related to the calculation of multilinear regression starting with the data of neat liquids (Eq. (3), on the right of the figure) and including progressively the data obtained with increasing  $\text{CO}_2$  percentage in the mobile phase, up to 95%  $\text{CO}_2$  (5% of modifier; on the left of the figure). For instance, it means that to obtain the  $R^2$  value for the point 90% of liquid, we added to the numerous values of retention obtained for all the pure liquids studied (data of Fig. 3), the retention values of the mobile phases composed by the mixtures 90/10 solvent/carbon dioxide. Then, each following point of Fig. 4 is obtained by adding to the previous retention data at each  $\text{CO}_2$ /modifier percentage, the retention values at the studied mixtures. Fig. 4 shows that reasonably good correlation coefficients are obtained for modifier percentage above 30% ( $R^2 > 0.8$ ). However, the correlation coefficient decreases dramatically for the lowest amounts of modifier, from 30 to 5%.

This result highlights the difficulty to calculate the HSP values for mixtures with lower amounts of modifier. This difficulty is either related to the higher compressibility of such fluids, or/and to the solubility changes in this area, which may be due to the formation of solvent clusters around the analyte. It was shown in supercritical fluid chromatography, that with low modifier percentages in carbon dioxide, a solvating sphere of modifier can surround the compounds. Thus in the immediate vicinity of the analyte (called the cybotactic region), the concentration of modifier is higher than in the bulk of the fluid [19]. Obviously, this must have a strong effect on the real solubility of the compounds, but this local environment is not easy to predict and describe with macromolecular data, such as the HSP, or any other type or polarity parameters.

Fig. 5 shows the changes in the coefficient values for the three computed Hansen parameters when the proportions of  $\text{CO}_2$  and

solvents are varied. All these coefficients decrease when adding carbon dioxide to the modifier.

This was expected from the respective values of the HSP parameters of acetonitrile, methanol, ethanol and carbon dioxide. The dispersion coefficient  $\delta D$  decreases rapidly upon addition of  $\text{CO}_2$ -solvent data, as the decrease is observed from 80% of liquid downwards. The other two are rather constant up to 40% of liquid and decrease only when the proportion of solvent is further reduced. This confirms the apparent changes in the compound solubility model below 40% of liquid mixed to carbon dioxide.

For the lowest modifier percentages, i.e. the highest carbon dioxide ones, the main Hansen parameter acting on retention/solubility is the dipole-dipole parameter. However, we have to keep in mind the very low regression coefficients obtained for these mixtures, suggesting limited interpretability of these models, which moderates this statement.

Nevertheless, almost of these results indicate reasonably good correlation between the chromatographic retention (measured from reverse phase supercritical and liquid chromatography) and the calculated HSP values either for liquid mixtures, or for rich liquid/carbon dioxide ones. It is then possible to predict solubility by choosing the right HSP: high  $\delta P$  and  $\delta D$  values should yield poor solubility, and vice versa.

Further developments could be done in the future, to back-calculate the accurate HSP values for low liquid content in carbon dioxide, based on retention values, and including more analytes with varied chemical structures.

#### 4. Extraction

First, we can notice the small amount of chlorophyll *a* extracted with acetonitrile, compared to methanol and ethanol extractions (Fig. 6). According to the retention model (Fig. 3), this solvent (mixed to carbon dioxide or used as pure liquid) yielded high chromatographic retention of chlorophyll *a*, and this is related to the low amount of chlorophyll extracted, whether in SFE or in PLE.

Between SFE points at 5, 30, and 70%, the worst extraction conditions are obtained at 5% modifier in  $\text{CO}_2$ , regardless of the modifier used. These experimental values of extracted amounts are in accordance with log  $k$  curves in Fig. 2. Indeed, log  $k$  at 5% was the highest retention point for each solvent (no measurement was possible with acetonitrile due to very high retention of chlorophylls). Conversely, SFE experiments using 30% and 70% modifier extracted a higher chlorophyll content for all modifiers. However, between

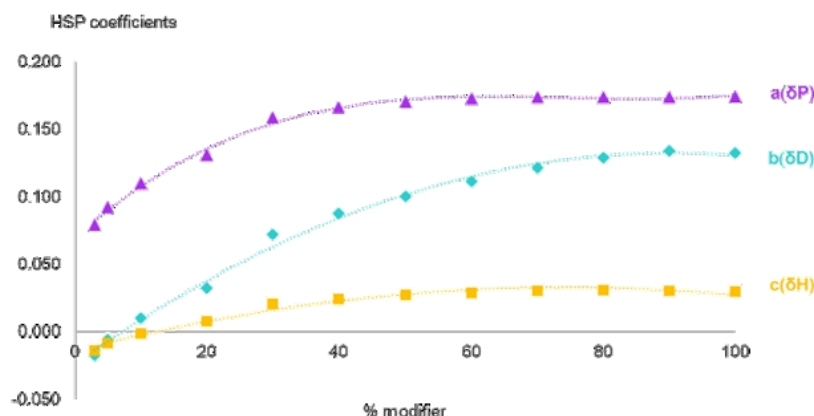


Fig. 5. Variations of the (a,b,c) HSP coefficients from the computed multilinear regression  $\log k = f(a\delta P + b\delta D + c\delta H)$ . Each point was computed from cumulative retention values of chlorophyll *a* measured with pure liquid mobile phases (100%) or progressively added with values from mixed  $\text{CO}_2$ /liquid mobile phases (see text for details).

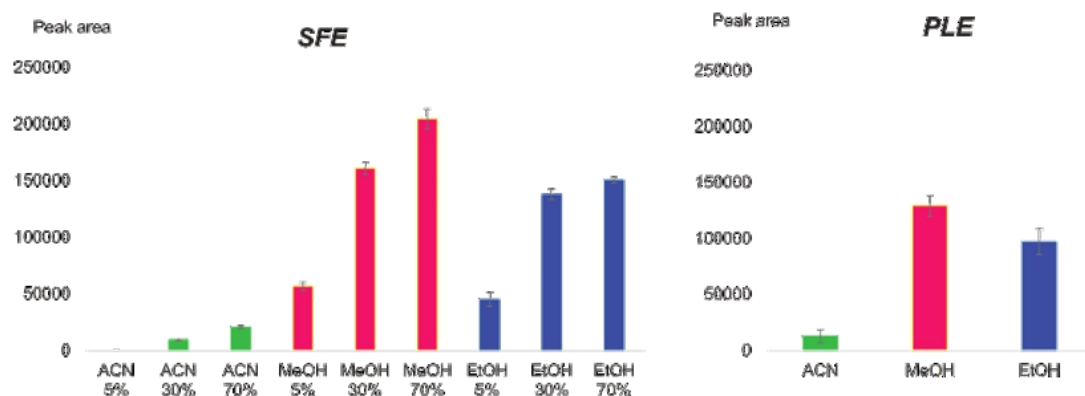


Fig. 6. Chlorophyll *a* content in rosemary extracts vs the extraction conditions both for SFE (left side) and PFE (right side), with different solvent compositions of extracting solvent.

these two points, the content extracted at 70% modifier is higher than the chlorophyll content extracted at 30% modifier for ACN, MeOH and slightly higher for EtOH. These results do not follow the retention model for acetonitrile and methanol (Fig. 2) since the measured chromatographic retention at 70% modifier was higher than the one at 30%. Differences at intermediate points could come from a lack of accuracy of the model. This may be because the model uses pure analyte molecules and then cannot predict all the intraparticle diffusional resistances (for the dried plant particles), which may occur into organelles (especially chloroplast) of plant cells, it could be some accessibility phenomena for chlorophyll compounds stored in chloroplast and the use of 70% of modifier would still be better to reach and solubilise them [38].

Besides, all PLE extracts all show lower content of extracted chlorophyll compared to SFE at both 30% and 70% for all three solvents. In that case, and whatever the intraparticle diffusivity, these results are in accordance with the retention model.

However, a difference is observed between the retention model and extraction content for methanol and ethanol. For percentages above 20% of modifier,  $\log k_{\text{ethanol}}$  was lower than  $\log k_{\text{methanol}}$ . This result, when related to the compound solubility, would mean that chlorophyll content would be lower for extractions with methanol than ethanol at 30, 70 and 100% of modifier but the contrary was observed. However, the retention model does not take into account the matrix effect, i.e. the ability to the fluid to penetrate the matrix. Fig. 7 shows the inlet pressure changes from the chromatographic measurements. For acetonitrile and methanol, the increase in pressure is regular and almost linear whereas for ethanol, inlet pressure strongly increases to reach very high values.

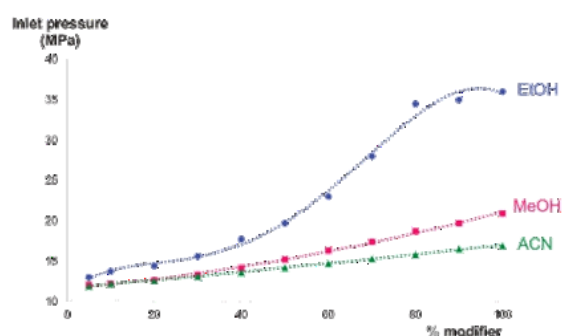


Fig. 7. Variation of the inlet pressure vs the modifier percentage in SFC system with the HSS C18 column.

Obviously, because the inlet pressure is related to the fluid viscosity, it shows the dramatic increase of viscosity with the addition of ethanol, and consequently, the decrease of both the diffusivity of the fluid, meaning a probable limitation of the fluid penetration in the dried plant, and of the diffusion coefficient of analytes, that also could limit the recovery of compounds when viscous solvent is mixed to carbon dioxide. Such behaviour, a decrease in the diffusion coefficient reducing the penetration capacity of the solvent and consequently the diminution in the extraction yield have been reported for chlorophyll in microalgae [39,40].

However, these diffusivity changes related to the viscosity variations do not explain the increased extraction recovery of chlorophylls between 30 and 70% of modifier that are observed whatever

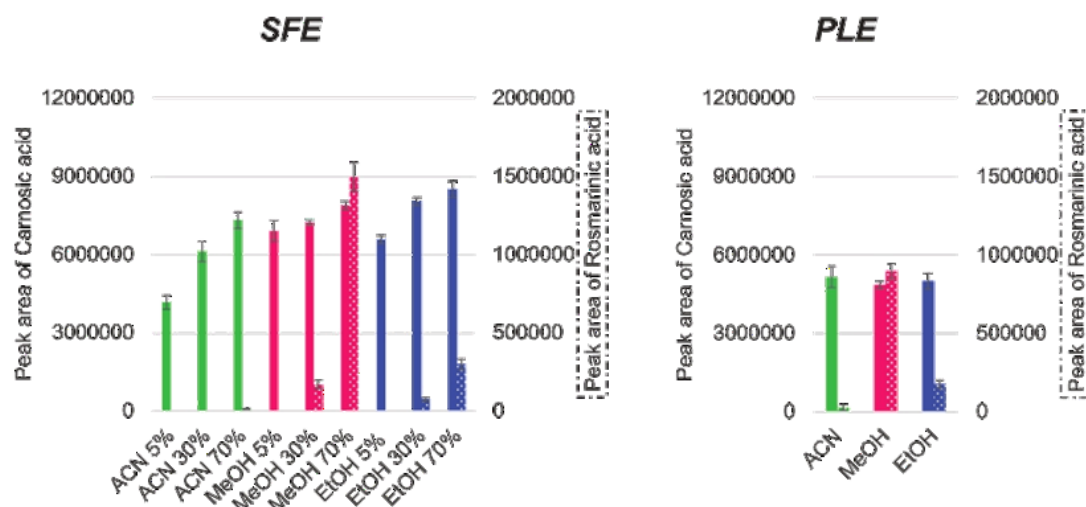


Fig. 8. Peak areas of Carnosic acid (solid colour) measured in SFC at 221 nm and rosmarinic acid (white dots) measured at 332 nm in function of extraction conditions for SFE (left side) and PFE (right side) (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

the modifier, acetonitrile, methanol or ethanol. An additional phenomenon should take place, which is neither related to the modifier nature, nor to the viscosity of the extraction solvent.

To conclude, the extraction experiments allowed us to correlate them with predicted conditions obtained by chromatographic technique. Nevertheless, even if most of the extraction results could be correlated to chromatographic observations, this study demonstrated the complexity of plant extraction and all the matrix effects that could impact the extraction. It is especially the case for conditions with low retention meaning high extraction capacity.

After focusing on model validation for chlorophyll extraction, the study was focused on the extraction of bioactive compounds of rosemary, in function of different conditions tested previously. The results are shown in Fig. 8. For any of the selective conditions for chlorophyll extraction (70% of modifier) or non-extraction (5% of modifier), carnosic acid was extracted. For the most selective SFE conditions, for instance low percentage of any modifier or with acetonitrile, the amounts of extracted carnosic acid were lower with regards to extraction conditions using higher modifier contents and/or alcohol modifiers. With SFE, extraction of carnosic acid was favoured by the use of ethanol compared to acetonitrile and methanol. However, in PLE extraction, the amount of extracted carnosic acid was similar with the three solvents. For rosmarinic acid, which is more polar compared to carnosic acid, the extraction selectivity was much higher. With the lowest modifier percentage up to 30%, no or small amounts of this compound were extracted. High methanol or ethanol proportions were required to achieve satisfactory extraction of rosmarinic acid. Whatever the ethanol amount in SFE and the extraction method (SFE and PLE), the amounts of rosmarinic acid extracted with ethanol remained lower than with methanol, which is in accordance with the higher polarity of methanol. Finally, the yield obtained for both acids from PLE remained below the best yields obtained from SFE. In that case, one can claim that it could be due to the difference of the extraction mode between PLE and SFE, as the first one was done in static conditions, whereas the second one was done in dynamic conditions.

To conclude, it is possible to extract selectively carnosic acid without chlorophyll with SFE using low modifier percentage or acetonitrile. However, due to the high polarity of rosmarinic acid, its extraction requires the use of high modifier percentage and polar solvent, also yielding the extraction of chlorophylls.

## 5. Conclusion

During this study, a SFC method was developed to predict extraction behaviour of chlorophyll *a*, and to easily find selective extraction conditions between pigments and bioactive compounds, rosmarinic and carnosic acids. This approach can be used either for liquid extraction by PLE with neat organic solvent or by SFE with mixtures of carbon dioxide and solvent in supercritical state.

Besides, correlation between the Hansen solubility parameters and the retention for chlorophyll *a* was studied. Rather satisfactory correlations were reported when using pure liquids or mixed  $\text{CO}_2$ /liquid mobile phases with modifier percentages higher than 30%. For the lower modifier contents, the use of the HSP model seems inadequate to describe the retention values, either due to erroneous calculation of the HSP values based on the volume fractions, or to inhomogeneity of such mobile phases, with significant clustering of the modifier around the analyte.

This retention model can be helpful to determine the fluid compositions allowing to reach selective extractions. For instance, the solubility of chlorophyll *a* was strongly reduced with acetonitrile, whether employed as neat solvent in PLE or mixed to  $\text{CO}_2$  in different proportions in SFE. Moreover, low modifier (5%) in carbon dioxide reduced chlorophyll solubility with regards to intermediate values of modifier (30%). However, we also observed, based on triplicate extractions with real samples, that some matrix effects can induce different results to the ones predicted from the chromatographic model, maybe due to different diffusivity of the solvents in the plant matrix. Nonetheless, selective conditions to extract carnosic acid without chlorophyll were identified thanks to this method (using 5% acetonitrile as modifier). However, it was harder to obtain selective conditions for rosmarinic acid due to its higher polarity. In order to extract it, higher modifier percentages or more polar solvents are required, which are also extracting significant amounts of chlorophyll. In addition, it was possible to extract most of the chlorophyll using 30% of ethanol as modifier in  $\text{CO}_2$ , showing that SFE can be a good alternative for more ecological extraction requiring low amounts of green solvents. The latter ( $\text{CO}_2$  and ethanol) are compatible with cosmetic regulations.

Finally, once different conditions are identified that can extract selectively the studied compounds, one of the solutions could be to use these different conditions successively on the same plant sample. Supercritical fluid extraction techniques use a dynamic extraction system which means that extraction conditions can be

changed at any time during the extraction process. This will be investigated in the future. However, this study also shown that the modelling from the chromatographic behaviour, could not take into account the various matrix effects, i.e. the fluid diffusivity into the complex samples, and the presence of other compounds in the plant.

### Declaration of Competing Interest

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

### Acknowledgements

This work was supported by the Centre-Val de Loire region, which provided the funding of the "Khloros" APR-IR project (2017).

### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461582.

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Une stratégie similaire à celle déjà présentée dans le chapitre 2 a été mise en place dans cette étude. La rétention de la chlorophylle *a* sur phase stationnaire C18, a été suivie en utilisant différents co-solvant (acétonitrile, méthanol et éthanol), et avec différents pourcentages (allant de 5 à 100% de co-solvant) en SFC (en tout cas avec l'appareillage utilisé pour la SFC, puisse qu'avec 100 de co-solvant, il s'agit alors de PLE plutôt que de SFE)). L'objectif de cette étude était de confirmer qu'une faible rétention (et donc une bonne solubilité dans la phase mobile) peut être corrélée avec une bonne condition en extraction dans des conditions similaires (en SFE et PLE pour des compositions sans CO<sub>2</sub>).

Des extractions en SFE et PLE ont confirmé les résultats obtenus en SFC et donc montré qu'il était possible de corréler des résultats en SFC avec des résultats en extraction (SFE ou PLE). Cependant des petites incohérences subsistent comme lors de l'extraction avec de l'éthanol ou méthanol qui montre des résultats contraires entre la chromatographie ( $\text{Log}k_{\text{méthanol}} < \text{log}k_{\text{éthanol}}$ ) et l'extraction (teneur en chlorophylle *a* avec éthanol > méthanol). Comme il s'agit de conditions favorables pour l'extraction de chlorophylles dans les deux cas, d'autres phénomènes liés à la matrice doivent rentrer en jeu lors de l'extraction qui ne peuvent être pris en compte à partir d'une analyse en SFC qui ne prend en compte que les variations d'interactions solutés/solvant

En parallèle de ces essais, les résultats obtenus en SFC ont été complétés par d'autres essais réalisés en CLHP avec différents solvants (ACN, MeOH, EtOH, MeTHF et PC). Toutes ces données de  $\text{log}k$  ont été corrélées (en utilisant une régression linéaire) aux paramètres d'Hansen ( $\delta P$ ,  $\delta D$  et  $\delta H$ ) à partir de toutes les compositions de phase mobile. Les résultats ont montré qu'il y avait une bonne corrélation entre les  $\text{log}k$  obtenus en CLHP et les paramètres de Hansen. Concernant ceux obtenues en SFC, les résultats sont corrélés mais pour de faibles teneurs en CO<sub>2</sub> (inférieures à 20%). Cela pourrait s'expliquer par une erreur de calcul des paramètres de Hansen car la fraction molaire du CO<sub>2</sub> supercritique, qui peut varier facilement en fonction des paramètres appliqués, n'a pas été prise en compte.

## **4. CONCLUSION**

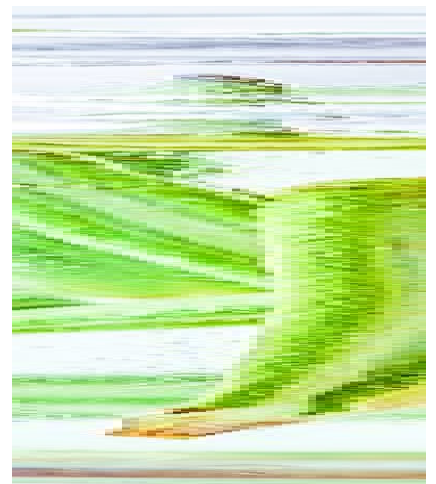
Ce chapitre a permis de présenter le romarin qui a été choisi comme plante afin de réaliser des conditions sélectives. A la suite de cela, un modèle a pu être développé afin de trouver à partir d'analyses en chromatographie des conditions d'extraction sélective.

Ce dernier article a permis de confirmer que des conditions d'extraction sélective pouvaient être obtenues par suivi chromatographique en milieu supercritique ou liquide. Il a donc été déterminé des conditions d'extraction sélective permettant d'extraire l'acide carnosique sans ou avec peu de chlorophylles, notamment en utilisant l'acétonitrile comme co-solvant.

Une partie de ces conclusions a donc été appliquée lors d'une extraction SFE afin de réaliser une extraction sélective des composés bioactifs et des pigments du romarin de façon séquencée. Les résultats sont présentés dans le prochain chapitre.



# CHAPITRE 4: Extraction sélective du romarin





## **1. INTRODUCTION**

Le précédent chapitre a permis d'identifier des conditions d'extraction sélective du romarin afin d'avoir d'un côté l'acide carnosique et d'un autre côté, les chlorophylles. Les travaux présentés dans ce chapitre visant à développer et optimiser un procédé d'extraction sélective s'appuient donc des précédents résultats obtenus.

Ces conditions utilisent différents solvants : du dioxyde de carbone, de l'acétonitrile et de l'éthanol. Dans le cas d'une transposition industrielle, il est important voire même impératif, d'utiliser des conditions compatibles avec les réglementations en vigueur. En cosmétique, l'utilisation de solvant vert pour l'extraction de molécules bioactives est vivement conseillée. L'acétonitrile n'est pas considéré comme un solvant vert. Dans ce chapitre, une alternative à l'utilisation de ce solvant a donc été envisagée. De par sa polarité, l'eau est un mauvais solvant pour l'extraction de chlorophylles. De plus, il a été vu qu'il améliore l'extraction de l'acide rosmarinique. L'optimisation des conditions d'extraction en utilisant un co-solvant composé d'éthanol et d'eau a donc été réalisée.

Lors de l'état de l'art sur l'extraction sélective du chapitre 1, un facteur important y est présenté. Il s'agit de la cinétique d'extraction. Dans le développement de ce procédé, les cinétiques d'extraction de différents composés seront donc prises en compte et utilisées afin d'optimiser l'extraction. Pour ce faire, le procédé d'extraction supercritique a été couplé en ligne avec un système de chromatographique par fluide supercritique. L'analyse régulière de la composition de l'extrait au cours de l'extraction permet d'avoir la quantité en différents biomarqueurs en fonction du temps et ainsi identifier si l'extraction est en train de se terminer ou non.

## **2. EXTRACTION SELECTIVE DU ROMARIN PAR SFE-SFC**

Ce chapitre de la thèse se focalise sur l'extraction sélective du romarin. Les points abordés sont le développement et l'optimisation du procédé d'extraction. Une fois celui-ci terminé, une analyse des différents extraits a été menée afin d'avoir une composition plus détaillée en métabolites.



Contents lists available at ScienceDirect

Journal of Chromatography A

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# Sequential extraction of carnosic acid, rosmarinic acid and pigments (carotenoids and chlorophylls) from Rosemary by online supercritical fluid extraction-supercritical fluid chromatography

Thibault Lefebvre, Emilie Destandau, Eric Lesellier\*

ICOA, UMR 7311, Université d'Orléans, rue de Chartres, BP 45067 Orléans, France

## ARTICLE INFO

## Article history:

Received 30 August 2020

Revised 6 November 2020

Accepted 9 November 2020

Available online xxx

## ABSTRACT

A high degree of selectivity is required during the plant extraction process in order to obtain extracts enriched in specific compounds or to avoid the extraction of unwanted ones. Rosemary is well known for its antioxidant compounds (carnosic acid, carnosol and rosmarinic acid). The plant also contains pigments (i.e. carotenoids, chlorophylls) which may cause a colour problem during the use of the extract in cosmetic formulations, for example. Supercritical fluid extraction is considered as a selective technique for plant extraction. Due to the physico-chemical properties of supercritical fluids, related to pressure, temperature and modifier addition, it is possible to carry out sequential extraction with successive conditions to collect different fractions that are rich either in pigments or in bioactive compounds.

The aim of this study was to selectively extract bioactive compounds (i.e. carnosic acid and rosmarinic acid) and pigments (carotenoids and chlorophylls) from rosemary using supercritical fluid extraction. The optimisation of the extraction method was carried out using supercritical fluid extraction online coupled with a supercritical fluid chromatography (SFE-SFC) system. Two columns of different polarities were coupled to achieve the separation of the targeted compounds every five minutes during the extraction.

Four fractions were obtained: a first one rich in carotenoids obtained with pure CO<sub>2</sub> (25°C and 20 MPa), a second rich in carnosic acid obtained with 3% polar modifier (EtOH:water 50/50 v/v), a third fraction rich in rosmarinic acid using 10% of the same modifier and a fourth fraction rich in chlorophylls with 30% of ethanol as modifier. These four samples were then analysed by UHPLC-DAD-ESI-QTOF-HRMS in order to identify other extracted compounds and to study how the selected conditions impacted their extraction.

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## 1. Introduction

Different parameters can be studied during the optimization of plant extraction. The main one is the extraction yield (extracted mass vs sample mass). However, this parameter is not specific to any particular compound. In some cases it can be useful to take other parameters into account to study the extraction selectivity, in order to obtain pure compounds [1–3], extract enriched in the targeted compounds, or to avoid the extraction of particular ones. For example, Reverchon et al. [4] used supercritical fluid extraction (SFE) with three different carbon dioxide densities in order to obtain a fractionated jasmine concrete without fatty acid esters. Morsy et al. [5] also improved the production of fatty acid-

free thymol-rich extracts at low temperature by using two different back-pressure conditions (10.4 and 16.7 MPa). Guedes et al. [6] studied the influence of temperature, pressure and ethanol percentage as modifier on the selectivity of carotenoid extraction over chlorophyll from *Cenodesmus obliquus*. The highest carotenoid to chlorophyll ratio was reached at 25 MPa and 60°C with pure carbon dioxide.

These three studies [4–6] highlighted the advantages of using supercritical fluid extraction in order to improve selectivity. First of all, supercritical carbon dioxide (Sc-CO<sub>2</sub>) can be tailored during extraction by modifying pressure, temperature (which induces density changes) and modifier (which modulates its polarity). Secondly, the technological configuration of extractor devices allows the application of different conditions and the collection of the different extracted fractions on the same plant sample [4,7–11]. These sequential extraction conditions are used to extract different compounds selectively by changing the CO<sub>2</sub> density or the modifier used.

\* Corresponding author. Institut de Chimie Organique et Analytique, (ICOA), rue de Chartres, 45067 Orléans, France. Phone: 33 2 28 49 45 88; Fax: 33 2 38 41 72 81.

E-mail address: [eric.lesellier@univ-orleans.fr](mailto:eric.lesellier@univ-orleans.fr) (E. Lesellier).

Rosemary (*Rosemarinus officinalis* Lamiaceae) is known for its content in antioxidant compounds such as rosmarinic acid, carnosic acid and carnosol [12,13]. Other biological activities can be attributed to these compounds such as astringent, anti-inflammatory, antibacterial and antiviral properties [14]. It has been reported that carnosic acid can degrade into carnosol [15,16]. This degradation has been studied in methanol and ethanol. Carnosic acid undergoes different reactions which transform it into quinone derivative and then into carnosol [16]. Rosmarinic acid seems to be more stable in similar conditions [16]. However, the extraction of these antioxidant compounds leads to the simultaneous extraction of the pigments in the aerial part, giving green extracts that are difficult to valorize (i.e. for cosmetic raw materials which must contain the lowest possible amount of chlorophyll pigments).

The aim of this study was to selectively extract bioactive compounds (carnosic and rosmarinic acids) and pigments (carotenoids and chlorophylls) from rosemary. Extracting these compounds separately may have several advantages. As the biological properties mainly come from carnosic acid, carnosol and rosmarinic acid, rosemary extracts are used in cosmetic formulations and pigments can interfere in the formulation process. This is especially the case for chlorophylls which may degrade into other forms (i.e. pheophytines) [17]. Because these products have different absorbance spectra, the colour of the final product can change over time. Furthermore, it can be useful to extract pigments separately to valorise them for other applications such as dyes.

In order to optimize our experimental conditions for selective extraction, it was advantageous to be able to analyze the extract online in order to reduce any risk of sample degradation, but also for the sake of convenience and time saving. Abrahamsson et al. [18] developed an online SFE/UV-Vis/ELSD device to monitor the extraction of carotenoids, chlorophyll *a*, ergosterol and total lipids from microalgae. Because of the overlap of the different UV-Vis spectra, it was necessary to use models (i.e. multivariate curve resolution by alternating least squares) to decompose the signal and thus obtain quantitative data for each of the compounds. It is also possible to couple chromatography (i.e. SFC) to an extraction instrument (i.e. SFE) to monitor solutes more selectively [19,20] (see Sánchez-Camargo et al. [21] for a review on coupling SFE with chromatographic techniques). We therefore used an online SFE-SFC system to meet our goal.

The study comprised the following stages. After developing the online system, the different extraction steps were optimised in order to obtain pigment- and bioactive-rich extracts. Three compounds were monitored to optimise the process: carnosic acid, rosmarinic acid and chlorophyll *a*. Finally, the enriched extracts were analysed and characterised by UHPLC-HRMS in order to study other predominant extracted compounds.

## 2. Material and methods

### 2.1. Solvents and chemicals

For all this study, the solvents used were methanol and ethanol, gradient grade (VWR International, Fontenay sous bois, France), and acetonitrile, gradient grade (Carlo Erba, Val de Reuil, France). Carbon dioxide (>99.5%) was from Air Liquide (Fleury-les-Aubrais, France). The water used was supplied by an Elga Purelab Flex station from Veolia (Antony, France). Formic acid was Normapur grade (VWR International, Fontenay sous bois, France).

In order to develop the analytical method, different standards were used. Carnosic and rosmarinic acid standards were supplied by Extrasynthèse (Genay, France). Chlorophyll *a* was obtained from ivy extract using standard conditions (protocol described in Section 2.2).

The dispersant used was diatomaceous earth (DE) from Fisher Scientific (Illkirch, France), general-purpose grade. No adsorption of the studied compounds was observed from experiments conducted with various sample/diatomaceous earth ratios (results not shown).

### 2.2. Plants and extraction methods

Two plants were used. Ivy leaves (*Hedera canariensis* Willd) for the production of a chlorophyll-rich extract were collected in Orleans (France). Rosemary leaves (*Rosemarinus officinalis*) for the study of the selective extraction of carnosic acid, rosmarinic acid and chlorophyll *a* were provided by one of our industrial partners, Alban Muller (Fontenay-sur-Eure, France) specialised in plant extraction.

Ivy was harvested and then dried using a Memmert Modell 500 dryer, at 50°C for 24 hours. The dried leaves were then ground using a Polymix PX-MFC 90 D grinder from Kinematica (Coueron, France). A previous study confirmed that the main chlorophyll pigments are chlorophylls *a* and *b* [17].

The production of ivy extract (used for the development of the analytical method) and the rosemary reference extract was obtained with a Branson 3510 ultrasonic bath. Both extracts were made using 1g of the plant and 15mL of ethanol. The mixture was then sonicated for 60 min. The extracts were centrifuged at 7000 rpm at 20°C for 10 min using a BR4i multifunction centrifuge (Thermo-electron, Nantes, France). The extracts were used no longer than one week in order to keep the extracts with the lowest amount of degradation products [17].

An online SFE-SFC device was developed in this study. A Waters MV-10 ASFE was used for supercritical fluid extraction. A UV detector was added between the oven and the BPR module in order to monitor the extraction kinetic. The detector was a Gilson 151 UV / VIS Multiwavelength Detector (GILSON, Villiers-le-Bel, France). This extractor was coupled with the JASCO system used in [22].

For all the extractions, extraction cells were prepared using 1g of plant mixed with 1g of dispersant (DE). Cotton was also used in order to fill the cell completely at the top and bottom. The cell used was a 5-mL extraction cell.

### 2.3. Sequential extraction

A four-step extraction was developed in order to extract different fractions from rosemary leaves. The first step using pure CO<sub>2</sub> at 25°C, 20 MPa during 20 min at 3.0 mL/min, extracted the majority of carotenoids. The second step using 3% of modifier (EtOH:Water 50/50 v/v) at 25°C, 10 MPa during 40 min at 3.0 mL/min, extracted the majority of carnosic acid. The third step using 10% of the same modifier at 25°C, 10 MPa during 50 min at 3.0 mL/min, extracted the majority of rosmarinic acid. The fourth and last step using 30% of modifier (EtOH) at 25°C, 10 MPa during 30 min at 3.0 mL/min, extracted chlorophylls.

The first three steps required the use of a make-up solvent in order to avoid precipitation right after the BPR module. 0.3 mL/min of EtOH was used for the first step and 0.3 mL/min of EtOH:Water 50/50 v/v for the second and third steps.

### 2.4. Analytical method development

In this study, two analytical methods were developed. For both methods, detection was performed using a PDA detector set at specific wavelengths: 221 nm to analyse carnosic acid, 332 nm for rosmarinic acid, 450 nm for carotenoids and 650 nm for chlorophyll *a*.

The first method was developed in order to evaluate the content in different bioactives of rosemary extracts. The method

was optimised on a UHPLC Ultimate 3000 Thermo Fisher system (Darmstadt, Germany). Major peaks were characterised using a maXis UHR-Q-TOF mass spectrometer (Bruker, Bremen, Germany). The column used was a Luna Omega Polar C18 (1.6  $\mu$ m, 100 x 2.1 mm). This RP-HPLC method uses two phases: (A) Water with 0.1% Formic acid and (B) Acetonitrile with 0.1% Formic acid. The following elution method was optimised in order to separate the maximum number of compounds: initially 15% of (B), then in 10 minutes to 100% of (B), maintained at 100% for 4 min, and finally equilibration at 15% during 2 min. The injection volume was 2  $\mu$ L, the flow rate 0.7 mL/min and the column temperature was set at 50°C. The use of this temperature, which decreases the mobile phase viscosity and compound solubility, allows a higher flow rate and hence a faster elution. The compounds were characterized by high resolution mass spectrometry using the following parameters: an ESI source working in positive and negative ionization mode, drying gas flow 9 L/min at 200°C, and nebulizing gas pressure 2 bar. The capillary voltage was set at 4.5 kV. This method is introduced in Section 3.1.

The second method is introduced in Section 3.2 below (Development of online SFE-SFC). It is a 5-minute method by SFC (coupled with SFE) in order to monitor the extraction kinetics of carnosic acid, rosmarinic acid and chlorophyll *a*. A JASCO SFC system was used (described in [22]). At the beginning of the development, the following initial conditions were used: Temperature: 25°C, Pressure: 10 MPa, Modifier percentage: 20%, Modifier: methanol/water 95/5 v/v, Injection volume: 5  $\mu$ L, Flow rate: 3.0 mL/min. Throughout the development, only isocratic conditions were applied. Different columns were tested: HSS C18 (5  $\mu$ m, 4.6 x 150 mm, Waters, St Quentin en Yvelines, France), Cosmosil Cholesterol (5  $\mu$ m, 4.6 x 250 mm, imChem, Voisins Le Bretonneux, France), BEH Xbridge C18 (5  $\mu$ m, 4.6 x 250 mm, Waters, St Quentin en Yvelines, France), Hypersil Gold silica (1.9  $\mu$ m, 3.0 x 100 mm, Darmstadt, Germany) and Viridis Silica (5  $\mu$ m, 4.6 x 250 mm, Waters, St Quentin en Yvelines, France). Two columns were selected and then coupled: Viridis Silica coupled with HSS C18. The final conditions were: Column oven temperature: 25°C; Pressure: 20 MPa; Modifier percentage: 23%.

### 3. Results and discussion

#### 3.1. UHPLC method development

##### 3.1.1. Chromatographic optimisation

In order to analyse rosemary extracts and evaluate their contents in different bioactive compounds, a UHPLC-DAD method was developed. Mixtures of acidified water and acetonitrile as chromatographic mobile phase were used in this method. Different gradients were tested in order both to separate the maximum number of compounds and to shorten the analysis time.

Various chromatographic profiles can be obtained by detecting at different wavelengths. Four profiles were acquired: at 221 nm in order to analyse and quantify carnosic acid, at 332 nm for rosmarinic acid, at 450 nm for carotenoids and at 650 nm for chlorophyllic compounds. During elution optimisation, the profile obtained at 221 nm was used to select the best separation conditions. Different gradients were tested with different starting points. The gradient time remained identical (from the initial percentage to 100% of acetonitrile in 10 min).

Fig. 1 shows different chromatographic profiles and their associated gradients (starting from 10, 15 and 20% of acidified acetonitrile). Increasing the initial percentage allowed faster elution, mainly for the first compounds eluted. However, at 20% the peaks eluted between 1 and 2 minutes appeared to be broader than the ones eluted afterwards. This phenomenon can be explained by a difference in viscosity between the mobile phase (in the present

case composed of Water:Acetonitrile 80/20 v/v at the beginning of the gradient) and the sample solvent, ethanol (the solvent used for the extraction of rosemary). Due to viscosity differences, the lack of diffusivity of the sample compounds into the mobile phase may reduce the equilibrium of the compounds between the different phases, leading to broader peaks. Reducing the elution of the first compounds by decreasing the initial acetonitrile content of the gradient helped to reduce this problem of diffusivity (equilibrium). This effect can also be decreased by changing the sample solvent. For example, it is possible to dilute rosemary extract with a solvent close to the mobile phase. However, this can lead to the non-solubility of less polar compounds in a water-rich solvent.

The carnosic acid peak was the most abundant peak in the profile at 221 nm. Its shape shows detection saturation. In order to improve the carnosic acid peak, the rosemary "standard" extract was diluted by a factor of two with acetonitrile before injection. This dilution was then applied to all the samples analysed with this method. An initial percentage of 15% acetonitrile was selected as the elution method.

##### 3.1.2. HRMS characterisation

This UHPLC method was applied on the same instrument but coupled with a maXis UHR-Q-TOF mass spectrometer. The most abundant compounds observed in UV for rosemary extract were identified according to the literature [23–25]. Table 1 summarizes all the characterisation data for 12 compounds. The compound numbers are also displayed in Fig. 2 in order to locate them on final chromatographic profiles.

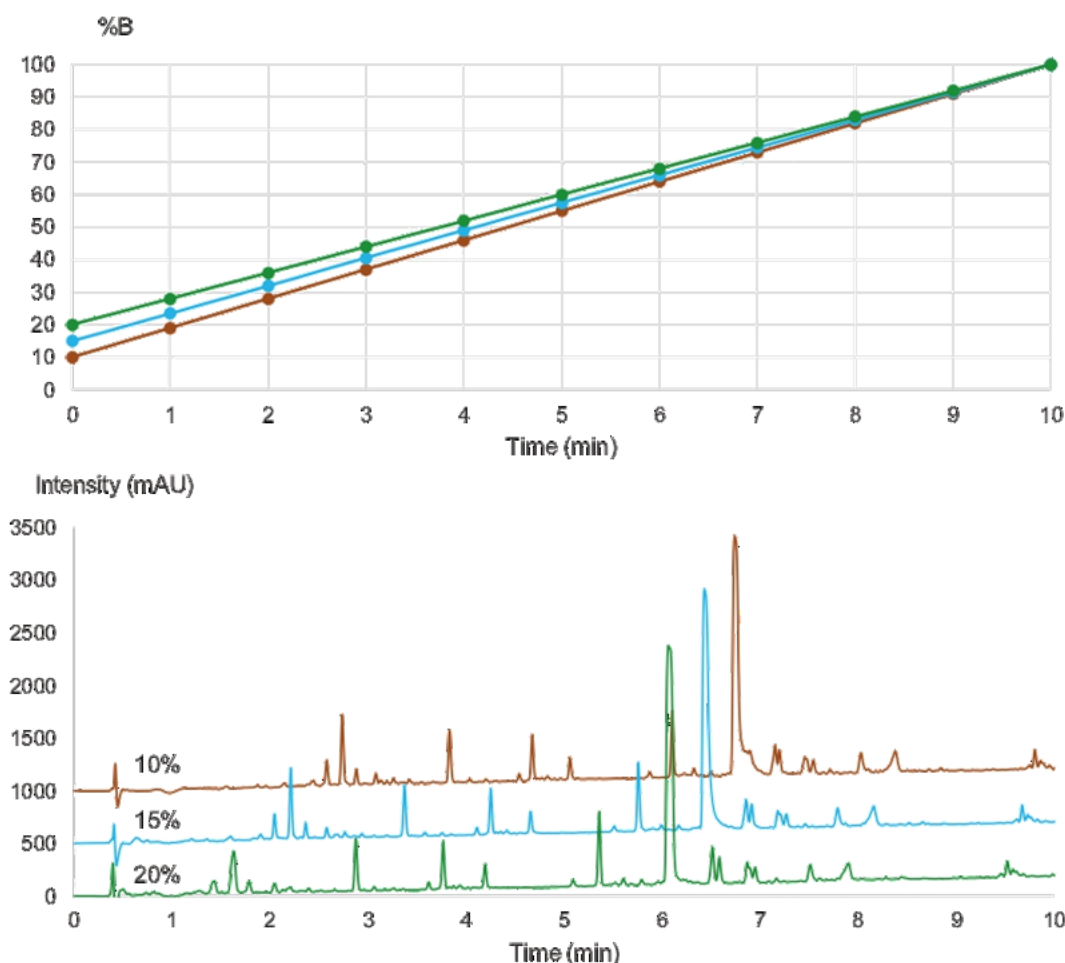
Two groups can be identified. The first one, with the most polar compounds, was composed of glycosylated flavonoids (Isorhamnetin-3-O-hexoside - n°1, Hispidulin-7-glucoside - n°3) and polyphenol (Rosmarinic acid - n°2). The second, less polar, group was composed of methyl ether flavonoids (Dihydroxydimethoxyflavone and Genkwanin), a less polar rosmarinic acid derivative (Rosmaquinone) and diterpene phenols (Carnosol - n°7 and Carnosic acid - n°8). Retention times were in line with the molecules' polarities in RP-HPLC: The more polar compounds were analysed faster than the less polar ones.

Mass detection parameters and negative ionization were chosen in order to characterise phenolic and phenolic diterpene compounds, but the chlorophylls (peaks 11 and 12) were detected through the analysis of protonated molecule ions  $[M+H]^+$ . However, the ionization mode used here (electrospray ionization) induced a higher degree of error in the mass results, going for instance from 907.52 for the theoretical mass to 970.53 for the measured one (Table 1). Nonetheless, both the visible spectra with a maximum of absorption at 650 nm, and our previous studies on retention of this chlorophyllic pigments [17,22], supported the identification of these native pigments, i.e. chlorophyll *a* and *b*. One carotenoid (n°9) around 10 min was detected thanks to its UV spectrum. However, it could not be characterised with these mass spectrometry conditions (ESI). According to the literature [26],  $\beta$ -carotene is the most abundant carotenoid in rosemary. An unidentified peak eluted around 11 min at 450 nm and 650 nm was not characterised but it is part of the chlorophyll family (similar UV spectra). This RP-UHPLC method was used during extraction optimisation (especially the carotenoid recovery step) and analysis of the rosemary final fractions obtained for each SFE selective condition.

#### 3.2. Development of online SFE-SFC

##### 3.2.1. SFC method development

The objective of this part was to develop a chromatographic method for the analysis of chlorophyll *a*, carnosic acid and



**Fig. 1.** UHPLC-DAD chromatograms of "standard" rosemary extract at  $\lambda = 221$  nm (bottom chart) with various elution starting points (upper chart with %A water + 0.1% FA and %B acetonitrile + 0.1% FA): 10% (brown); 15% (blue); 20% (green). Column: Luna Omega Polar (1.6  $\mu$ m, 100  $\times$  2.1 mm). For other analytical conditions, see the experimental section.

**Table 1**

Peak assignments of "standard" Rosemary extracts identified using UHPLC-DAD-ESI-QTOF-HRMS and wavelength used for their quantification in different samples.

Compound number	RT (min)	Measured $m/z$ [M-H] <sup>-</sup>	Ion Formula	Theoretical $m/z$ [M-H] <sup>-</sup>	Mass difference [ppm]	Name	Quantification wavelengths (nm)
1	2.07	477.103583	C <sub>22</sub> H <sub>21</sub> O <sub>12</sub>	477.10385	0.6	Isorhamnetin-3-o-hexoside	332
2	2.24	359.077028	C <sub>18</sub> H <sub>13</sub> O <sub>8</sub>	359.077241	0.6	Rosmarinic acid	332
3	2.39	461.108674	C <sub>22</sub> H <sub>21</sub> O <sub>11</sub>	461.108935	0.6	Hispidulin-7-glucoside	332
4	4.29	313.071483	C <sub>17</sub> H <sub>13</sub> O <sub>6</sub>	313.071762	0.9	Dihydroxy-dimethoxyflavone	332
5	4.71	283.060973	C <sub>16</sub> H <sub>11</sub> O <sub>5</sub>	283.061197	0.8	Genkwanin	332
6	5.58	343.155264	C <sub>20</sub> H <sub>23</sub> O <sub>5</sub>	343.155097	-0.5	Rosmaquinone	332
7	5.79	329.175713	C <sub>20</sub> H <sub>23</sub> O <sub>4</sub>	329.175833	0.4	Carnosol	221
8	6.46	331.191033	C <sub>20</sub> H <sub>27</sub> O <sub>4</sub>	331.191483	1.4	Carnosic acid	221
9	9.93					Carotenoid	450
Compound number	RT (min)	Measured $m/z$ [M+H] <sup>+</sup>	Ion Formula	Theoretical $m/z$ [M+H] <sup>+</sup>	Mass difference [ppm]	Name	Quantification wavelengths (nm)
10	11.09					Chlorophyll derivative	
11	12.59	907.534909		907.521854	-14.4	Chlorophyll b	650
12	13.38	893.554631	C <sub>35</sub> H <sub>71</sub> MgN <sub>4</sub> O <sub>6</sub>	893.54259	-13.5	Chlorophyll a	650

rosmarinic acid compatible with SFE-SFC coupling. The study of carnosol was not selected as, from Fig. 1, this compound was found in small amounts in the rosemary extract. The main constraint on the development of this method is the analysis time. In order to follow the extraction kinetic, it is necessary to analyse as many fractions as possible to obtain the most accurate kinetic curves. It

is therefore interesting to use the fastest separation method. This method was developed using isocratic conditions in order to avoid time-consuming equilibration. Based on previous work [27], the modifier used was MeOH:Water 95/5 v/v. Methanol is a classical solvent used in SFC and the addition of water helps improve the shape of the rosmarinic acid peak.

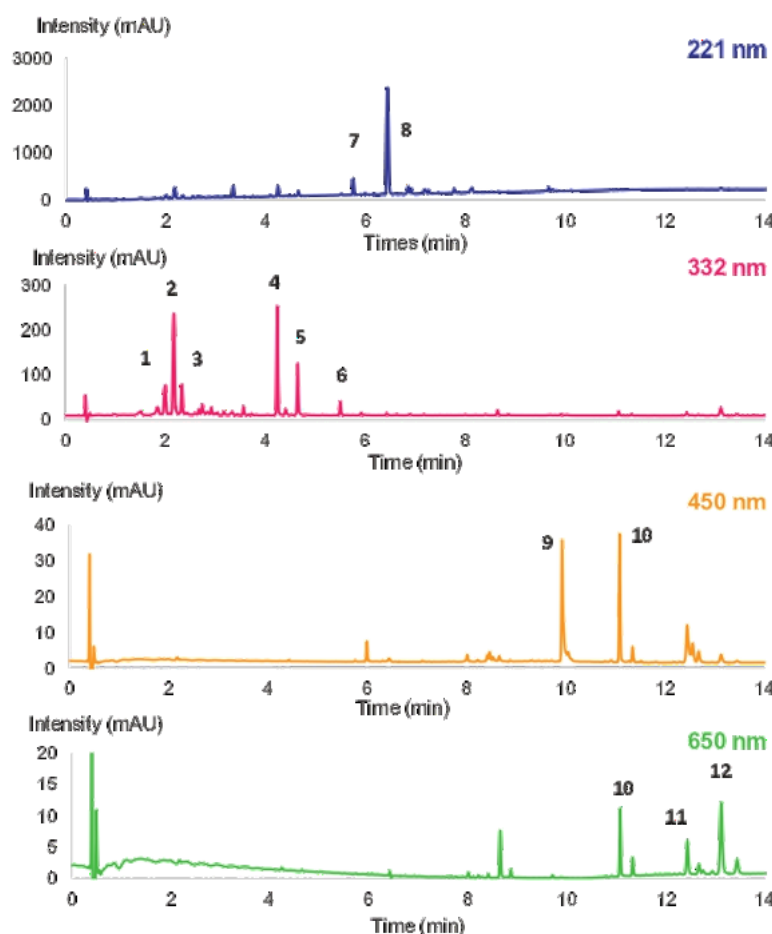


Fig. 2. UHPLC-DAD chromatograms of "standard" rosemary extracts at different wavelengths (221 nm – blue, 332 nm – pink, 450 nm – orange, 650 nm – green) and identified compounds mentioned in Table 1 labelled from 1 to 11.

The first step was to screen and select the stationary phase. However, chlorophyll *a*, rosmarinic acid and carnolic acid are very different compounds in terms of polarity ( $\text{Log } P_{\text{Carnolic acid}} = 5.14$ ,  $\text{Log } P_{\text{Rosmarinic acid}} = 1.82$  and  $\text{Log } P_{\text{Chlorophylls}} > 8$ ). Hence, it is difficult to find a stationary phase which can interact with all of them without the use of an elution gradient. However, unlike RP-HPLC, all kinds of stationary phases, from classical octadecyl bonded silica to pure silica, are suitable with SFC using the same mobile phase. Different columns were tested with the initial conditions (3 mL/min, 20% modifier, 10 MPa and 25°C). Results showed that no column was able to analyse these 3 compounds under the same analytical conditions.

On the one hand, non-polar phases such as HSS C18 or Cholesterol phase separated chlorophyll (*a* and *b*) and carnolic acid but not rosmarinic acid. In addition, Cholesterol phase displayed too high a retention for chlorophyll compounds. On the other hand, polar phases such as silica strongly retained rosmarinic acid but not chlorophylls and carnolic acid. Both BEH and Hypersil Gold Silica showed a broad peak for rosmarinic acid while the Viridis silica phase gave the best results. Consequently, both kinds of stationary phase (polar and non-polar) needed to be used to achieve the separation of all the studied compounds. Two columns were thus selected, the HSS C18 and the Viridis Silica, connected end-to-end.

When using a two-column isocratic system in HPLC, the order has no influence on the separation. However, in SFC, there is a pressure gradient from the back pressure regulator to the pumps. This gradient has a direct influence on the density of supercritical

$\text{CO}_2$ , which is a compressible fluid. The density will be higher in the first column than in the second column, because the pressure (and the fluid density) is higher at the entrance of the column located first. This means that the time spent in the column for different compounds will be shorter when the column is set in the first position. Both positions were tested and the best results were obtained using HSS C18 in the first position and the Viridis Silica in the second.

Using supercritical fluid allows us to use other parameters such as pressure and temperature in order to optimise separation. Increasing the pressure will increase the density of the supercritical fluid and thus increase the interaction between analytes and mobile phase. The difference between 10 and 20 MPa was significant for rosmarinic acid: the retention time was reduced from 7.5 min at 10 MPa to 5.1 min at 20 MPa. Carnolic acid and chlorophyll *a* were only slightly impacted. 20 MPa was therefore selected for the following steps. Varying the temperature did not show any significant improvement; a temperature of 25°C was therefore retained.

In order to reduce the analysis time, the modifier percentage was increased. However, it is necessary to keep carnolic acid and chlorophyll *a* separated (using 20% modifier,  $t_{\text{Carnolic acid}} = 2.2$  min and  $t_{\text{Chlorophyll } a} = 3.0$  min). Chlorophylls absorb at around 650 nm and 415 nm, but also a little at lower wavelengths and especially at 221 nm. It is thus important to keep them separated from carnolic acid to avoid inducing errors during the quantification of the phenolic terpene compound. The best compromise between separation and analysis time was obtained with 23% of modifier.

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T. Lefebvre, E. Destandon and E. Lesellier

Journal of Chromatography A xxx (xxxx) xxx

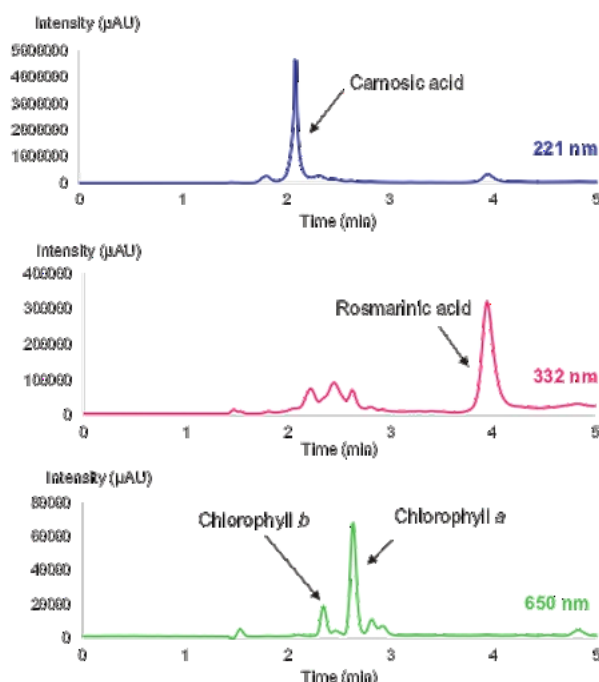


Fig. 3. SFC-DAD chromatograms of "standard" rosemary extract using HSS C18 and Viridis Silica columns with methanol/water 95/5 v/v as modifier at 221 nm; 332 nm and 650 nm. For other analytical conditions, see the experimental section.

All these conditions (pressure, modifier and modifier percentage) offered the best compromise between separation of the different compounds and an analytical time below five minutes, which is required for the online study. Fig. 3 shows the chromatograms

obtained with the optimised parameters for the analysis of a rosemary extract by SFC.

### 3.2.2. Development of the SFE-SFC online system

Usually in SFE, the supercritical fluid goes through the extraction cell, solubilises solutes from the plant sample, continues its way to the BPR module and finally, the extract is collected after depressurisation. In order to change the density of supercritical carbon dioxide ( $\text{Sc-CO}_2$ ), pressure and temperature can be used. Another solvent (called modifier) can be added to  $\text{Sc-CO}_2$  to further modulate its polarity. When the extract reaches the BPR module,  $\text{Sc-CO}_2$  vaporises due to the change in pressure (atmospheric pressure). The extract is then solubilised in the modifier, which remains in a liquid state. However, when no or only a low amount of modifier is used, another solvent needs to be added after the extraction cell and before the BPR module in order to avoid precipitation of the extract at atmospheric pressure (Fig. 4).

A Waters MV-10 ASFE was used for supercritical fluid extraction. A UV detector was added between the oven and the BPR module in order to monitor the extraction kinetic. It was set at 450 nm in order to evaluate the compounds that absorb at this wavelength (mainly carotenoid pigments). This monitoring is complementary to the online SFC devoted to the analysis of rosmarinic, carnosic acids and chlorophylls.

To monitor the extract composition during the extraction process, the SFE system was modified in order to circulate a non-depressurized extract through an injection loop connected to a six-port switching valve configuration (Fig. 4). This switching valve can be considered as an SFC injector with two different positions: "Load" and "Inject" positions. When this valve changes position, extract present in the injection loop is then diffused in the SFC mobile phase flow which passes through the switching valve as well. Fig. 4 shows the SFE-SFC online system with the two six-port

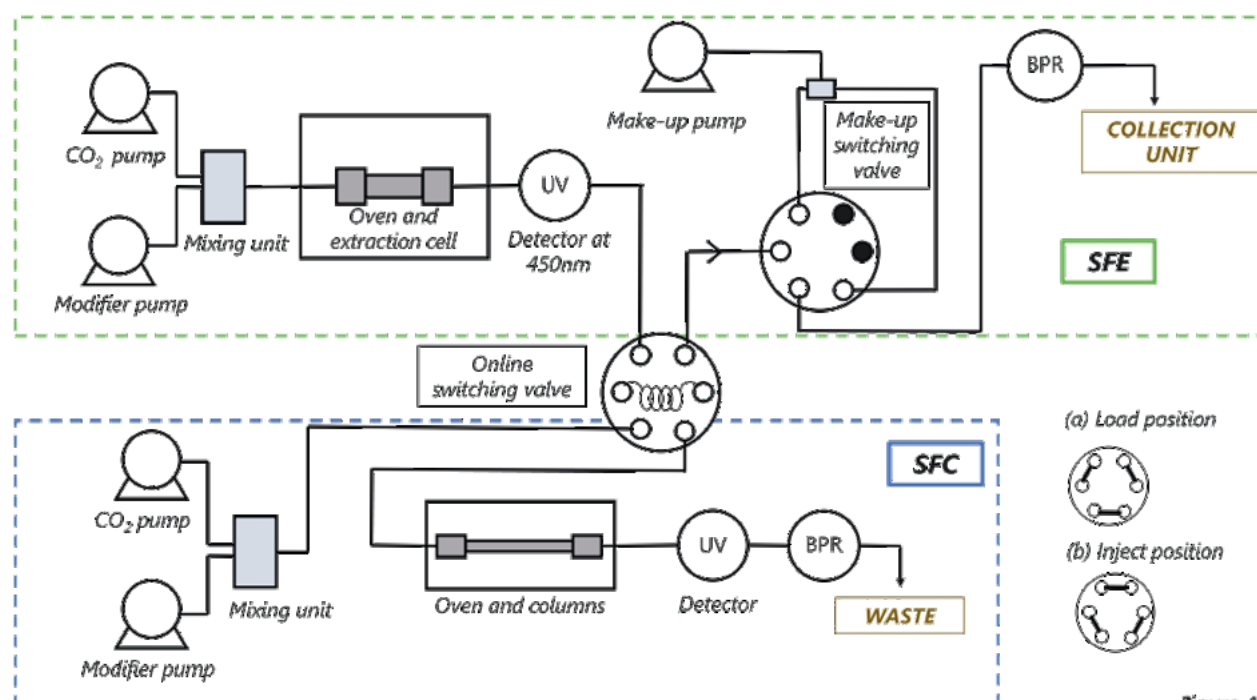


Figure 4

Fig. 4. Schematic diagram of the on-line SFE/SFC system. Supercritical Fluid extractor MV-10 coupled to a JASCO SFC system using an automatic high pressure six-port switching valve equipped with a 5  $\mu\text{L}$  injection loop. (a) Six-port switching valve configuration in position "load". (b) Six-port switching valve configuration in position "inject".

switching valve configurations. In this way it is possible to analyse the composition of the extract every five minutes.

The advantage of coupling an SFE device with an SFC instrument is that both equipment and modules are compatible especially with respect to the maximum pump pressure. In addition, during the monitoring of the extraction kinetic, the extract is never depressurised, which means that an extract composed of modifier and Sc-CO<sub>2</sub> is analysed. This effect guarantees the integrity of the extract and other modifications which can occur during depressurisation are avoided. During the depressurisation of supercritical fluid, carbon dioxide can spray liquid extract which may cause its partial evaporation. It has been observed that this effect can cause a low repeatability of the process due to changes in concentration [17].

### 3.2.3. Selective extraction development

The aim of this study was to selectively extract different bioactive compounds (carnosic and rosmarinic acids) and different pigments (carotenoids and chlorophylls). It is possible to use different successive conditions during SFE in order to favour the extraction of specific compounds.

Different extraction steps were then independently optimised in order to obtain different rich fractions. In a first step, carotenoid pigments with the lowest amount of bioactive compounds (carnosic acid) were extracted. Then, the recovery of chlorophyllic compounds was optimised based on previous work [27]. Finally, the extraction of bioactive compounds was optimised.

#### • Carotenoid extraction

SFE is a suitable technique for the extraction of carotenoids [28,29]. The higher pressure resulting in a higher Sc-CO<sub>2</sub> density favours their recovery. However, these conditions are also suitable for the extraction of carnosic acid, due to the close polarity of these compounds [27,30].

This extraction step was optimised by determining the carotenoid and carnosic acid content in two successive extraction steps. The first, pure CO<sub>2</sub>, step was carried out for 20 minutes applying different pressures (10 and 20 MPa) and temperatures (25 and 50°C) with 0.3 mL/min of EtOH as make-up. The second step, allowing the extraction of carnosic acid, using 3% modifier at 10 MPa and 25°C (with 0.3 mL/min of the same solvent as the modifier as make-up) was then applied for 40 minutes (the optimisation is described in the next part).

In order to evaluate the selectivity of carotenoid and carnosic acid between the first and the second step, the ratio of each content (content in the first step fraction over content in the second step fraction) was calculated for both compounds. These ratios were based on peak areas normalized with the collected volume using the method explained in a previous study [27]. This ratio indicates if the majority of each compound is in the first or the second fraction. Results are presented in Fig. 5.

The ratios of carnosic acid content (in blue) are higher when extraction is at a higher pressure (20 MPa), whatever the temperature. In addition, all the ratios for this compound were below 1, meaning that a higher content was extracted during the second step, using modifier, than during the first one, with pure CO<sub>2</sub>.

Carotenoid ratios followed a similar trend. However, one condition (25°C and 20 MPa) seemed to give the most selective extraction with a ratio above 1. These conditions gave the highest density of the supercritical fluid, which increases the carotenoid solubility. This is in line with what has been observed in other studies [28,29].

Finally, in order to extract the maximum of carotenoid pigment with the lowest possible amount of carnosic acid, 25°C and 20 MPa during 20 min was selected as the best method based on the experimental work conducted.

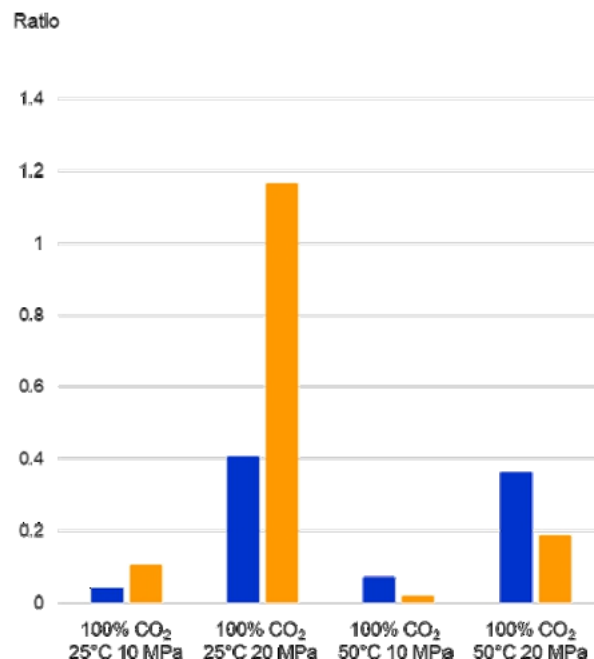


Fig. 5. Carnosic acid (blue) and carotenoid (orange) peak area (obtained by UHPLC) ratios calculated between the first pure CO<sub>2</sub> step in different conditions (see horizontal axis) and the second extraction step using 3% EtOH/water 50/50 v/v at 25°C and 10 MPa.

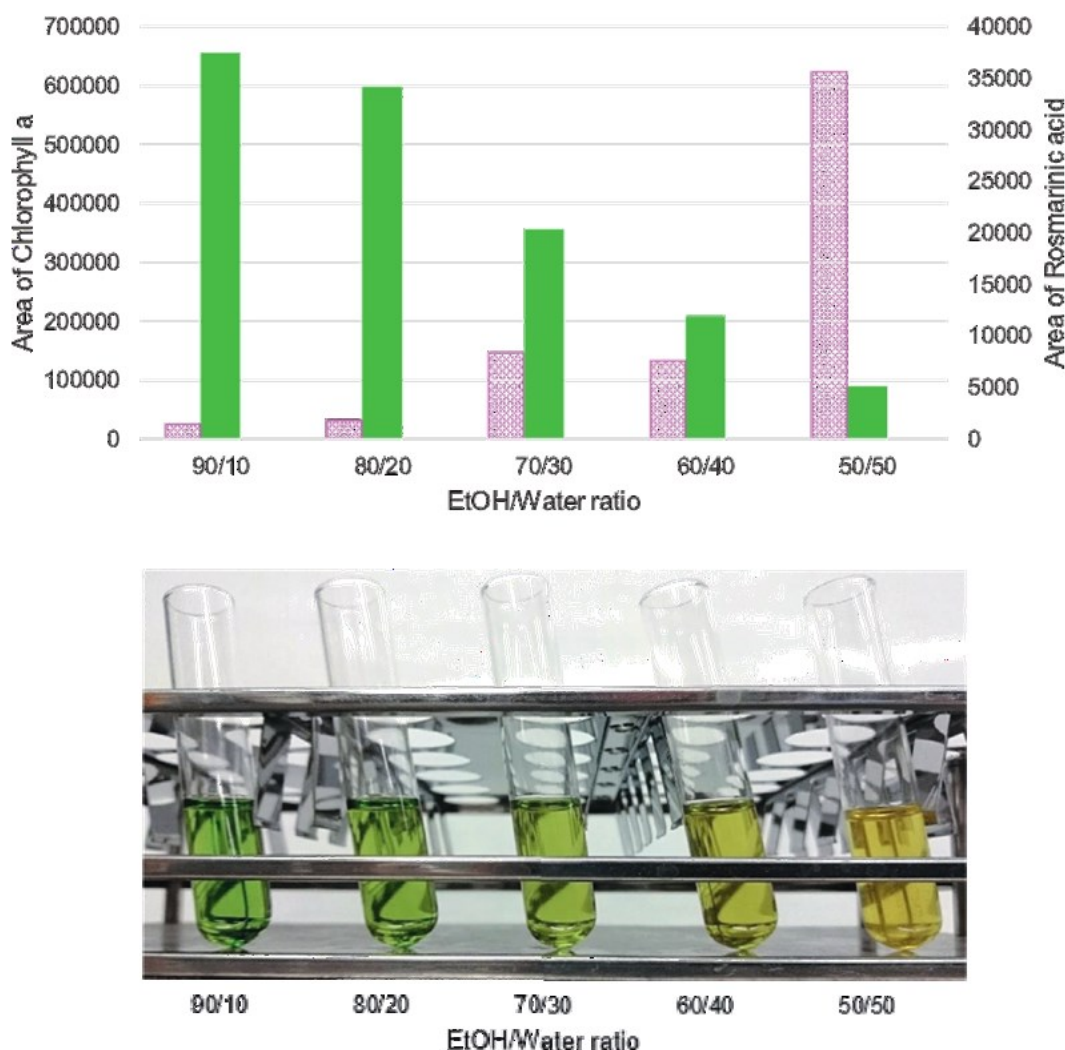
#### • Chlorophyll extraction

It is also possible to extract chlorophyllic pigment by using pure CO<sub>2</sub> [31]. However, it requires a high pressure (50 MPa), and the extraction recovery is much lower than the one obtained with ethanol extraction. Due to the pressure limit of the SFE-SFC system used, the outlet pressure for SFE cannot be set above 20 MPa. At this pressure, the use of a modifier such as ethanol is needed to extract chlorophylls [7,32,33]. In addition, previous work that studied the solubility of chlorophyll *a* into Sc-CO<sub>2</sub> chromatographically using different ethanol percentages (from 5 to 100%) confirmed that chlorophyll *a* can be extracted by using a relatively low modifier percentage (5%) [27]. However, the best chlorophyll extraction was obtained using 30% (v/v) ethanol with carbon dioxide. This study also confirmed that using 30% of ethanol, carnosic and rosmarinic acid were extracted. In order to avoid their extraction, this condition (30% of ethanol) was applied only as a last step (after the extraction of bioactive compounds) to recover chlorophylls and other remaining solutes.

#### • Bioactive compounds extraction

A step had to be developed to extract bioactive compounds after the extraction of carotenoids (extracted with pure CO<sub>2</sub>) and before the extraction of chlorophylls (extracted with 30% EtOH). As described previously, carnosic acid can be extracted using only Sc-CO<sub>2</sub> but rosmarinic acid requires a polar solvent as modifier. To do so, a polar modifier needs to be identified in order to extract both of these compounds without chlorophylls. EtOH:Water mixtures can be used for rosmarinic acid recovery [34]. Fortunately, chlorophyll *a* is non soluble in water because of its low polarity ( $\log K_{chlorophyll} > 8$ ).

Different EtOH:Water ratios were tested using standard conditions (5% modifier, 25°C, 10 MPa and 3mL/min total flow for 60 min). Fig. 6A shows the chlorophyll *a* and rosmarinic acid content as a function of different EtOH:Water ratios. Carnosic acid was extracted with any conditions and is therefore not represented in the figure.



**Fig. 6.** A: Peak area of chlorophyll *a* (solid green colour) at  $\lambda = 650$  nm and rosmarinic acid (pink stripes) at  $\lambda = 332$  nm obtained by online SFC using different EtOH/water v/v compositions as modifier (5% in  $\text{CO}_2$ ) in SFE. B: photograph of collected extracts.

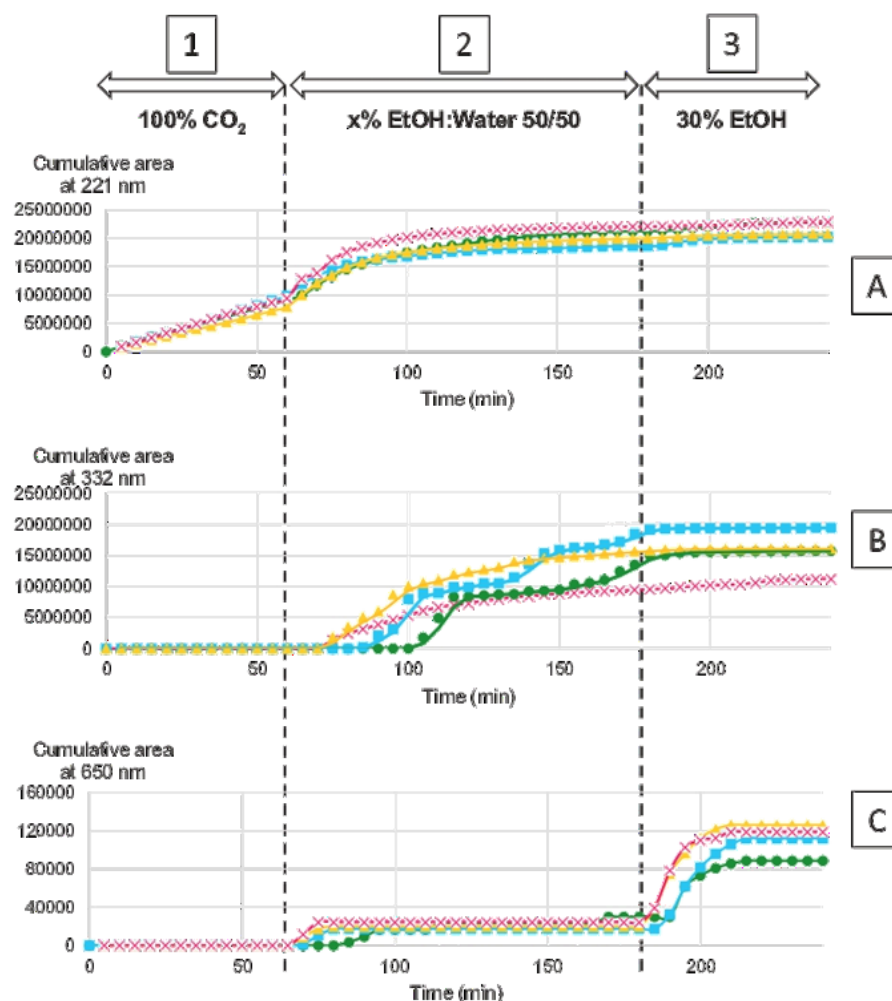
Using an EtOH:Water ratio of 90/10 extracted chlorophyll *a* and a low amount of rosmarinic acid. The chlorophyll *a* content decreased when the water proportion increased whereas the contrary was observed for rosmarinic acid. Finally, using a 50/50 ratio extracted a low content of chlorophyll *a* and a higher content of rosmarinic acid. A mixture of EtOH: Water 50/50 was therefore selected in order to recover bioactive compounds and limit the chlorophyll content, as can be seen on Fig. 6b.

In a second stage, the percentage of modifier needed to be optimised for the extraction of bioactives. Different percentages from 3 to 15% were tested. This optimisation was carried out after a first step with pure carbon dioxide to remove carotenoids, and was followed by a 30% ethanol step to extract chlorophylls. The following parameters and duration were applied: 25°C, 10 MPa, total flow of 3.0 mL/min, first step during 60 min, second step during 120 min and third step during 60 min. The extraction kinetics are plotted in Fig. 7 using the sum of the different areas obtained by SFC online every five minutes.

The extraction of carnosic acid (A) started during the first step (pure  $\text{CO}_2$ ), as shown above. The second step allowed extraction of the majority of carnosic acid; no additional compound was extracted with a higher modifier percentage in step 3.

Rosmarinic acid (B) was not extracted during the first step, with pure  $\text{CO}_2$ . During the second step, its extraction kinetic was slower than the one observed with carnosic acid. Different trends were visible with different modifier percentages. Using 3 and 15% of modifier seemed to give the lowest rosmarinic acid yield, whereas the highest amounts were obtained for intermediate modifier percentages of 5 and 10%. This could be due to solubility differences related to the modifier percentages, with an optimum reached for 5 to 10%. Moreover, the extraction kinetics appeared to differ at low and high percentages, with a regular improvement in the extracted amount at 10 and 15%, showing classical mass transfer between the plant and the extraction fluid, whereas for lower percentages, the extraction kinetic appeared to follow steps, with more or less identical profiles, which are probably due to lower diffusivity of the fluid into the dried plants, explaining some shifts in the amount of rosmarinic acid extracted, both toward higher percentages and carnosic acid.

Another interesting difference was observed for rosmarinic acid (B). The varied modifier percentages led to different extraction delays at the beginning of extraction (after 60 minutes). With 15% and 10% of modifier, the extraction of this compound started around 70 min, with 5% of modifier extraction started around



**Fig. 7.** Extraction kinetics of carnosic acid (A), rosmarinic acid (B) and chlorophyll a (C) obtained using online SFE-SFC for three different steps at 25°C and 10 MPa (1: pure CO<sub>2</sub>; 2: x% of EtOH:Water 50/50 v/v; 3: 30% EtOH). Percentages of EtOH:Water 50/50 v/v modifier used during the second step are 3% (green dots), 5% (blue squares), 10% (yellow triangles) and 15% (red cross) v/v.

85 min and with 3%, extraction started around 100 min. Different hypotheses may explain this phenomenon. The first reason could be the extraction competition which can occur between different compounds. For example, compounds such as carnosic acid or other compounds are strongly extracted at 60 minutes, leading to a “saturating” extraction solvent, and preventing the extraction of rosmarinic acid during a few minutes. The second reason could be linked to the location of these two compounds in plant cells. Rosmarinic acid may be in a deeper and harder to reach location which may require a longer time for the solvent to penetrate into the cells and solubilise the compound. Whatever the reason, from all these results, it is possible to use this time difference in order to produce extracts with different compositions.

For chlorophyll a (Fig. 7C), no green pigments were extracted during the first step, and only a low amount during the second step. This is in line with what was found during optimisation of the EtOH:Water ratio. The majority of these pigments were extracted in 30 minutes during the last step with 30 % of ethanol. This duration was used in the final extraction.

### 3.2.4. Characterisation of different SFE fractions

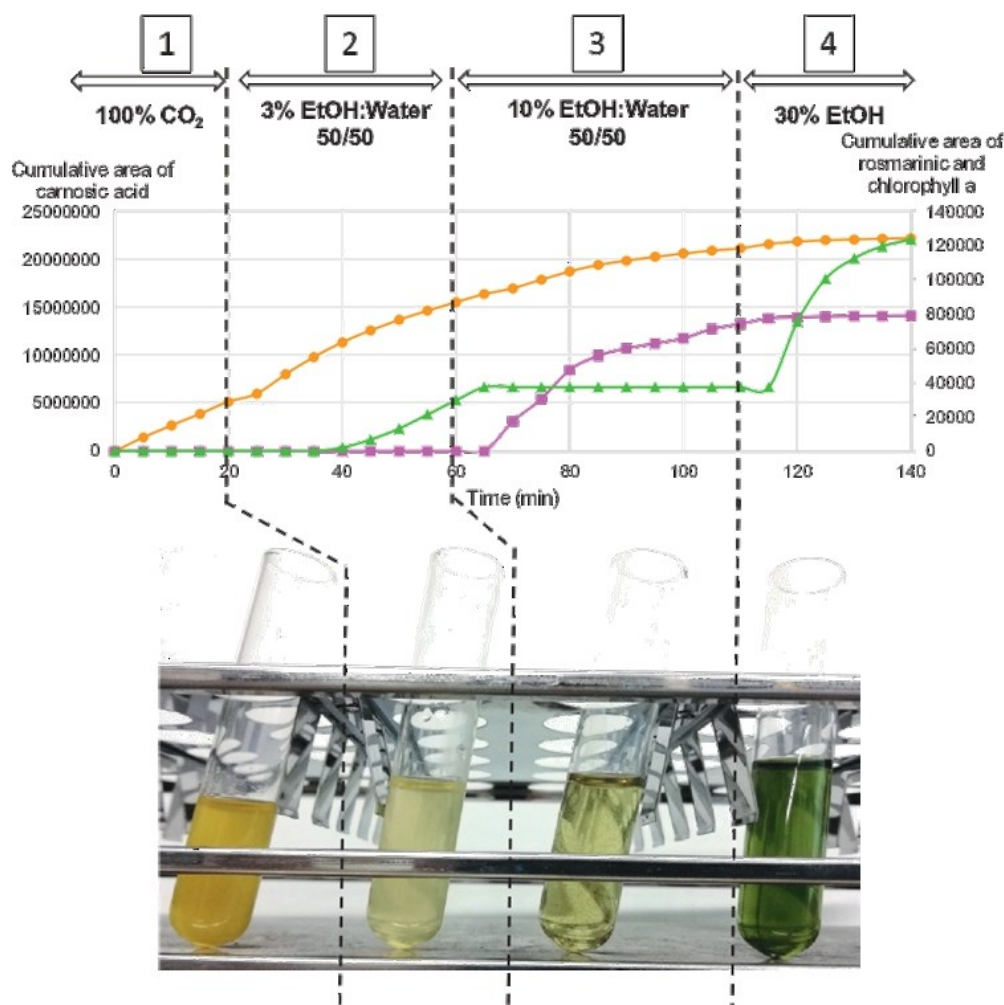
Initially, three fractions were collected: One rich in carotenoids (with pure CO<sub>2</sub>), one rich in bioactive compounds (carnosic and rosmarinic acids with EtOH:water), and one rich in chlorophylls

(with 30% Ethanol). However, the delay observed in the second step allowed the collection of two extracts during this second step: one rich in carnosic acid and the other rich in rosmarinic acid. Four fractions were then collected during the final and optimised extraction. After the pure CO<sub>2</sub> step, 3% modifier was used during 40 min, then 10% modifier during 50 min, before the last step with 30% of ethanol.

The extraction kinetics, from SFE-SFC online quantification (see Fig. 3), and a photograph of the collected fractions are shown in Fig. 8. These four samples were analysed by UHPLC-DAD-HRMS (see Fig. 2) and the identified compounds from Table 1 were quantified using peak area at different wavelengths. Table 2 shows the distribution of different compounds in the four fractions (in percentage). In addition, global yield (expressed in mg/g of plant used which is 1.0 g) is displayed.

In the pure CO<sub>2</sub> fraction, compounds from 6 (rosmaquinone) to 9 (carnosic acid) were detected. Carotenoid and carnosic acid were found and the extraction percentages are in accordance with previous results (Fig. 5). Carnosol was also observed in lower amounts. Rosmaquinone was also quantified in this first step.

During the second extraction step (3% EtOH:Water 50/50 v/v), the same compounds were detected. Carotenoids were still present in this fraction, but in a lower amount (47% vs 53% in first fraction). A similar observation was made for rosmaquinone. The



**Fig. 8.** Extraction kinetics of carnosic acid (orange circles), rosmarinic acid (pink squares) and chlorophyll *a* (green triangles) obtained using online SFE-SFC through four different steps (1: pure CO<sub>2</sub> at 25°C and 20 MPa; 2: 3% of EtOH:Water 50/50 v/v 25°C and 10 MPa; 3: 10% of EtOH:Water 50/50 v/v 25°C and 10 MPa; 4: 30% EtOH 25°C and 10 MPa). Bottom of the Fig.: Photograph of the four collected fractions.

**Table 2**  
Percentages of compounds in different SFE fractions and global yield (mg/g).

	100% CO <sub>2</sub>	3% EtOH/water 50/50	10% EtOH/water 50/50	30% EtOH
1 Isorhamnetin-3-O-hexoside	0	0	77	23
2 Rosmarinic acid	0	0	78	22
3 Hispidulin-7-glucoside	0	0	79	21
4 Dihydroxy-dimethoxyflavone	0	0	73	27
5 Genkwanin	0	9	66	25
6 Rosmaquinone	13	6	81	0
7 Carnosol	13	35	9	43
8 Carnosic acid	33	49	15	3
9 Carotenoid	53	47	0	0
11 Chlorophyll <i>b</i>	0	0	0	100
12 Chlorophyll <i>a</i>	0	0	0	100
Global yield (mg/g)	28	35	202	115

opposite was observed for carnosic acid. A higher amount was observed in the second fraction (49 vs 33% in the first fraction). The carnosol content also increased. Moreover, a low percentage (6%) of genkwanin was detected.

The third fraction (obtained with 10% EtOH:Water 50/50 v/v) was much richer in more polar compounds (from 1 to 5). This fraction contained more than 75% of total isorhamnetin-3-O-hexoside, rosmarinic acid, hispidulin-7-glucoside, dihydroxy-

dimethoxyflavone and rosmaquinone. Genkwanin was also more abundant in this third fraction than in the previous one (66% vs 9% in the second fraction). On the other hand, carnosic acid and carnosol contents were lower in this fraction. Finally, no carotenoid was detected.

The fourth and last step (30% EtOH) was rich in chlorophylls and contained lower amount of bioactive compounds. However, this fraction still had around 20% of polar compounds (1 to 5). The

content in less polar compounds (i.e. carnosic acid, rosmarinone) was once again reduced. Oddly, the carnosol content was higher in this fraction.

Differences can be observed between all the fractions. It has been confirmed that the majority of bioactive compounds are extracted during hydro alcoholic steps and that they can be extracted selectively over pigments.

In terms of global yield, the third and last fractions have the highest yield (respectively 202 mg/g and 115 mg/g). This may be explained by the extraction of a higher percentage (10 and 30%) of rosemary phenolic compounds when the duration of the extraction process is longer (50 and 30 min). Total SFE extracts (four fractions together) indicate a higher extraction yield than with "standard" ultrasonic extraction (380 mg/g for SFE vs 118 mg/g for "standard" US). However, because ultrasonic extraction was not optimised, these two extraction modes cannot be totally compared in terms of efficiency.

#### 4. Conclusion

This study developed a selective sequential supercritical fluid extraction in order to produce different rich extracts. This optimization was conducted using an online SFE-SFC system, which was also developed during this study.

Four fractions were collected and analysed. The first and fourth fractions are respectively carotenoid- and chlorophyll-rich while the second and third are bioactive-rich. Each fraction can be used separately or by mixing them. For example, if the objective is to produce chlorophyll-free extract, the first three fractions can be mixed together, whereas fractions 2 and 3 can be mixed to group the bioactive compounds in order to simplify an industrial process.

Using Sc-CO<sub>2</sub> alone or with a modifier allowed the sequential (and selective) extraction of compounds with different polarities. This study also highlighted another phenomenon related to the extraction kinetic, namely the delay in extraction which could be due to the diffusion of solvents into the sample, in our case favouring the extraction selectivity.

Finally, this work offers extraction conditions which can be considered as green, thanks to the use of green solvents (carbon dioxide, ethanol and water), thus complying with various regulations such as those in the cosmetic field.

#### Declaration of Competing Interest

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

#### CRediT authorship contribution statement

**Thibault Lefebvre:** Conceptualization, Investigation, Methodology, Visualization, Writing - original draft. **Emilie Destandau:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing - review & editing. **Eric Lesellier:** Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Visualization, Writing - review & editing.

#### Acknowledgements

This work was supported by the Centre-Val de Loire region, which provided the funding of the "Khloros" project APR-IR (2017).

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### **3. CONCLUSION**

L'optimisation des conditions d'extraction en SFE a été permise grâce au couplage de l'extracteur supercritique avec de la SFC en ligne. Celui-ci a permis d'analyser la composition de l'extrait pendant l'extraction toutes les cinq minutes. Les cinétiques des trois composés cibles (acide carnosique, acide rosmarinique et chlorophylle *a*) ont pu être suivies au cours du temps. Cela était possible grâce au développement d'une méthode d'analyse rapide en SFC permettant la séparation de ces trois marqueurs en moins de cinq minutes. Compte tenu des différences de polarité entre l'acide rosmarinique et la chlorophylle *a* et de l'impossibilité d'utiliser un gradient d'élution, le retour à l'équilibre d'un gradient étant incompatible avec la fréquence d'échantillonnage, deux colonnes de différentes polarités (silice et greffée C18) ont été couplées. Afin de valider ce système, celui-ci a été comparé avec une cinétique réalisée en analysant de façon séparée (en offline) les différentes fractions. Les résultats étaient similaires et ont donc permis de valider l'utilisation de ce couplage pour la suite des travaux. Cette validation n'a pas été présentée dans l'article.

Durant l'optimisation du procédé d'extraction sélective, un mélange d'éthanol et d'eau a été utilisé. Il s'agissait du meilleur compromis pour l'extraction de l'acide carnosique et rosmarinique sans celle des chlorophylles. Un mélange à 50/50 a été déterminé comme le mélange optimum. La mesure des cinétiques d'extraction a permis de mettre en avant des phénomènes intéressants comme des différences de début d'extraction pour les deux composés bioactifs. Ces phénomènes donnent la possibilité de gagner en sélectivité et de passer d'une fraction riche en composés bioactifs (contenant les deux acides) en deux fractions respectivement riches l'une en acide carnosique et l'autre en acide rosmarinique. En fonction de futurs besoins, il sera toujours possible de réunir ses fractions entre elles ou non.

A la fin de cette étude, quatre fractions ont été réalisées : riches en (1) caroténoïdes, (2) acide carnosique, (3) acide rosmarinique et (4) chlorophylles. Ces fractions ont été analysées par UHPLC-UV-HRMS afin de comparer leur composition en différents composés autres que les deux initialement étudiés. Les résultats ont montré que les composés moins polaires (excepté les chlorophylles) sont extraits durant la première et deuxième étape de l'extraction comme les caroténoïdes, l'acide carnosique et le carnosol. Seulement à partir de la troisième étape (10% du co-solvant

EtOH/Eau), des composés plus polaires, dont des composés glycolysés ont été extraits en quantité majoritaire. La dernière étape (30% EtOH) contient ce qui n'a pas été extrait précédemment en composés polaires, et les chlorophylles.

Ce procédé d'extraction supercritique a donc permis d'extraire sélectivement des composés bioactifs sans chlorophylles et les chlorophylles avec peu de composés bioactifs. D'autres paramètres peuvent être optimisés comme le débit de solvant. De plus, il serait intéressant de voir si les résultats sont transposables sur un extracteur à une plus grande échelle afin d'étendre cette extraction vers un procédé industriel.



## CONCLUSIONS ET PERSPECTIVES



## Conclusion

L'objectif principal de ce travail de thèse était de trouver et d'appliquer des conditions d'extraction sélective dans le but de produire des extraits riches en composés bioactifs et en chlorophylles à partir de ressources végétales.

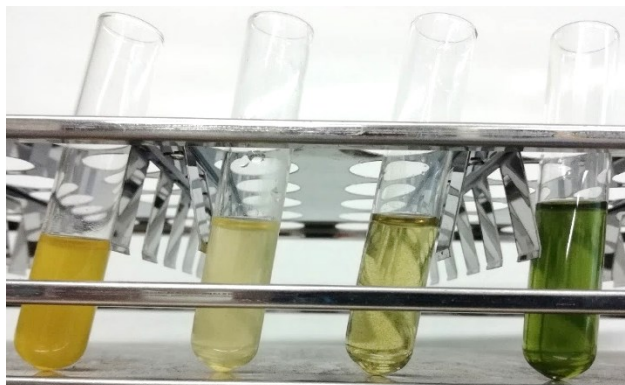
Le chapitre 1 a permis de faire un état de l'art des différentes possibilités afin d'avoir séparément des composé bioactifs et les chlorophylles. Notre choix s'est porté sur l'extraction sélective. Une review a présenté une vue d'ensemble des différentes sélectivités obtenues avec plusieurs techniques d'extraction (US, MO, PLE et SFE). L'extraction par fluide supercritique a alors montré un grand potentiel.

Dans le chapitre 2, une première étude a permis d'identifier des conditions afin de produire un extrait riche en chlorophylles qui a servi par la suite de « standard » de chlorophylles. Il s'est avéré qu'il était quasiment inévitable de subir une dégradation des chlorophylles. Cependant, certaines conditions favorisent l'apparition des produits de dégradation comme l'évaporation de l'extrait ou encore l'utilisation de microondes lors de l'extraction. Ensuite, ces chlorophylles et leurs produits de dégradations ont été analysés par SFC et différentes conditions ont été appliquées. Cela a permis d'étudier leur comportement chromatographique dans le but de trouver des conditions de séparation optimales.

Un modèle a été développé dans le chapitre 3 afin de trouver par chromatographie (SFC et CLHP) des conditions transposables en extraction (SFE et PLE). Ce modèle a donc pu être validé et les quelques résultats divergents peuvent être expliqués notamment pas des effets liés à la matrice végétale. Les résultats de rétention ont également pu être corrélés au modèle de solubilité de Hansen. Il est possible de corréliser les logk des chlorophylles avec les paramètres d'Hansen lorsque des solvants liquides sont utilisés. Il est aussi possible de corréliser les résultats avec des compositions contenant du dioxyde de carbone. En revanche, il n'est pas possible de trouver une corrélation lorsque la teneur en CO<sub>2</sub> est supérieure à 70% (donc des teneurs en co-solvant inférieures à 30%).

Les conditions sélectives obtenues précédemment ont donc été utilisées dans le dernier chapitre afin de produire les extraits riches en composés bioactifs (acides carnosique et rosmarinique) et d'autres, riches en pigments (caroténoïdes et

chlorophylles). Le chapitre 4 a donc permis de développer une extraction séquencée par fluide supercritique afin de produire différents extraits. Ce développement a été appuyé par la mise en place d'un système SFE-SFC en ligne. Les dernières expérimentations de cette thèse ont permis la production de quatre fractions (Figure 7) : une première fraction riche en caroténoïdes, une seconde riche en acide carnosique, une troisième riche en acide rosmarinique et une dernière riche en chlorophylles.



*Figure 7: Photographie des quatre fractions obtenues en SFE avec de gauche à droite la fraction riche en: caroténoïdes, acide carnosique, acide rosmarinique et chlorophylles*

## Perspectives

Cette thèse présente un travail à but d'application, compte tenu du sujet et du partenaire industriel associé. Pendant ces trois années, de nombreux développements et les choix fait ont été réalisés dans l'optique de transposer le procédé à une plus grande échelle. Il aurait donc été intéressant d'utiliser les conditions trouvées précédemment, sur un autre système d'extraction de plus grande dimension avec une quantité plus importante de plante.

Les quatre dernières fractions obtenues ont été analysées chimiquement afin d'avoir la composition de chacune d'elles. Cependant, il aurait été intéressant d'évaluer l'activité (essentiellement le pouvoir antioxydant) de ces fractions et de les comparer avec un extrait non sélectif comme un extrait de romarin éthanolique obtenu par ultrasons.

Comme étudié dans le chapitre 1, il existe de nombreux autres solvants ayant différentes propriétés. L'extraction par SFE développée dans cette thèse utilise majoritairement du CO<sub>2</sub> supercritique mais aussi de l'éthanol et de l'eau. D'autres solvants, tout aussi écologiques, peuvent aussi être étudiés. Certains d'entre eux (le propylène carbonate et le 2-méthyltetrahydrofurane) ont d'ailleurs été testés dans le chapitre 3 lors de la corrélation entre les différentes rétentions obtenues par CLHP et SFC et le modèle de solubilité d'Hansen [96]. Les NaDES montrent aussi des propriétés intéressantes en terme de sélectivité due à leurs compositions qui peuvent être modulées. Cela pourra être intéressant d'étudier différents solvants de cette famille dans le futur. De plus, ces solvants représentent un engouement et un potentiel industriel important compte tenu des différents brevets apparus ces dernières années.

Dans le premier chapitre, d'autres solutions ont été présentées afin de retirer les chlorophylles d'un extrait de plante (piégeage par un adsorbant, ou séparation par outils de purification comme la CPC).

Concernant l'utilisation d'un adsorbant, il est possible de le coupler avec une technique d'extraction en ligne afin de rester dans un seul et unique procédé. La difficulté de ce procéder sera d'affiner la sélectivité de l'adsorbant pour que celui-ci piège uniquement les pigments (chlorophylliens principalement) et pas d'autres

composés d'intérêts. De plus, il est aussi intéressant de récupérer ces pigments. Il faudra donc trouver des adsorbants permettant tout de même dans certaines conditions, de désorber les chlorophylles sans les dégrader. Cela représente un bon potentiel dans la problématique de la thèse et pourrait mériter un développement.

Concernant l'utilisation de la CPC, la possible récupération des deux phases liquides contenant des composés d'intérêt différents (bioactifs dans une phase et chlorophylles dans l'autre) représente son principal avantage. L'extrait riche en chlorophylle peut être injecté dans le système biphasique ou être utilisé comme une des deux phases tandis que l'autre phase pourrait extraire soit les bioactifs soit les chlorophylles pour assurer leur séparation et leur valorisation. Concernant l'aspect économique, ce procédé ne consomme que des solvants moins onéreux que des adsorbants solides et souvent en moins grande quantité que la chromatographie en phase liquide préparative. Cette technique peut donc être utilisée pour répondre à la problématique de cette thèse. Cependant, l'un des freins à son utilisation industrielle peut être la dimension des colonnes actuelles qui limitent la quantité d'extrait traitée en une seule étape et surtout la possibilité de générer des systèmes biphasiques composés uniquement de solvants verts.

Cette thèse a permis d'établir une nouvelle approche dans le développement et l'optimisation de procédé d'extraction. De plus, les différentes perspectives ont ouvert encore plus le champ des possibles afin de produire des extraits sélectifs.





# ANNEXES (1/2) : TABLEAUX CITES DANS L'ARTICLE DU CHAPITRE 1

## *Selectivity in plant extraction and the influence of modern extraction techniques (UAE, PLE, MAE and SFE): A review*

Table 01. Table of names, logP and formulas of lipids cited in review (part 1)

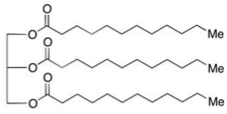
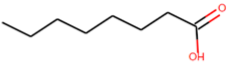
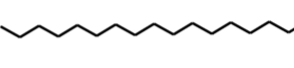
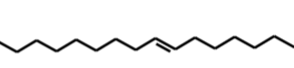
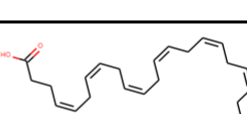
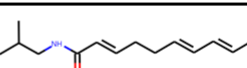
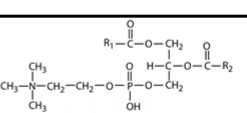
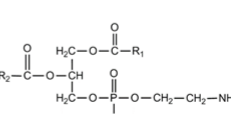
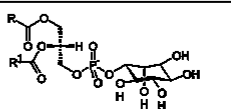
LIPIDS			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Glycerides (mainly triglycerides)	Triaurin (C12:0)	11.7	
	FFA C8:0	2.4	
	FFA C18:0	6.3	
	FFA C18:1 Octadecenoic acid	6.1	
	DHA C22:6	6.5	
Free Fatty acids	Spilanthol	3.2	
	Phosphatidylcholine (i.e. C16:0 and C18:1)	11.2	
	Phosphatidylethanolamine (i.e. C15:0 and C20:0)	11.7	
	α-phosphatidylinositol (i.e. C20:0 and C20:4)	9.9	

Table 02. Table of names, logP and formulas of lipids cited in review (part 2)

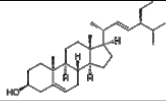
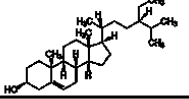
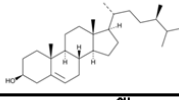
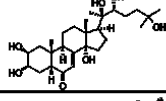
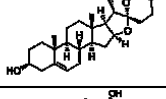
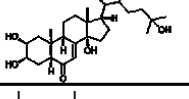

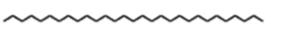
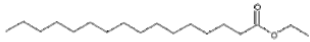
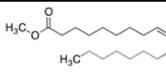

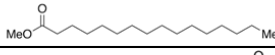
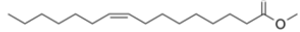
LIPIDS			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Non-saponifiable (waxes, steroids, alkanes)	Stigmasterol	7.8	
	$\beta$ -sitosterol	8.0	
	Methylcholesterol	7.6	
	20-hydroxyecdysone	1.9	
	Diosgenin	5.7	
	$\beta$ -ecdysone	2.7	
	Squalene	10.6	
	Nonacosane	11.6	
Fatty esters	Ethyl palmitate	6.0	
	Methyl oleate	6.2	
	Ethyl eicosenoate	7.4	
	Methyl palmitate	5.6	
	Methyl palmitoleate	5.4	

Table 03. Table of names, logP and formulas of terpenes cited in review (part 1)

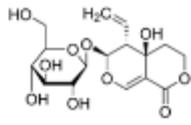
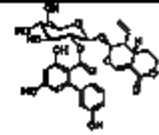




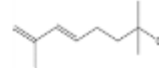
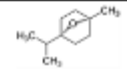


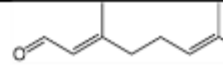
TERPENES			
FAMILY	COMPOUND	LOG P	FORMULA
Monoterpenoids	Swarfumarin	-2.5	
	Amarogenin	1.1	
Monoterpenes	Limonene	3.3	
	Limonene oxide	2.5	
	Linalool	2.7	
	Sabinene	3.0	
	Ocimene	2.7	
	1,8-Cineole (also known as eucalyptol)	2.7	
	δ-3-carene	3.0	
	α-pinene	3.0	
	Geraniol (also known as citral)	2.9	

Table 04. Table of names, logP and formulas of terpenes cited in review (part 2)




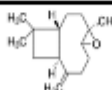
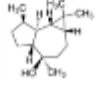
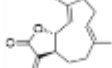
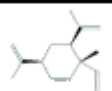

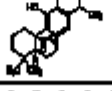


TERPENES			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Sesquiterpenes	Artemisinin	2.4	
	$\alpha$ -caryophyllene	4.7	
	$\alpha$ -farnesene	5.2	
	Caryophyllene oxide	3.9	
	Globulol	3.5	
	Germacranolides (i.e. costunolide)	3.6	
	$\beta$ -elemene	4.7	
Diterpenes	Carnosic acid	4.3	
	Carnosol	4.3	
	Phytol	6.4	
	Germaphenol	6.1	

Table 05. Table of names, logP and formulas of terpenes cited in review (part 3)

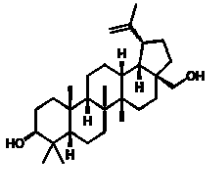
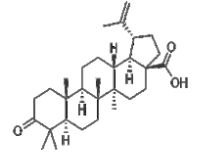
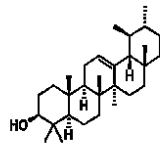
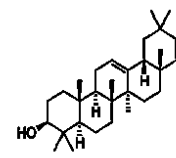
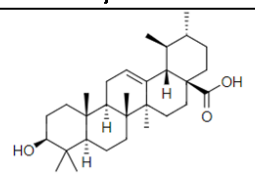
TERPENES			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Triterpenes	Betulin	7.0	
	Betulonic acid	7.3	
	$\alpha/\beta$ -amyrin	8.0/7.0	  <i>Alpha</i> <i>Beta</i>
	Ursolic acid	7.1	

Table 06. Table of names, logP and formulas of coumarins cited in review

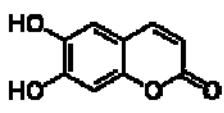
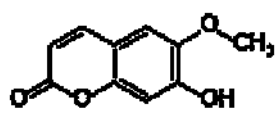
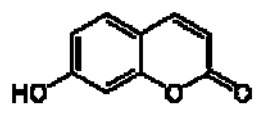
COUMARINS		
COMPOUNDS	LOG P	FORMULAS
Esculetin	1.2	
Scopoletin	1.5	
7-hydroxy coumarine	1.5	

Table 07. Table of names, logP and formulas of pigments cited in review

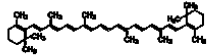
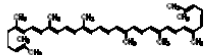
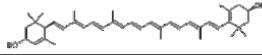
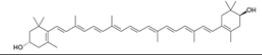
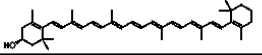
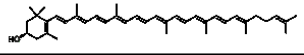
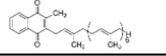
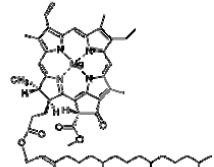
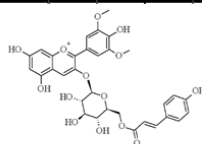
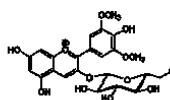
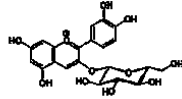
PIGMENTS			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Carotenoids	$\beta$ -carotene	12.6	
	Lycopene	12.9	
	Lutein	10.4	
	Zeaxanthin	10.5	
	Cryptoxanthin	11.6	
	Rubixanthin	11.7	
	Menaquinon-7	8.9	
Chlorophylls	Chlorophyll a	12.8	
Anthocyanins	Malvidin-3-O-(6-p-coumaroyl)glucoside	2.7	
	Malvidin-3-O-glucosides	0.7	
	Cyanidin-3-O-glucoside	0.4	

Table 08. Table of names, logP and formulas of cannabinoids and alkaloids cited in review

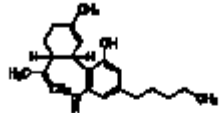
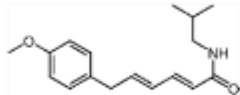

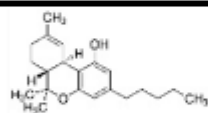

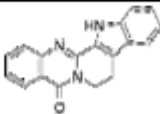
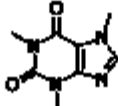
CANNABINOIDS AND ALKALOIDS		
COMPOUNDS	LOG P	FORMULAS
Cannabidiol (CBD)	5.8	
Piperovaline	3.1	
Pipercalcixidine	3.5	
Tetrahydrocannabinol	5.7	
Evodiamine	3.3	
Rutascarpine	3.1	
Caffein	-1.0	

Table 09. Table of names, logP and formulas of polyphenols cited in review (part 1)

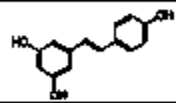
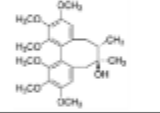
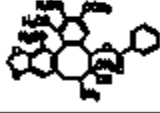
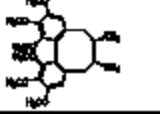
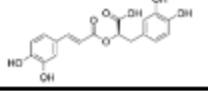
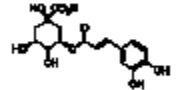
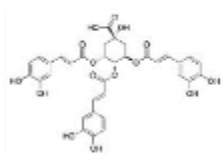
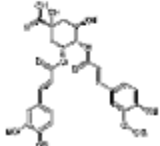
POLYPHENOLS			
FAMILY	COMPOUNDS	LOG P	FORMULAS
<b>Stilbenes</b>	Resveratrol	3.0	
<b>Lignans</b>	Schizandrin	3.9	
	Schisantherin a	5.0	
	Deoxyschizandrin	4.8	
<b>Phenols</b>	Rosmarinic acid	1.8	
	Chlorogenic acid (3-caffeoylquinic acid)	-0.8	
	Tricaffeoylquinic acid	2.7	
	4,5-dicaffeoylquinic acid	1.0	

Table 10. Table of names, logP and formulas of polyphenols cited in review (part 2)

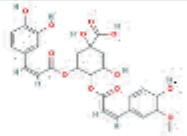
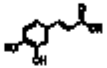
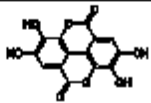



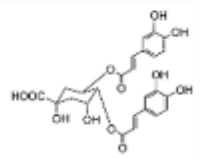
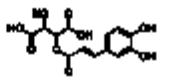
POLYPHENOLS			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Phenols	3-cafeyloyl-4-feruloylquinic acid	1.3	
	Caffeic acid	1.2	
	Ellagic acid	1.3	
	Galic acid	0.6	
	Thymol	2.8	
	Ecklonoside	-3.2	
	Cyanine	1.0	
	Caffeic acid	-0.4	

Table 11. Table of names, logP and formulas of polyphenols cited in review (part 3)

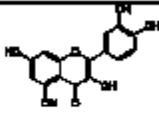
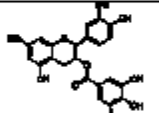
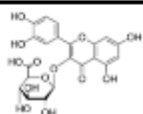
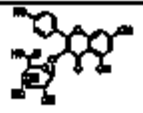
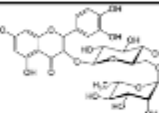
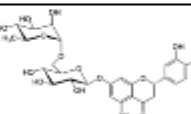
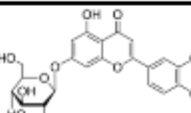
POLYPHENOLS			
FAMILY	COMPOUND	LOG P	FORMULA
Flavonoids	Quercetin	2.0	
	Quercetin	0.6	
	Quercetin-3-O-β-D-glucuronide	-0.4	
	Kaempferol-3-O-β-D-glucuronide	-0.2	
	Rutin	-1.7	
	Heperidin	-1.2	
	Luteolin-7-O-glucoside	-0.2	

Table 12. Table of names, logP and formulas of polyphenols cited in review (part 4)

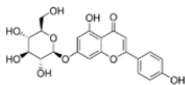
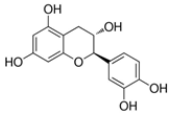
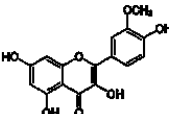
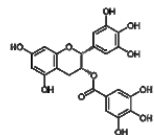
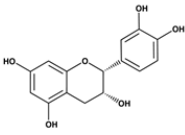
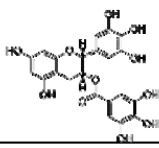
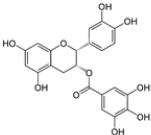
POLYPHENOLS			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Flavonoids	Apigenin-7-O-glucoside	0.0	
	Catechin (CT)	1.5	
	Isorhamnetin	2.3	
	Epigallocatechin (EGC)	1.3	
	Epicatechin (ECT)	1.5	
	Epigallocatechin gallate (EGCG)	2.2	
	Epicatechin gallate (ECG)	2.5	

Table 13. Table of names, logP and formulas of polyphenols cited in review (part 5)

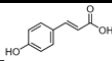

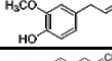
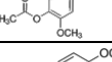
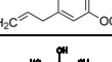
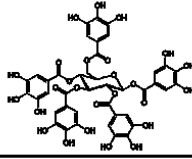
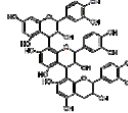
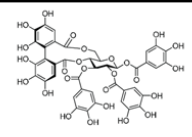
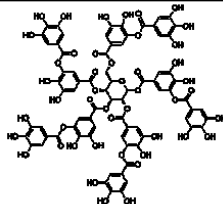
POLYPHENOLS			
FAMILY	COMPOUNDS	LOG P	FORMULAS
Phenylpropenes	P-coumaric acid	1.5	
	Trans-ferulic acid	1.5	
	Eugenol	2.1	
	Eugenyl acetate	2.3	
	Methyl eugenol	2.4	
Tannins	Hydrolysable tannins (i.e. 1.2.3.4.6-Pentagalloyl glucose)	1.7	
	Condensed tannins (i.e. Procyanidin C1)	4.4	
	Tellimagrandin 2	1.7	
	Tannic acid	4.8	

Table 14. Table of names, logP and formulas of carbohydrates and polysaccharides cited in review

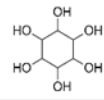
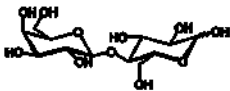

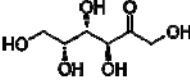
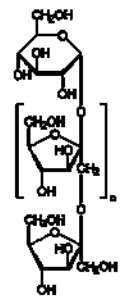
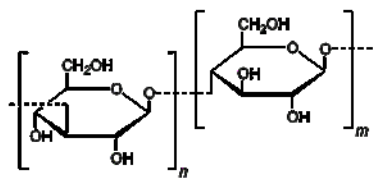
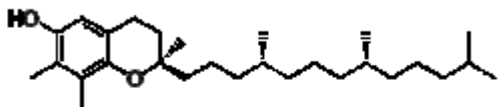
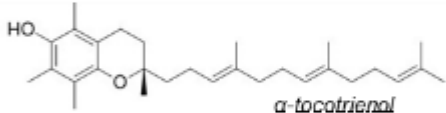
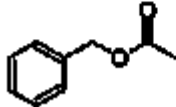


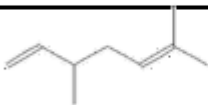

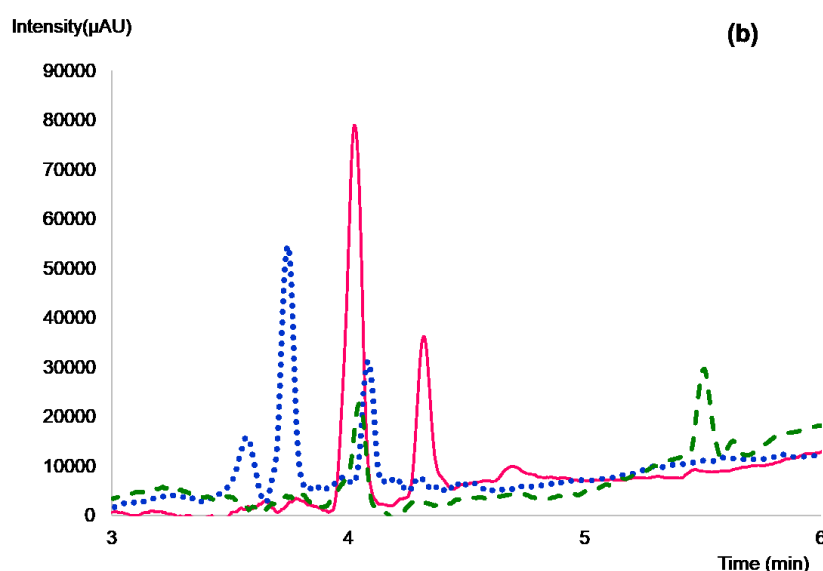
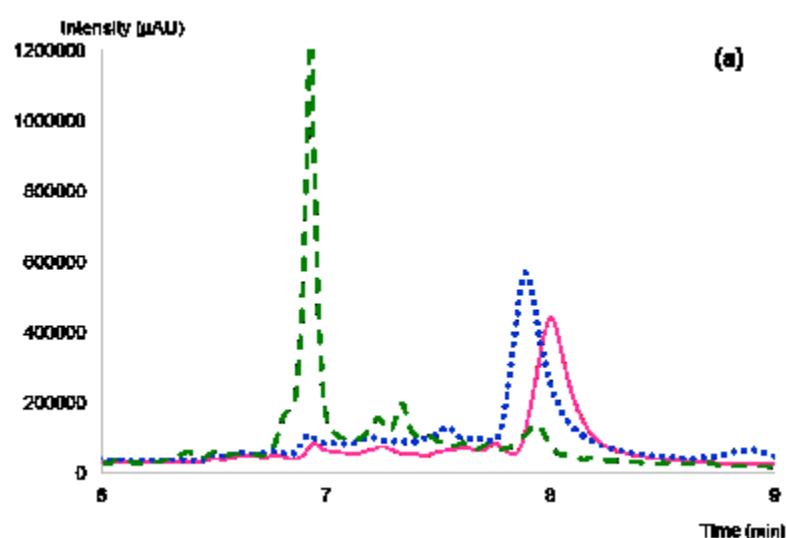
CARBOHYDRATES		
COMPOUNDS	LOG P	FORMULAS
Inositols	-3.8	
Lactose	-5.4	
Galactose	-3.4	
Tagatose	-3.4	
POLYSACCHARIDES		
Inulin (i.e. n=35)	-83.7	
$\beta$ -glucan (i.e. n=1 et m=2)	-7.6	

Table 15. Table of names, logP and formulas of other molecules cited in review

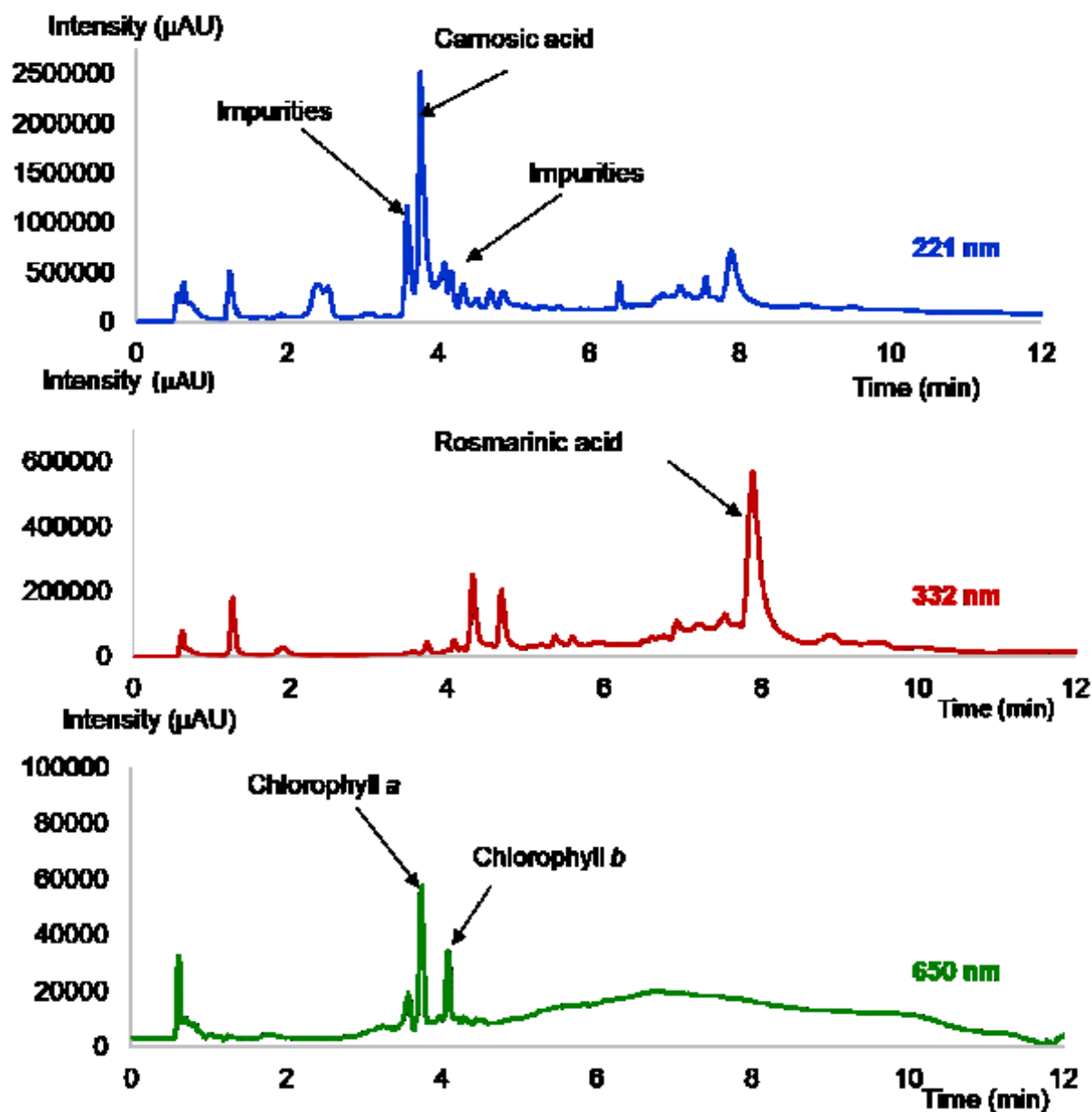
OTHERS		
COMPOUNDS	LOG P	FORMULAS
<b><math>\gamma</math>-tocopherols</b>	8.5	
<b><math>\gamma/\alpha</math>-Tocotrienol</b>	8.3/8.6	
<b>Benzyl acetate</b>	1.7	
<b>3,9-dodecadien</b>	3.3	
<b>Bicyclo[10.1.0]undec-1-ene</b>	4.1	
<b>3,6-Dimethyl-1,5-heptadiene</b>	3.2	
<b>Octane</b>	3.4	

## ANNEXES (2/2) : FIGURES ET TABLEAUX CITES EN SUPPLEMENTARY DATA DANS L'ARTICLE DU CHAPITRE 3

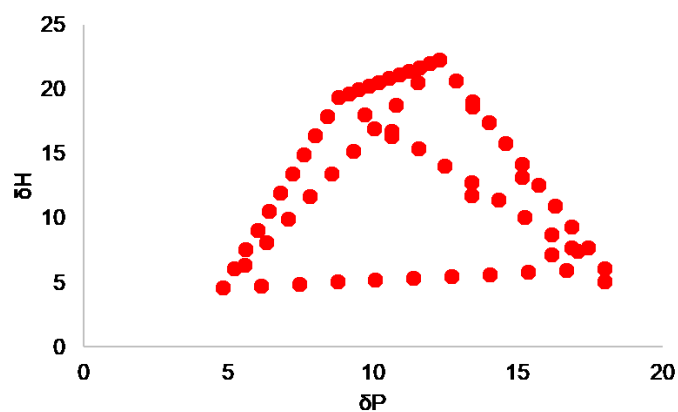
*Supercritical fluid chromatography (SFC) development of a  
predictive analytical tool to selectively extract bioactive  
compounds by pressurised extraction techniques (SFE and  
PFE)*



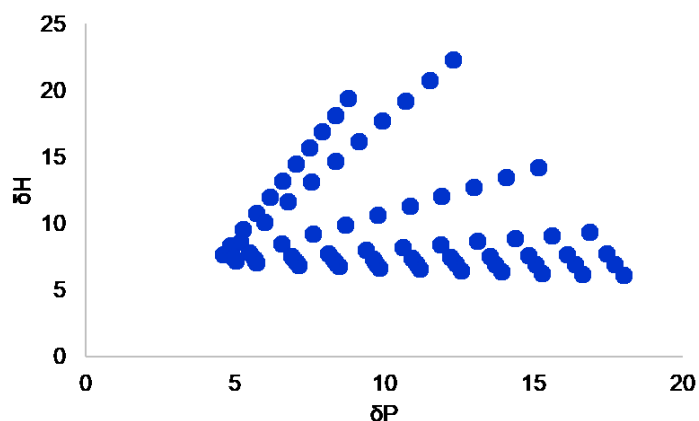
S1. SFC Chromatograms of (a) rosmarinic acid at 331 nm and (b) chlorophylls at 650 nm, with varied modifiers: methanol (pink solid line); methanol/water 95/5, v/v (blue dotted line); methanol/water/citric acid 95/5/0.1 v/v/v (green dashes). Column: Torus DIOL. For other analytical conditions, see section 2.4.



S2. SFC Chromatograms of carnosic acid, rosmarinic acid and chlorophylls on Torus DIOL with methanol/water 95/5 v/v as modifier. Other conditions described in section 2.4



S3. Distribution of  $\delta H$  and  $\delta P$  for all liquid mixtures



S4. Distribution of  $\delta H$  and  $\delta P$  for all supercritical carbon dioxide mixtures

S5. HSP calculated for different methanol-ethanol mixtures

Solvent	$\delta D$	$\delta P$	$\delta H$
100% MeOH	15.10	12.30	22.30
90:10 MeOH-EtOH	15.17	11.95	22.01
80:20 MeOH-EtOH	15.24	11.60	21.72
70:30 MeOH-EtOH	15.31	11.25	21.43
60:40 MeOH-EtOH	15.38	10.90	21.14
50:50 MeOH-EtOH	15.45	10.55	20.85
40:60 MeOH-EtOH	15.52	10.20	20.56
30:70 MeOH-EtOH	15.59	9.85	20.27
20:80 MeOH-EtOH	15.66	9.50	19.98
10:90 MeOH-EtOH	15.73	9.15	19.69
100% EtOH	15.80	8.80	19.40

S6. HSP calculated for different ethanol-acetonitrile mixtures

Solvent	$\delta D$	$\delta P$	$\delta H$
100% EtOH	15.80	8.80	19.40
90:10 EtOH-ACN	15.75	9.72	18.07
80:20 EtOH-ACN	15.70	10.64	16.74
70:30 EtOH-ACN	15.65	11.56	15.41
60:40 EtOH-ACN	15.60	12.48	14.08
50:50 EtOH-ACN	15.55	13.40	12.75
40:60 EtOH-ACN	15.50	14.32	11.42
30:70 EtOH-ACN	15.45	15.24	10.09
20:80 EtOH-ACN	15.40	16.16	8.76
10:90 EtOH-ACN	15.35	17.08	7.43
100% ACN	15.30	18.00	6.10

S7. HSP calculated for different methanol-acetonitrile mixtures

Solvent	$\delta D$	$\delta P$	$\delta H$
100% MeOH	15.10	12.30	22.30
90:10 MeOH-ACN	15.12	12.87	20.68
80:20 MeOH-ACN	15.14	13.44	19.06
70:30 MeOH-ACN	15.16	14.01	17.44
60:40 MeOH-ACN	15.18	14.58	15.82
50:50 MeOH-ACN	15.20	15.15	14.20
40:60 MeOH-ACN	15.22	15.72	12.58
30:70 MeOH-ACN	15.24	16.29	10.96
20:80 MeOH-ACN	15.26	16.86	9.34
10:90 MeOH-ACN	15.28	17.43	7.72
100% ACN	15.30	18.00	6.10

S8. HSP calculated for different 2-methyltetrahydrofuran-methanol mixtures

Solvent	$\delta D$	$\delta P$	$\delta H$
100% MeTHF	16.80	4.80	4.60
90:10 MeTHF-MeOH	16.63	5.55	6.37
80:20 MeTHF-MeOH	16.46	6.30	8.14
70:30 MeTHF-MeOH	16.29	7.05	9.91
60:40 MeTHF-MeOH	16.12	7.80	11.68
50:50 MeTHF-MeOH	15.95	8.55	13.45
40:60 MeTHF-MeOH	15.78	9.30	15.22
30:70 MeTHF-MeOH	15.61	10.05	16.99
20:80 MeTHF-MeOH	15.44	10.80	18.76
10:90 MeTHF-MeOH	15.27	11.55	20.53
100% MeOH	15.10	12.30	22.30

S9. HSP calculated for different 2-methyltetrahydrofuran-ethanol mixtures

Solvent	$\delta D$	$\delta P$	$\delta H$
100% MeTHF	16.80	4.80	4.60
90:10 MeTHF-EtOH	16.70	5.20	6.08
80:20 MeTHF-EtOH	16.60	5.60	7.56
70:30 MeTHF-EtOH	16.50	6.00	9.04
60:40 MeTHF-EtOH	16.40	6.40	10.52
50:50 MeTHF-EtOH	16.30	6.80	12.00
40:60 MeTHF-EtOH	16.20	7.20	13.48
30:70 MeTHF-EtOH	16.10	7.60	14.96
20:80 MeTHF-EtOH	16.00	8.00	16.44
10:90 MeTHF-EtOH	15.90	8.40	17.92
100% EtOH	15.80	8.80	19.40

S10. HSP calculated for different 2-methyltetrahydrofuran-acetonitrile mixtures

Solvent	$\delta D$	$\delta P$	$\delta H$
100% MeTHF	16.80	4.80	4.60
90:10 MeTHF-ACN	16.65	6.12	4.75
80:20 MeTHF-ACN	16.50	7.44	4.90
70:30 MeTHF-ACN	16.35	8.76	5.05
60:40 MeTHF-ACN	16.20	10.08	5.20
50:50 MeTHF-ACN	16.05	11.40	5.35
40:60 MeTHF-ACN	15.90	12.72	5.50
30:70 MeTHF-ACN	15.75	14.04	5.65
20:80 MeTHF-ACN	15.60	15.36	5.80
10:90 MeTHF-ACN	15.45	16.68	5.95
100% ACN	15.30	18.00	6.10

S11. HSP calculated for different propylene carbonate mixtures with ethanol, methanol and acetonitrile

Solvent	$\delta D$	$\delta P$	$\delta H$
80:20 PC-EtOH	19.20	16.20	7.20
50:50 PC-EtOH	17.90	13.40	11.80
20:80 PC-EtOH	16.60	10.60	16.30
100% EtOH	15.80	8.80	19.40
80:20 PC-MeOH	19.00	16.90	7.70
50:50 PC-MeOH	17.60	15.20	13.20
20:80 PC-MeOH	16.10	13.40	18.70
100% MeOH	15.10	12.30	22.30
50:50 PC-ACN	17.70	18.00	5.10
100% ACN	15.30	18.00	6.10

S12. HSP calculated for different carbon dioxide-ethanol mixtures

Modifier in CO2	$\delta D$	$\delta P$	$\delta H$
5% EtOH	16.28	4.62	7.72
10% EtOH	16.25	4.84	8.33
20% EtOH	16.20	5.28	9.56
30% EtOH	16.15	5.72	10.79
40% EtOH	16.10	6.16	12.02
50% EtOH	16.05	6.60	13.25
60% EtOH	16.00	7.04	14.48
70% EtOH	15.95	7.48	15.71
80% EtOH	15.90	7.92	16.94
90% EtOH	15.85	8.36	18.17
100% EtOH	15.80	8.80	19.40

S13. HSP calculated for different carbon dioxide-methanol mixtures

Modifier in CO2	$\delta D$	$\delta P$	$\delta H$
5% MeOH	16.24	4.80	7.86
10% MeOH	16.18	5.19	8.62
20% MeOH	16.06	5.98	10.14
30% MeOH	15.94	6.77	11.66
40% MeOH	15.82	7.56	13.18
50% MeOH	15.70	8.35	14.70
60% MeOH	15.58	9.14	16.22
70% MeOH	15.46	9.93	17.74
80% MeOH	15.34	10.72	19.26
90% MeOH	15.22	11.51	20.78
100% MeOH	15.10	12.30	22.30

S14. HSP calculated for different carbon dioxide-acetonitrile mixtures

Modifier in CO2	$\delta D$	$\delta P$	$\delta H$
20% ACN	16.10	7.12	6.90
30% ACN	16.00	8.48	6.80
40% ACN	15.90	9.84	6.70
50% ACN	15.80	11.20	6.60
60% ACN	15.70	12.56	6.50
70% ACN	15.60	13.92	6.40
80% ACN	15.50	15.28	6.30
90% ACN	15.40	16.64	6.20
100% ACN	15.30	18.00	6.10

S15. HSP calculated for different carbon dioxide-acetonitrile:methanol 95/5 v/v mixtures

Modifier in CO2	$\delta D$	$\delta P$	$\delta H$
10% ACN-MeOH 95:5	16.20	5.73	7.08
20% ACN-MeOH 95:5	16.10	7.06	7.06
30% ACN-MeOH 95:5	16.00	8.39	7.04
40% ACN-MeOH 95:5	15.90	9.73	7.02
50% ACN-MeOH 95:5	15.80	11.06	7.01
60% ACN-MeOH 95:5	15.69	12.39	6.99
70% ACN-MeOH 95:5	15.59	13.72	6.97
80% ACN-MeOH 95:5	15.49	15.05	6.95
90% ACN-MeOH 95:5	15.39	16.38	6.93
100% ACN-MeOH 95:5	15.29	17.72	6.91

S16. HSP calculated for different carbon dioxide-acetonitrile:methanol 90/10 v/v mixtures

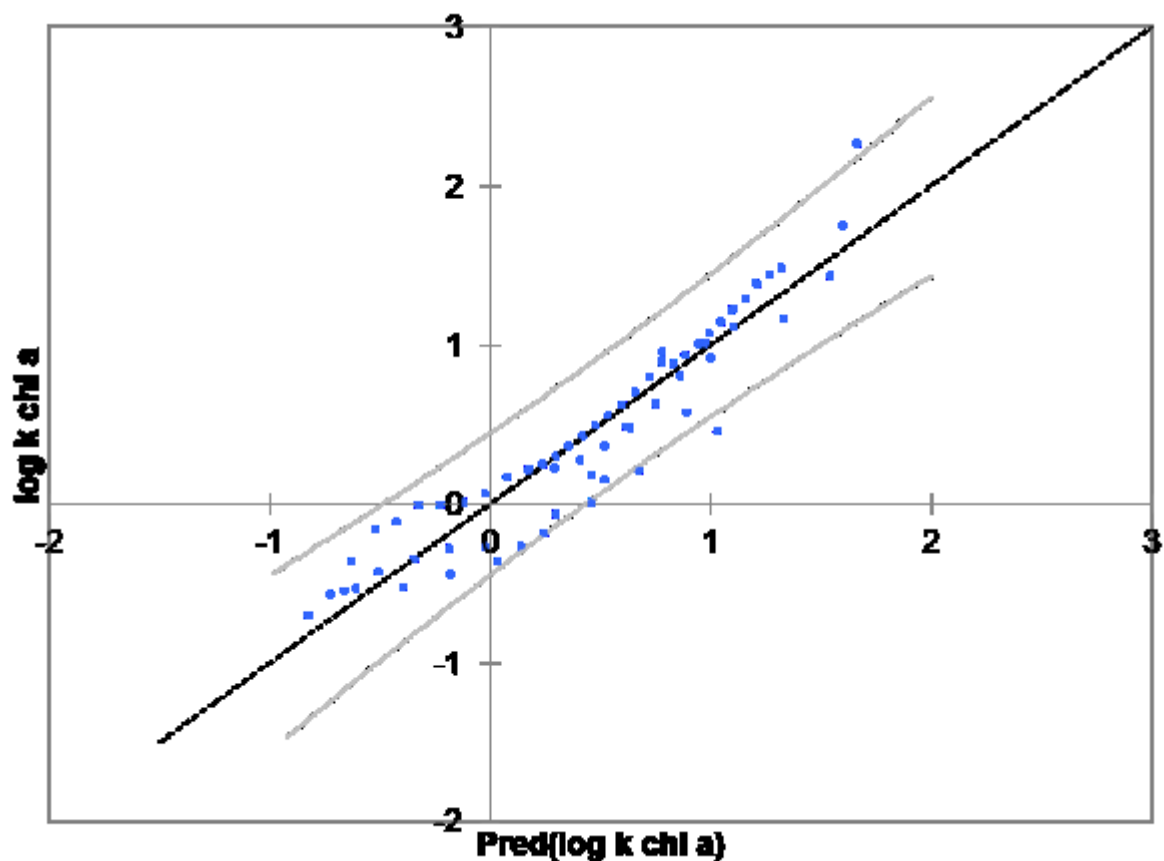
Modifier in CO2	$\delta D$	$\delta P$	$\delta H$
10% ACN-MeOH 90:10	16.20	5.70	7.16
20% ACN-MeOH 90:10	16.10	7.01	7.22
30% ACN-MeOH 90:10	15.99	8.31	7.29
40% ACN-MeOH 90:10	15.89	9.61	7.35
50% ACN-MeOH 90:10	15.79	10.92	7.41
60% ACN-MeOH 90:10	15.69	12.22	7.47
70% ACN-MeOH 90:10	15.59	13.52	7.53
80% ACN-MeOH 90:10	15.48	14.82	7.60
90% ACN-MeOH 90:10	15.38	16.13	7.66
100% ACN-MeOH 90:10	15.28	17.43	7.72

S17. HSP calculated for different carbon dioxide-acetonitrile:methanol 80/20 v/v mixtures

Modifier in CO2	$\delta D$	$\delta P$	$\delta H$
5% ACN-MeOH 80:20	16.25	5.02	7.21
10% ACN-MeOH 80:20	16.20	5.65	7.32
20% ACN-MeOH 80:20	16.09	6.89	7.55
30% ACN-MeOH 80:20	15.99	8.14	7.77
40% ACN-MeOH 80:20	15.88	9.38	8.00
50% ACN-MeOH 80:20	15.78	10.63	8.22
60% ACN-MeOH 80:20	15.68	11.88	8.44
70% ACN-MeOH 80:20	15.57	13.12	8.67
80% ACN-MeOH 80:20	15.47	14.37	8.89
90% ACN-MeOH 80:20	15.36	15.61	9.12
100% ACN-MeOH 80:20	15.26	16.86	9.34

S18. HSP calculated for different carbon dioxide-acetonitrile:methanol 50/50 v/v mixtures

Modifier in CO2	$\delta D$	$\delta P$	$\delta H$
5% ACN-MeOH 50:50	16.25	4.94	7.46
10% ACN-MeOH 50:50	16.19	5.48	7.81
20% ACN-MeOH 50:50	16.08	6.55	8.52
30% ACN-MeOH 50:50	15.97	7.63	9.23
40% ACN-MeOH 50:50	15.86	8.70	9.94
50% ACN-MeOH 50:50	15.75	9.78	10.65
60% ACN-MeOH 50:50	15.64	10.85	11.36
70% ACN-MeOH 50:50	15.53	11.93	12.07
80% ACN-MeOH 50:50	15.42	13.00	12.78
90% ACN-MeOH 50:50	15.31	14.08	13.49
100% ACN-MeOH 50:50	15.20	15.15	14.20



S19. Values of  $\log k$  experimental vs  $\log k$  predicted from the regression coefficients obtained from retention in liquid mobile phases of figure 6

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**Production d'extraits de plantes non colorés enrichis en composés bioactifs. Investigation des techniques d'extraction modernes (US, MO, SFE, PLE)**

**Résumé :**

Dans le domaine de la cosmétique, de nombreuses matières premières sont utilisées et les extraits naturels issus de plantes en représentent une grande partie. Les extraits végétaux peuvent avoir des activités différentes : antioxydantes, anti-vieillessement.... Cependant, leur couleur essentiellement dû aux pigments peut être un frein à leur utilisation car ils peuvent avoir des problèmes de stabilité. Leur dégradation se traduit par un changement de couleur ce qui peut être incompatible avec leur formulation.

L'objectif de la thèse est de trouver des conditions sélectives afin d'extraire des composés d'intérêts sans pigments (principalement chlorophylliens).

Dans un premier temps, une étude de la stabilité des chlorophylles a permis d'identifier des conditions d'extraction et de stockage afin d'identifier et de quantifier les chlorophylles natives et leurs produits de dégradation en fonction des différentes approches opératoires. Cela a permis d'identifier des conditions d'extraction et de stockage limitant leurs dégradations. Dans un second temps une modélisation a été réalisée, principalement en chromatographie supercritique, pour déterminer des conditions d'extraction sélective, avec des fluides supercritiques, de molécules antioxydantes (acide carnosique et rosmarinique) d'une part et des pigments chlorophylliens d'autre part à partir de romarin, sans avoir besoin d'effectuer de nombreuses expériences d'extraction. Finalement, une extraction par CO<sub>2</sub> supercritique séquencée utilisant des conditions déterminées par le précédent modèle a été développée. Un système extraction/chromatographie en ligne a été développé afin d'appuyer ce développement par des études de cinétiques d'extraction. Cette extraction a permis de réaliser quatre fractions respectivement riches en caroténoïdes, acide carnosique, acide rosmarinique et chlorophylles.

Mots clés : Extraction sélective, Pigments, Romarin, Phytochimie, Chimie analytique,

**Production of non-colored plant extracts enriched with bioactive compounds. Investigation of modern extraction techniques (UAE, MAE, SFE, PLE)**

**Summary :**

In the field of cosmetics, many raw materials are used and natural extracts from plants represent a large part of them. Plant extracts can have different activities: antioxidants, anti-aging ... However, their color mainly due to pigments can be an obstacle to their use because they can have stability problems. Their degradation results in a change of color which may be incompatible with their formulation.

The objective of the thesis is to find selective conditions in order to extract compounds of interest without pigments (mainly chlorophylls).

Initially, a study of the stability of chlorophylls allowed identification of extraction and storage conditions in order to identify and quantify native chlorophylls and their degradation products according to the different operating approaches. This made it possible to identify extraction and storage conditions limiting their degradation. Secondly, modeling was carried out, mainly by supercritical chromatography, to determine selective extraction conditions, with supercritical fluids, of antioxidant molecules (carnosic and rosmarinic acids) on the one hand and chlorophyll pigments on the other hand from rosemary, without the need for numerous extraction experiments. Finally, a sequential supercritical CO<sub>2</sub> extraction using conditions determined by the previous model was developed. An online extraction/chromatography system was developed to support this development through extraction kinetics studies. This extraction allowed the realization of four fractions respectively rich in carotenoids, carnosic acid, rosmarinic acid and chlorophylls.

Keywords : Selective extraction, Pigments, Rosemary, Phytochemistry, Analytical chemistry



Institut de Chimie Organique et Analytique  
Université d'Orléans, CNRS UMR 7311, BP 6759  
45067 Orléans Cedex 2, France

