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Mahmoud HAMZAOUI

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Développements méthodologiques en Extraction de Partage Centrifuge (EPC). Application au fractionnement et à la purification de substances naturelles végétales et issues des biotechnologies blanches

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Jury

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Pr. Dominique PAREAU	École Centrale Paris	Rapporteur
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Romain REYNAUD	Soliance	Examinateur
Pr. Jean-Hugues RENAULT	Université de Reims	Directeur de thèse
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Développements méthodologiques en Extraction de Partage Centrifuge (EPC). Application au fractionnement et à la purification de substances naturelles végétales et issues des biotechnologies blanches.

Institut de Chimie Moléculaire de Reims (UMR CNRS 7312, Groupe « Isolement & Structures »)

Résumé : Les travaux présentés dans ce manuscrit s'inscrivent dans une optique d'étude et de caractérisation d'un prototype Extracteur de Partage Centrifuge (FCPE) son application au fractionnement et à la purification de produits naturels à partir des extraits végétaux ou fermentaires. La première partie des travaux de thèse consiste aux développements méthodologiques réalisés en EPC en mode de déplacement par échange d'ions fort (SIX-CPE) pour l'intensification de l'extraction et de la purification de composés ioniques naturels (glycyrrhizine, sinalbine, acide itaconique). Les développements méthodologiques étaient focalisés en particulier sur l'étude hydrodynamique des phases en écoulement à l'intérieur de la colonne EPC durant les différentes étapes du processus de séparation, sur la détermination des facteurs limitants ainsi que sur les conditions opératoires optimales conduisant à des taux de recouvrement, productivité et pureté satisfaisants. La deuxième partie des travaux est consacrée à l'investigation des potentialités de l'extracteur FCPE en tant qu'instrument permettant de simplifier des extraits naturels complexes par extraction fractionnée sélective et enrichissement des fractions en composés ciblés. Deux méthodes sont en particulier présentées, utilisant comme support méthodologique un extrait éthanolique de l'écorce d'un arbre sub-saharien, *Anogeissus leiocarpus* Guill. & Perr. (Combretaceae). La première a été développée en combinant l'EPC à un mode original d'élution tri-phasique séquentiel balayant un large spectre de polarité. La seconde a également été développée par pH-zone-refining, basée sur le changement du coefficient de distribution (K_D) des composés ciblés (polyphénols et dérivés de l'acide ellagique notamment) par une variation du pH. Les fractions générées à l'issue de ce procédé ont été analysées pour leur potentiel antioxydant par analyse chimique (piégeage du radical DPPH).

Mots clés : Extraction de partage centrifuge, intensification, glycyrrhizin, sinalbine, acide itaconique, polyphénols, acide éllagique, DPPH, echange d'ion, three-phase solvent systems, pH-Zone refining.

Methodological developments of Centrifugal Partition Extraction (CPE). Application to fractionation and purification of the natural substances derived from plants and white biotechnology. *(Institute of Molecular Chemistry Reims (UMR CNRS 7312), Groupe: "Isolation & Structures")*

Abstract: This work presented in this manuscript deal with the study and the characterization of the new Centrifgal Partition Extractor (FCPE) and its application to the fractionation and the purification of natural products from plant and microbial extracts. In the first part of this work, the potential of the extractor CPE combined with the Ion-Exchange (SIX-CPE) mode to simultaneously extract and purify natural ionized compounds (glycyrrhizin, sinalbine and itaconic acid). Methodological developments were focused in particular on the study of the hydrodynamic behavior of the liquid phases inside the CPE partition cells during the different stages of the separation process, the determination of the limiting factors, as well as, the optimal operating conditions leading to optimal product recovery, productivity and purity. The second part of the work is devoted to the investigation of the potentialities of the FCPE extractor to simplify complex natural extracts by fractional extraction and selective enrichment of fractions in target compounds. Two methods are developed using an ethanolic extract of the bark of a sub- Saharan tree, Anogeissus leiocarpus Guill. & Perr. (Combretaceae) as methodological support. The first was developed by combining the EPC to an original sequential three-phase elution method with a wide range of polarity. The second was also developed in displacement pH-zone refining mode, based on the change of the distribution coefficient (KD) of the target compounds (polyphenols and ellagic acid derivavtives) by a changing the pH. Fractions generated at the end of this process were analyzed for their antioxidant potential by chemical analysis (DPPH radical scavenging).

Key words: Centrifugal partition extraction, intensification, glycyrrhizin, sinalbin, itaconic acid, polyphenols, ellagic acid, DPPH, ion-exchange, three-phase solvent systems, pH-zone refining.

A

Mes parents,

Mes sœurs

Ma femme,

Ma future petite fille « KENZA »

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Liste des publications et des posters

Articles :

1) Intensified extraction of ionized natural products by ion pair centrifugal partition extraction.

Mahmoud Hamzaoui, Jane Hubert, Jamila Hadj-Salem, Bernard Richard, Dominique Harakat, Luc Marchal, Alain Foucault, Catherine Lavaud, Jean-Hugues Renault; *Journal of Chromatography A*, Volume 1218, Issue 31, **2011**, Pages 5254–5262.

2) Strong ion exchange in centrifugal partition extraction (SIX-CPE): Effect of partition cell design and dimensions on purification process efficiency.

Mahmoud Hamzaoui, Jane Hubert, Luc Marchal, Alain Foucault, Romain Reynaud, Jean-Hugues Renault; *Journal of Chromatography A*, Volume 1247, **2012**, Pages 18–25.

3) Stepwise Elution of Three Phase Solvent System in Centrifugal Partition Extraction: A New Strategy for the Fractionation and Phytochemical Screening of Crude Bark Extracts.

Mahmoud Hamzaoui, Jean-Hugues Renault, Romain Reynaud, Jane Hubert; *Phytochemical Analysis*, **2013**, DOI 10.1002/pca.2418.

4) New perspectives for microbial glycolipid fractionation and purification processes

Jane Hubert, Karen Plé, Mahmoud Hamzaoui, Gladys Nuissier, Imane Hadef, Romain Reynaud, Arnaud Guilleret, Jean-Hugues Renault; *Comptes Rendues Chimie*, Volume: 15, Issue 1**, 2012**, Pages: 18-28.

5) Polyphenol purification by solid support-free liquid-liquid chromatography (CCC, CPC).

Jane Hubert, Karen Plé, Mahmoud Hamzaoui, Jean-Hugues Renault, *Handbook of Natural Products - Phytochemistry, Botany, Metabolism*, Chapter V. 189, **2013**.

6) A gradient elution method in centrifugal partition chromatography for the separation of a complex sophorolipid mixture obtained from Candida bombicola yeasts.

Alexis Kotland, Imane Hadef, Jean-Hugues Renault, Mahmoud Hamzaoui, Agathe Martinez, Arnaud Guilleret, Romain Reynaud, Jane Hubert; *Journal of Separation Science*, **2013**, (Accepted article)

Posters:

1) Intensified extraction of ionized natural products by ion pair centrifugal partition extraction.

Mahmoud Hamzaoui, Jane Hubert, Jamila Hadj-Salem, Bernard Richard, Dominique Harakat, Luc Marchal, Alain Foucault, Catherine Lavaud, Jean-Hugues Renault; in *CCC2010 symposium*, **2010** (Lyon, France) and *PREP symposium*, **2011** (Boston, USA)

2) Intensified extraction of ionized natural products by strong ion-exchange centrifugal partition extraction.

Mahmoud Hamzaoui, Jane Hubert, Luc Marchal, Alain Foucault, Romain Reynaud, Jean-Hugues Renault; *SPICA*, **2012** (Bruxuelles, Belgium)

3) Stepwise Elution of Three Phase Solvent System in Centrifugal Partition Extraction: A New Strategy for the Fractionation and Phytochemical Screening of Crude Bark Extracts.

Mahmoud Hamzaoui, Jean-Hugues Renault, Romain Reynaud, Jane Hubert; *SPICA*, **2012** (Bruxelles, Belgium)

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INTRODUCTION GÉNÉRALE

Introduction générale

Ces dernières années, les substances d'origine végétale ont suscité un intérêt croissant dans de nombreux domaines. Cet intérêt est directement lié à un public de plus en plus réticent vis à vis des produits synthétiques ou contenant des molécules issues de la pétrochimie. Dans ce contexte, certains secteurs comme l'industrie pharmaceutique, cosmétique ou agro-alimentaire sont revenus vers des matières premières naturelles pour mettre en œuvre leurs procédés de fabrication. L'enjeu majeur réside dans l'exploitation et l'incorporation de ces molécules naturelles aux caractéristiques chimiques et biologiques variées, dans des préparations médicamenteuses, formulations cosmétiques ou produits alimentaires.

Tout comme les métabolites secondaires issus des plantes (composés phénoliques, terpénoïdes et alcaloïdes), les molécules issues des biotechnologies (peptides, protéines, antibiotiques, glycolipides, acides carboxyliques, acides gras …) sont également de plus en plus valorisées dans les secteurs industriels cités ci-dessus. En effet, l'accroissement de la demande énergétique mondiale (pétrole et ressources naturelles fossiles) et les problèmes environnementaux (rejets industriels) exigent une diminution de l'utilisation des ressources limitées et polluantes. A l'heure actuelle, les biotechnologies blanches, par exemple, qui consistent à utiliser des systèmes biologiques (bactéries, enzymes) pour fabriquer, transformer ou dégrader des molécules valorisables dans l'industrie ou pour produire de l'énergie à partir de la biomasse [1, 2], constituent une alternative intéressante pour remplacer, à terme, les produits issus de la pétrochimie [3-5]. Un progrès important a été réalisé au niveau de la recherche et du développement des procédés de fermentation, notamment au niveau de la sélection de nouvelles souches bactériennes et de l'intensification de la production de ces bioproduits.

Dans le but de valoriser les métabolites naturels issus des végétaux ou des biotechnologies, ceux-ci doivent d'abord être séparés de leur matrice d'origine. De nombreuses étapes d'extraction et de purification sont nécessaires pour l'isolement de ces métabolites et freinent encore souvent leur production à grande échelle. Un des enjeux majeurs des sciences séparatives consiste donc en l'innovation technologique et le développement de nouveaux équipements et méthodologies permettant d'extraire et de purifier ces molécules avec une productivité satisfaisante. Les techniques d'extraction liquide-liquide présentent un intérêt industriel qui n'est plus à démontrer, tant les procédés utilisant ce concept pour les opérations unitaires d'extraction sont nombreux. Nous pouvons citer à titre d'exemple les extracteurs de type mélangeurs-décanteurs, les extracteurs

centrifuges (Podbielniak®, Quadronic®) ou les colonnes d'extraction liquide-liquide (Sheibel®, Kühni®, *etc*…). Ces équipements, de part leurs caractéristiques propres, présentent des champs d'applications spécifiques. Les extracteurs centrifuges en particulier permettent d'appliquer des débits élevés en limitant les phénomènes d'entraînement lors de l'utilisation de systèmes émulsifs et/ou présentant de faibles différences de masse volumique. Ces extracteurs sont aussi caractérisés par des temps de séjours très courts (de l'ordre de la seconde) et par des productivités élevées. Généralement, avec ces technologies, le nombre d'unités de transfert « NTU » ne dépasse pas la dizaine. Par ailleurs, les techniques de chromatographie liquide-liquide sans support solide (Chromatographie à Contre-courant « CCC » et Chromatographie de Partage Centrifuge « CPC ») se sont également développées, au départ essentiellement dans les laboratoires académiques pour la purification de substances naturelles. Bien que relevant du même processus que l'extraction liquide-liquide, ce type de technique générant plusieurs centaines de plateaux théoriques rentre bien dans les critères définissant la chromatographie.

Il apparaît cependant que des appareils « intermédiaires », c'est-à-dire générant aux alentours de 100 plateaux théoriques, sont encore très peu développés. Ils permettraient pourtant de lever certains verrous technologiques rencontrés en extraction liquide-liquide, tels que la faible sélectivité et le manque d'efficacité des colonnes classiquement utilisées dans l'industrie, mais aussi en chromatographie liquide-liquide comme le coût élevé des opérations de purification et le manque de productivité des systèmes chromatographiques fréquemment utilisés dans les industries chimique, pharmaceutique, cosmétique, etc …

L'Extraction de Partage Centrifuge (EPC) a récemment été co-développée par l'UMR 6144 (GEPEA, Saint-Nazaire), la société Rousselet-Robatel Kromaton (Annonay, France), et l'UMR 7312 (ICMR, Reims) et c'est dans ce cadre que s'inscrit ce projet de thèse. L'objectif était d'étudier les aspects fondamentaux de l'Extraction de Partage Centrifuge et son potentiel dans l'extraction, le fractionnement, voire la purification de métabolites de faible poids moléculaire issus d'extraits végétaux ou de milieux fermentaires.

Ce projet de thèse a fait intervenir plusieurs partenaires académiques et industriels:

- Notre unité, l'Institut de Chimie Moléculaire de Reims (UMR CNRS 7312) et plus particulièrement le groupe « Isolement & Structures », pour les développements méthodologiques en EPC et la comparaison des procédés développés.

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- L'équipe de GÉnie des Procédés Environnement et Agroalimentaire (GEPEA) de L'UMR CNRS 6144 (Saint-Nazaire, France), pour la visualisation des écoulements et l'étude hydrodynamique des phases liquides lors des procédés extractifs développés en EPC.
- Rousselet-Robatel Kromaton (Annonay, France) pour la mise à disposition d'un appareil prototype d'extraction de partage centrifuge (FCPE300®).

Ce manuscrit s'articule autour de trois parties distinctes. Le premier chapitre présente une synthèse bibliographique visant à décrire les aspects fondamentaux de l'extraction et de la chromatographie liquide-liquide sans support-solide. Il s'agira de décrire les différentes méthodes et de classifier les principaux équipements utilisés pour l'extraction et la purification des métabolites secondaires végétaux ou microbiens. Les techniques de chromatographie liquide-liquide sans support solide seront également développées en mettant l'accent sur la CCC et la CPC et leur évolution qui a mené au développement de l'EPC et à son positionnement par rapport aux autres techniques séparatives liquide-liquide.

Le second chapitre présente les résultats des développements méthodologiques réalisés en EPC en mode de déplacement par échange d'ions fort (SIX-CPE) pour l'intensification de l'extraction et de la purification de composés ioniques naturels. Ces premiers travaux ont été réalisés en utilisant comme supports méthodologiques la glycyrrhizine, une saponine ionique et biologiquement active présente dans les racines de réglisse (*Glycyrrhiza glabra L.*) [6-8] ; la sinalbine, un glucosinolate naturellement présent dans les grains de moutarde blanche (*Sinapis. alba L.*) ; et l'acide itaconique, un diacide organique hydrophile produit par fermentation à l'échelle industrielle. Ces premiers développements méthodologiques étaient focalisés en particulier sur l'étude hydrodynamique des phases en écoulement à l'intérieur de la colonne EPC durant les différentes étapes du processus de séparation, sur la détermination des facteurs limitants (masses injectées, volumes traités, complexité de la composition des extraits naturels, durée de l'expérience) ainsi que sur les conditions opératoires optimales conduisant à des taux de recouvrement, productivité et pureté satisfaisants.

Enfin, un troisième chapitre est consacré à l'investigation des potentialités de l'extracteur de partage centrifuge en tant qu'instrument permettant de simplifier des extraits naturels complexes

par extraction fractionnée sélective et enrichissement des fractions en composés ciblés. Deux méthodes sont en particulier présentées, utilisant comme support méthodologique un extrait éthanolique de l'écorce d'un arbre sub-saharien, *Anogeissus leiocarpus* Guill. & Perr. *(Combretaceae*) fourni par la société Soliance. Une première méthode de fractionnement des différentes familles moléculaires de cet extrait (phénols, tannins, triterpènes, saponines, etc.) a été développée en combinant l'EPC à un mode original d'élution tri-phasique séquentiel balayant un large spectre de polarité. Une seconde méthode de fractionnement a également été développée par pH-zone-refining, basée sur le changement du coefficient de distribution (K_D) des composés ciblés (polyphénols et dérivés de l'acide ellagique notamment) par une variation du pH (protonation et déprotonation des métabolites). Les fractions générées à l'issue de ce procédé ont été analysées pour leur potentiel antioxydant par analyse chimique (piégeage du radical DPPH).

L'extracteur de partage centrifuge prototype est ainsi présenté comme un outil prometteur permettant d'intensifier des opérations de purification de composés ioniques naturels et de fractionnement sélectif d'extraits naturels complexes, le rapprochant des chromatographes CPC en termes de recouvrement et de pureté, et des extracteurs liquide-liquide classiques en termes d'application de débits (de 20 à 50 mL/min en moyenne), de volumes d'entrée, des masses chargéées et donc de productivité.

♣ *NB : Pour des raisons de commodité les références bibliographiques de la partie « Introduction générale » sont à la page 82.*

CHAPITRE 1

EXTRACTION DE PARTAGE CENTRIFUGE: QUAND LA CHROMATOGRAPHIE LIQUIDE-LIQUIDE REJOINT L'EXTRACTION LIQUIDE-LIQUIDE

CHAPTER 1

LIQUID-LIQUID CENTRIFUGAL PARTITION EXTRACTION: TOWARDS FILLING THE TECHNOLOGICAL GAP BETWEEN LIQUID-LIQUID EXTRACTION AND LIQUID-LIQUID CHROMATOGRAPHY

Chapitre 1 : Extraction de partage centrifuge: Quand la chromatographie liquide-liquide rejoint l'extraction liquide-liquide

Introduction du chapitre 1:

Ce chapitre de synthèse bibliographique intitulé « *Liquid-liquid centrifugal partition extraction: Towards filling the technological gap between liquid-liquid extraction and liquid-liquid chromatography* » sera publié comme une revue scientifique. Cette revue vise à décrire les aspects fondamentaux de l'extraction et de la chromatographie liquide-liquide sans support solide et s'articule autour de trois parties principales. La première partie décrit l'extraction liquide-liquide, les paramètres fondamentaux et les bases théoriques intervenant lors de la mise en œuvre d'un procédé d'extraction liquide-liquide, les caractéristiques des différents équipements d'extraction liquide-liquide, ainsi que les méthodes appliquées aux extraits végétaux et aux molécules issues des biotechnologies blanches. Dans une seconde partie, les techniques de chromatographie liquide-liquide sans support solide sont développées en mettant l'accent sur la CCC (Chromatographie à Contre-Courrant) et la CPC (Chromatographie de Partage Centrifuge). Les principes et les bases théoriques de ces deux techniques, les différents modes de développements et leurs applications pour l'isolement et la purification de métabolites naturels issus d'extraits naturels complexes sont en particulier décrits. La dernière partie est centrée sur l'évolution de ces chromatographes liquide-liquide sans support solide (CCC et CPC) et sur l'apparition récente de l'EPC (Extracteur de Partage Centrifuge). Les caractéristiques de l'EPC, son dimensionnement particulier et son positionnement par rapport aux autres techniques séparatives liquide-liquide incluant les extracteurs liquide-liquide et les chromatographes liquide-liquide sont également discutés.

I. Liquid-liquid centrifugal partition extraction: Towards filling the technological gap between liquid-liquid extraction and liquid-liquid chromatography

I.1. Introduction

Liquid–liquid extraction techniques are widely used in many fields including the pharmaceutical, cosmetic and food industries. In natural product research, numerous liquid-liquid extraction methods have been successfully to obtain molecules of high added values from natural sources such as crude plant extracts (steroids, phenols and polyphenols, terpenoids, alkaloids, polysaccharides, peptides, proteins,…) [9-14] or microbial fermentation broths (carboxylic acids, glycolipids, amino acids, peptides, antibiotics,…) [15-18]. The aim of liquid-liquid extraction methods is to liberate the target compound(s) from the whole initial matrix in sufficient quantities either for a direct commercialization, further purification experiments, structural analyses or biological assays. Liquid-liquid extraction methods are generally recommended for processing large quantities of raw materials with low cost equipment and low energy consumption. They are also particularly appropriate for the extraction of some fragile metabolites such as antibiotics, polyphenols, alkaloids, proteins and peptides which could be degraded by using distillation or crystallization. The increasing interest for the extraction and purification of natural products is accompanied by the development and implementation of many new liquid-liquid separation technologies and devices at different scales ranging from laboratory and pilot scale to industrial scale.

Numerous liquid-liquid extraction devices are now available and extensively used industrially. They include for instance the mixer-settlers, centrifugal contactors (Podbielniak®, Quadronic®) or liquid-liquid extraction columns (Sheibel®, Kühni® ...). These instruments are suitable for specific applications according to their own characteristics. Centrifugal extractors for instance allow the application of high flow rates with minimum hydrodynamic disturbance (flooding and bleeding phenomena) especially when using liquid phases with low density differences or with low surfactant properties. They are also characterized by very short residence time (5 to 10 seconds) and high productivities. Generally, the number of transfer units "NTU" in the existing liquid-liquid extraction devices does not exceed ten [19, 20]. Thus, these extraction techniques are thus, unfortunately, not sufficiently efficient to obtain pure compounds. They often enable the simplification of raw material composition, but in most cases, additional chromatographic techniques, such as preparative HPLC, Sephadex, silica gel column chromatography or liquid-liquid chromatography are required to achieve

the full purification of target compounds. Solid support-free liquid-liquid chromatographic systems, including Countercurrent Chromatography "CCC" and Centrifugal Partition Chromatography "CPC", have been extensively developed in the last thirty years. These systems are currently mostly used for the purification of natural products [21, 22] due to the absence of solute-irreversible absorption and to their high NTU (≈ 1000), these techniques are generally highly selective.

The aim of the present review was to highlight the technological gap existing between the current liquid-liquid extraction techniques, which are able to generate around 10 NTU and the liquid-liquid chromatographic techniques, which generate between 500 and 1000 NTU. Despite the efforts made in the development of both liquid-liquid extraction and chromatographic instruments, this technological gap is increasing. Indeed, the use of liquid-liquid extraction in the field of natural product research is limited to the extraction of compounds having the same physico-chemical properties. Such processes can be a starting step to rapidly recover some molecular families but without selectivity. Besides, liquid-liquid chromatographic systems (CPC and CCC) are characterized by a high selectivity but remain laboratory or pilot-scale techniques with quite low productivity. Intermediate devices, generating around 50-100 NTU are still poorly developed (Figure I.1). In this context, the Fast Centrifugal Partition Extractor (FCPE300®), recently designed by Rousselet-Robatel Kromaton® [23, 24] appears as an interesting devices which could resolve some technological gaps encountered either in upstream operations for the capture and concentration of analytes or in downstream operations for purification or polishing.

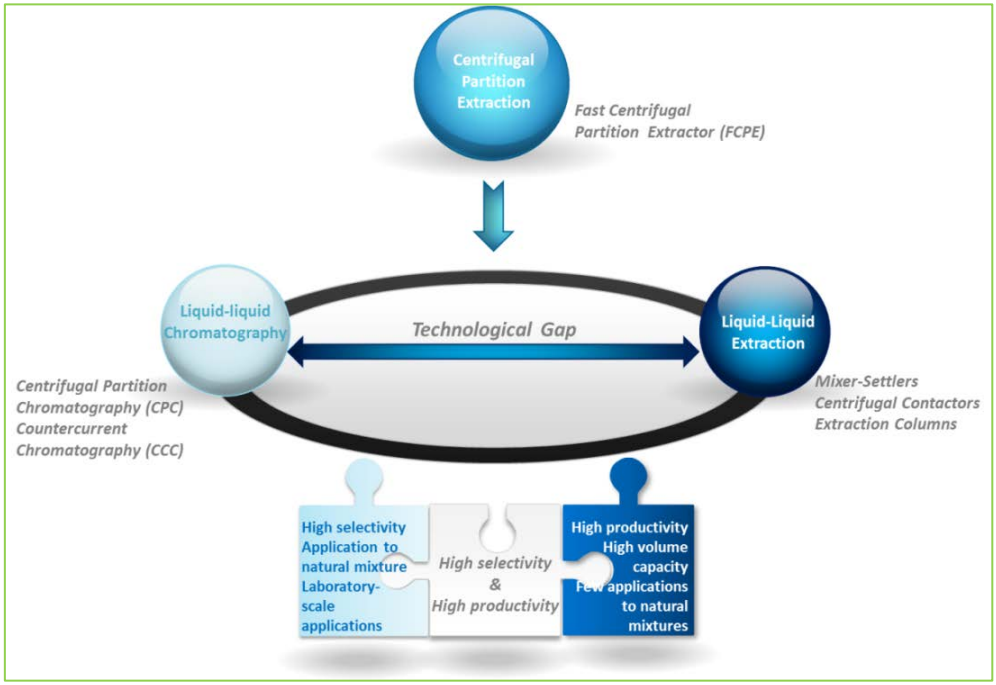

Figure I. 3: Liquid-liquid separation processes : current situation

I.2. Liquid-liquid extraction processes: Fundamentals, devices and practice in downstream processes

I.2.1. Principle and definitions

A liquid-liquid extraction process, also called "solvent extraction" or "liquid-liquid partitioning", is a separation method based on the distribution of analytes between at least two immiscible or partially miscible liquid phases in equilibrium after a mixing operation. This process is dedicated to the separation and/or concentration of analytes from raw materials [25, 26]. Its principle relies on the differences in the chemical and physicochemical properties of the target analytes including the solubility, polarity and hydrophobic/hydrophilic character. The transfer of analytes between the liquid phases and their resulting distribution are principally governed by the thermodynamic rules firstly developed by Josiah Willard Gibbs in the 1870's and known as the "Gibbs phase rules". These rules have allowed constructing phase diagrams (unary, binary and ternary diagrams) to represent and interpret liquid phase equilibrium. To understand the distribution of different analytes in a solvent system, it is necessary to refer to the chemical potential (μ). Indeed, the system is at thermodynamic equilibrium when the chemical potential a considered analyte is the same in each phase (equation 1). The chemical potential is usually expressed using the molar fraction (x_i) or directly expressed using the molar concentration (C_{aq}) and (C_{org}) of the analyte in the aqueous and organic phases, respectively (equations 2 and 3).

$$\mu_{aq} = \mu_{org}; \qquad Equation\ 1$$

$$Aqueous\ phase: \quad \mu_{aq} = \mu^0_{aq} + RT \ln C_{aq}\,; \qquad Equation\ 2$$

$$Organic\ phase: \quad \mu_{org} = \mu^0_{org} + RT \ln C_{org}\,; \qquad Equation\ 3$$

μ^0_{aq} , μ^0_{org} are the standard chemical potentials (considering that the analyte under examination is infinitely diluted at unit concentration (C_0 = 1), and standard pressure (P° = 10^5 Pa).

Besides, the Gibb's energy in the aqueous phase $(G_{aq})_{(T,P)}$ and that in the organic phase $(G_{org})_{(T,P)}$ at equilibrium can be expressed as given in equation 4 and 5, respectively:

$$Aqueous\ phase: \quad G_{aq\,(T,P)} = G^0_{aq}(T) + RT \ln C_{aq}; \qquad Equation\ 4$$

$$Organic\ phase: \quad G_{org_{(T,P)}} = G^0_{org}(T) + RT \ln C_{org}\,; \qquad Equation\ 5$$

At the thermodynamic equilibrium the differential Gibb's energy ($\Delta G_{(T,P)}$) is minimal and the chemical potentials of the analyte are equal in both phases (equation 6) :

$$\mu_{aq} = \mu_{org} = \Delta G_{(T,P)}; \qquad Equation\ 6$$

And therefore

$$\Delta G_{(T,P)} = G_{aq} - G_{org} = 0; \qquad Equation\ 7$$

$$\Delta G_{(T,P)} = \left[\Delta G^0 + RT \ln\left(\frac{C_{org}}{C_{aq}}\right)\right] = \mu_{aq} - \mu_{org} = 0; \qquad Equation\ 8$$

Therefore the standard differential Gibb's energy (ΔG^0) of the solutes can be expressed by the following equation (9):

$$\Delta G^0 = \left(\mu_{aq} - \mu_{org}\right) = -RT \ln\left(\frac{C_{org}}{C_{aq}}\right); \qquad Equation\ 9$$

The distribution coefficient (K_D) is a very important parameter in liquid-liquid extraction processes. K_D is also known as "partition coefficient" but "distribution coefficient" is the recommended term in the IUPAC terminology [27]. As given in equation 10, K_D is defined as the ratio between the concentration of the analyte in the upper phase (generally the organic phase) (C_{org}) and its concentration in the lower phase (generally the aqueous phase) (C_{aq}) at thermodynamic equilibrium.

$$K_D = \frac{C_{org}}{C_{aq}}; \qquad Equations\ 10$$

The distribution coefficient (K_D) is expressed for specific conditions of temperature and pH. Remembering equation 5, the standard Gibb's energy can thus also be expressed as given in equation 11.

$$\Delta G^0 = \left(\mu_{aq} - \mu_{org}\right) = -RT \ln K_D\,; \qquad Equation\ 11$$

Therefore the distribution of an analyte in a solvent system depends on its chemical potential (μ). Indeed, each analyte will be transferred from the phase in which the chemical potential is the highest to the phase in which the chemical potential is the lowest, until these chemical potentials become equivalent. For instance, when the chemical potential of an analyte in the aqueous phase is greater than that in the organic phase, its concentration is higher in the organic phase than in the aqueous phase and therefore $K_D > 1$ and *vice versa*.

I.2.2. Separation factor (*α*) and recovery (*R*)

In certain applications, it is important not only to recover a target analyte from the initial phase, but also to separate it from other analytes present in the initial phase. The selectivity of a given solvent for an analyte (1) compared to an analyte (2), characterized by their distribution coefficients K_{D_1} and K_{D_2} , respectively, is determined by the separation factor ($\alpha_{1,2}$) (equation 12).

$$\alpha_{1,2} = \frac{K_{D_1}}{K_{D_2}}; \qquad Equation\ 12$$

The separation factor measures the relative enrichment of analyte (1) in the extract phase, compared to analyte (2), after one theoretical stage of extraction and values must be greater than $\alpha_{1,2} = 1$ to achieve an acceptable selectivity.

The recovery *(R)* is another important term in liquid-liquid extraction. It refers to the ratio between the amount of extracted analyte (Q_f) in the organic phase and its total amount in the original phase (often in the aqueous phase) (Q_i) at equilibrium. It is expressed as given in equation 13:

$$R = \frac{Q_f}{Q_i}; \qquad Equation\ 13$$

I.2.3. Solvent systems in liquid-liquid extraction processes

In liquid-liquid extraction processes, at least two immiscible or partially miscible liquid phases are required to perform the partition of the analytes initially present in a crude mixture. The selection of a solvent system is a primordial step to be developed. This selection is firstly based on the physico-chemical characteristics of the analytes including their solubility, distribution coefficient, polarity, and complex formation ability. Secondly, the selection of a suitable solvent system depends on the intrinsic characteristics of the individual solvents which constitute the solvent systems, for instance the viscosity, density, compressibility or interfacial tension.

Two main classes of solvent systems are used: the aqueous two-phase solvent systems (also called aqueous biphasic systems) and the aqueous/non-aqueous solvent systems (also called aqueous/organic systems). Aqueous two-phase systems were firstly reported by Beijerinck in 1896 [28] and mainly developed by Albertsson [29-32]. They are formed by mixing two polymers solutions, or one polymer solution and one salt solution at appropriate concentrations (above critical concentration) [33, 34] and at a particular temperature. The aqueous/non-aqueous solvent systems

are the most commonly used solvent systems. They are based on the formation of two or more liquid phases by mixing organic solvents with water.

Ideally, two solvents involved in an extraction process are immiscible. However, in some extraction processes partially miscible solvents are used. For these systems, and particularly when the analyte concentration in the system is high, triangular or ternary phase diagrams, as shown in figure I.2, are used. In such diagrams, solvent concentrations are usually expressed in mole or mass fractions. Figure (I.2.a) shows the phase diagram of an analyte, its initial solvent (1) and its extracting solvent (2). Such phase diagram represents all possible compositions of the three components by the area within the triangle. The composition of the mixture at the point (H) on the diagram is such that the content of (A) is proportional to HL, the content of solvent (1) is proportional to (HJ) and the content of solvent (2) is proportional to (HK). The curve separating the two diagram regions (one phase and two-phase regions) is called the binodal solubility curve. The area under the curve represents the two-phase region. Any mixture represented by a point within this region will split into two phases in equilibrium. For a mixture at point (H), the composition of the two phases are represented by the points (P) and (Q) which are obtained from the intersection of the binodal solubility curve with the tie line (PQ) passing through it (H). The point (F) on the binodal solubility curve is called the plait point. The area above the binodal solubility curve represents the single-phase region where all three components in the system are mutually miscible.

Typical liquid-liquid extraction processes use binary solvent systems where the aqueous phase is directly contacted with an organic solvent (acetates, ketones, esters or alcohols) to extract the analyte. Ternary systems are of two types. The first type (type 1) uses one binary pair with limited miscibility (figure I.2.b). This is the case of water/acetic acid/methyl isobutyl ketone for the extraction of acetic acid [35]. The second type (type 2) consists in two binary pairs having limited miscibility (figure 2.c). This is the case of water/ hydroxybenzene/methyl isobutyl ketone or water/methyl ethyl ketone/chlorobenzene for the extraction of hydroxybenzene or chlorobenzene, respectively [36, 37].

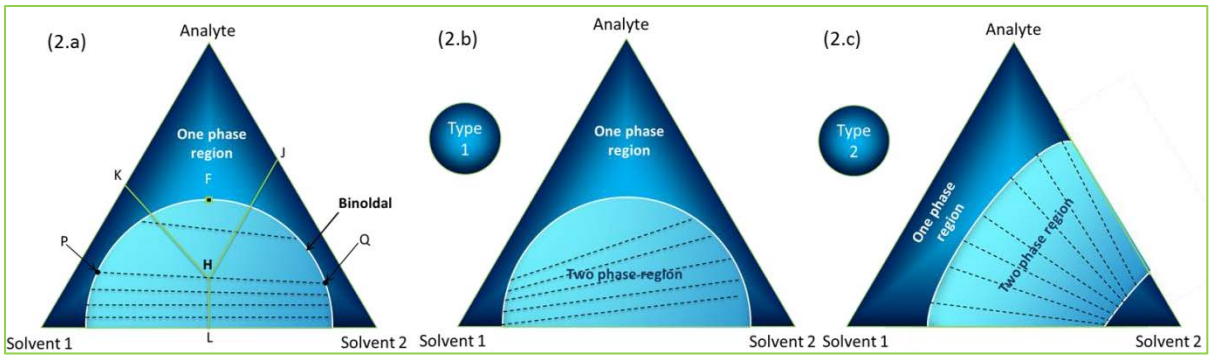

Figure I. 4: Phase diagram of the mostly used liquid-liquid extraction ternary solvent systems; (2.a): general description of the phase diagram construction; (2.b): type 1 ternary systems; (2.c): type 2 ternary systems

Very few liquid-liquid extraction processes have been developed using three-liquid phases in equilibrium. A first category of three-phase solvent system consists of a mixture of organic solvents and water, such as methyl *ter*-butyl ether/methyl acetate/acetonitrile/water [38, 39]. A second category is composed of an aqueous two-phase solvent systems mixed to an organic solvent [40] such as butyl acetate, poly(ethylene glycol), ammonium sulphate ($(NH_4)_2SO_4$) and water [41].

A major advantage in liquid-liquid extraction relies on the availability of numerous solvents which possess a wide range of physico-chemical properties (density, viscosity, miscibility, dielectric constant, volatility) and thus enable the solubilization of compounds within a wide polarity range (alkanes, ethers, esters, alcohols, water). However, the major limitation for applications at the industrial scale is principally linked to the use of toxic organic solvents which derive from the petrochemical industry such as hexane, cyclohexane, chloroform, and dichloromethane. This limitation is more noticeable when liquid-liquid extraction is applied to pharmaceuticals, cosmetic and food products [42, 43]. Indeed, few organic solvents are tolerated because of their toxicity, and they are classified into four main categories (Table I.1.) [44].

Solvents classes	*Example*
Class 1 **Solvents should not be used in the manufacture of drugs because of their acute toxicity and their adverse effect on the environment.**	Benzene, carbon tetrachloride, 1,1-dichloroethane, 1,2-dichloroethane, and 1,1,1-trichloroethane.
Class 2 **Solvents should be limited in pharmaceutical products because of their inherent toxicity.**	Acetonitrile, methanol, cyclohexane, hexane, 1,4-dioxane, pyridine, toluene, xylene, chloroform, cumene, 1,2-dichloroethane, dichloromethane, N,N-dimethylacetamide, N,N-dimethylformamide, 2-ethoxyethanol, ethylenglycol, formamide, methylbutyl ketone, methylcyclohexane, N-methylpyrrolidone, nitromethane, tetrahydrofurane, tetralin, 1,1,2-trichloroethene.
Class 3 **Solvents may be regarded as less toxic and of lower risk to human health. This class includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of them.**	Acetic acid, heptane, acetone, isobutyl acetate, anisole, isopropyl acetate, 1-butanol, methyl acetate, 2-butanol, 3-methyl-1-butanol, butyl acetate, methylethyl ketone, methyl *ter*-butyl ether, methylisobutyl ketone, dimethyl sulfoxide, 2-methyl-1-propanol, ethanol, pentane, ethyl acetate, 1-pentanol, ethyl ether, 1-propanol, 2-propanol, formic acid, ethyl formate, propyl acetate.
Class 4 **The solvents may also be used because no adequate toxicological data were found.**	1,1-diethoxypropane, methylisopropyl ketone, 1,1-dimethoxymethane, methyltetrahydrofuran, 2,2-dimethoxypropane, petroleum ether, isooctane, trichloroacetic acid, isopropyl ether, trifluoroacetic acid.

Table I. 1 : An updated guidance and recommendations for organic solvents use in pharmaceutical, cosmetic and food industries as given by the Food and Drug Administration (FDA) (2012) [44]

Faced to the increasing regulatory constraints, other alternatives based on the use of bio-based solvents are currently being developed [45]. In fact, the use of green solvents in liquid-liquid extraction is an emerging alternative [46]. Recently, for instance, rice bran oil, soybean oil, sunflower oil and sesame oil were successfully used by Keshav et *al.* as natural non-toxic solvents to achieve the liquid-liquid extraction of carboxylic acids (propionic acid, itaconic acid, succinic acid, citric acid, picolinic acid) from aqueous phases using different extractants (trioctylamine, tributylphosphate and trioctylmethylammonium chloride (Aliquat 336®)) [46, 47]. Some other available “green solvents” are summarized in Table I.2

Ester	*Alcohol*	*Terpene*
Methyllaurate	Ethanol	α-Pinene
Ethylmyrisate	Butanol	β-Pinen
Ethyl (oleate, linoleate, linolenate)	Isoamyl alcohol	d-Limonene
Glyceroltriacetate	Ethylene glycol	Terpinolene
Ethyl lactate	Propylene glycol	p-Cymene
Dimethyl (adipate (DMA), glutarate (DMG), succinate (DMS))	1,3-propanediol	β-Myrcene
	Furfuryl alcohol	
(Methyl, ethyl, n-propyl, iso-propyl, n-butyl) acetate.	Glycerol carbonate	

Table I. 2: Available green solvents (non-exhaustive list) [48]

Another interesting alternative is the use of ionic liquids to develop green liquid-liquid extraction processes [49-52]. For instance, amino acid ionic liquids with different amino acid anions (1-ethyl-3-methylimidazolium alanine [EMIM]Ala and 1-ethyl-3-methylimidazolium lysine [EMIM]Lys) as extractants diluted in *N*,*N*-dimethylformamide (DMF) were investigated in the selective liquid-liquid separation of *α*-tocoperol from its mixture with methyl linoleate [53]. The extraction of natural phenols such as tyrosol and *p*-hydroxybenzoic acid from aqueous phases was also investigated by using a series of 1-(n-alkyl)-3-methylimidazolium tetrafluoroborates [C_nMIM][BF_4] (n = 1, 3, 6, 8, 10) and hexafluorophosphates [C_nMIM][PF_6] (n = 6, 10)) [54], as well as the ionic liquid extraction of carbohydrates including xylose, fructose, glucose and sucrose from aqueous phases [55]. The interesting feature of ionic liquids is their immeasurably low vapor pressure in contrast to traditional volatile organic compounds (VOCs) [45]. They also provide other attractive properties, such as chemical and thermal stability, nonflammability, high ionic conductivity, and a wide electrochemical potential window. However, as they are not volatile, their total elimination, especially during drying of pharmaceutically active compounds, is unfortunately very difficult. Similarly, in case of overheating, degradation of these solvents may occur instead of evaporation, what can lead to other toxic by-products.

I.2.4. Liquid-liquid extraction methods

I.2.4.1. Standard liquid-liquid extraction processes

A standard extraction process is exclusively based on the distribution of the analytes between two immiscible phases. It can be achieved in batch or continuously, and in different two-

phase contacting modes according to the flow directions of the phases. The three main contacting modes are countercurrent, cross-current and co-current. In countercurrent extraction, the aqueous (P_{aq}) and the organic (P_{org}) phases pass each other in a countercurrent flow (Figure I.3.a). The two phases are mixed with droplets of one phase suspended in the other, but the phases are separated before leaving each stage. The cross-current extraction mode (Figure I.3.b) consists in a series of discrete extraction stages. As in the first case, the phase containing the target analytes, for instance the aqueous phase, contacts with the organic phase to perform mass transfer, but the aqueous phase exiting from one extraction stage is contacted again with another fresh organic phase in a subsequent stage. The extracts can be collected separately or combined for further processing. Using the cross-current extraction method is generally less often used as it requires more important solvent volumes. When using the co-current contacting mode (Figure I.3.c), the two phases flow through the column side by side but with different velocities [56].

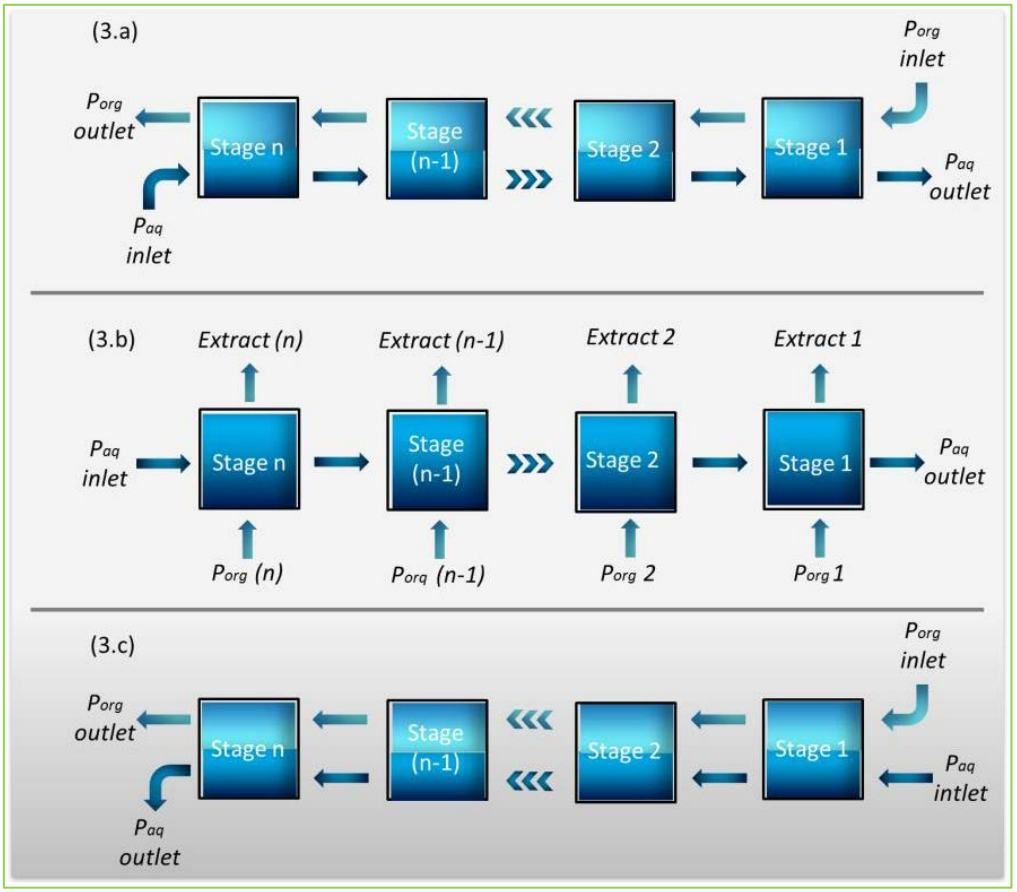

Figure I. 5: Standard liquid-liquid extraction two-phase contacting modes. (3.a) countercurrent (3.b): cross-current, (3.c): co-current

I.2.4.2. pH-based liquid-liquid extraction processes

The concept of pH-based liquid-liquid extraction derived from the acid-base character of the analytes to be separated, *i.e.* their pK_a. Two main methods are well known in pH-based liquid-liquid extraction: dissociative extraction and pH-swing extraction.

The dissociative extraction method typically involves partitioning of weak organic acids or bases between an aqueous phase and an immiscible organic phase (figure 4). The affinity of each analyte for one phase depends upon its electric charge [57-60]. Generally, aqueous phases interact much more with charged species and non-ionic forms partition in the organic phase. The pH of the injected mixture must be adjusted according to the pK_a of the target analytes to achieve a selective extraction (Figure I.4) [61]. As an example, for an organic acid [RCOOH] dissolved in an aqueous solution, the amount of dissociated state relative to the non-dissociated state is described in equation 13.

$$\frac{[RCOO^-]}{[RCOOH]} = 10^{pH-pK_a} \qquad Equation\ 13$$

Extraction of an organic acid in an aqueous phase from an organic mixture is greatly facilitated by operating at a pH above its pK_a. In the opposite case, extracting an organic acid from an initial aqueous mixture to an organic phase is favored by operating at a pH below its pK_a to promote its protonated form [RCOOH]. Dissociative extraction processes were applied for instance to the separation of a mixture of *m*-cresol (K_a = 9.8 10^{-11}) and *p*-cresol (K_a = 6.7 10^{-11}) based on the difference between their acid dissociation constant [51], to the selective extraction of substituted phenols (phenol-*o*-chlorophenol; *p*-chlorophenol-2,4-dichlorophenol; *o*-cresol-6-chloro-*o*-cresol) and (*m*-cresol-2,6-xylenol and *p*-cresol-2,6-xylenol) having closed boiling points [62], to for the extraction of natural alkaloids from *Aconitum leucostomum* [12]. Dissociative liquid-liquid extraction methods using relatively inexpensive buffer medium of various pH have also been investigated for the selective isolation and purification of primary, secondary, and tertiary amines [63].

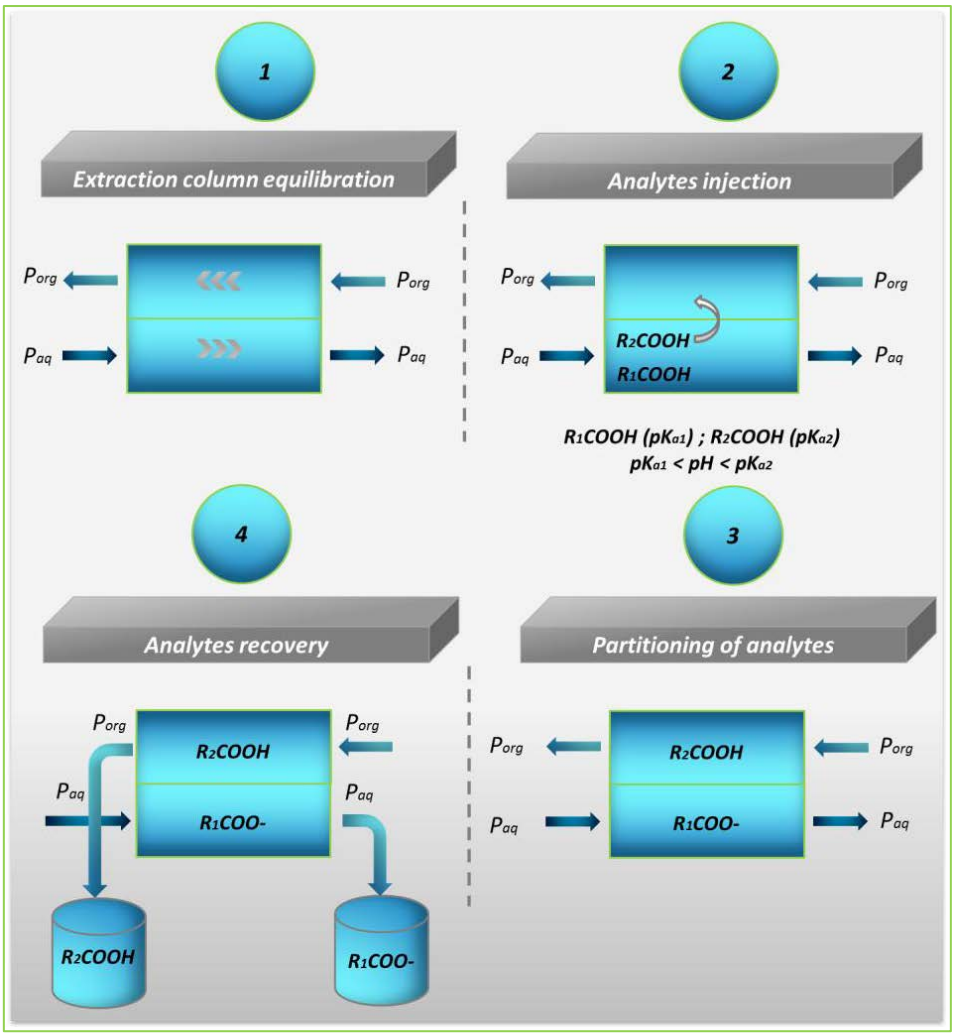

Figure I. 6: Dissociative liquid-liquid extraction procedure

The pH-Swing extraction method is used to extract and purify ionizable analytes based on dissociative extraction concepts but in an extraction/back-extraction scheme (figure I.5). Each extraction operation is carried out at a specific pH. For example, an analyte is extracted in its protonated form in an organic phase. This analyte is then back-extracted into a fresh aqueous phase in its ionized form by pH adjustment. This pH-Swing extraction method can result in both high recovery and selectivity if the non-desired analytes are not ionizable or have pK_a values that differ greatly from those of the target analytes. This method is for instance commonly used to recover and purify antibiotics, alkaloids and other complex organic compounds with ionizable functions [64].

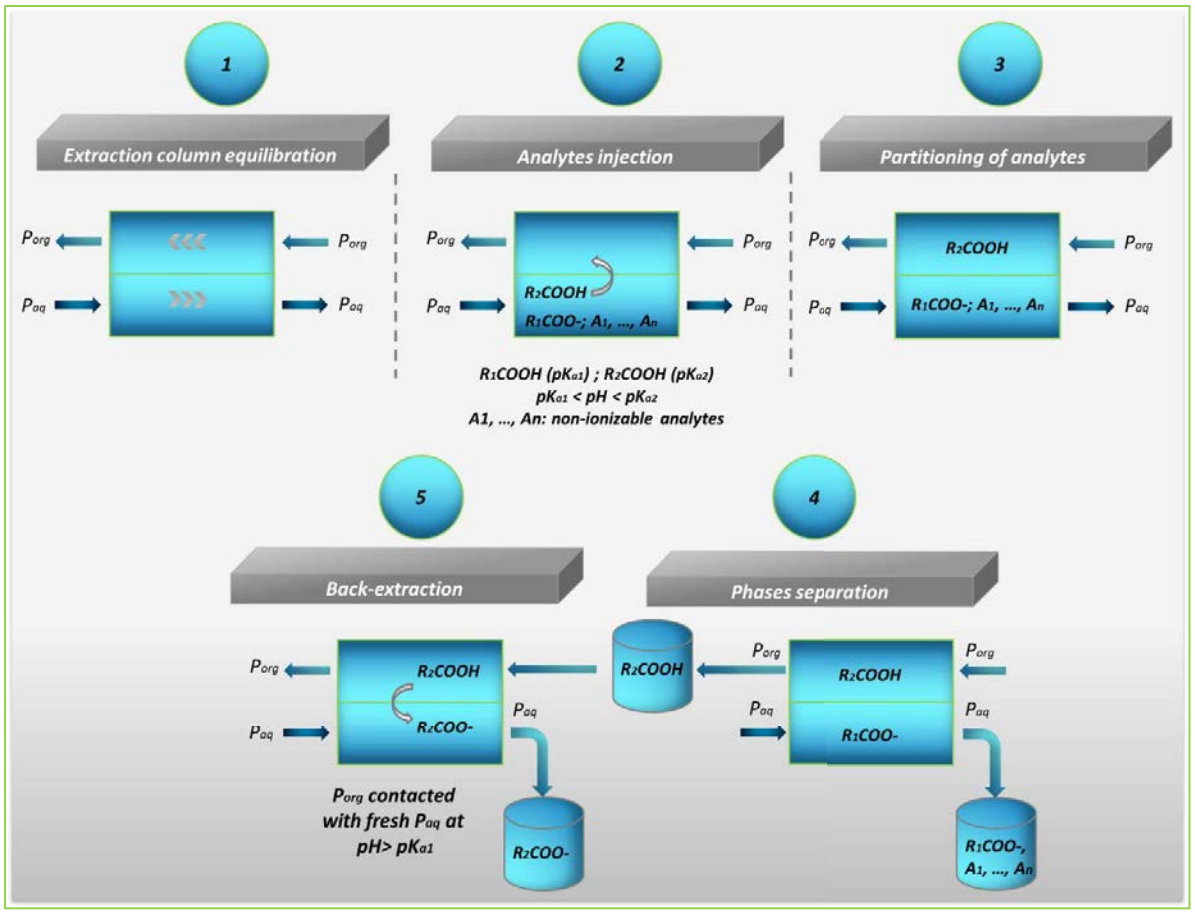

Figure I. 7: pH-swing extraction procedure

I.2.4.3. Reactive extraction processes

Reactive extraction is a separation process based on the reactions between an extractant and the target analytes through the formation of reversible adducts or molecular complexes (Figure I.6). Smith and Page [65] have first pointed out that long chain aliphatic amines are able to form hydrophobic ion pairs with organic acids which can be extracted by organic solvents. King et al. [66-68] have then investigated the formation of these ion-pairs with different long chain amines and long chain alkyl phosphine oxides. Reactive extraction is a highly interesting alternative for the selective extraction of analytes presenting specific functional groups capable of complexation. The commonly used extractants are phosphorous compounds, such as trioctylphosphine oxide (TOPO), di-(2-ethylhexyl)-phosphoric acid (DEHPA) or tributylphosphate (TBP); aliphatic secondary amines such as *N*-lauryl-*N*-tri-alkyl-methylamine (Amberlite-LA2®); ternary amines such as tri-*n*-octylamine (TOA); and quaternary amines such as methyl trioctylammonium chloride (Aliquat336®). These extractants are generally soluble in organic solvents and react with analytes initially present in the aqueous phase. Several studies have reported the use of such reactive extraction processes. The most

extensively studied compounds were carboxylic acids obtained by fermentation [69-72]. For instance, Keshav et *al.*, have examined the recovery of propionic acid by reactive extraction using (TOA) as an extractant in different organic phases including *n*-heptane, petroleum ether, ethyl acetate and oleyl alcohol [73]. Similarly, Sharma et *al*., have recovered a variety of organic acids from aqueous acid mixtures by adding TOA in various organic solvents [74]. Siebold et *al.*, have studied the separation of lactic acid from citric acid and acetic acid by using various extractants [75]. More recently, the reactive extraction of succinic acid was studied by mixing aqueous succinic acid solutions with 448 different amine-solvent mixtures as extractants in mixer-settler operations. The extracting solutions were composed of either one amine and one solvent (208 reactive extraction systems) or two amines and two solvents (240 reactive extraction systems). The maximum extraction yields of succinic acid were obtained with trihexylamine dissolved in 1-octanol or with dihexylamine and diisooctylamine dissolved in 1-octanol and 1-hexanol with more than 95% yields [76]. Reactive extraction was also useful to recover bioproducts obtained by fermentation of the *Escherichia coli W3110* modified strain. For instance, L-phenylalanine was extracted from the cell free medium by using (DEHPA) dissolved in kerosene in a countercurrent mode. After back-extraction, L-phenylalanine was enriched by a factor of four [77]. Some antibiotics were also extracted by reactive extraction processes and the most extensively investigated antibiotic was penicillin [78-81]. For instance, a method using secondary amines (DEHPA) as extractants was developed to successfully extract penicillin G while avoiding its decomposition. The extraction equilibrium and the back-extraction process of certain *β*-lactam antibiotics such as 7-aminocephalosporanic acid, 7-aminodeacetoxy cephalosporanic acid, 6-aminopenicillanic acid, cephalosporin-C and cephalexin from aqueous solution of phosphate and carbonate buffers were also studied by using quaternary ammonium (Aliquat336®) dissolved in *n*-butylacetate over an aqueous phase pH range of 5–10 [82].

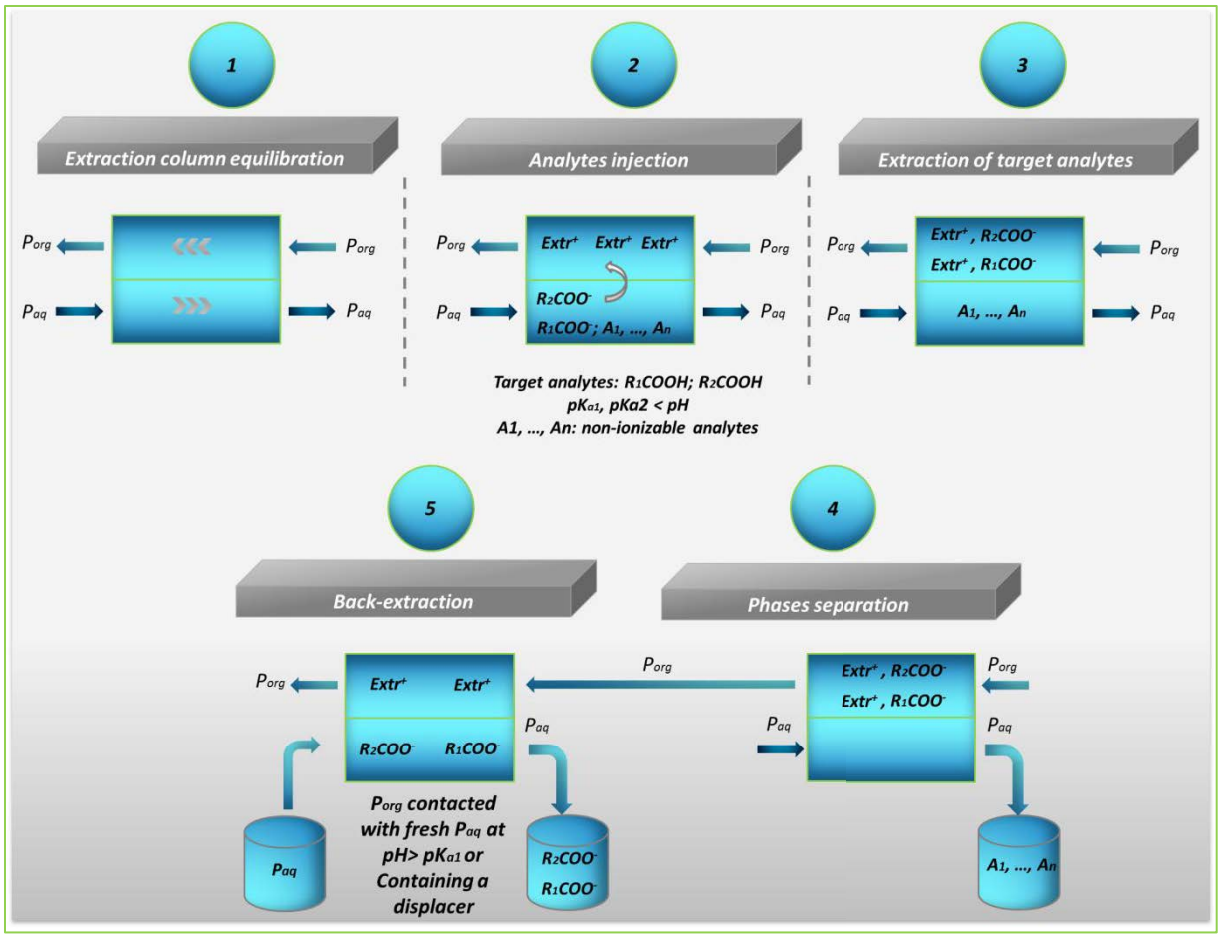

Figure I. 8: Reactive extraction process. $Extr^+$: lipophilic anion-extractant; R_1COOH, R_2COOH: target ionizable analytes; A_1,..., A_n: non-ionic or non-ionizable compounds

I.2.5. Liquid-liquid extraction equipment

I.2.5.1. Classification of liquid-liquid extraction equipment

Liquid-liquid extraction devices are well described in several reviews and book chapters [37, 83, 84]. Their classification is generally based on the method which is applied to disperse the liquid phases and/or how the countercurrent flow is generated (gravity or centrifugal force). Liquid-liquid extraction devices can also be divided into three main categories according to the contactor design: column extractors, mixer-settler extractors and centrifugal extractors. In this review, the different instruments will be classified according to the nature of the theoretical stages generated during the extraction process. Two types of apparatus are distinguished: The **differential contactors** with no discrete stages and the **stagewise contactors** with discrete and individualized theoretical stages (Figure I.7).

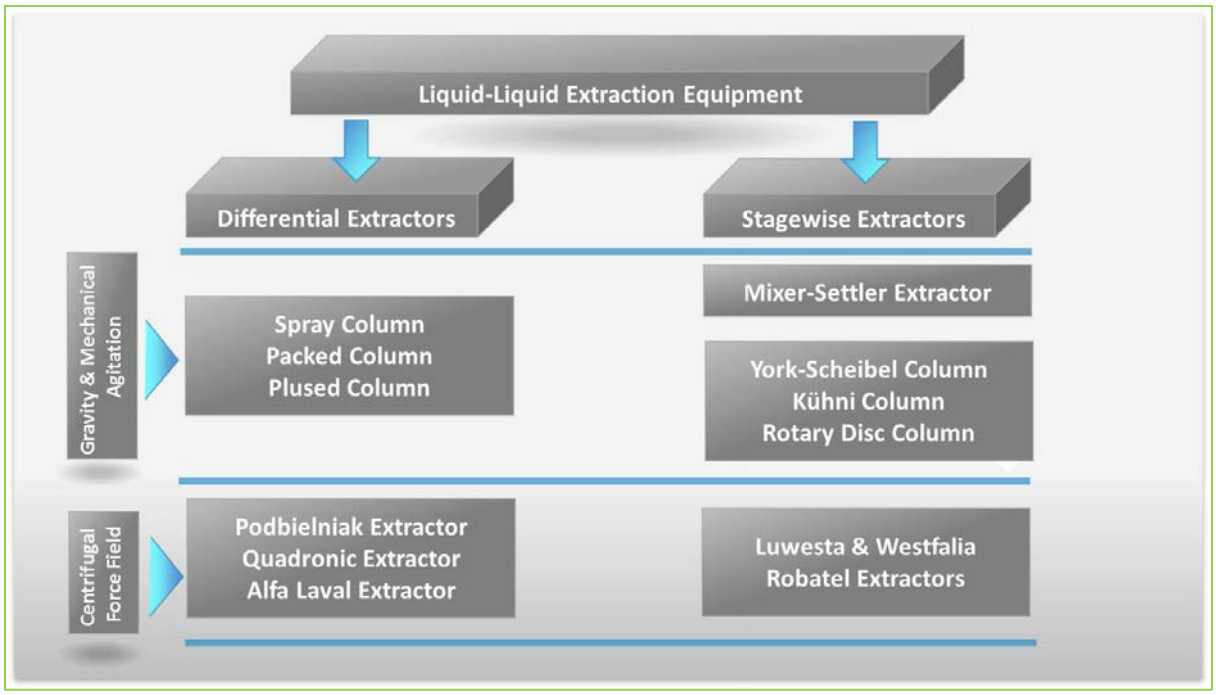

Figure I. 9: Classification of liquid-liquid extraction devices

I.2.5.2. Differential extractors

- ***Differential extraction columns***

Differential extractors are designed to provide a constant contact between the two liquid phases. Usually, the apparatus consists in vertical columns or centrifugal extractors in which the dispersed phase (mobile phase) circulates through the continuous phase (stationary phase) in a counter-current flow. The extraction is achieved continuously along the contactor and the phase separation is carried out at the end. The differential liquid-liquid extractors have no discrete stages but the number of stages is determined by the height of a theoretical stage (HTS) also called equivalent height equivalent to a theoretical plate (HETP) or height of transfer unit (HTU). The HTS is one of the major parameters determining the efficiency and performance of a liquid-liquid extractor. Here are given some examples of the most commonly used differential liquid-liquid extractors.

Spray columns consist in a vertical tower in which the dispersed and continuous phases flow counter-currently (Figure I.8). The low-density phase is dispersed through nozzle tips located at the lower part of the column. The dispersed phase drops rise through the descending continuous phase (aqueous phase) and coalesces at the interface at the upper end of the column. Spray columns are still used in the industry for simple operations such as washing and neutralization [85]. For instance, they are used to extract proteins and enzyme with aqueous two-phase systems [86-88].

The principle of **pulsed columns** is similar to that of spray columns. The phases are continuously and counter-currently pumped into a vertical extraction column (Figure I.8). The main difference is the application of a mechanical energy to pulse the liquids up and down. Pulsed columns contain typically an active mixing section in the central parts of the cylinders and two settling sections at both ends of the column [89]. The greater the pulse, the smaller the drops, resulting in an improved separation interfaces and mass transfer [89, 90]. The flow of such extraction equipment is often limited due to flooding phenomenon, hold up or emulsion [91]. Pulsed columns are currently used in mineral extraction, nuclear effluent treatment and fuel processing [92].

Packed columns are commonly used at the industrial scale. They are filled with some packing metallic, ceramic or polymer materials to provide a sinuous path for the two liquid phases [85, 93]. This packing guarantees a constant contact between the liquid phases and ensures a good mixing of the dispersed phase with the continuous phase (Figure I.8). Generally the packed columns are not characterized by a good efficiency due to their high HTS. Only a few studies have reported the use of packed columns for the separation of natural products. For instance, the fractionation of shark liver oil was investigated by subcritical 1,1,1,2-tetrafluoroethane (R134a) as a solvent in a pilot-scale packed column and a good separation of squalene from triglycerides was achieved. Nevertheless, when applying this method to a model mixture of oleic acid and squalene, a poor separation was obtained [94]. More recently, the performance of a pilot scale packed column was evaluated for the continuous countercurrent extraction of human immunoglobulin G (IgG) from a Chinese hamster ovary cell supernatant enriched with pure proteins using aqueous two-phase system (phosphate-rich or polyethylene glycol 3350 (PEG)-rich phase). As a result, an IgG recovery of 85% and with a purity of 84% were obtained [95].

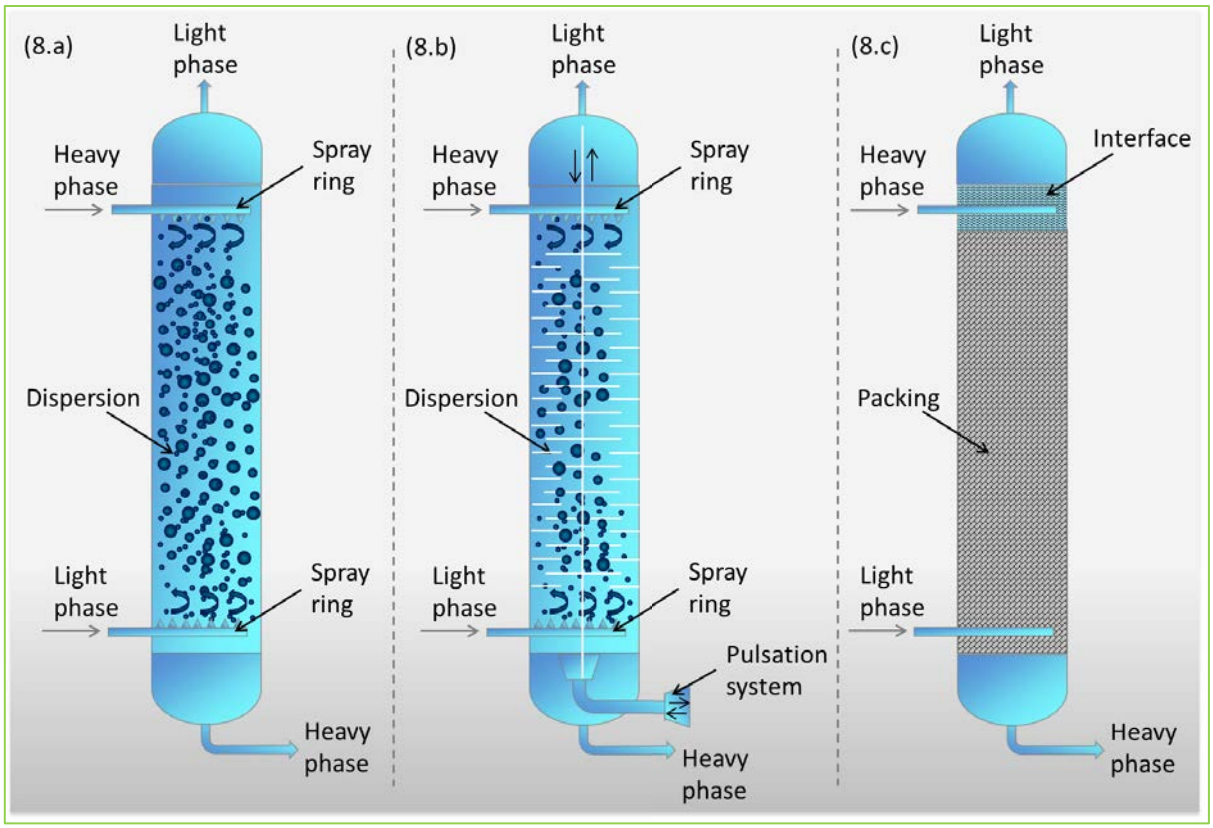

Figure I. 10: Differential liquid-liquid extraction columns. (a): spray column; (b): pulsed column and (c): packed column

- ***Differential centrifugal contactors***

The concept of using a centrifugal force to achieve countercurrent mixing and separation of liquid phases was developed in the early 1930s [83, 96-98]. In differential centrifugal extractors (Figure I.9), no discrete stage can be recognized. These devices are usually designed as rotating columns (spinning rotors) made of several concentric perforated cylinders and working as "rotating perforated plate columns". The liquid phases move radially in a countercurrent flow mode under the effect of the applied centrifugal force, which can reach up to 300-400 *g*. Differential centrifugal contactors are efficient systems for the selective extraction of several analytes on the basis of their distribution coefficients. A variety of differential centrifugal contactors are commonly used in the industry, including the Podbielniak® extractors [20, 97, 99-101] (Figure I.9.a), the Quadronic® extractor [97, 98, 101] (Figure I.9.b), and the Alfa-Laval® extractor [97, 101] (Figure I.9.c).

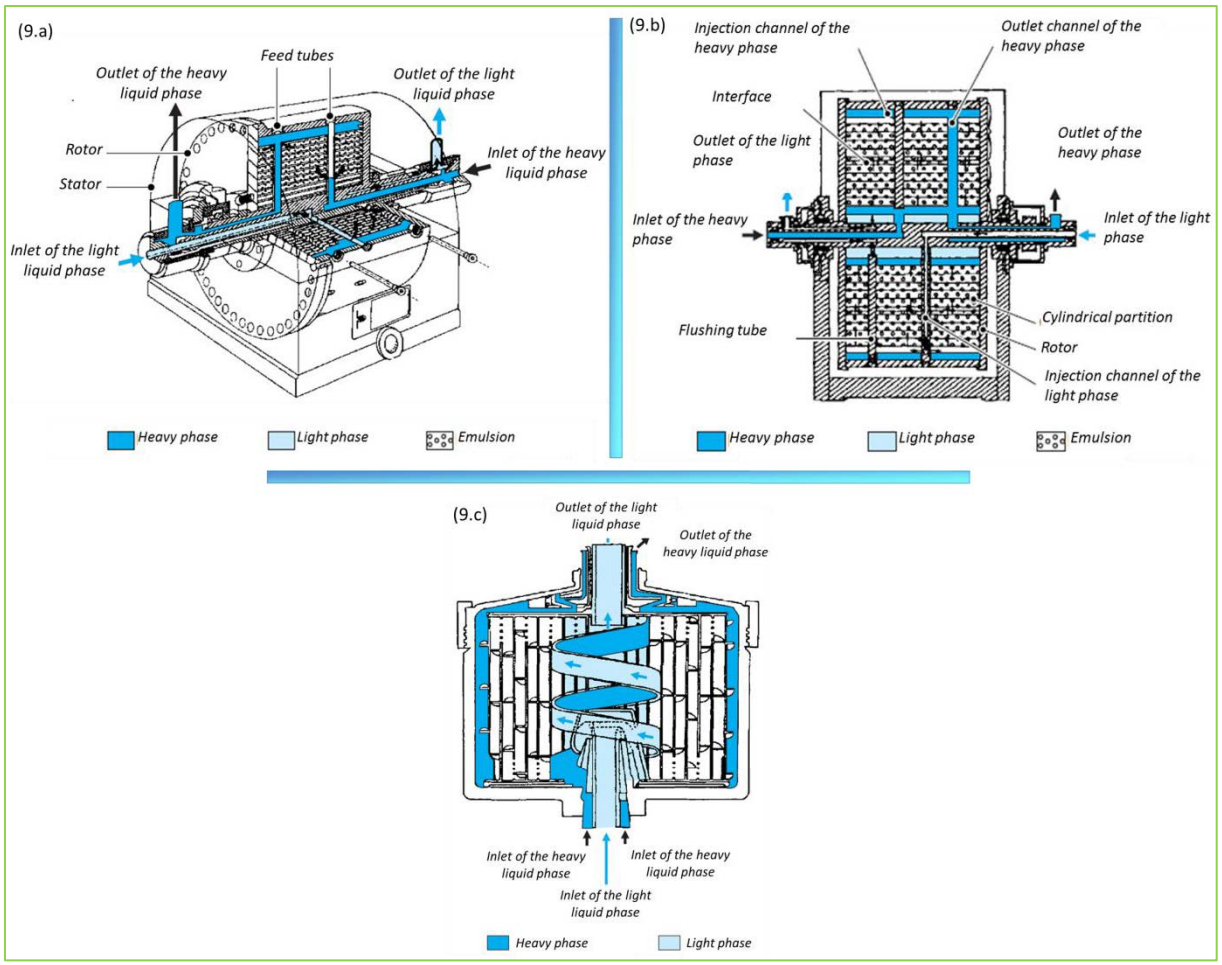

Figure I. 11: Differential centrifugal liquid-liquid extractors [101]. (a): Podbielniak® extractor, (b): Quadronic® extractor, (c): Alfa-Laval® extractor.

I.2.5.3. Stagewise extractors

- ***Mixer-settlers contactors***

Mixer-settlers are the simplest liquid-liquid extraction apparatus. They are composed of a rectangular or cylindrical mixing chamber and a large gravity settling chamber (Figure I.10.a) [34, 37]. In a standard mixer-settler separation process, the two phases enter the mixing part where they are shacked thanks to an impeller. Then, they are transferred into the settling part where they are allowed to separate by gravity due to their density differences. Each mixer-settler unit provides a single extraction stage. Depending on the application, this liquid-liquid extraction procedure can require the use of only one mixer-settler unit (single stage) or several units (multistage) arranged in "cascade" (Figure I.10.b) [101, 102] or "battery" (Figure I.10.c) [101, 103] to guarantee a high separation process efficiency. A mixer-settler operation can be performed batch wise or continuously [104]. Continuous mixer-settlers are particularly interesting for applications requiring several equilibrium stages and especially long residence times due to slow extraction kinetics or high fluid

viscosities. However, this can be a major drawback if the target compounds are potentially degradable over time. Another limiting condition for using such process is when stable emulsions are formed [105]. Mixer-settler extractors are typically used for the recovery of metal ions from aqueous solutions, uranium enrichment and fuel processing [106]. A few examples of applications to the purification of biomolecules such as α-amylase can also be found in the literature [107, 108].

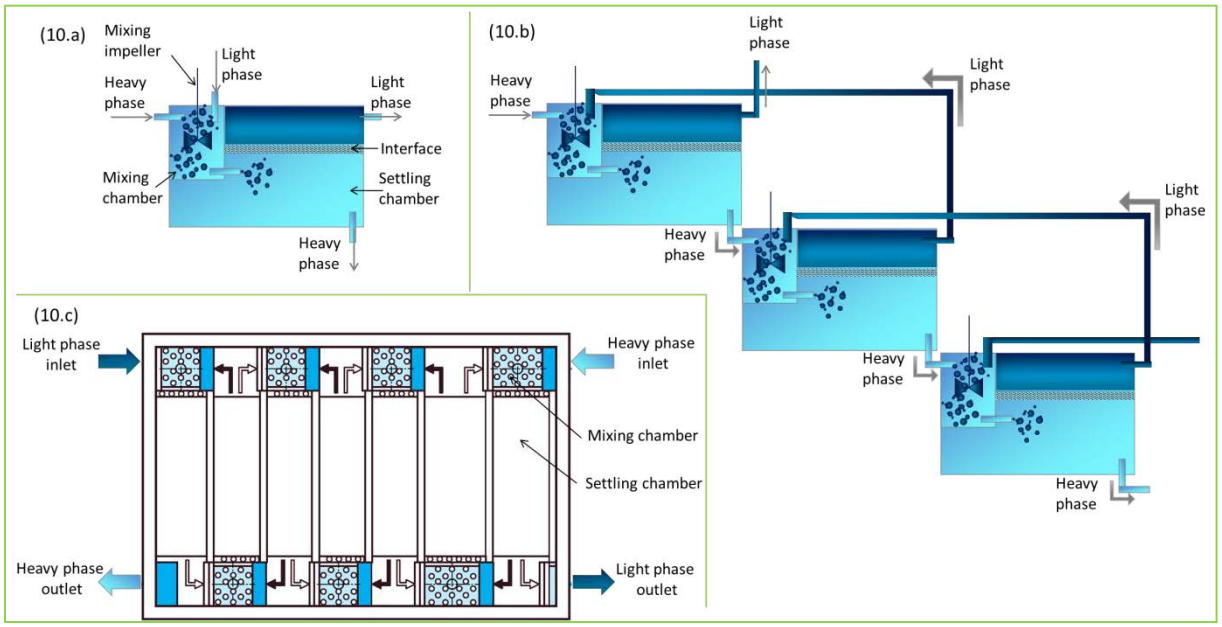

Figure I. 12: Mixer-settler contactors [101]. (a): mixer-settler unit, (b): multistage mixer-settler cascade, (c): multistage mixer-settler battery.

- ***Stagewise liquid-liquid extraction columns***

The York-Scheibel extraction column also known as the "**Scheibel column**" (Figure I.11.a) is considered as the oldest liquid-liquid extraction equipment [109, 110]. This is a multistage and agitated countercurrent liquid-liquid contactor having internal paddle connected to a central rotating shaft. The mixing impellers of each stage are separated by partition sections. This design provides successive stacked mixer-settlers units, one above the other [111]. The design, separation efficiency and hydrodynamic behavior of Scheibel extraction columns have been intensively studied [112-114]. The high efficiency of such column provides 3 to 5 "NTU" per meter of height. However, it decreases very rapidly for diameters greater than 1 m. The applied flow rates for the Scheibel column range usually from 10 to 25 m^3/h [115].

Kühni extraction columns are designed similarly to the Scheibel columns (Figure I.11.b) [116]. The main difference is based on the structure of their stator-discs which are made of

perforated plates in the Kühni extraction columns [97, 117]. The extraction performances of Kühni columns were evaluated by studying several operating parameters including mass transfer of analytes between the liquid phases, drop formation during the experiments, hydrodynamic of the steady-state (hold-up of the dispersed phase, flooding of the continuous phase), and the effect of agitation intensity [116, 118]. For instance, the reactive extraction of penicillin G using Amberlite LA2® as an extractant dissolved in *n*-butyl acetate and its back-extraction were investigated in a Kühni column at different operating conditions (pulsation frequencies, impeller speeds) and compared to other extraction columns. The Kühni column exhibited the highest extraction and back-extraction performance due to the obtained specific interfacial area [119].

Rotating Disc contactors (RDC) (Figure I.11.c) are other stagewise liquid–liquid extractors. Their design was firstly proposed by Reman et *al*. in 1951's [120]. The RDC columns are counter-currently operating extractor with the low density dispersed phase moving from the bottom to the top of the column, and the continuous high density phase moving from the top to the bottom. The cylindrical tube contains an agitator made of a series of rotating flat discs, which are fixed on the central shaft [121]. Flood conditions in the RDC column, mass transfer efficiency of each stage and scale-up conditions have been intensively studied in order to better understand the hydrodynamic parameters providing an efficient separation at different scales [122-124]. Among the available designs of RDC columns, the Perforated Rotating Discs Contactors (PRDC) are more efficient than simple RDC extractors [125]. Another interesting apparatus derived from the RDC columns is the Asymmetric Rotating Discs Contactors (ARDC) in which the shaft with the rotating discs is asymmetric to the vertical axis of the column [126]. The main applications of these contactors are found in the petroleum industry for furfural and sulfur dioxide extraction, solfolane extraction and caprolactam purification [127]. RDC contactors have also been applied to the recovery and purification of proteins from their initial natural mixtures, such as the cutinase produced by *Escherichia coli* [128-130]. In this study, intracellular cutinase was directly extracted by the organic phase composed of Di-2-ethylhexyl sodium sulfosuccinate (AOT) dissolved in isooctane from a complex biological aqueous media of *Escherichia coli* disrupted cells. The optimized conditions for the direct extraction of the enzyme led to an extraction yield of 54.4% [130].

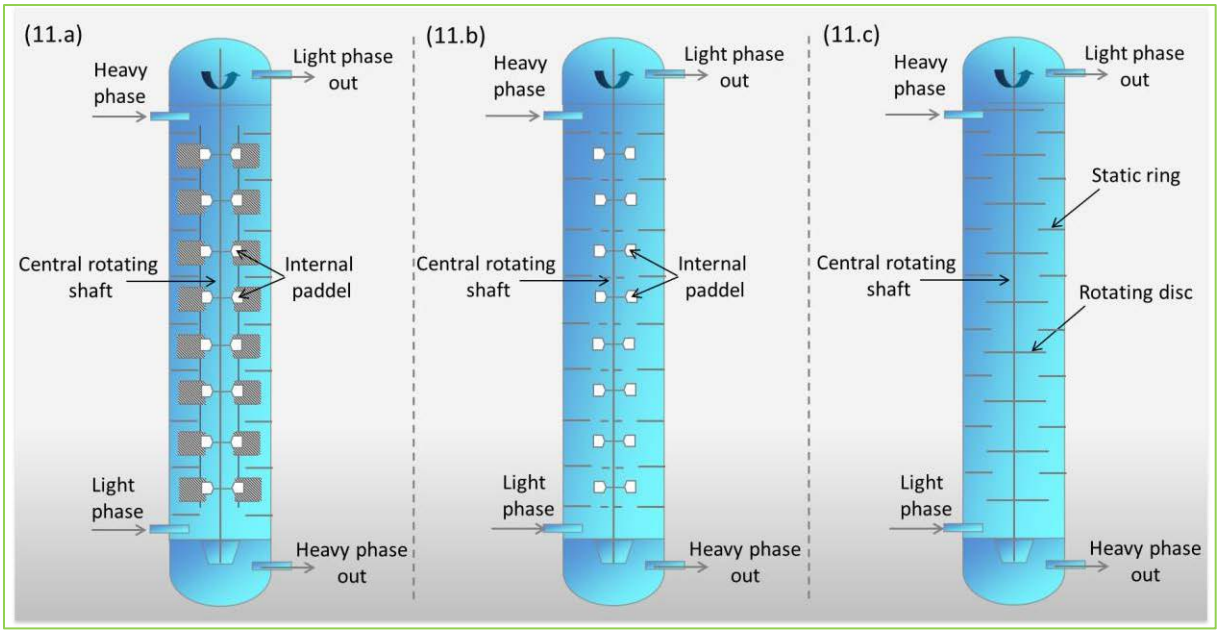

Figure I. 13: Stagewise extraction columns. (a): Sheibel column, (b): Kühni column, (c): Rotating disc column (RDC)

- ***Stagewise centrifugal contactors***

Stagewise centrifugal contactors include the Robatel® extractors, the Luwesta® and the Westfalia® contactors. **Robatel®** Extractors consist in several chambers stacked vertically and designed for countercurrent extraction. Each chamber (discrete stage) is physically divided into three sections. The first section is the central part of the rotating drum, where a mixing disc ensures the mixing of the liquid phases. The second section is the settling part of the extractor where the rotating disc sends the two phases to be separated thanks to the centrifugal force. Finally, the third section consists in a weir system which ensures the flow of the phases to the next stage. Two main classes of Robatel® apparatus are designed: monostage and multistage centrifugal extractors. In the first design, the contactor is made of a turbine located at the bottom of a rotating bowl. This turbine is used to aspire the liquid-liquid mixtures into the centrifugal bowl. The phases are then separated thanks to the centrifugal force field generated by the rotating bowl. These machines can provide flow rates ranging from 6 to 50 m^3/h. In multi-stage centrifugal extractors, each stage consists in a mixing chamber and a decantation chamber with an inter-stage in which the liquids circulate in a countercurrent flow. One physical stage (mixer and settler chamber) is nearly equivalent to one theoretical stage; therefore the number of theoretical stages generated during the extraction corresponds to the number of mixer-settler pieces installed in the rotating bowl (up to 12 stages) [131, 132]. This type of contactor with four stages and a capacity of 10.2 L is used for instance for the

extraction of antibiotics, novobiocin, fusidin, and penicillin from their aqueous culture medium using butyl acetate as organic phase [133].

The **Luwesta® extractors** are liquid-liquid contacting instruments, which operate in several distinct mixing and settling chambers. The liquid phases enter at the top of the extractor under pressure and are forced to flow counter-currently. They are simultaneously pumped and centrifuged by angled baffles. In each chamber (each stage) the two liquid phases are firstly mixed as they passed through a distributor ring. Then they are settled in the chamber by a centrifugal force field, and finally separated by a weir system and directed to the next chamber. A series of apparatus with flow rates ranging from 1.25 to 120 m^3/h and leading to residence time per stage from 4 to 25 s have been designed. A Luwesta EG 2006 extractor was used for instance to extract active compound allopregnane-3(*β*),16(*α*)-diol-20-one from pig suprarenal glands dissolved in 60% methanol aqueous phase by using petroleum ether as organic phase at flow rate of 0.6 m^3/h [134]. The Luwesta® extractor also exists in a monostage version, called the Westfalia TA®, which can operate in series.

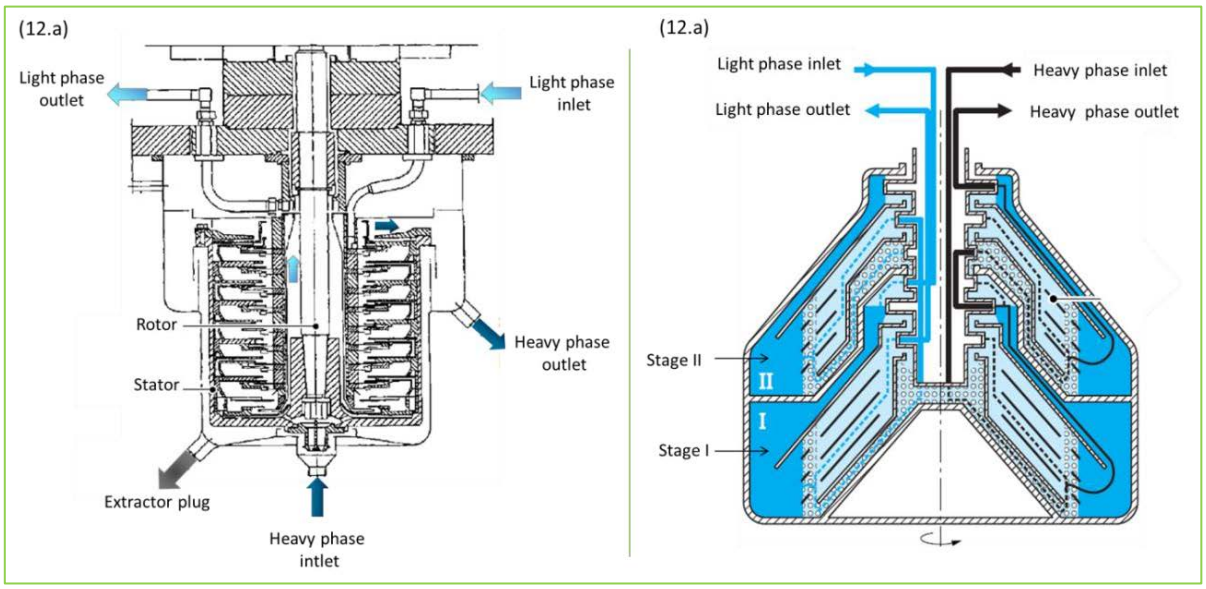

Figure I. 14: Stagewise centrifugal extractors [101]. (a): Robatel extractor, (b): Luwesta extractor

I.2.6. Current status of liquid-liquid extraction devices: what needs?

The above section has outlined the main types of commercially available liquid-liquid extraction devices. The choice of a liquid-liquid extractor depends on many factors including the

required number of theoretical stages, required residence time (extraction kinetics and analyte stability), the required productivity rate, as well as the ability to handle high or low density difference, and low interfacial tension. In Table I.3 are summarized and compared the general characteristics of all liquid-liquid extractors presented above.

Type of extractor	*General features*	*Fields of industrial applications*
Spray column **Pulsed column** **Packed column**	Simple construction Low to medium mass –transfer efficiency Suitable to systems with low to moderate interfacial tension Can handle high production rates.	Petrochemicals Chemicals Foods
York-Scheibel column **Kühni column** **RDC and ARDC columns**	Moderate to high efficiency (several "NTU" possible in a single column) Moderate capital cost Suited to low to moderate viscosity Suited to systems with moderate to high interfacial tension Can handle moderate production rates	Petrochemicals Nuclear Chemicals Metallurgicals Foods Pharmaceuticals
Mixer-settlers extractors	High stage efficiencies with long residence time Can handle high viscosity liquids Can handle very high production rates	Petrochemicals Nuclear Chemicals metallurgicals
Centrifugal extractors	Allow short contact time especially for unstable analytes Minimum space requirements Can handle systems with low density difference or tendency to easily emulsify	Petrochemicals Chemicals Pharmaceuticals Nuclear

Table I. 3: Common liquid-liquid extraction equipment: features and fields of industrial applications

In view of these data, it clearly appears that most liquid-liquid extraction instruments are used in the petrochemical and mineral industries, for nuclear effluent treatment and fuel processing. Some of these extractors including the Podbielniak®, Quadronic®, and Robatel® extractors are also used in biotechnological downstream processes for the recovery of amino acids, carboxylic acids, and proteins from fermentation mediums. However, globally, any of these systems are suitable for the selective extraction of natural products from plant sources. Indeed, the current extractors are not able to efficiently separate high-valuable compounds such as polyphenols, steroids, fatty acids, and peptides from complex natural plant extracts. The main limitation of the existing liquid-liquid extraction devices is the lack of selectivity. The recovery of secondary metabolites from crude extracts of natural origin is currently performed at the laboratory-scale using solid support free

liquid-liquid chromatographic systems including the hydrodynamic Countercurrent Chromatography (CCC) and hydrostatic centrifugal partition chromatography (CPC) [21, 135, 136]. These technologies are of great interest due to the quite high selectivity and efficiency (about several hundred NTU) that can be reached.

The versatility of the CPC technique and its low solvent consumption has led researchers to explore its potential when it is used in the extraction mode and to compare it to conventional liquid-liquid extraction techniques [137-139]. The use of CPC chromatographs as liquid-liquid extractors was for instance firstly investigated by Menges et *al*., in 1992 for the extraction of non-ionic surfactants from waste water [137]. CPC was also developed to extract nonyphenol ethoxylate (NPEO) present at very low concentration (16.5 g/L) in 20 L of an aqueous solution. A total of 86 % of NPEO was recovered with only 30 mL of stationary phase (ethyl acetate) when working at a flow rate of 5 mL/min. Liu et *al*., have then investigated the ability of CPC to extract parts-per-billion levels of 13 phenols and 20 organochlorine pesticides [138]. More recently, CPC was applied to the extraction of *β*-carotene produced by *Dunaliella salina* [139].

The following sections are focused on the fundamental aspects and principles of CCC and CPC chromatographic techniques, their development modes and applications to the separation of bioactive natural products from plants and microbial fermentation media. The evolution of the CCC and CPC machines and scale-up operations towards industrial applications will also be described. At the interface between liquid-liquid extraction and liquid-liquid chromatography, we will introduce the newly developed laboratory-scale Fast Centrifugal Partition Extractor (FCPE300®), its characteristics and main differences as compared to classical CPC chromatographs. The positioning of the FCPE300® extractor will be discussed, as well as, its potentiality towards filing the technological gap between liquid-liquid extraction and liquid-liquid chromatography.

I.3. Solid support-free liquid-liquid chromatography: Efficient liquid-liquid contacting devices for the fractionation and purification of natural metabolites

I.3.1. General aspects of solid support free liquid-liquid chromatography

Solid support-free liquid-liquid chromatography is a unique form of chromatography which uses a column filled with a liquid stationary phase. The separation is achieved on the basis of partition of the analytes between at least two immiscible liquid phases. One phase is maintained inside the column due to the application of a centrifugal force field while the other phase is pumped

through it and used as mobile phase. Because of the absence of solid support, these techniques are advantageous over other chromatographic methods by avoiding analyte adsorption, degradation or contamination. Countercurrent chromatographs (CCC) (hydrodynamic machines) and centrifugal partition chromatographs (CPC) (hydrostatic machines) are the two major types of solid support free liquid-liquid chromatographic systems being used today. CCC was firstly introduced by Ito, Harada and Aoki in 1966 [140], while CPC was introduced by Nunogaki in 1982 [141]. CCC instruments are composed of a so-called "multilayer coil" made of a long piece of continuous tubing wrapped around a holder in multiple layers. The resulting multiple coils can be connected in series to increase the total volume of the instrument. The coil is submitted to a centrifugal force field and rotates around its own axis while simultaneously rotating around the central axis of the system. This results in stationary phase retention and partitioning of the analytes between the two liquid phases [142]. In contrast, CPC is hydrostatic and uses a spinning rotor, made of a series of interconnected cells circumferentially engraved on stacked disks in rotation around a single axis. In CPC, the centrifugal force applied to maintain one phase stationary inside the column remains constant in intensity and direction [143]. In both CCC and CPC systems, the mobile phase is pumped through the stationary phase, and the hydrodynamic equilibrium between the liquid phases depends on the intensity of the centrifugal force, on the flow rate of the mobile phase, and on the physic-chemical properties of the biphasic solvent system (density, viscosity, interfacial tension) [144-147]. When the lower phase of the biphasic solvent system is used as the stationary phase, the experiment is performed in the "ascending mode" in CPC or "tail to head" in CCC. When the lower phase is used as the mobile phase, the experiment is performed in the "descending mode" in CPC or "head to tail" in CCC. CCC and CPC are very interesting in terms of selectivity, sample loading capacity, and scale-up ability [148, 149] and have both been mainly applied to the purification of natural products [21, 150-152]. Another interesting aspect of these techniques is the ability to change the role of the two phases during the run or in independent experiments, the stationary phase becoming mobile and *vice versa*.

I.3.2. Definitions and theoretical aspects

I.3.2.1. Stationary phase retention factor (*Sf*)

The stationary phase retention factor is a crucial parameter in CCC and CPC separation experiments. Neglecting the sample volume, CPC and CCC column volumes (V_C) correspond to the sum of the stationary and mobile phase volumes, V_S and V_M, respectively (equation 13).

$$V_C = V_M + V_S \ ; \qquad Equation\ 13$$

The volume of the stationary phase retained in the partition cells of CPC columns or in the coiled tube of CCC instruments can be defined by a "stationary phase retention factor" (*Sf*) expressed in percentage (equation 14) [148].

$$Sf = 100 \times \frac{V_S}{V_C}; \qquad Equation\ 14$$

The greater the *Sf*, the greater the capacity of the column, and therefore the better the resolution and quality of the separation. The stationary phase retention factor can be affected by several operating conditions, such as the nature of the solvent system, the flow rate of the mobile phase or the rotation speed which all can cause a significant loss of stationary phase in the form of "**bleeding**" or "**flooding**" [146, 153].

Flooding phenomena is a known limiting factor encountered in liquid-liquid separation processes, especially when the phases flow counter-currently [154]. In CPC and CCC, flooding of the stationary phase can be encountered especially when a high mobile phase flow rate is applied and/or when the injected sample is highly concentrated. Indeed the injection of a high sample amount can modify the thermodynamic equilibrium between the mobile and the stationary liquid phases. A zone containing the concentrated sample can move through the column, disturbing the percolation of the mobile phase and pushing the stationary phase out of the column [146, 155]. A "bleeding" of stationary phase corresponds to a discontinuous or continuous loss of small volume of stationary phase during the experiment. This stationary phase loss can strongly affect the separation efficiency by reducing the available stationary phase inside the column and therefore the whole resolution of the separation process. The stationary phase loss depends on the equilibrium between the liquid phases (difference of phase densities, interfacial tension, etc...) and particularly on the physico-chemical proprieties of the injected sample, especially its surfactant characteristics.

I.3.2.2. Retention volume (V_R) retention factor (k') and separation factor (α)

The **retention volume** of an analyte (V_R) corresponds to the mobile phase volume required for its elution. It can be expressed as given in equation 15. K_D represents the distribution coefficient of the target analyte between the stationary and mobile phases.

$$V_R = V_M + K_D V_S \ ; \qquad Equation\ 15$$

The **retention factor** (k') represents the time during which an analyte is retarded inside the stationary phase in comparison to the velocity of the mobile phase. It can be related to the analyte distribution coefficient as given in equation 16 [156].

$$k' = \frac{(V_R - V_M)}{V_M} = K_D\left(\frac{V_S}{V_M}\right); \qquad Equation\ 16$$

The **separation factor** (α), also called "selectivity", is an important parameter which reflects directly the quality of the separation process. It corresponds to the ratio between the retention factors of two analytes eluted successively peaks (Equation 17). A satisfying (α) value is higher than 1 [156].

$$\alpha = \frac{k'_2}{k'_1} = \frac{K_{D_2}}{K_{D_1}}; \qquad Equation\ 17$$

I.3.2.3. Column efficiency (*N*) and resolution (*Rs*)

The efficiency of a CPC or CCC column is reported as the NTU and it can be determined for a Gaussian peak as given in equation 18. (W) corresponds to the peak width.

$$N = 16\left(\frac{V_R}{W}\right)^2; \qquad Equation\ 18$$

The resolution (Rs) also reflects the quality of a separation process and it can be determined as given in equation 19.

$$R_S = {}^1\!/_4\sqrt{N}(\alpha - 1)\left(\frac{k'_1}{1 + k'_1}\right); \qquad Equation\ 19$$

I.3.3. Solvent systems in solid support-free liquid-liquid chromatography

One of the key factors for a successful CCC or CPC separation relies on the choice of a suitable biphasic solvent system. A good solvent system should ideally dissolve and efficiently partition the target analytes between the two immiscible liquid phases while displaying a sufficient stability at the hydrodynamic equilibrium. Generally, the distribution coefficient K_D of a compound in a biphasic solvent system should range between 0.5 and 2 [157]. A too high K_D value results in broad chromatographic peaks and long elution duration while a too low K_D value results in a poor separation resolution. The stability of a solvent system is reflected by its settling time, which should

ideally be shorter than 30 s when measured in a test tube. Finally, the selected solvent system should result in similar volumes of each immiscible phase in order to optimize their use and avoid solvent waste.

I.3.3.1. Biphasic ternary solvent systems in CCC and CPC separation

The most commonly used biphasic solvent systems in CCC and CPC experiments are made of three solvents (ternary solvent systems). A large choice of ternary solvent systems is available in the literature, and sometimes several systems can be suitable for a same application [158]. The solvent systems composed of an organic phase and an aqueous phase are the most popular in conventional CCC or CPC processes. Three types of ternary solvent systems can be distinguished according to their phase diagrams (Figure I.13).

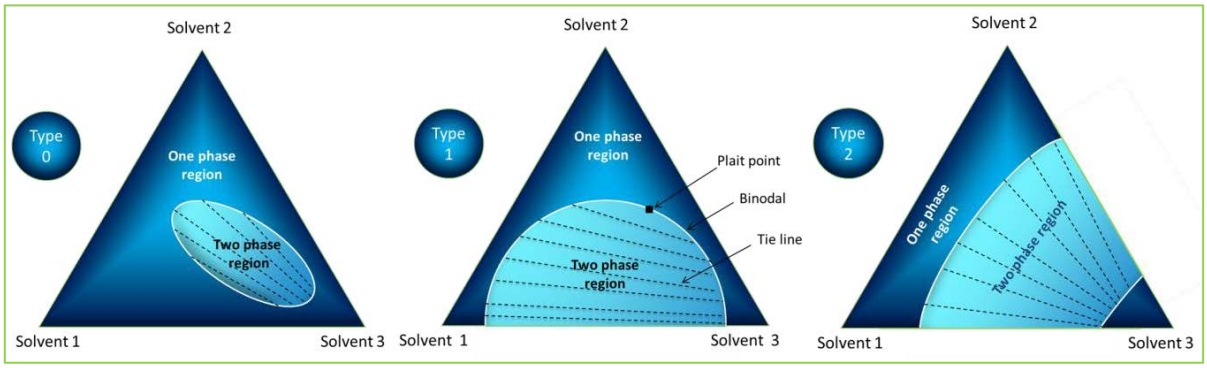

Figure I. 15: Phase diagrams for ternary solvent systems: equilateral triangle representation

Type 0 solvent systems are composed of three solvents fully miscible by pairs but for which a zone exists in the ternary diagram where a biphasic system occurs. This is the case of the solvent system composed of tertrahydrofuran (THF), dimethylsulfoxide (DMSO) and water [155, 159]. Most solvent systems are conformed to type 1 (for instance chloroform/methanol/water), composed of one solvent miscible to the two other immiscible solvents. In the corresponding ternary diagrams (Figure I.13), the binodal is the border line separating the monophasic and the biphasic zones. Along the tie-line, any point corresponds to the same composition of the left and right phases, but with different volume ratios. The plait point indicates that the compositions of the left and right phases become identical and partition coefficients converge to 1.

A few systems composed of three solvents result in a type 2 ternary diagram. This is the case for instance of ethyl acetate/*n*-butanol/water for which the binodal is in two parts without plait point [159]. Some examples of ternary solvent systems are given in Table I.4.

Type	Examples		
	Solvent 1	Solvent 2	Solvent 3
0	Tetrahydrofurane	Dimethylsulfoxide	Water
1	1,2-Dichloroethane, Benzene, Chloroform,	Formic acid,	Water
	Cyclohexane, Hexane, Toluene, Diethyl ether, Chloroform, 1,2-Dichloroethane, Isoamyl alcohol, *n*-Butanol, Methyl iso-butyl ketone, Ethyl acetate	Acetic acid	Water
	Heptane, Hexane	Benzene	Dimethylformamide, Ethylene glycol
	Cyclohexane, Heptane	Toluene	Acetonitrile, Dimethylsulfoxide, Sulfolane
	Toluene, Chlorobenzene, Methyl ter-butyl ether, Methyl isobutyl ketone, Ethyl acetate	Acetonitrile	Water
	Toluene, Chloroform, Methyl isobutyl ketone Diethylether, Cyclohexane, Ethylacetone	Acetone	Water Ethylene glycol
	Heptane	1-Butanol	Acetonitrile, Ethylene glycol
	Toluene	2-Butanol	Water
	Toluene, Benzene, 1-Butanol,	1-Propanol	Water
	Chloroform, Diisopropyl ether, Heptane, Benzene	2-Propanol	Water
	Hexane, Chloroform, Diethylether, Ethylacetate	Ethanol	Water
	Heptane, Hexane	Ethanol	Acetonitrile
	Heptane, Toluene, Chloroform, 1,2-Dichloromethane, Ethylacetate, 1-Butanol	Methanol	Water
	Hexane, Heptane	Methanol	Acetonitrile
	1,2-Dichloroethane, Carbontetrachloride, Chloroform, Methylenechloride	Tetrahydrofurane	Water
2	Hexane, Heptane, Carbontetrachloride, Chloroform, Ethyl acetate, Toluene, Benzene	1-Butanol	Water
	Ethyl acetate, Benzene,	2-Butanol	Water

Table I. 4: Composition and classification of ternary biphasic solvent systems commonly used in CPC and CCC separation processes

I.3.3.2. Other solvent systems used in CCC and CPC separation

In addition to “ternary solvent systems”, four or more solvents can also be combined to perform suitable biphasic solvent systems. For instance, the Arizona-Margraff liquid systems combining *n*-hexane, ethyl acetate, methanol, and water in different proportions are widely used in CCC and CPC experiments, mainly because they enable the separation of compounds with in a large polarity range [155, 160, 161]. The OKA solvent range composed of *n*-hexane/methanol/*n*-butanol and water, the HBAW range composed of *n*-hexane, *n*-butanol, acetonitrile and water, and finally the

acetone range composed of n-heptane, toluene, acetone and water, are also used to develop CCC and CPC separations [155]. A fifth solvent, such as *n*-butanol, can further be added to increase the polarity of the system [162].

Ternary solvent systems composed of *n*-hexane, acetonitrile and water in combination with a fourth solvent like methyl acetate, ethyl acetate or methyl *ter*-butyl ether can also lead to three-phase solvent systems [163]. More recently, aqueous two-phase system, firstly designed for peptide and protein separation, has been used in CCC and CPC [164]. These aqueous two-phase systems present the advantage of being low cost and environmentally safe, providing higher polarity compared to the conventional organic/aqueous systems and being suitable for the separation of highly polar natural products. Another original solution consists in the use of salting-out gradients in CPC by using solvent systems composed for instance of chloroform/*n*-butanol/LiCl solution and/or phosphate buffer. These types of solvent systems have been used for instance to purify chlorogenic acid derivatives [165].

I.3.4. CCC and CPC development modes and their applications

I.3.4.1. Elution methods in CCC and CPC

- ***Isocratic and gradient elution modes***

In a CCC or CPC experiment performed in the elution mode, the selectivity depends only on the relative affinity of the analytes for each of the liquid phases. An elution mode can be achieved isocratically, when the composition of the mobile phase remains unchanged during the overall process. It can be either a normal or reversed phase elution depending on the nature of the mobile phase. In a "normal phase" mode, the analytes elute in an increasing order of polarity and in the opposite case, in a "reverse phase" mode, they elute in a decreasing order of polarity. The isocratic elution is considered as the simplest and mostly used development mode in CCC and CPC processes. It was applied to the fractionation of crude plant mixtures or even to the purification of different classes of secondary metabolites such as terpenoids, flavonoids, coumarins, carotenoids, and alkaloids [21, 22, 151, 166].

By contrast to the isocratic elution mode, the gradient elution mode is applied by changing gradually the mobile phase composition during the separation process. The use of gradient elution is recommended when the isocratic mode is not effective, particularly when the target analytes exhibit large differences in their respective distribution coefficient (K_D). The main limitation of gradient elution in CCC and CPC arises from the few solvent systems adapted for this kind of procedure. In fact, ternary diagrams should have converging tie-lines in order to keep the composition of the

stationary phase (the convergence point) unchanged regardless that of the mobile phase. This is the case of type 2 solvent systems [167]. Several examples of gradient elution methods applied to the separation of natural products, mainly phenolic compounds, can be found in the literature [167-174].

- ***Dual-mode and multiple dual-mode elution methods***

A Dual-Mode (DM) consists in switching the role of the two liquid phases during an experiment, the stationary phase becoming the mobile phase and *vice versa* [148, 175, 176]. The DM can be very useful to elute compounds having a strong affinity for the stationary phase. Several examples have demonstrated the importance of this mode in reducing process time duration and in enhancing the recovery of compounds poorly separated by other classical elution operations [175]. The multiple dual-mode (MDM) (Figure I.13) is derived from the DM elution procedure. It corresponds to several successive DM steps, *i.e.* in several phase inversion cycles during the experiment [143, 177-179]. The MDM can also be performed with sample re-injection performing a semi-continuous separation process. It was applied for instance to the separation of a model mixture of acenaphthylene and naphthalene [180]. More recently, the MDM was applied to the chiral CCC separations of two racemic mixtures, (±)-*N*-(3,4-*cis*-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,5-dinitrobenzamide and *N*-(3,5-dinitrobenzoyl)-(±)-leucine, using (S)-naproxen *N*,*N*-diethylamide as chiral selector [177].

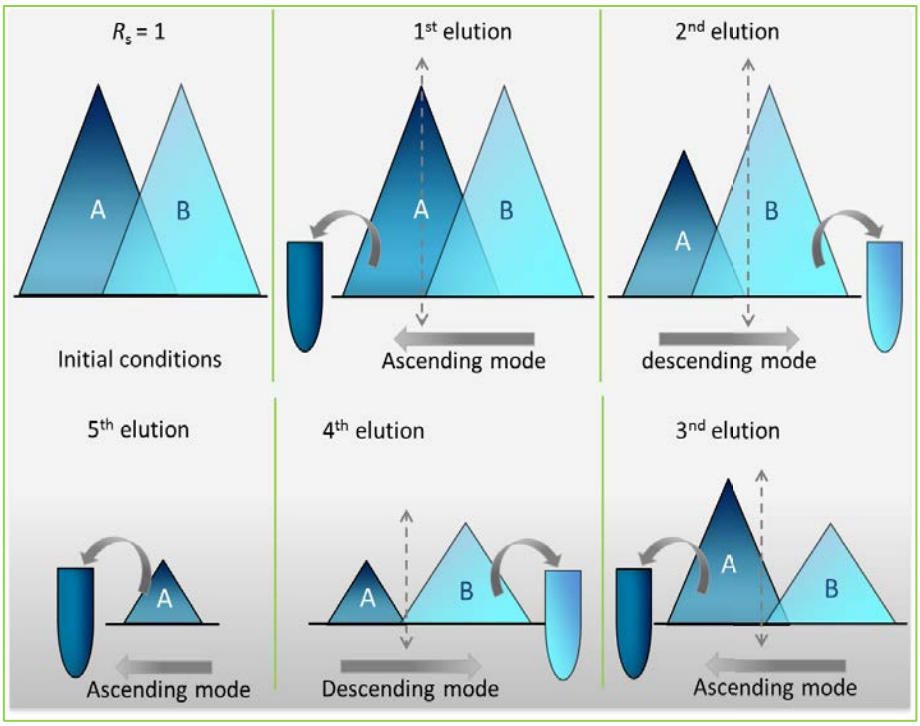

Figure I. 16: The MDM procedure of the separation of two poorly resolved analytes

- ***Elution-extrusion method***

The elution-extrusion method (Figure I.15) was firstly reported by Conway in 1990 [181]. It consists in a discontinuous recovery and separation of compounds which are highly retained in the liquid stationary phase. After injecting a sample mixture, the analytes presenting an affinity for the mobile phase are firstly eluted in a classical elution mode, and in a second step, the stationary phase is completely extruded without mixing the separated bands in order to exit the analytes which were retained inside the column [182]. This mode is rapid and efficient for the screening of complex mixtures containing analytes within a large polarity range [183-186].

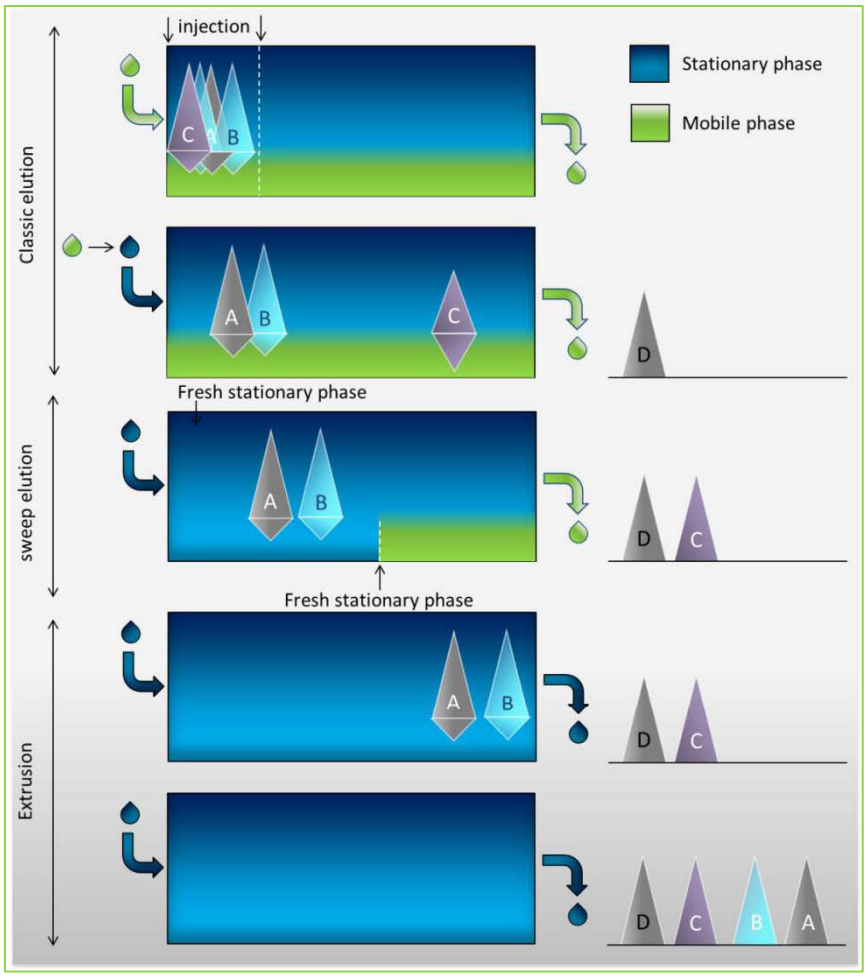

Figure I. 17: Elution-extrusion procedure

I.3.4.2. Displacement methods in CPC and CCC

Two major types of displacement methods exist in CCC and CPC, the "**pH-zone-refining**" mode (similar to the dissociative extraction and pH-swing separation processes described in liquid-liquid extraction, see section I.2.3) and the weak or strong "**ion-exchange**" mode (similar to the reactive extraction processes described in liquid-liquid extraction, see section I.2.3). Both liquid-liquid extraction and solid support-free chromatography are liquid-liquid separation processes, therefore methods used in liquid-liquid extraction can also be applied in liquid-liquid chromatography. Globally, in both displacement modes, the liquid stationary phase contains a substance (a retainer: H^+ or OH^-, or an ion-exchanger) which enables the capture of the target analytes (extraction step). In a second step, the mobile phase is supplied by a displacer agent, *i.e.* a substance presenting a stronger affinity for the retainer/exchanger than the captured analytes, resulting in a step-by-step transfer of the analytes from the stationary to the mobile phase (back-extraction step). These operations (extraction/back-extraction) are continuously repeated through the CPC column. Thus, several

equilibrium states between the (analytes/exchanger) and (displacer/exchanger) occur along the separation process. The displacement mode is highly efficient because it involves a strong competition between the analytes. The displaced analytes progress along the column as adjacent pure rectangular boundary zones (non-Gaussian peaks of high concentration and purity) forming a so-called "isotachic train". The overlapping zones between two successive bands in the isotachic train is called "shock layer" and its width represents the loss in yield achieved in displacement chromatography. In order to obtain a maximum productivity, it is necessary to reduce the width of these transition zones.

- ***pH-zone-refining displacement mode***

The pH-zone-refining mode was firstly introduced by Ito et *al*. in 1994 [187]. The separation of analytes in pH-zone-refining is based on the combination of the acidity strength (K_a) and the hydrophobic/hydrophilic character (K_D) of each dissolved analyte. Indeed the driving force in a pH-zone-refining process relies on the modification of the ionization state of the analytes. The development of a pH-zone-refining method requires, in addition to the selection of a suitable solvent system, the selection of an appropriate substance to play the role of retainer in the stationary phase, and of a displacer agent which will permit their back-extraction into the mobile phase. The velocity of the displaced analytes and their concentration is normally exclusively controlled by the displacer concentration, regardless of the mobile phase flow rate. The extraction mechanisms of ionizable analytes in the stationary phase and their displacement into the mobile phase have been described in several reviews [188, 189]. The isotachic train obtained in the pH-zone-refining mode for the separation of three organic acids is shown in figure I.16.a. In each analyte-zone (Figure I.16.b), the analyte in the mobile phase at position 1 is protonated by acidic pH and transferred into the organic stationary phase at position 2; as the sharp acid border moves forwards, the analyte molecule is exposed to high pH at position 3, deprotonated and transferred back into the aqueous mobile phase at position 4, where it quickly migrates through the sharp acid border in position 1 to repeat the above cycle [188].

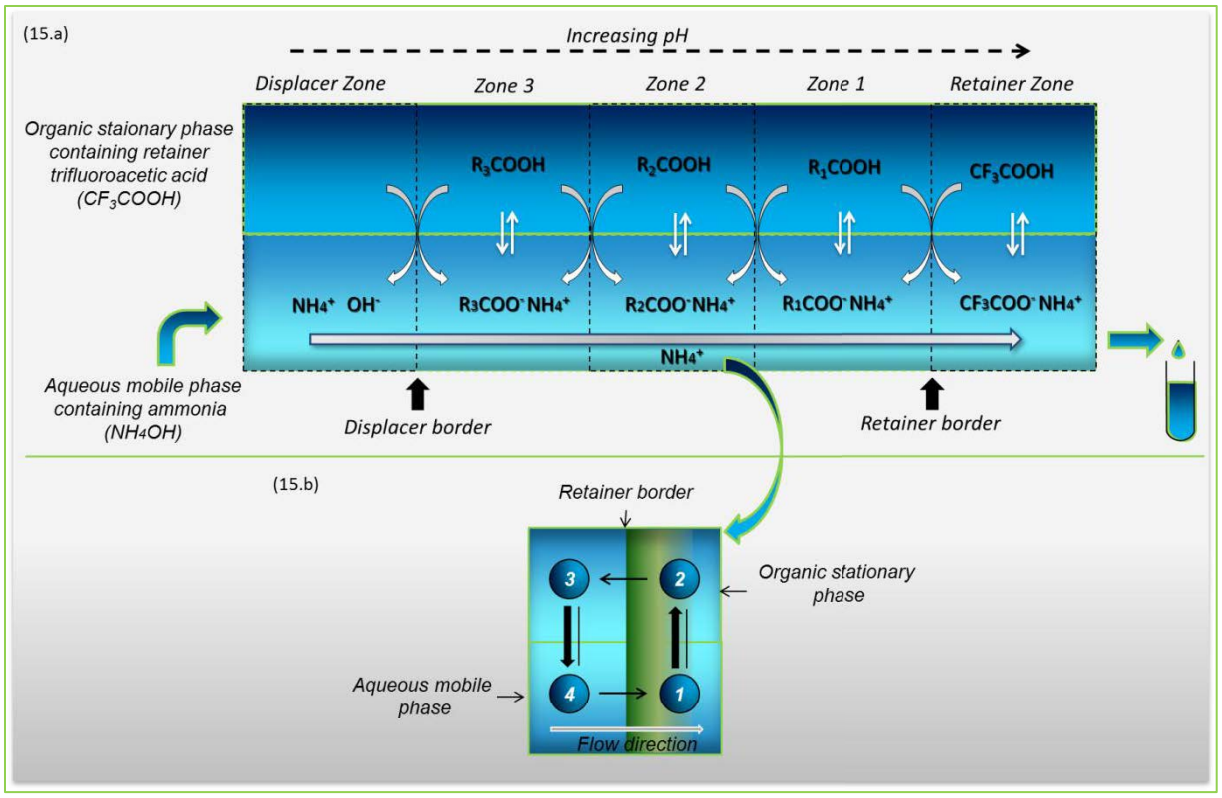

Figure I. 18: (a): The isotachic train formation in pH-zone-refining displacement mode for the separation of three organic acid: R1COOH (pKa-1), R2COOH (pKa-2) and R3COOH (pKa-3) using trifuoroacetic acid (CF3COOH, pKa-r) as acidic retainer in the organic stationary and ammonia (NH4OH, pKa-d) as basic displacer in the aqueous mobile phase $pK_{a\text{-}d} < pK_{a\text{-}1} < pK_{a\text{-}2} < pK_{a\text{-}3} < pK_{a\text{-}r}$*; (b): the pH analyte-zone mechanism [188].*

According to the acid character of the target analytes, the implementation of the process will be different as described in Table I.5. The displacer must be soluble in the mobile phase to elute the analytes according to their pK_a and hydrophobicity [189]. Ito et *al*. have developed the equation showing the relationship between the pH of the mobile phase in each analyte zone ($pH_{z\text{-}a}$) and the other thermodynamic parameters (equation 20).

$$pH_{z-a} = pK_a + \log\left[\left(\frac{K_{D-s}}{K_s}\right) - 1\right]; \qquad Equation\ 20$$

Acid-base character of the desired analytes	***Pumping mode (chlorinated solvents except)***	***Selection criteria of retainer and displacer***
Basic analytes	Descending mode	Displacer in the aqueous mobile phase: hydrophilic strong acid (hydrochloric acid: HCl, methanesulfonic acid: MSA)

	Ascending mode	Retainer in the organic stationary phase: lipophilic strong base (triethylamine: TEA)
		Displacer in the organic mobile phase: lipophilic strong base
		Retainer in the stationary phase aqueous: hydrophilic strong acid
Acidic analytes	Descending mode	Displacer in the aqueous mobile phase: hydrophilic strong base (NH_4OH)
		Retainer in the organic stationary phase: lipophilic strong acid (trifluoroacetic acid: TFA)
	Ascending mode	Displacer in the organic mobile phase: lipophilic strong acid
		Retainer in the aqueous stationary phase: hydrophilic strong base

Table I. 5: Retainer and displacer selection in pH-zone-refining displacement mode based on the nature of the target analytes and on the pumping mode

The pH-zone-refining mode has been widely applied to the purification of alkaloids by using biphasic solvent systems containing typically methyl *ter*-butyl ether and water. Experiments were mainly performed in the descending mode, using a lipophilic strong base (triethylamine for instance) as retainer to maintain the alkaloids in the stationary phase, and a hydrophilic strong acid (HCl for instance) as displacer to gradually ionize the alkaloids and decrease their K_D [190-194]. The pH-zone-refining mode was also applied to the purification of a few phenolic compounds [195-199], ionizable triterpenoids [200], peptides [201], and amino acids [202].

- ***Ion-exchange displacement mode***

The ion-exchange displacement mode was firstly introduced in 1998 by Chevolot et *al.* [203] for the purification of sulfated polysaccharides (fucans) using a lipophilic secondary amine (Amberlite-LA2). Renault et *al*., have then significantly contributed to the development of this mode on CPC instruments, particularly the strong ion-exchange mode. In the ion-exchange displacement mode, a cationic (or anionic) exchanger is introduced in the stationary phase in order to capture all anionic (or cationic) analytes present initially in the crude sample *via* the formation of ion pairs. This mode mainly concerns analytes whose electric charge is invariant during the purification process.

Then the displacer introduced in the mobile phase will force the analytes to competitively progress along the column according to their affinity for the ion-exchanger. Narrow shock layers between analytes are always observed. When experimental conditions are well optimized (exchanger/displacer concentrations, flow rate of the mobile phase) ionic analytes elute from the column as rectangular adjacent and concentrated zones (isotachic train) as given in (Figure I.17) [204]. Two main ion-exchange CPC methods have been described, the Weak Ion-eXchange (WIX) and the Strong Ion-eXchange (SIX) methods [205]. More recently, a third method combining SIXCPC and WIXCPC named “Mixed Ion-eXchange CPC” (MIXCPC) was reported for the purification of peptides [204].

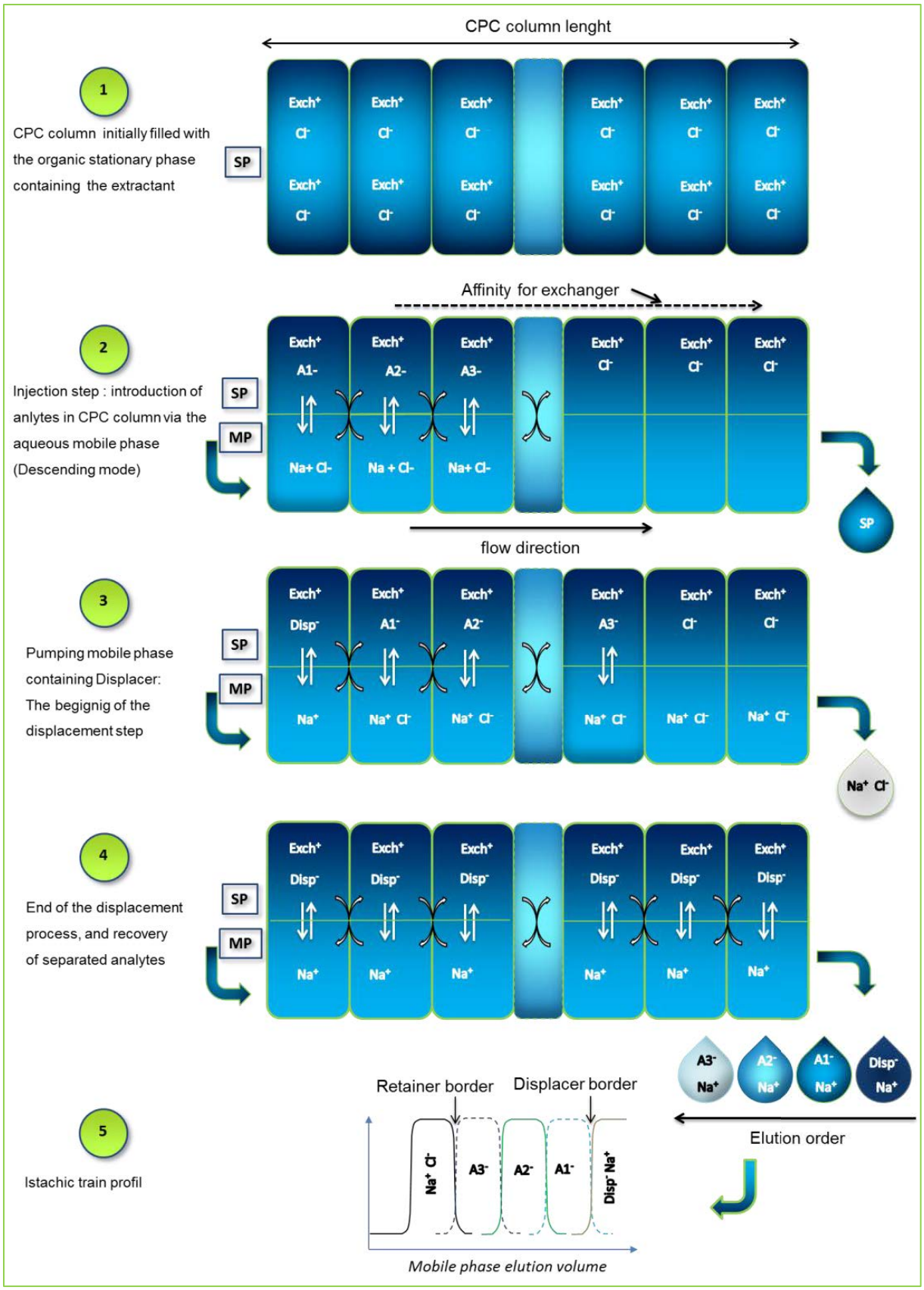

Figure I. 19: Schematic representation of the ion-exchange displacement mode mechanism for the separation of ionic analytes and the isotachic train formation

In the **weak ion-exchange mode**, the exchanger is a species ionized in a narrow range of pH. Two main classes of weak exchanger can be distinguished. The first class includes cationic weak exchangers such as Amberlite LA2®, a high molecular weight secondary amine miscible with most common organic solvents and immiscible with aqueous solutions (Figure I.18). For this type of exchangers, the pH is adjusted to ensure the ionization of the secondary amine as a quaternary ammonium form. The second class include the anionic weak exchangers, such as di-(2-ethylhexyl)phosphoric acid (DEHPA), an organophosphorus compound which is widely used for instance in liquid-liquid extraction of uranium and rare earth metals. In this case the exchange site ensuring the ion-pair formation is the phosphate functional group. The weak ion-exchange CPC mode has been successfully applied to the separation of a mixture of partially depolymerized fucans, a family of polysulfated polysaccharides extracted from brown seaweed [203] and also to the fractionation of low molecular weight heparins [206]. The weak-ion exchange was also applied to the separation and the preconcentration of metal ion using CPC [207-213] and CCC instruments [207, 214, 215].

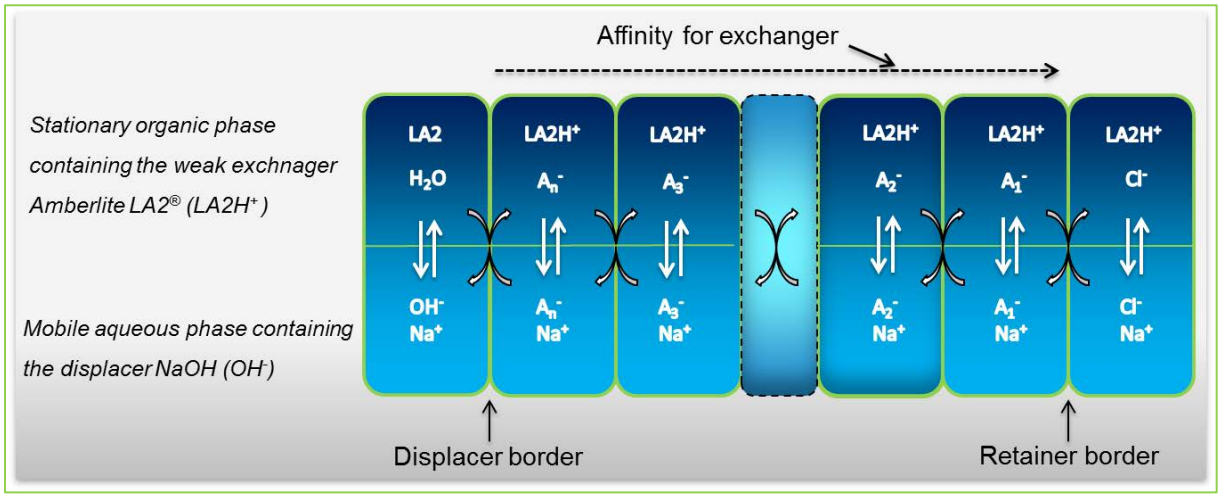

Figure I. 20: Isotachic train formation in weak-anion exchange CPC process (A_1, A_2, A_3, ..., A_n) using the Ambelite LA2® as anionic-exchanger in the organic stationary phase and sodium hydroxide (NaOH) as a displacer in the aqueous mobile phase

In the **strong ion-exchange mode**, the exchanger is permanently ionic and keeps its positive or negative charge at any pH value [205]. Strong ion-exchangers can be anionic or cationic. Anionic exchangers are principally quaternary amines salts, such as benzalkonium chloride or methyltrioctylammonium chloride (Aliquat 336®). When using this mode, ionic analytes are directly captured into the stationary phase by forming stable ion-pairs with the exchanger. The displacer-free mobile phase is not able to elute the extracted analytes. However, when adding the displacer, which

generally presents a strong affinity for the exchanger, the captured analyte return to the mobile phase (Figure I.19). The separation mechanisms in strong ion-exchange centrifugal partition chromatography have been well-described and modeled by Maciuk et *al*. using the separation of phenolic acid regioisomers as methodological support [216]. SIXCPC was also successfully applied to the purification of glucosinolates using the solvent system ethyl acetate/*n*-butanol/water (3:2:5, v/v), the lipophilic anion-exchanger Aliquat 336® in the organic stationary phase and sodium iodide (NaI) as displacer in the aqueous mobile phase [217-219]. The purification of rosmarinic acid from *Lavandula vera* cell culture at the multi-gram scale using the solvent system chloroform/*n*-butanol/water (4.5:1:4.5, v/v), benzalkonium chloride as a lipophilic exchanger and sodium iodide as a displacer [220] and anthocyanins from *Vitis vinifera* was also reported [221].

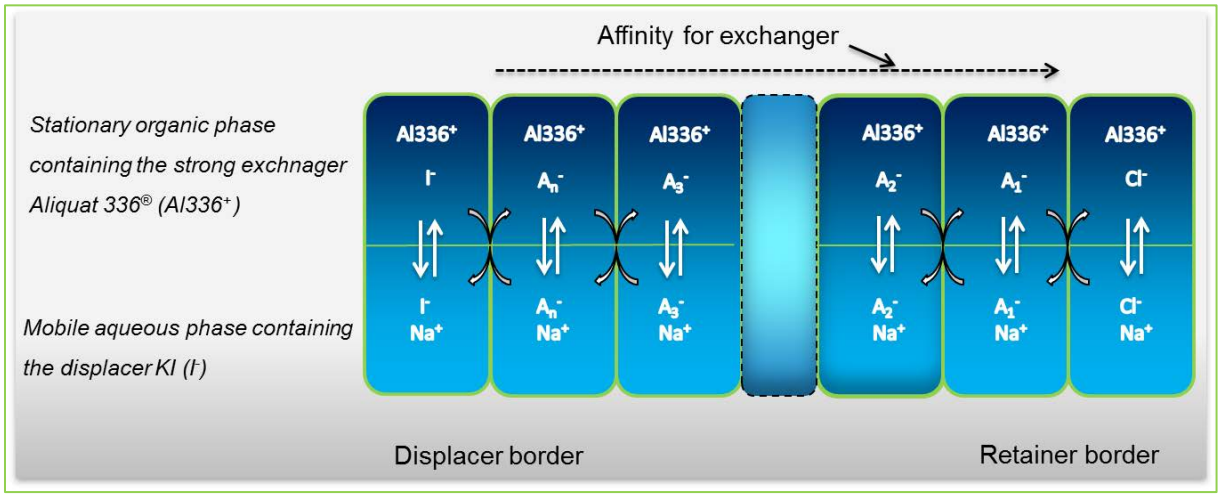

Figure I. 21: Isotachic train formation in strong-anion exchange CPC process (A_1, A_2, A_3, ..., A_n) using the Aliquat 336® as strong anionic-exchanger dissolved in the organic stationary phase and potassium iodoides (KI) as a displacer in the aqueous mobile phase

The **Mixed Ion-eXchange CPC mode** (MIXCPC) is an original displacement method which was recently developed by Boudesocque et *al.* [204, 222]. In this mode the column containing the stationary phase is divided into two zones, each one containing a different exchanger ionization state. The back-extraction step can be achieved by carrying out a sequential use of different displacers (Figure I.20). This methodology was applied to separate a model mixture of five dipeptides of different polarity (Gly-Gly, Gly-Tyr, Ala-Tyr, Leu-Val and Leu-Tyr). The upper phase of the biphasic solvent system methyl *ter*-butyl ether/acetonitrile/*n*-butanol/water (2:1:2:5, v/v) was used as stationary phase to dissolve the cation-exchanger (DEHPA). This stationary phase was segmented into two sections with different triethylamine concentrations. The sequential pumping of the

aqueous mobile phase containing different displacers (Ca^{2+} followed by H^+) allowed the separation of the model mixture in a single run [204].

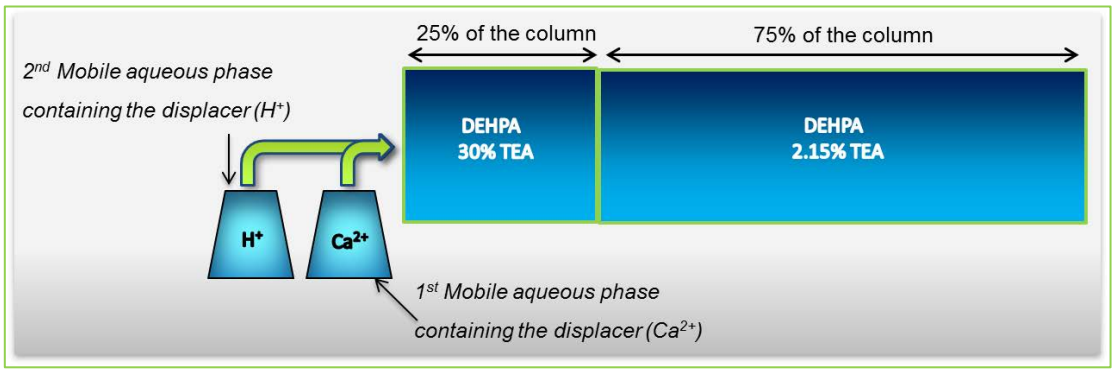

Figure I. 22: Column segmentation in a mix ion-exchange CPC displacement mode (MIXCPC)

I.4. Evolution of CCC and CPC instruments

All currently available CCC and CPC instruments derive from the *Craig-Post apparatus*, which was firstly described by Lyman Creighton Craig and Otto Post in 1949 [223]. It consists in a series of connected glass tubes designed and arranged to perform sequences of mixing and settling steps between two immiscible phases. The analytes are separated in different glass tubes in a countercurrent distribution. Two models containing respectively 30 cells and 220 cells are shown in figure I.21.a-b [224]. The next progression after the *Craig-Post* apparatus was the apparition of Droplet Counter-Current Chromatography (DCCC) (Figure I.21.c) introduced by Tanimura et *al*. in 1970 [225]. It consists in a series of vertical tubes connected top to bottom by capillaries. This design enables one liquid phase to be retained in the vertical tubes while the other liquid phase is pumped through the stationary phase as droplets. The separation of analytes is based on their K_D. The stationary phase retention is governed by the gravity, resulting in poor efficiency and extremely low flow rates [225]. Various CCC and CPC instruments have then been proposed to improve the performance of separation processes.

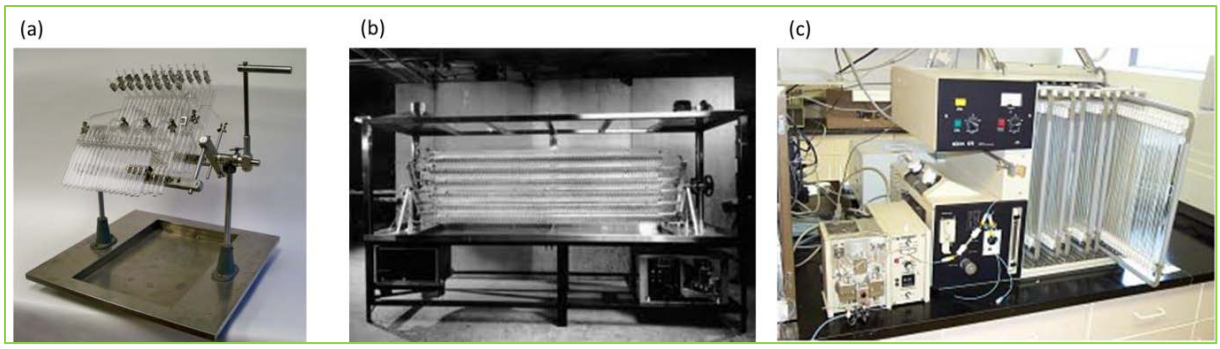

Figure I. 23: (a) Double-row, 30 cells Craig countercurrent separation train, (b) the large 220-cell Craig unit countercurrent extraction train at the Rockefeller Institute Circa [224], (c): Droplet Countercurrent Chromatograph: Buechi_DCCC® [225]

I.4.1. Evolution of CCC instruments and scale-up for industrial separation operation

Prototype CCC instruments have been firstly developed by Ito et *al*. in 1966 [140]. They included a variety of rotating coil instruments describing planetary and non-planetary rotation around a central axis (revolution or solar axis). The first series of CCC instruments called "Coil Planet Centrifuges" was developed to investigate the effect of the rotation axis inclination towards the revolution axis of the cylindrical column on the stationary phase retention and on the separation efficiency [140]. Despite the variety of rotating coil tubing configurations, very few have been commercialized due to too long separation times, poor loading capacities and poor yields. CCC has become popular with the development of the *J*-type coil centrifuge, developed in the 1980's and renamed later "High Speed Countercurrent chromatography (HSCCC)". Indeed, the separation methods developed on HSCCC instruments were radically improved in terms of resolution, separation times and sample loading capacity [151, 226]. The most modern HSCCC instruments (Figure I.22) contain multiple (two or three) separation coils that can be connected either in series (only one inlet and one outlet) or in parallel (multiple inlets/outlets). In practice it is usually most convenient to connect multiple columns in series [145, 181]. Recently, four vertical coils symmetrically arranged around the centrifuge axis have been developed with a total column volume of 1600 mL [227].

Intense research efforts have been made to improve the design of CCC instruments. For instance, the length of tubing, the sample loading capacities, flow-rate, revolution speed or the orientation of the coil, have been studied [181, 228]. Different tubing geometries (round and rectangular, normal and twisted) have also been investigated [229]. High performance countercurrent chromatography (HPCCC) is another hydrodynamic CCC variant. The particular

feature is that HPCCC is designed to generate stronger centrifugal force field as compared to HSCCC. For instance, a comparison study was recently realized between both instruments, indicating that the increase in *g*-level (240 x *g* for HPCCC and 80 x *g* for HSCCC) and the larger column bore enabled a ten-fold increase in productivity, due to increased mobile phase flow rates and higher stationary phase retention [230].

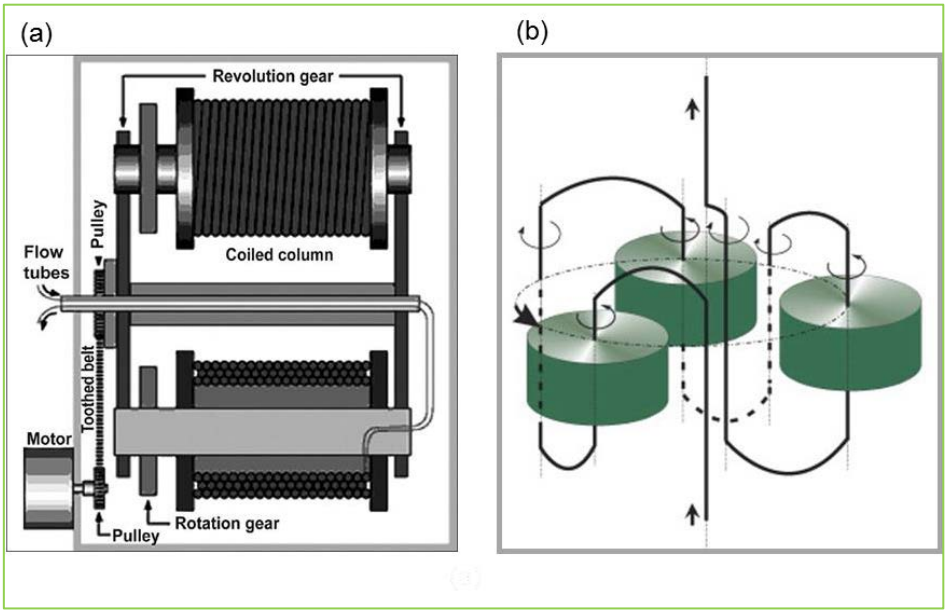

Figure I. 24: (a): High speed countercurrent chromatography column; (b): Three-coil high speed countercurrent chromatograph configuration

Scale-up of countercurrent chromatography has been mainly described by Sutherland et al. [231, 232]. The scale-up feasibility requires the development of robust and efficient CCC instruments having high column capacities with special geometries. Two Maxi-CCC centrifuges having dual coils tubing operating in parallel or in series with column volume capacities of 4.6 L and 18 L have been recently developed by Dynamic Extraction Ltd [231, 233]. The first device was applied to the pilot-scale production of glucoraphanin from broccoli seeds [234, 235] and of honokiol from *Magnolia officianalis* Rehd. et Wils. [236, 237].

I.4.2. Evolution of CPC instruments and scale-up for industrial separation operation

The first CPC instrument design was inspired from droplet countercurrent chromatography and Helix-CCC introduced in 1970s and known today as Toroidal coil CCC [238, 239]. These

instruments yielded thousands NTU but required very long run time to achieve separation. In 1982, Nunogaki et *al*. described the first Centrifugal Partition Chromatograph (CPC) [141]. The CPC column consisted in twenty cartridges, in which the partition cells and ducts were engraved. The first commercial device called "CPC LLN" was marketed in the late 80s by Sanki engineering Ltd. Since, CPC apparatus have been significantly improved and the rotors are now made of superposed partition disks separated by sheets of Teflon® (Figure I.23.a). This type of columns are currently manufactured by Rousselet-Robatel Kromaton® [24], Armen Instruments [240] and Sanki [241]. Another CPC design known as the Partitron® was developed by Margraff et *al*. where the rotor consisted in a titanium monobloc wherein the partition cells and ducts are pierced (Figure I.22.b) [242].

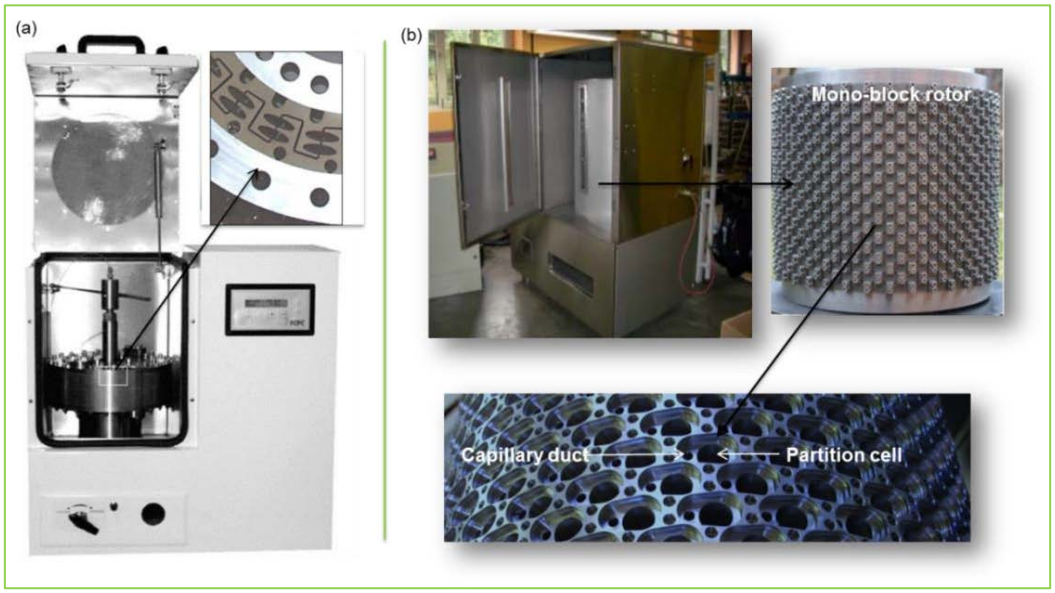

Figure I. 25: Centrifugal partition chromatographs (a): with "sandwich" column configuration, (b): "monobloc" column configuration

The major improvements realized recently in CPC design were focused on the geometry of the partition cells. Marchal et *al*., was intensively studied the effect of partition cell geometry on the improvement of the hydrodynamic behavior of the phases inside the cells during experiments [243]. The control of flow patterns and the *Coriolis* force inside various cell geometries and when varying operating conditions, especially, mobile phase flow rate and rotation speed of the rotor was carried out by visualization experiments. The principal objective is to select a suitable geometry for an optimal mobile phase dispersion and therefore for an efficient transfer and separation performance [243-245]. The first cells (Figure I.24.a) were rectangular cells with an axis parallel to the centrifugal force and the ducts are inserted laterally to the base of the cells. In this configuration, the mobile phase jet was directed to the wall of cells and the dispersion of the mobile phase was not optimal

[141, 243]. This geometry was then improved firstly by connecting the ducts to the centers of the base of the cells. (Figure I.24.b), and then by inclining the cells to perform a *Z*-cell configuration (Figure I.24.c). New generations of cell geometry called "Twin-cells" (Figure I.24.d), with oval symmetric or asymmetric geometries were developed [246, 247]. One Twin-cell forms one physical transfer unit with one cell playing the role of mixing chamber and the other one achieving the settling of the two phases. With this configuration the mobile phase dispersion was significantly increased and the hydrodynamic of the phases during the experiments was improved [248]. Advances in the development of robust laboratory-scale CPC instruments have allowed the development of new pilot and industrial CPC instruments of higher column volume capacities. A laboratory scale CPC instrument with a column capacity of 245 mL was firstly scaled-up to a column of 5.4 L and then to the Partitron® of 25 L [242, 249].

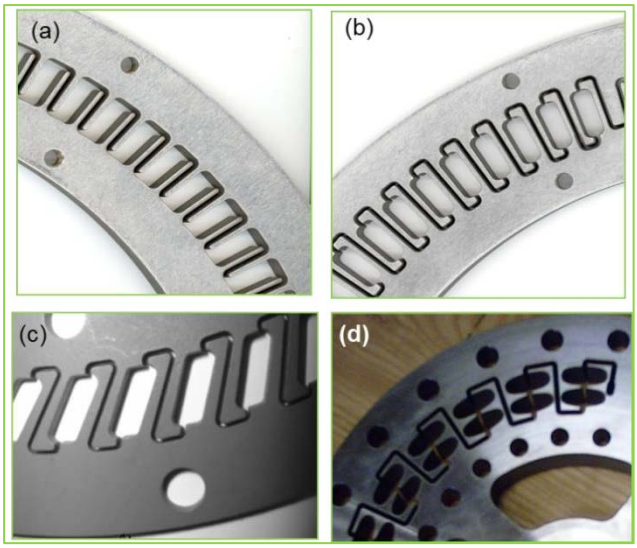

Figure I. 26: Different CPC cell geometries. (a) : rectangular cell geometry used in the HPCPC® chromatograph manufactured by Sanki Engineering Co. Ltd [141, 149, 250], (b): rectangular cell used in the FCPC chromatographs®, (c): Z-cell configuration used in FCPC® chromatographs manufactured by Kromaton® [24], (d): modern cell configuration with "Twin-cell" geometry [247, 251]

Several CPC scale-up operations were achieved using the Armen Elite Continuum® devices with a column capacity of 12.5 L and the Kromaton FCPC® with a column capacity of 5.4 L. The Armen device was used by Sutherland et *al.* to scale-up the separation of two proteins, lysozyme and myoglobin, using an aqueous two-phase system comprising 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4. The separation was optimized on a laboratory-scale CPC of 500 mL by injecting 176 mg of the protein mixture and then the separation was linearly scaled-up on the pilot-scale device by injecting 2.2 g [252]. The pilot-scale Kromaton FCPC5000® device with a column volume of 5700 mL

was used to scale-up the separation of several natural products from their complex mixtures. It was used for instance to isolate 70.3 g of pure sinalbin from 341 g of white mustard seed extract (*Sinapis alba* L.) by applying the strong ion-exchange displacement mode. Due the versatility and flexibility of CCC and CPC, the column volume can be easily extended by a simple addition of partition discs (CPC), or by extending the coil tubing (CCC). Marchal et *al*. have significantly contributed to a better understanding of hydrodynamics and mass transfer phenomena in an attempt to optimize CPC partition cell design and size [146, 149, 243, 245, 246].

I.4.3. Centrifugal partition extraction (CPE) : An interesting positioning between liquid-liquid extraction and liquid-liquid chromatography

As described in the two previous sections of the present review, liquid-liquid extractors are currently clearly distinguished from liquid-liquid chromatographs with respect to equipment and application areas. On the one hand, liquid-liquid extraction processes are more dedicated to industrial-scale applications as the devices are designed to generate around 10 NTU, to allow the application of high flow rates (in the m^3/h order) and the treatment of high sample quantities (several tones/year). However, despite their high productivity, liquid-liquid extraction processes are strongly lacking of selectivity and thus are not appropriate for the separation of structurally related compound mixtures as encountered in complex natural extracts. On the other hand, solid support-free liquid-liquid chromatographic systems including CCC and CPC have proven to be very efficient for the purification of natural products. These techniques can generate around 1000 NTU and are today well-recognized for their high selectivity. However, despite the efforts made to improve the geometry and design of CCC and CPC devices for a better productivity, these techniques remain mostly used by academic researchers on a laboratory-scale.

In an attempt to combine the benefits of liquid-liquid extraction processes with those of solid support-free liquid-liquid chromatography, a new device named “Fast Centrifugal Partition Extractor” (FCPE300®) was recently developed by Rousselet-Robatel Kromaton. With several hundred of NTU, this first laboratory-scale prototype of 303 mL capacity was designed to be more selective than classical liquid-liquid extractors while providing a higher productivity than classical liquid-liquid chromatographs. The FCPE300® design was directly inspired from CPC rotors. Its column is composed of 7 partition disks (Figure I.25) engraved with a total of 231 oval and symmetric twin-cells (33 twin-cells per disk).

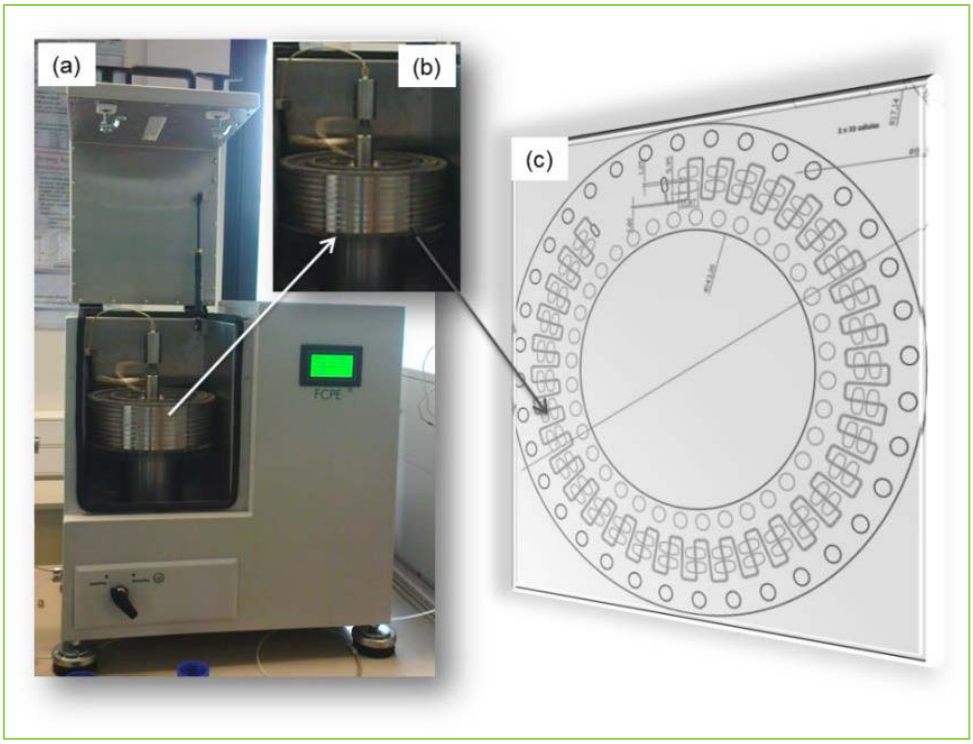

Figure I. 27: (a) The laboratory-scale Centrifugal Partition Extractor FCPE300®; (b): The rotor, (c): Design of the partition disks containing the Twin-cells

The particularity of the FCPE300® as compared to a CPC of equivalent column capacity (Table I.6) is that the cells are of larger volume (≈ 1 mL against ≈ 0.1 mL) and connected together by larger ducts (0.8 mm against 0.6 mm). These characteristics have a direct impact on the number of physical plates and NTU. Indeed, the NTU in CPC and CPE is not only related to the number of physical plates (number of partition cells) but also to several hydrodynamic parameters such as the mobile phase flow rate, rotation speed or stationary phase retention volume (solvent system). Increasing the mobile phase flow rate while maintaining a stable pressure and stationary phase retention inside a CPC or CPE column results for instance in an increase in the number of NTU due to a better mixing of the liquid phases and therefore in a better mass transfer [243, 253, 254]. The resulting number of NTU is thus greater than the number of physical plates [242]. However, a compromise between the mobile phase flow rate and the associated pressure drop and stationary phase retention volume requires a specific geometry and number of partition cells as demonstrated by Foucault [246]. Besides, reducing the number of partition cells in the FCPE300® as compared to a CPC apparatus of equivalent column volume results in partition cells and ducts of larger volume in CPE, and consequently in a decrease of the generated pressure drop inside the system. Thus the FCPE300® tolerates the application of higher flow rates than CPC, what provides interesting perspectives in

terms of separation productivity. What remains unknown is the ability of the FCPE300® to achieve the separation of complex matrices with the same selectivity as obtained from CPC.

Characteristics	*Lab-scale FCPC200 Kromaton®*	*Lab-scale FCPE300 Kromaton®*
Process parts	*Inox, Teflon®*	*Inox*
Number of partition disks	*20*	*7*
Number of partition cells	*1320*	*231*
Number of cells per disk	*66*	*33*
Total rotor volume (mL)	*205*	*303.5*
Dead volume (mL)	*32.3*	*72.8*
Partition cell volume (mL)	*0.130*	*0.986*
Usual operating pressure (bar)	*40-50*	*6-11*

Table I. 6: Comparison of the main characteristics of the FCPC200® chromatograph and the FCPE300® extractor

I.5. Conclusion

This first chapter has reported an updated overview of the principal liquid-liquid extraction processes and devices currently used to achieve specific extractive operations with high productivity at the industrial scale. The limitations of liquid-liquid extraction processes have been highlighted particularly their lack of selectivity for the purification of analytes from complex natural mixtures. This review has also described the current solid support-free liquid-liquid chromatographic techniques, their applications in the field of natural product purification and the efforts devoted to scale-up and industrialize the CCC and CPC devices. In view of these data, it appears clearly that a significant gap existing between liquid-liquid extraction and liquid-liquid chromatography must be filled by new instruments combining both selectivity and high productivity. In this context, the prototype laboratory-scale Centrifugal Partition Extractor (CPE) was recently designed to be positioned at the interface between extraction and chromatography techniques.

I.6. References

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CHAPITRE 2:

EXTRACTION DE PARTAGE CENTRIFUGE EN MODE D'ÉCHANGE D'IONS FORT (SIX-CPE): INTENSIFICATION DE LA PURIFICATION DE MÉTABOLITES NATURELS IONIQUES

CHAPTER 2:

CENTRIFUGAL PARTITION EXTRACTION IN THE STRONG ION-EXCHANGE MODE (SIX-CPE): AN INTENSIFYING APPROACH FOR THE PURIFICATION OF IONIC NATURAL METABOLITES

Chapitre 2 : Extraction de partage centrifuge en mode d'echange d'ions fort (SIX-CPE): intensification de la purification de metabolites naturels ioniques

Introduction du chapitre 2

Le projet de thèse qui m'a été confié avait pour but d'étudier les principes fondamentaux et les applications potentielles de l'extraction de partage centrifuge. La conception particulière d'un extracteur de partage centrifuge par rapport aux chromatographes de partage centrifuge, en particulier le nombre réduit de cellules ayant un volume plus grand, nous a incité tout d'abord à explorer les performances du prototype FCPE300® développé par Rousselet-Robatel Kromaton® dans différents modes déjà développés en CPC (élution et déplacement). La performance et l'efficacité des techniques de séparation liquide-liquide sont directement liées au nombre de plateaux théoriques générés. Etant donné que le nombre de plateaux théoriques des chromatographes liquide-liquide sans support solide type « CPC » est généralement égal au nombre de cellules de partage que ces instruments contiennent, il convenait de penser dans un premier temps qu'une diminution du nombre de cellules devait affecter significativement la performance des processus de séparation réalisés en EPC en termes d'efficacité, de sélectivité et de résolution. L'objectif de ces premiers travaux de thèse a donc été d'étudier l'utilisation de l'extracteur de partage centrifuge en mode d'échange d'ions fort dans une optique de purification et d'isolement de métabolites secondaires d'intérêts. Nous avons fait le choix de travailler sur des extraits naturels complexes d'origine végétale, c'est le cas de l'extrait de réglisse (*Glycyrrhiza. Glabra* L.) pour extraire la glycyrrhizine (une saponine ionique), et de l'extrait de moutarde blanche (*Sinapis. Alba* L.) pour extraire la sinalbine (un glucosinolate ionique) et sur un milieu fermentaire pour extraire l'acide itaconique, un C_5 diacide carboxylique issus des biotechnologies blanches. Ce chapitre sera divisé en trois parties distinctes pour chacun des supports méthodologiques étudiés. Les résultats de ces travaux ont déjà conduit à la publication de deux articles dans des journaux scientifiques à comité de lecture international et à la rédaction d'un troisième article qui vient d'être soumis dans le « *Journal of Chemical Technology and Biotechnology* ». Ce chapitre reprend ces trois publications qui seront présentés en anglais.

CHAPITRE 2 - PARTIE 1 :

INTENSIFICATION DE LA PURIFICATION D'UNE SAPONINE IONIQUE (GLYCYRRHIZINE) À PARTIR D'UN EXTRAIT RACINAIRE DE RÉGLISSE PAR EXTRACTION DE PARTAGE CENTRIFUGE EN MODE ÉCHANGE D'IONS FORT

CHAPTER 2 - PART 1:

INTENSIFIED PURIFICATION OF A NATURAL IONIC SAPONIN (GLYCYRRHIZIN) FROM A LICORICE ROOTS EXTRACT BY STRONG ION-EXCHANGE CENTRIFUGAL PARTITION EXTRACTION (SIX-CPE)

II. Intensification de la purification d'une saponine ionique (glycyrrhizine) à partir d'un extrait racinaire de réglisse par extraction de partage centrifuge en mode échange d'ions fort

Introduction Chapitre 2 - Partie 1

Cette étude présente les potentialités de l'extracteur de partage centrifuge pour l'extraction et la purification de la glycyrrhizine, une saponine naturelle ionique présente en quantité significative dans les racines de réglisse (*Glycyrrhiza glabra L.*, Fabaceae) et qui serait responsable de ses propriétés anti-inflammatoire [6, 7]. Le choix de ce premier modèle a été défini en accord avec notre partenaire industriel (Soliance). Etant donné la nature ionique de la glycyrrhizine, les premiers développements méthodologiques de séparation ont été réalisés en mode de déplacement par échange d'ions fort. Cette étude s'est également attachée à caractériser les propriétés intrinsèques de l'extracteur, en particulier le volume interne exact de sa colonne, le volume des canaux reliant les cellules de partage ainsi que sa capacité de rétention de phase stationnaire en fonction du débit de la phase mobile et de la vitesse de rotation pour plusieurs systèmes de solvants bi-phasiques. Le mode de déplacement par échange d'ions fort a tout d'abord été appliqué à la glycyrrhizine standard pure (98 %) en vue d'étudier la faisabilité de de la méthode sur ce métabolite et d'optimiser les conditions opératoires (débit de la phase mobile, ratio (extractant/analyte) et ratio (extractant/déplaceur), volume injecté). La méthode a été ensuite appliquée sur l'extrait hydroalcoolique (EtOH 80 %) brut de réglisse (*Glycyrrhiza. glabra L.*, Fabacée) tout en visant l'optimisation des principaux paramètres (débit de la phase mobile et masse de l'extrait injectée) pour obtenir une productivité élevée et une pureté satisfaisante. Ces résultats sont présentés et discutés en détails dans les paragraphes suivants.

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Intensified extraction of ionized natural products by ion pair centrifugal partition extraction

Mahmoud Hamzaoui [a], Jane Hubert [a,*], Jamila Hadj-Salem [a], Bernard Richard [a], Dominique Harakat [a], Luc Marchal [b], Alain Foucault [b], Catherine Lavaud [a], Jean-Hugues Renault [a]

[a] UMR CNRS 6229, Université de Reims Champagne-Ardenne, Bât. 18, Moulin de la Housse, BP 1039, 51687 Reims, Cedex 2, France
[b] UMR CNRS 6144, Université de Nantes, Laboratoire GEPEA, CRTT, 44602 Saint-Nazaire, France

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ABSTRACT

The potential of centrifugal partition extraction (CPE) combined with the ion-pair (IP) extraction mode to simultaneously extract and purify natural ionized saponins from licorice is presented in this work. The design of the instrument, a new laboratory-scale Fast Centrifugal Partition Extractor (FCPE300®), has evolved from centrifugal partition chromatography (CPC) columns, but with less cells of larger volume. Some hydrodynamic characteristics of the FCPE300® were highlighted by investigating the retention of the stationary phase under different flow rate conditions and for different biphasic solvent systems. A method based on the ion-pair extraction mode was developed to extract glycyrrhizin (GL), a biologically active ionic saponin naturally present in licorice (*Glycyrrhiza glabra* L., Fabaceae) roots. The extraction of GL was performed at a flow rate of 20 mL/min in the descending mode by using the biphasic solvent system ethyl acetate/*n*-butanol/water in the proportions 3/2/5 (v/v/v). Trioctylmethylammonium with chloride as a counter-ion (Al336®) was used as the anion extractant in the organic stationary phase and iodide, with potassium as counter-ion, was used as the displacer in the aqueous mobile phase. From 20 g of a crude extract of licorice roots, 2.2 g of GL were recovered after 70 min, for a total process duration of 90 min. The combination of the centrifugal partition extractor with the ion-pair extraction mode (IP-CPE) offers promising perspectives for industrial applications in the field of natural product isolation or for the fractionation of natural complex mixtures.

II.1. Introduction

Centrifugal partition chromatography (CPC) refers to a particular type of support-free liquid–liquid chromatographic techniques where solutes are separated on the basis of their partition coefficients between two immiscible solvent systems [1] and [2]. The particularity of CPC systems arises from their column which contains a series of interconnecting cells mounted circumferentially on stacked disks in rotation around a single axis [1]. One liquid is maintained inside the column as the stationary phase by a constant centrifugal force field while the other phase is pumped through it. This technique is very attractive in terms of selectivity, sample loading capacity and scaling-up ability [3] and [4]. CPC has been widely used for the extraction and purification of natural products [5], [6], [7] and [8]. Many processes and equipment are currently being developed to transpose these techniques at the industrial scale [2], [9] and [10]. The main challenge is to reduce the process duration and consequently to increase flow rates and mass sample loading.

In this study, we used an original device, the Fast Centrifugal Partition Extractor (FCPE300®) that has recently been designed by Rousselet Robatel Kromaton (Angers, France). This laboratory-scale extractor has evolved from centrifugal partition chromatography (CPC), but its column contains less cells of larger volume (231 twin cells, each twin cell representing a single mixing and settling stage). The aim of this work was to examine the potential of the FCPE300® apparatus for the extraction and purification of natural ionic compounds. For this purpose, a method based on the ion-pair extraction mode was developed on the centrifugal partition extractor (IP-CPE). The inspiration for ion-pair extraction came from a strong ion exchange method previously developed in combination with centrifugal partition chromatography (SIXCPC) for the isolation and purification of glucosinolates from white mustard and broccoli seed aqueous extracts [11] and rosmarinic acid from *Lavandula vera* cell cultures [12]. After investigating the effect of flow rates on the retention of the stationary phase for different biphasic solvent systems, the IP-CPE method was applied to the extraction of glycyrrhizin (GL), an ionized triterpene saponin present in 1–12 % of the dry hydro-alcoholic extracts of licorice roots (*Glycyrrhiza glabra* L., Fabaceae) [13] and [14]. GL comprises two molecules of glucuronic acid linked to the glycyrrhetic acid aglycone moiety and occurs naturally in a salt form (calcium, magnesium or potassium) (Figure II.1).

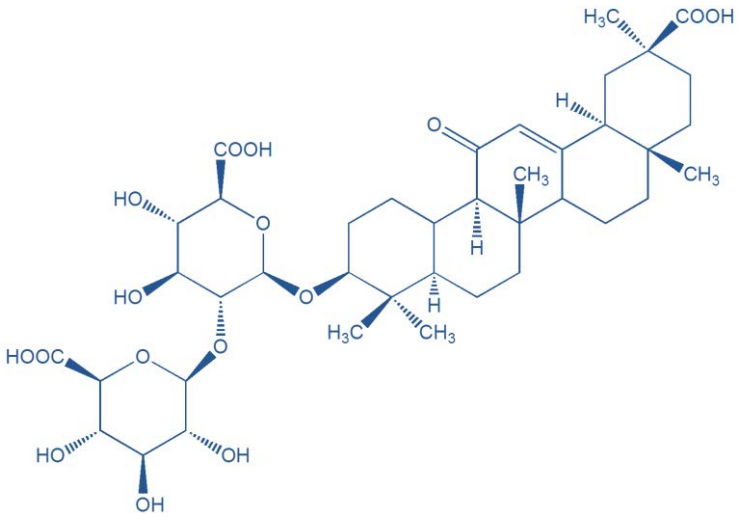

Figure II. 1: Glycyrrhizin: (3β,18α)-30-hydroxy-11,30-dioxoolean-12-en-3-yl-2-O-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid

Various other secondary metabolites are present in licorice roots, among which flavanones (liquiritin and glabrol derivatives), isoflavanes (glabridine and derivatives) and chalcones (isoliquiritin, licuraside) [15], [16] and [17]. Several studies conducted on animal models and in humans have demonstrated the hepatoprotective, anti-inflammatory, anti-allergic, anti-ulcer and antimicrobial

activities of GL and licorice root extracts [18] and [19]. Several extraction and separation methods for GL have been developed, including macro-porous resin separation, microwave-assisted extraction, pressurized hot water extraction, as well as high-speed and multi-stage countercurrent chromatography [20], [21], [22] and [23]. However, at present, the purification of GL requires several steps and its recovery is generally only about 20–30% [24]. We have applied the IP-CPE method to recover GL from 20 g of a crude extract of licorice roots in only one lab-scale operation. This innovative combination offers promising perspectives for industrial application due to its potential in terms of process duration, mass sample loading, extraction recovery and purity.

II.2. Experiments

II.2.1. Reagents

All analytical grade solvents were used: ethyl acetate (EtOAc), *n*-heptane, *n*-butanol (*n*-BuOH), acetonitrile (CH_3CN), methanol (MeOH), ammonia (NH_4OH) and chloroform ($CHCl_3$) were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Aliquat336® (trioctylmethylammonium chloride, Al336) was purchased from Sigma–Aldrich (Saint-Quentin, France) as a mixture of C_8 and C_{10} chains with C_8 predominating. Potassium iodide was obtained from Prolabo (Fontenay, France). The standard molecule of GL (monoammonium salt, purity >98%, MW = 839 $g.mol^{-1}$) was purchased from Extrasynthèse (Genay, France).

II.2.2. Instrumentation

The laboratory-scale Fast Centrifugal Partition Extractor FCPE300® (Kromaton Technology, Angers, France) was equipped with a rotor made of 7 circular partition disks containing a total of 231 partition twin cells (Figure II.2). The distance from the center of the rotor to the center of twin cells is 98.8 mm, and the total diameter of the rotor is 250 mm. The volume of the column is about 300 mL (see Section II.3.1). The twin cells are connected together by ducts of 0.8 mm internal width. Rotation speed can be adjusted from 200 to 2000 rpm, producing a relative centrifugal acceleration in the partition cell up to 437 × g. The mobile phase was pumped either in the ascending or in the descending mode with low residual pulsation through a KNAUER Preparative Pump 1800® V7115 (Berlin, Germany). This pump can deliver constant flow up to 1000 mL/min at a maximum pressure of 400 bars. The system was equipped with an Isco500D Syringe Pump of 507 mL cylinder capacity delivering flow rates up to 204 mL/min at pressures up to 262 bars (Teledyne IscoInc., Lincoln, Nebraska, USA). The system was coupled to a UVD 170S detector (Dionex, Sunnivale, CA, USA)

equipped with a preparative flow cell (6 µL internal volume, 2 mm path length). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). All experiments were conducted at room temperature (20 ± 2 °C).

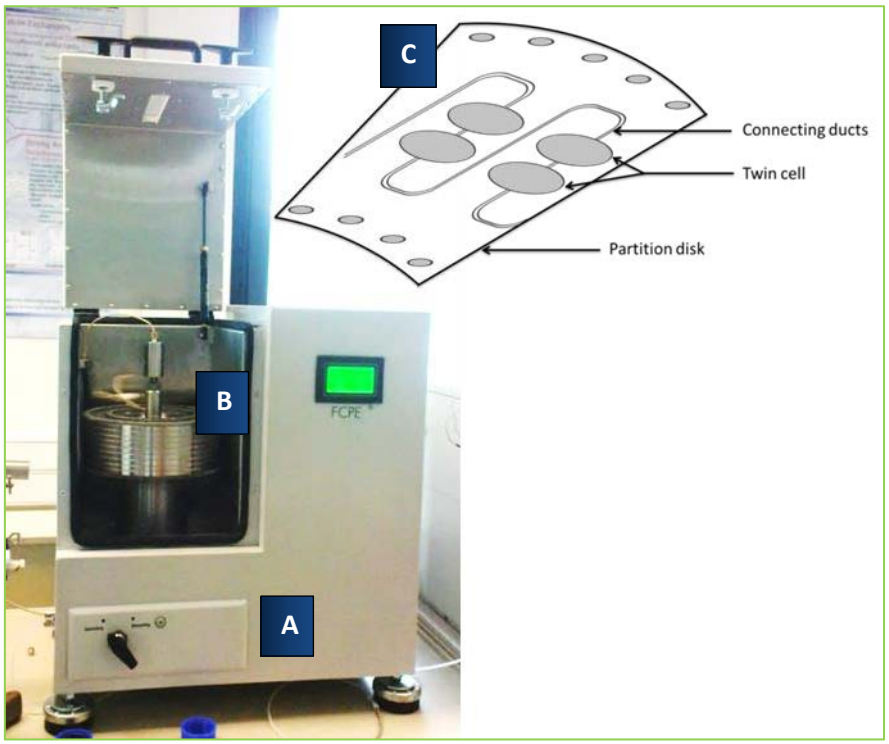

Figure II. 2: Kromaton Technology FCPE300®. (A) Laboratory-scale extractor design, (B) partition disks and (C) geometry of the twin partition cells

II.2.3. Characterization of the Fast Centrifugal Partition Extractor (FCPE300®)

The volume of the FCPE300® column was measured by using a highly stable biphasic solvent system *n*-heptane/water. The column was filled with the aqueous phase saturated with *n*-heptane in the ascending mode at 50 mL/min (column rotation speed: 200 rpm) and subsequently flushed with the organic phase saturated with water in the descending mode. The column volume was measured as the quantity of the aqueous phase evacuated from the column. The experiment was repeated 10 times. Stationary phase retention was evaluated at different flow rates by using the biphasic solvent systems *n*-heptane/water (1/1, v/v), *n*-heptane/MeOH (1/1, v/v), *n*-butanol/water (1/1, v/v), EtOAc/water (1/1, v/v), M*t*BE/water (1/1, v/v), and EtOAc/*n*-butanol/water (3/2/5, v/v/v). Each solvent system was prepared in a separatory funnel. The solvent mixture was vigorously shaken and allowed to settle until phase separation. The column was filled with the organic stationary phase and the rotation speed was adjusted to 1000 rpm. The aqueous mobile phase was then pumped in the

descending mode at flow rates ranging from 10 to 300 mL/min in independent experiments without sample injection. For each condition, the percentage of the stationary phase retention was determined by measuring the volume of the stationary phase remaining in the column relative to the total column capacity. All experiments were repeated in the ascending mode and the results were not significantly different.

II.2.4. Influence of flow rates on the extraction profile of GL

An ion pair extraction method was developed by using the solvent system EtOAc/*n*-butanol/water (3/2/5, v/v/v). The objective here was to determine the optimum flow rate for a short and efficient extraction of GL. The column was filled with the organic stationary phase containing the extractant Aliquat336® (Al336). This lipophilic quaternary ammonium salt is able to extract anionic compounds in the organic stationary phase while keeping a good retention of the stationary phase, in spite of its surfactant properties [12]. GL standard (200 mg, 0.238 mmol) was dissolved in 19 mL of the fresh aqueous mobile phase and 1 mL of the organic phase. As GL contains three potential ionic sites and Al336 only one ionic site, a large excess of Al336 was added to the stationary phase to ensure the complete extraction of GL. The concentration of Al336 was fixed at about 9 mM (molar ratio $n_{Al336}/n_{GL} \approx 7$). The solution was adjusted to pH = 7 and loaded into the column.

The mobile phase was pumped in the descending mode at 10, 20 or 50 mL/min in independent experiments (Table II.1). The concentration of Al336 was adjusted to the stationary phase retention value obtained for each flow rate tested in order to keep the molar ratio nAl336/nGL constant. After eluting one column volume, potassium iodide (KI) was added to the mobile phase for the back extraction step at a molar ratio $n_{Al336}/n_{KI} = 1$. Iodides were selected as displacer agents due to their higher affinity for the extractant Al336 than GL, resulting in a step-by-step transfer of GL from the stationary to the mobile phase and its progression along the column. The extraction/back extraction process of ionic analytes inside the FCPE300® column is described in Figure II.3. As the analytes are expelled from the stationary phase by the displacer, a series of successive bands is formed and moves along the column at the velocity of the displacer front. This series of bands is called the isotachic train. Each rectangular band refers to analytes of high concentration and high purity. The UV spectral profiles monitored at 252 nm were compared between each flow rate condition.

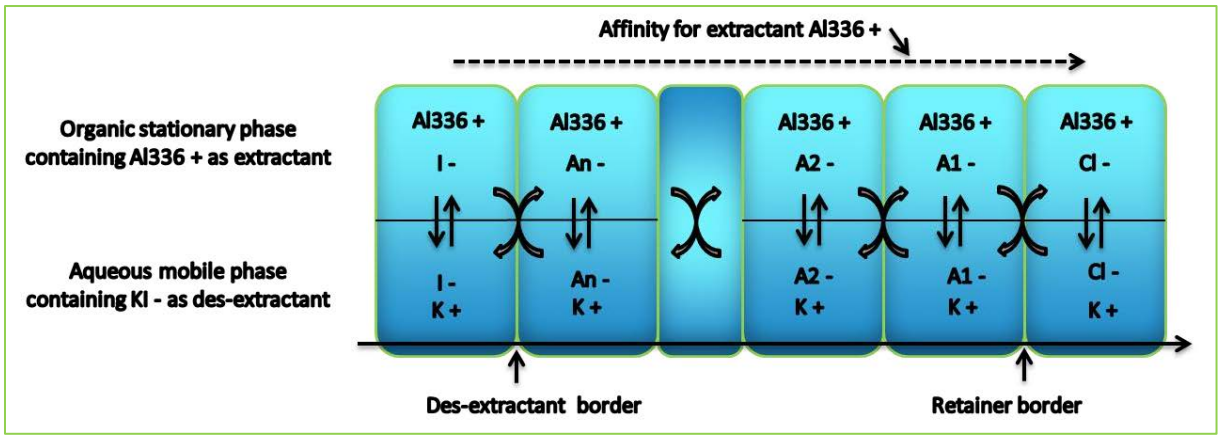

Figure II. 3: Progression of the isotachic train during the ion-pair extraction/back extraction process inside the FCPE300® column. Separation of nA− anions (A_{1-}, A_{2-},…, A_{n-}) using ($Al336^+$) as anion-extractant and iodides (I^-) as displacer (Cl^-: retainer and K^+: displacer counter-ion)

	Experiment	Flow rate (mL/min)	Loaded volume (mL)	GL standard (mg), (mmol)	Stationary phase retention (%)	$C_{Al336} = C_{KI}$ (mM); n_{Al336}/n_{GL}	GL back extraction duration (min)	Total process duration (min)	GL outlet concentration (mM)
Flow rate variation experiments	1	10	20	200 (0.238)	67.21	8.7 (7.4)	7.5	75	3.17
	2	20	20	200 (0.238)	64.9	9.0 (7.4)	3.5	38	30.9
	3	50	20	200 (0.238)	62.43	9.3 (7.4)	1.5	23	30.9
Loaded volume experiments	1’	20	20	131 (0.156)	64.7	3.3 (4.2)	3.3	39	2.34
	2’	20	1000	131 (0.156)	63.9	3.3 (4.2)	2.8	85	278
	3’	20	2000	131 (0.156)	63.2	3.3 (4.2)	2.4	136	3.25

Table II. 1 : Experimental conditions for EPC process development.CAl336: extractant concentration; C_{KI} = displacer concentration; n_{Al336}/n_{KI} = extractant/displacer molar ratio. See ***Figure II.5*** *for the effect of the flow rate on the extraction profile of GL*

II.2.5. Concentration profile of GL inside the FCPE300® column before the back extraction process

Three concentrations of Al336 (3.3 mM, 10 mM or 30 mM) were preliminary tested in order to determine the optimum molar ratio n_{Al336}/n_{GL} which is required to achieve the complete extraction of GL. For that we have examined the condition where all the sites of Al336 are occupied by GL (as reflected by an excess of GL out of the column) and subsequently calculated the molar quantities of Al336 and GL remaining inside the column in this particular situation.

The concentration profile of GL inside the FCPE300® column was then determined by extrusion [25]. After loading 200 mg of GL standard into the system, one column volume of the aqueous mobile phase (without KI) was pumped in the descending mode at 10 mL/min. The organic stationary phase was then extruded by pumping a new column volume of the aqueous mobile phase in the ascending mode at 10 mL/min. The amount of glycyrrhizin was determined by HPLC in the successive fractions (20 mL each) collected at the column outlet.

II.2.6. Influence of the inlet sample volume

The influence of the feed volume on the extraction profile and outlet concentration of GL was investigated. The concentrations of Al336 in the stationary phase and of KI in the mobile phase were fixed at 3.3 mM (n_{Al336}/n_{GL} = 4.2). In three independent experiments, GL standard was dissolved in 20 mL, 1000 mL or 2000 mL of the aqueous mobile phase and loaded into the CPE column. Experimental conditions are summarized in Table II.1. The displacer KI was added at a molar ratio n_{Al336}/n_{KI} = 1 and pumped at 20 mL/min until all GL was back extracted.

II.2.7. Preparation of the crude extract of licorice roots

Powdered licorice roots (510 g, *G. glabra* L.) were added to 14 L of an aqueous 80% ethanol solution. The mixture was stirred for 4 h at 70 °C and filtered through a sintered glass funnel. The supernatant was recovered and concentrated under vacuum to dryness. The resulting crude extract (175.3 g) was analyzed by HPLC (see Section 2.10) to determine the GL content.

II.2.8. Influence of extractant and displacer concentrations on the extraction profile of GL from a crude extract of licorice roots

Several extractant/GL molar ratios (n_{Al336}/n_{GL}) (3; 11; 22; 44) were tested. For each condition, 3 g of the crude licorice root extract (0.46 mmol of GL) was dissolved in 20 mL of the mobile phase and maintained at pH = 7. The aqueous mobile phase was pumped in the descending mode at 20 mL/min with a column rotation speed of 1000 rpm. During this extraction step, anionic and neutral lipophilic compounds were retained by the stationary phase while cationic and neutral hydrophilic compounds were eluted. The mobile phase was then supplemented with the displacer KI. Different extractant/displacer (n_{Al336}/n_{KI}) molar ratios (2:1) were also tested.

II.2.9. Influence of increasing crude extract sample loading mass

The crude extract of licorice roots (3 g, 10 g, and 20 g) was dissolved in 20 mL, 100 mL or 400 mL of the aqueous mobile phase, respectively. The mobile phase was progressively pumped from 0 to 20 mL/min in 2 min to maintain the hydrodynamic equilibrium inside the column. Fractions were collected every minute.

II.2.10. TLC, HPLC and MS experiments

All fractions were checked by TLC on Merck 60 F254 silica gel plates, developed with $CHCl_3$/MeOH/water (70/30/2.5, v/v/v) and using 50% sulfuric acid in EtOH as spray reagent. Quantitative analyses were performed on a Waters HPLC system (Saint-Quentin, France) equipped with a 600E pump, a 717plus autosampler and a Jasco CO965 column oven. The chromatographic column (Luna, 250 mm × 4.6 mm, 5 µm, Phenomenex, Le Pecq, France) was maintained at 22 °C. The mobile phases were 0.025% TFA in water (solvent A) and acetonitrile (solvent B). The separation was performed at 1 mL/min with a gradient elution starting with 80% solvent A for 10 min. Then solvent B increased to 60% in 40 min, to 100% in 1 min and was maintained for 7 min. The injection volume was 15 µL. The system was coupled to a Waters® 996 photodiode array detector monitored at 252 nm. Data acquisition was controlled by the Empower 2 Software (Waters). Calibration curves were established by serial dilution of three independent stock solutions (0.1, 0.5, 1, 1.5, and 2 g/L) and by plotting the peak area recorded from HPLC chromatograms as a function of GL concentration. The identity of GL in the crude extract of licorice roots was confirmed on the basis of retention time and compared with the corresponding standard.

Mass spectrometry experiments were carried out to check the fractions of interest and to identify the major compounds present in the licorice crude extract together with GL. Samples were directly infused in a quadrupole time-of-flight hybrid mass spectrometer (QTOF micro®, Micromass, Manchester, UK) equipped with an electrospray source. The mass range of the instrument was set at m/z 100–1650 and scan duration was set at 1 s in the positive and negative ion modes. The capillary voltage was 3000 V, the cone voltage was 35 V, and the temperature was 80 °C.

II.3. Results and discussion

II.3.1. FCPE300® characteristics

The volume of the FCPE300® column was measured as 303.5 ± 1.3 mL. Figure II.4 represents the percentage of the stationary phase retention inside the FCPE300® partition cells as a function of flow rates for different biphasic solvent systems. After biphasic system equilibration, a low and constant pressure drop (ΔP = 2–6 bars) was measured in the different operating conditions. A linear relationship between the flow rate and the stationary phase retention was observed for all of the solvent systems tested. This linearity is in accordance with previous results obtained on CPC systems [26]. The intercepts of the linear regression curves (≈76%) with the ordinate axis, defined as the theoretical retention of the stationary phase at zero flow, allowed the calculation of the dead volume of the column. This volume was 73 mL (24% of the total column volume). A stationary phase retention higher than 50% was obtained when working up to 80 mL/min for the six solvent systems tested (Figure II.4).

For the *n*-heptane/water system, which displayed the highest stability, the stationary phase retention was 72% at 50 mL/min and still 55% at 300 mL/min. For the less stable *n*-butanol/water system, the stationary phase retention was 61% at 50 mL/min, 26% at 200 mL/min and only 9% at 300 mL/min, indicating poor retention when working at flow rates higher than 50 mL/min. For the intermediary *n*-heptane/MeOH, EtOAc/water, M*t*BE/water and EtOAc/*n*-butanol/water solvent systems, the stationary phase retention was maintained higher than 50% when working up to 100 mL/min. However, when increasing the flow rate to 200 mL/min, the stationary phase retention was 52% for the M*t*BE/water solvent system, and only 29% for the EtOAc/*n*-butanol/water solvent system. For experiments carried out in the ascending mode, the stationary phase retention when using the n-heptane/water system was 71% at 50 mL/min and decreased to 46.6% at 300 mL/min. With the EtOAc/*n*-butanol/water solvent system, the stationary phase retention was 45.9% at 100 mL/min and only 14.3% when increasing the flow rate to 200 mL/min. The slope of the different

linear regressions indicate that the FCPE300® column can work at flow rates ranging from 10 to 100 mL/min for the most common biphasic solvent systems.

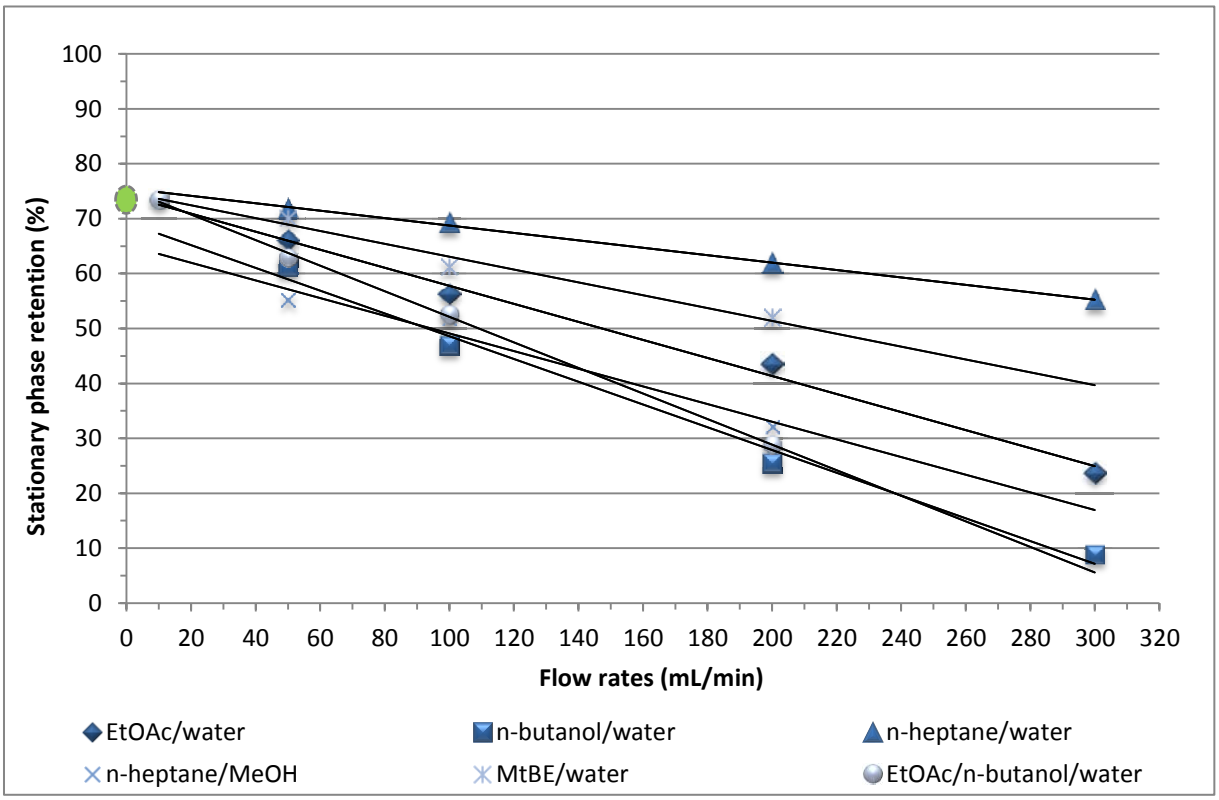

FIGURE II. 4: Influence of flow rates on the stationary phase retention for different biphasic solvent systems at a rotation speed of 1000 rpm. Intercept: (y-axis with convergent lines) = volume of connecting ducts (24% of total column volume)

II.3.2. Influence of flow rates on the extraction profile of GL

Our objective was to find the optimum flow rate to achieve a short and efficient extraction of GL. Increasing the flow rate leads to a better process efficiency and shorter separation, but it reduces the stationary phase retention (and thus the extractor capacity) especially in the presence of amphiphilic species [27]. The biphasic solvent system EtOAc/*n*-butanol/water (3/2/5, v/v/v) and the extractant Al336 were selected on the basis of a previous method developed for the purification of glucosinolates (another class of ionized heterosides) by ion-exchange centrifugal partition chromatography [11]. This choice was validated by qualitative GL partition coefficient measurements by TLC. The extraction profiles of 200 mg GL standard were examined at 10, 20, and 50 mL/min. As illustrated in Figure II.5 and Table II.1, working at 10 mL/min allowed a 100% recovery of GL after 75 min. In this condition GL was back extracted during 7.5 min (outlet concentration = 3.2 mM) and the stationary phase retention was 67.2% (experiment 1). At 20 mL/min, a total GL recovery was

possible in 38 min and back extracted in a total of 3.5 min (outlet concentration = 3.1 mM). The stationary phase retention was 64.9% (experiment 2). At 50 mL/min, total GL recovery was complete after 23 min and back extracted during 1.5 min (outlet concentration = 3.1 mM). The stationary phase retention was 62.4% (experiment 3).

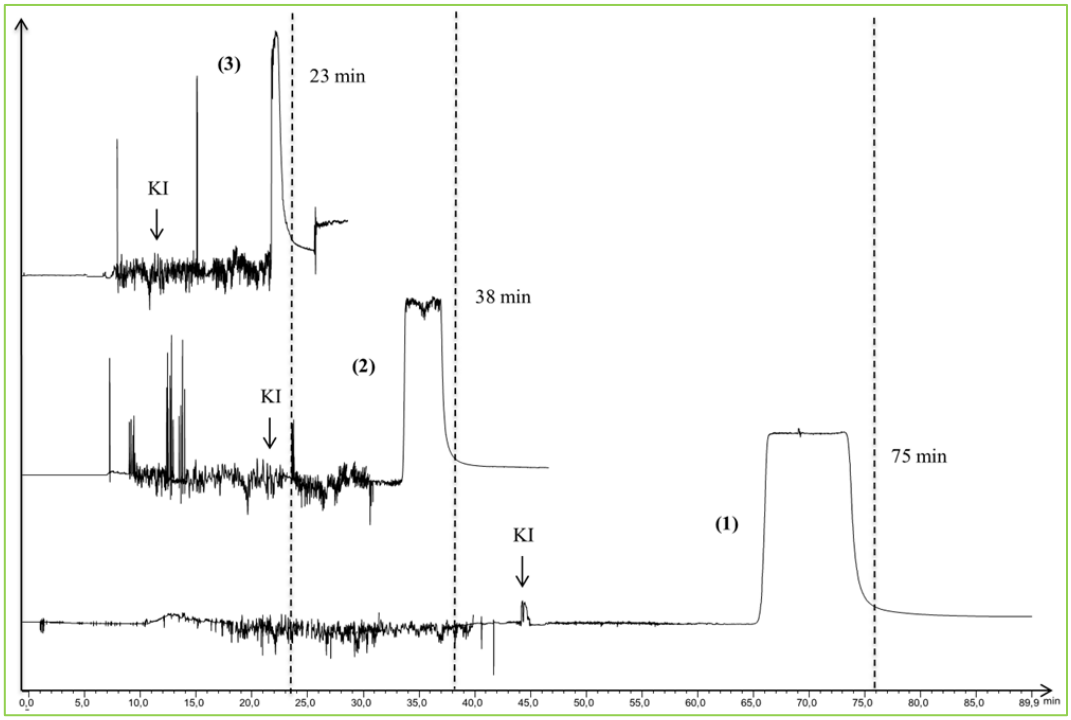

Figure II. 5 : Influence of the flow rate on the extraction profile of GL. (1) 10 mL/min, (2) 20 mL/min and (3) 50 mL/min. Experimental conditions: rotation speed = 100 rpm; loading of 200 mg GL standard; n_{Al336}/n_{KI} = 1; n_{Al336}/n_{GL} = 7.4; UV absorbance monitored at 252 nm. Dash line = end of GL back-extraction

The reduction of the total IP-CPE process duration is proportional to the increased flow rate from 10 to 20 mL/min, but not from 20 to 50 mL/min. This could suggest a kinetic limitation during the back extraction of the retainer (Cl^-). These results highlight the capacity of the FCPE300® to work at high flow rates while maintaining high retention of the stationary phase and the compressive character of the shocklayers, *i.e*. thin analyte border zones in the isotachic train. This feature is interesting since the flow rates represent a major limiting factor in support free liquid–liquid separation systems [10] and [28].

Due to the chemical complexity of licorice root composition and due to the surfactant character of GL [29] and [30], we decided to fix the flow rate at 20 mL/min when applying the process to the crude extract of licorice roots. In fact, working at 20 mL/min offers the best

compromise in terms of extraction process duration, GL outlet concentration, and stationary phase retention.

II.3.3. Concentration profile of GL in the FCPE300® partition cells before the back extraction step

The longitudinal concentration profile of GL inside the FCPE300® column after extraction in the organic stationary phase, without any back extraction process, was determined using the extrusion procedure. The column was filled with the organic stationary phase containing 3.3 mM, 10 mM or 30 mM of Al336. In each experiment 200 mg of GL standard (0.238 mmol) were injected. When working with 10 mM or 30 mM of the extractant Al336, no GL exited from the column, indicating that all GL was retained in the stationary phase by the extractant. When working with 3.3 mM of Al336, an excess of GL (69 mg) was recovered out of the column, indicating that only 131 mg (0.156 mmol) of GL were effectively extracted. Considering that the retention volume is 204 mL at 10 mL/min, the amount of extractant inside the column is 0.67 mmol. We can conclude that 0.67 mmol of Al336 are necessary to achieve the complete extraction of 0.156 mmol of GL. A minimum molar ratio n_{Al336}/n_{GL} = 4.3 is thus necessary to quantitatively extract GL. As illustrated in Figure.II.6, the extrusion profile of GL was characterized by three distinct zones. The first 20 mL extruded from the column contained the greatest amount of GL (Figure II.6, zone 1). This indicates that the amount of GL is higher in the first partition cells at the head of the column. The fractions recovered during the subsequent 12 min (120 mL) contained a constant amount of GL (Figure II.6, zone 2), reflecting a homogeneous partition of GL inside the column. Finally a gradual decrease in GL concentration was observed during the last 10 min (100 mL) of the extrusion process (Figure II.6, zone 3). This could be due to dispersion phenomena between the stationary phase containing the extractant-GL ion pairs and the freshly pumped mobile phase.

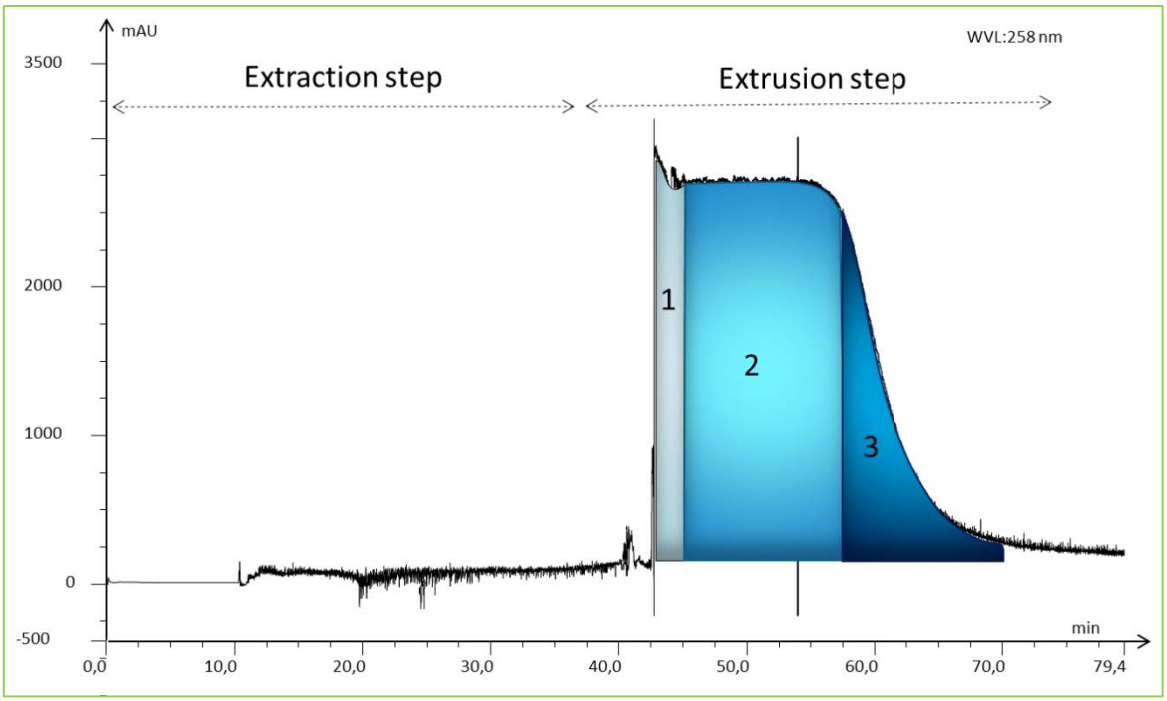

Figure II. 6: Concentration profile of GL inside the FCPE300® column. Loading of 131 mg of GL standard. The organic stationary phase was extruded by pumping the aqueous mobile phase in the descending mode at 10 mL/min. Rotation speed: 1000 rpm. Zone 1: first fraction extruded; zone 2: constant GL concentration; zone 3: gradual decrease of GL concentration

II.3.4. Influence of the inlet sample volume

As in CPC systems, sample loading is a crucial step in centrifugal partition extraction. Indeed, the sample can disrupt the hydrodynamic equilibrium of the biphasic solvent system [31]. This disruption may result from the nature of the sample (viscosity, surfactant properties), its concentration or from the injection procedure. As shown in Figure II.7, when 131 mg of GL standard are loaded in 20 mL (7.8 mM) of the aqueous mobile phase, all GL was recovered in a total of 66 mL (outlet concentration = 2.4 mM). When the same quantity of GL standard was directly pumped with 1 L (0.156 mM) or 2 L (0.078 mM) of the aqueous mobile phase, GL was recovered in a total of 56 mL (outlet concentration = 2.8 mM) and 48 mL (outlet concentration = 3.2 mM), respectively. The outlet concentration of GL was significantly increased as a function of the feed volume. This concentration effect obtained by displacement processes has already been described [32]. In all systems based on affinity chromatography or more precisely on displacement processes, the action of the displacer enables the solutes to exit from the column at high concentration due to competition for the adsorption sites in the stationary phase. This phenomenon is interesting for the analysis of contaminants which are generally present at low concentrations in high volumes of a liquid phase or

for the separation of critical trace elements such as in proteomic applications [32]. Here the results reflect the capacity of the extractor to pump large volumes of the mobile phase with very low analyte concentrations without affecting the formation of lipophilic ion pairs between the anionic analytes and the extractant. This also indicates that the volume of the feeding phase only affects the overall duration of the extraction process.

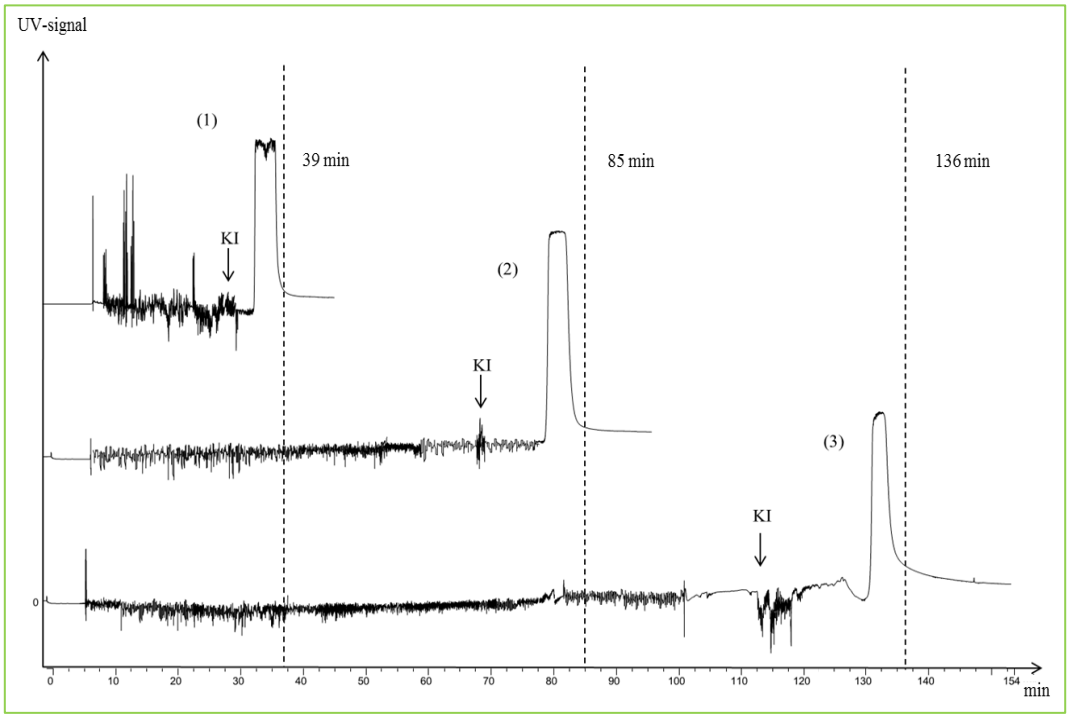

Figure II. 7: Influence of the inlet sample volume on the quality of GL extraction. (1) 20 mL; (2) 1 L and (3) 2 L. Loaded sample mass 200 mg of GL standard; flow rates = 20 mL/min, rotation speed = 1000 rpm. $n_{Al336}/n_{KI} = 1$; $n_{Al336}/n_{GL} = 4.2$; *UV absorbance monitored at 252 nm. Dash line = end of GL back-extraction*

II.3.5. Influence of the ratio extractant/displacer on the quality of GL extraction starting from a crude extract of licorice roots

The amount of GL in the crude extract of licorice roots was 12.8% on a dry weight basis, as determined by HPLC analyses. A large number of secondary metabolites have been identified in licorice roots, among which more than 24 saponin derivatives, 45 flavonoid derivatives and 18 phenolic compounds [15] and [30]. All these compounds are potentially ionisable and thus can interact competitively with the extractant. In this section, influences of n_{Al336}/n_{KI} and of n_{Al336}/n_{GL} on the quality of GL extraction were investigated. The extraction profiles obtained for each experiment (see Table II.2) are given in Figure II.8.

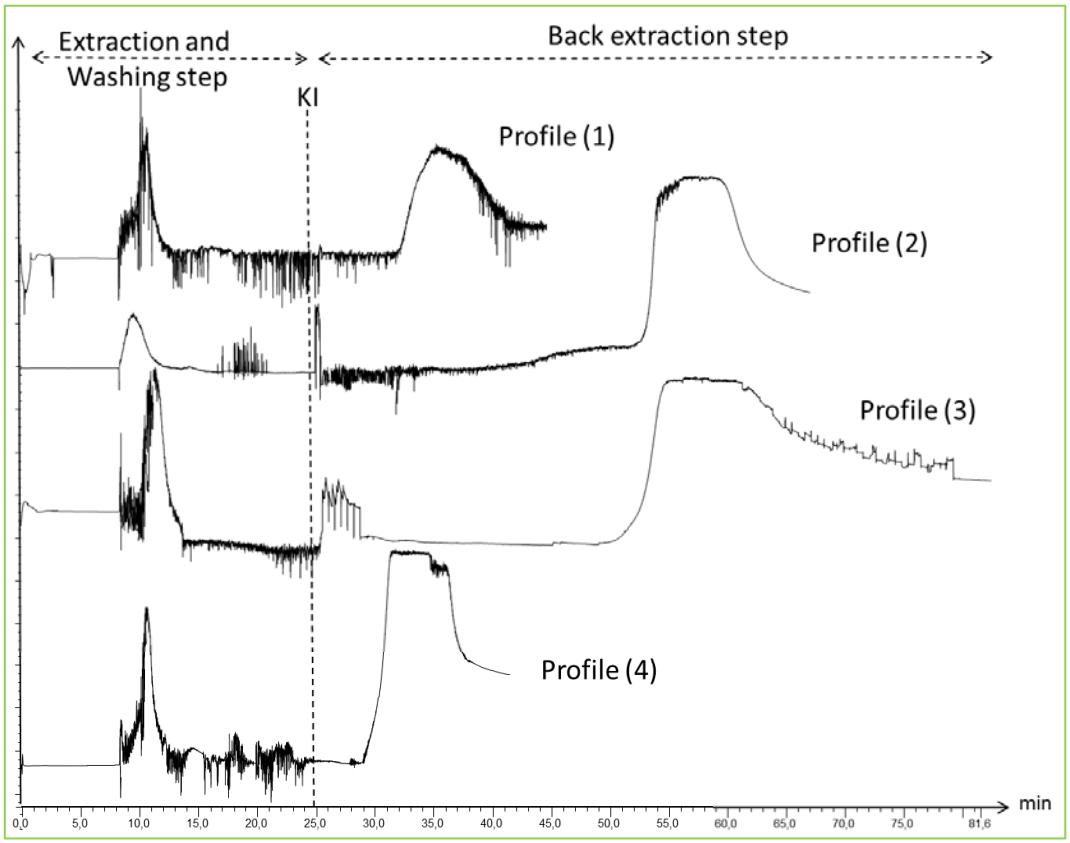

Figure II. 8: Influence of extractant/displacer (n_{Al336}/n_{KI}) and extractant/GL (n_{Al336}/n_{GL}) molar ratios on the quality of GL extraction. Flow rate: 20 mL/min; rotation speed: 1000 rpm; loading of 3 g of the crude extract of licorice roots. From profile (1) to profile (4): experiments with various ratios (n_{Al336}/n_{KI}) and (n_{Al336}/n_{GL}) (summarized in Table 2). UV absorbance monitored at 252 nm; dash line = start of back-extraction step

As a minimum molar ratio n_{Al336}/n_{GL} = 4.3 is necessary to fully extract standard GL, we used 12 mM Al336 (stationary phase retention at 20 mL/min = 197 mL) and a ratio n_{Al336}/n_{KI} = 1. In these conditions a significant quantity of GL was lost, as confirmed by TLC and HPLC analyses of the first collected fractions. This indicates that all GL was not captured by the extractant and that the exchange sites available for GL were insufficient. As a result, an isotachic train profile without clearly defined concentration plates was obtained (Figure II.8, profile 1). This loss of GL was attributed to the presence of other ionic compounds naturally present in the crude extract and competitively retained by the extractant in the stationary phase. When increasing the extractant concentration up to 48 mM, GL was fully retained and further back extracted, as shown by the characteristic back extraction profile in the form of a well-structured isotachic train. However, the residence time of GL inside the column was too long (60 min, Figure II.8, profile 2). When reducing the displacer

concentration (ratio n_{Al336}/n_{KI} = 20.5, n_{KI}/n_{GL} = 1), the separation of GL from the other analytes was improved but the global process duration was dramatically increased, thus decreasing the productivity of the process (75 min, Figure II.8, profile 3). The optimum molar ratios were found to be n_{Al336}/n_{GL} = 13 (Al336 concentration = 31 mM) and n_{Al336}/n_{KI} = 1. In these conditions, the amount of extractant was high enough to recover all GL and provided efficient separation of GL from the other major flavonoids present in the licorice crude extract. The equimolar ratio n_{Al336}/n_{KI} allowed the reduction of the total process time to only 37 min (Figure II.8, profile 4).

Experiment	C_{Al336} (mM)	Molar ratio (n_{Al336}/n_{KI})	Molar ratio (n_{Al336}/n_{GL})	Total process time (min)	Analyte back-extraction (min)
1	12	1	5	40	8.3
2	48	1	20.5	60	7.6
3	12	25.5	5	75	15
4	31	1	13	37	5.6

Table II. 2: Experimental conditions for the influence of extractant and displacer concentrations on the extraction profile of GL from 3 g of crude extract of licorice roots. GL concentration was estimated at 0.46 mmol; C_{Al336}: extractant concentration; C_{KI} = displacer concentration; n_{Al336}/n_{KI} = extractant/displacer molar ratio; n_{Al336}/n_{GL}: extractant/GL molar ratio. See Fig. 8 for the effect n_{Al336}/n_{KI} and nAl336/nGL molar ratios on the quality of GL extraction

II.3.6. Increasing mass sample loading

The objective here was to investigate the ability of the FCPE300® to achieve the extraction of GL when loading up to 20 g of the crude licorice root extract. The stationary phase retention was reduced from 46.1% to 43.6%, 39.4% and 0% (total flooding of the stationary phase) when increasing sample mass from 3 g to 10 g, 20 g and 25 g, respectively. This phenomenon, previously observed for the purification of heparins [33] by ion-exchange CPC, is probably accentuated by the surfactant character of GL. As the outlet concentration of GL is directly correlated to the displacer concentration, we decided to reduce the KI concentration by a factor of 2 (molar ratio n_{Al336}/n_{KI} = 2). In this way, the back extraction of GL and other analytes was more progressive. The extraction profile of GL obtained by injecting 20 g of the crude extract is presented in Figure II.9. A 45 min extraction/washing step was necessary to retain all GL in the stationary phase while eluting all cationic and neutral hydrophilic compounds out of the column.

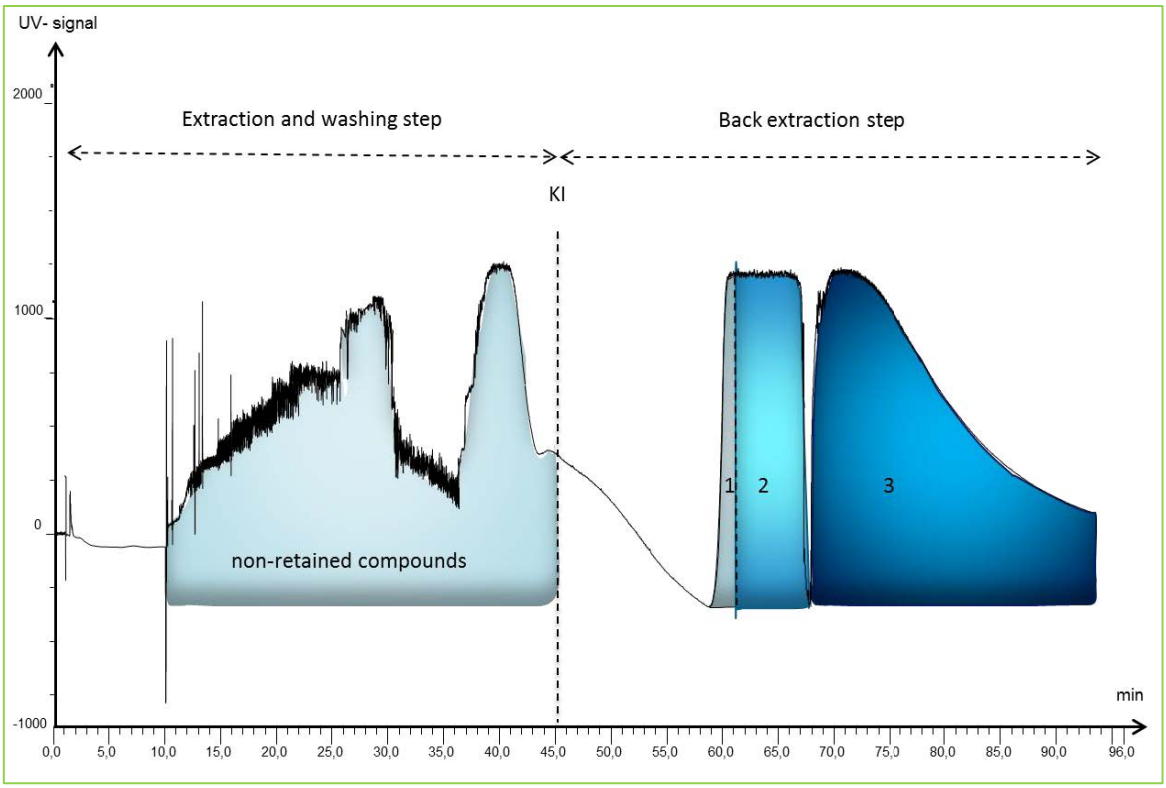

Figure II. 9: Extraction profile of GL by IP-CPE, after loading of 20 g of a crude licorice root extract. Flow rate: 20 mL/min; rotation speed: 1000 rpm; C_{Al336} = 183 mM; C_{KI} = 91 mM. (1): Zone of (liquiritin/isoliquiritin) apioside, (2): zone of GL, (3): zone of the undefined compound. Dash line = start of back-extraction step; UV absorbance monitored at 252 nm

After the mobile phase was supplemented with the displacer (KI), we observed the formation of an isotachic train which could be divided into 3 zones: the first zone (from 59 to 61 min, i.e. 40 mL) corresponded to the back extraction of a major flavonoid of licorice roots. MS analyses revealed a molecular ion at m/z 589 in the positive ion mode and at m/z 549 in the negative ion mode, what corresponded respectively to the $[M+K]^+$ and $[M-H]^-$ of liquiritin apioside (or its isomer isoliquiritin apioside). The second zone (from 61 to 69.5 min, *i.e*. 170 mL) corresponded to the back extraction of GL. HPLC analyses revealed that GL was well-separated from the other analytes. In total, 2.21 g of GL were obtained, corresponding to a GL recovery of 86.5%. As shown in Figure II.10, 1.5 g of the GL pool (from fraction 66 to 70) was obtained with a mean purity of 86.3%. The maximum purity was 97% in fraction 68 (240 mg).

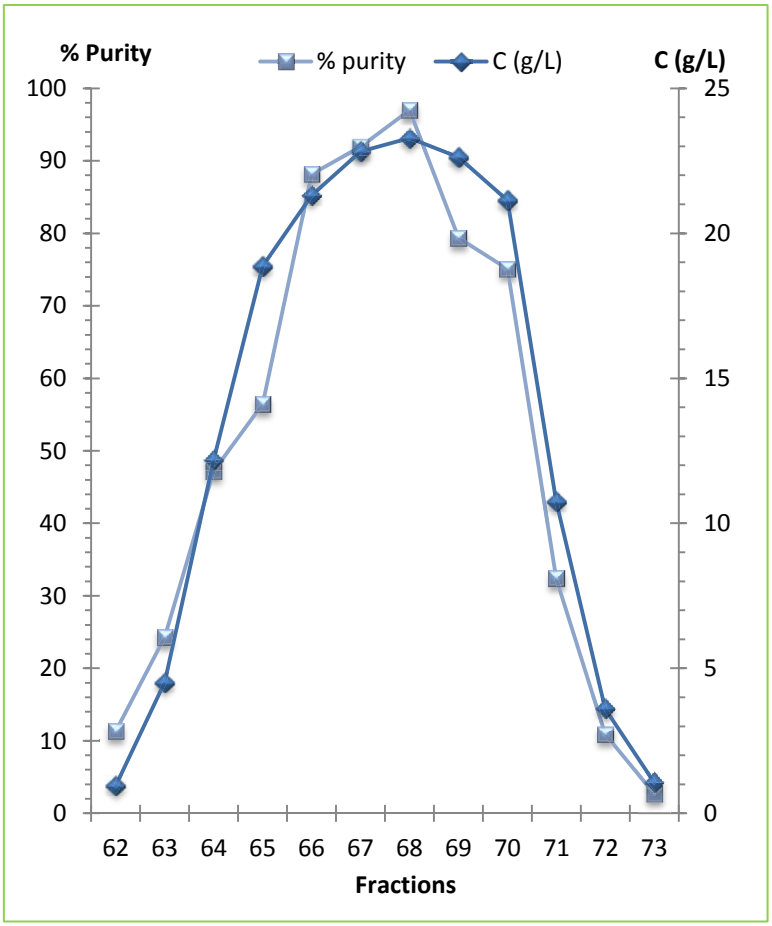

FIGURE II. 10: *Fractogram of GL, purity (%) and concentration (g/L)*

The identity of GL was confirmed by MS analyses. Molecular ions were detected at m/z 861 in the positive ion mode and at m/z 821 in the negative ion mode, what corresponded to the $[M+K]^+$ adduct and to the $[M-H]^-$ parent ion of GL, respectively. The retention of the stationary phase was 39.4% at the end of the experiment.

These results show that the IP-CPE method allowed the simultaneous extraction and purification of GL from 20 g of a complex licorice root extract in a single run. In comparison, in a recent study dedicated to the separation of GL by counter-current chromatography in the elution mode, the process duration was 350 min to yield 42.2 mg of pure GL from 130 mg of a crude extract of licorice roots with stationary phase retention of 18.1% [12]. The FCPE300® exhibits not only an interesting potential in terms of extraction yield and productivity, but also in terms of purification when working in the ion-pair mode. By introducing exchange sites in the liquid stationary phase, the ion-pair mode results in a succession of extraction/back extraction steps, thus improving separation efficiency.

II.4. Conclusion

The aim of the present study was to examine the extraction and purification capacity of the lab-scale liquid–liquid FCPE300® extractor when working in the ion-pair extraction mode. This extractor can work at flow rates ranging from 10 to 100 mL/min while retaining high volumes of the stationary phase. By using the IP-CPE method, 2.21 g of the bioactive saponin glycyrrhizin were extracted and purified with good recovery (86.5%) and in one step starting from 20 g of a crude extract of licorice roots. The next step will be to evaluate the robustness and efficiency of the FCPE extractor in other development modes (elution mode, pH-zone refining, etc.) for the extraction of small molecules naturally occurring in complex mixtures and belonging to different chemical classes.

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CHAPITRE 2 - PARTIE 2

EXTRACTION DE PARTAGE CENTRIFUGE EN MODE D'ÉCHANGE D'IONS FORT: EFFET DE LA CONCEPTION ET DE LA GÉOMÉTRIE DES CELLULES SUR LA PERFORMANCE DU PROCÉDÉ DE PURIFICATION

CHAPTER 2 - PART 2

STRONG ION-EXCHANGE IN CENTRIFUGAL PARTITION EXTRACTION: EFFECT OF PARTITION CELL DESIGN AND DIMENSIONS ON PURIFICATION PROCESS EFFICIENCY

III. Extraction de partage centrifuge en mode d'échange d'ions fort: effet de la conception et de la géométrie des cellules sur la performance du procédé de purification

Introduction du chapitre 2- Partie 2:

Cette seconde partie présente une étude approfondie concernant l'influence de la géométrie des cellules et de la conception de l'extracteur de partage centrifuge sur les performances de purification de métabolites naturels ioniques, avec comme point d'appui et de comparaison un système CPC équipé d'une colonne de volume équivalent à la colonne EPC. Le but principal de cette étude était, dans un premier temps, d'étudier l'effet de la diminution du nombre de cellules, et donc du nombre de plateaux théoriques, puis de l'augmentation du débit de la phase mobile sur les performances de l'EPC en mode échange d'ions fort. Le support méthodologique utilisé est la sinalbine, un glucosinolate présent en quantité significative dans les graines de moutarde blanche (*Sinapis Alba* L.) et dont la purification avait déjà été optimisée par CPC au laboratoire à différentes échelles [217, 218]. La purification des glucosinolates ayant des propriétés physico-chimiques particulières (hydrophilie élevée, caractère émulsif,…), est difficile et peu productive par un fractionnement classique liquide-liquide séquentiel. Ce fractionnement est généralement suivi de plusieurs étapes de purification utilisant les techniques chromatographiques d'adsorption classiques (sur colonne de silice, par CLHP préparative ou par chromatographie Flash,…).

Une étude hydrodynamique a été réalisée à l'aide d'outils de visualisation de l'écoulement de la phase mobile à travers la phase stationnaire et ce à chaque étape du procédé (extraction/ back-extraction) afin d'identifier les paramètres responsables d'éventuelles pertes de phase stationnaire. L'influence du débit de la phase mobile sur la qualité de la séparation a été déterminée en contrôlant principalement le taux de recouvrement de la sinalbine, sa pureté et la productivité globale du procédé. Ainsi, la productivité du procédé développé par EPC a pu être comparée avec les résultats obtenus par CPC pour la purification du même métabolite à l'échelle laboratoire et pilote. Cette comparaison, nous a permis de positionner notre extracteur par rapport à d'autres équipements de séparation liquide-liquide.

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Strong ion exchange in centrifugal partition extraction (SIX-CPE): Effect of partition cell design and dimensions on purification process efficiency

Mahmoud Hamzaoui[a], Jane Hubert[a,*], Romain Reynaud[c], Luc Marchal[b], Alain Foucault[b], Jean-Hugues Renault[a]

[a] UMR CNRS 7312, Université de Reims Champagne-Ardenne, Bât. 18, Moulin de la Housse, BP 1039, 51687 Reims Cedex 2, France
[b] UMR CNRS 6144, Université de Nantes, Laboratoire GEPEA, CRTT, 44602 Saint-Nazaire, France
[c] Soliance France, Route de Bazancourt, 51110 Pomacle, France

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ABSTRACT

The aim of this article was to evaluate the influence of the column design of a hydrostatic support-free liquid–liquid chromatography device on the process efficiency when the strong ion-exchange (SIX) development mode is used. The purification of *p*-hydroxybenzylglucosinolate (sinalbin) from a crude aqueous extract of white mustard seeds (*Sinapis alba* L.) was achieved on two types of devices: a centrifugal partition chromatograph (CPC) and a centrifugal partition extractor (CPE). They differ in the number, volume and geometry of their partition cells. The SIX-CPE process was evaluated in terms of productivity and sinalbin purification capability as compared to previously optimized SIX-CPC protocols that were carried out on columns of 200 mL and 5700 mL inner volume, respectively. The objective was to determine whether the decrease in partition cell number, the increase in their volume and the use of a "twin cell" design would induce a significant increase in productivity by applying higher mobile phase flow rate while maintaining a constant separation quality. 4.6 g of sinalbin (92% recovery) were isolated from 25 g of a crude white mustard seed extract, in only 32 min and with a purity of 94.7%, thus corresponding to a productivity of 28 g per hour and per liter of column volume (g/h/L_v). Therefore, the SIX-CPE process demonstrates promising industrial technology transfer perspectives for the large-scale isolation of ionized natural products.

III.1. Introduction

Support-free liquid–liquid separation techniques are promising technologies in cosmetic and pharmaceutical industries for the purification of high added-value bioactive compounds of natural origin [1], [2] and [3]. These technologies offer clear advantages in terms of column capacity, target compound recovery, selectivity, resolution, and process duration compared to more conventional purification techniques using for instance silica gel or RP-18 chromatographic solid supports [4] and [5]. Depending on the nature of the target compounds, different methods based on elution, polarity gradient, displacement by pH-zone refining or ion-exchange have been developed [3], [6], [7], [8] and [9]. Previous studies have in particular demonstrated that strong ion-exchange combined to centrifugal partition chromatography (SIX-CPC) was an efficient method for the purification of ionic compounds. For example, the glucosinolates sinalbin and glucoraphanin were successfully isolated by SIX-CPC using a 200 mL laboratory-scale column [10]. However, experiments were limited to a maximum flow rate of 2 mL/min to retain the stationary phase inside the column, resulting in a

recovery of 2.4 g pure sinalbin in 170 min. This SIX-CPC method was then transposed at a larger scale by using a 5.7 L pilot CPC column [11]. Experiments performed at 50 mL/min resulted in a one-step purification of 70.3 g sinalbin in 160 min. More recently, centrifugal partition extraction (CPE) has been presented as a highly productive solid support-free liquid–liquid separation process when combined to the strong ion-exchange mode (SIX-CPE) [12]. The main difference between CPE and CPC columns relies on the partition cell design. For an equivalent column capacity, the CPE rotor contains less partition cells of larger volume with an oval twin cell design and interconnected in series by larger ducts.

The purpose of this study was to investigate if this particular geometry of the CPE column would allow an equivalent separation quality in the ion-exchange mode with an increase in the mobile phase flow rate. The model used for this study was sinalbin, a glucosinolate extracted from white mustard seeds [10].The influence of increasing the flow rate on the hydrodynamics of the two liquid phases in the CPE column was investigated in order to determine the operating limits and to define the optimal conditions for both productivity and purity.

III.2. Experimental

III.2.1. Reagents

Ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), acetic acid, acetonitrile (CH_3CN), methanol (MeOH), aqueous ammonia (25%), silver nitrate and chloroform ($CHCl_3$) were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). All solvents were of analytical grade. Aliquat336® (trioctylmethylammonium chloride, Al336) was purchased from Sigma–Aldrich (Saint-Quentin, France) as a mixture of C_8 and C_{10} chains with C_8 predominating. Potassium iodide (KI) was obtained from Prolabo (Fontenay, France). Deionised water was used to prepare all aqueous solutions. Pure sinalbin (SNB) was obtained from previous work carried out in our laboratory [11].

III.2.2. Apparatus: Fast Centrifugal Partition Extractor FCPE300®

The extraction process was developed on a lab-scale Fast Centrifugal Partition Extractor (FCPE300®, Kromaton Technology, Angers, France) containing a rotor of 7 circular partition disks engraved with a total of 231 twin partition cells arranged circumferentially and connected together by ducts. The stationary phase was maintained inside the column by a centrifugal force field generated by rotation around a single central axis. The volume of the rotor is 303.5 ± 1.3 mL [12]. The

rotation speed could be adjusted from 200 to 2000 rpm, producing a relative centrifugal acceleration in the partition cell up to 437 × *g*. The mobile phase was pumped through the stationary phase either in the ascending or in the descending mode with low residual pulsation through a KNAUER Preparative Pump 1800® V7115 (Berlin, Germany). The system was coupled to a UVD 170S detector (Dionex, Sunnivale, CA, USA) equipped with a preparative flow cell (6 µL internal volume, 2 mm path length). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). All experiments were conducted at room temperature (20 ± 2 °C).

III.2.3. Visual-CPC instrument

The visualization experiments were carried out using an individual transparent disk engraved with twin-cells of the same geometry of our FCPE300®. The engraved disk was clamped between a stainless steel disk and a glass ring in a rotor. The instrument named "Visual-CPC" was equipped with an asynchronous camera and a stroboscope triggered at the same frequency as the rotor. The rotor was connected to the chromatographic system through 1/16 peek tubing (Upchurch Scientific, Oak Harbor, WA, USA) and two rotary seals (Tecmeca, Epernay, France) (internal diameter = 2.54 cm). The rotational speed could be adjusted from 0 to 3000 rpm. A slightly modified Videostrobe system was used (SysmatIndustrie, St Thibault des Vignes, France), consisting of a TMC-9700 progressive scan CCD digital color camera (Pulnix, Sunnyvale, CA, USA.) with an asynchronous shutter, two Phylec stroboscopic units (Sysmat) and a VLS7T optical speed sensor (Compact, Bolton, UK) which triggered both the stroboscopes and the camera. To freeze the rotor in its movement, a flash of 2 microseconds was used. The camera was equipped with a 18–108 mm F 2.5 TV Zoom lens. Images were directly recorded on a computer.

III.2.4. Preparation of the crude extract of white mustard seeds

White mustard seeds (1.5 kg, *Sinapis alba* L. variety Concerta, Alpha semences, Douai, France) were immersed in a 6-fold excess (w/v) of boiling water. After decreasing the temperature to 70 °C, the mixture was stirred for 4 h. The supernatant was promptly recovered from the warm mixture by filtration (Whatman No. 4 filter paper). Mucilage was then precipitated by adding a 1-fold (v/v) excess of ethanol three times. After solid–liquid separation using a laboratory-scale Westfalia Separator type KA 1 (Château-Thierry, France), the filtrate was evaporated to dryness under vacuum. A 102 g crude glucosinolate extract was recovered and stored at −20 °C until use.

III.2.5. SIX-CPE process description

The biphasic solvent system (2 L) was composed of EtOAc/*n*-BuOH/water in the proportions 3:2:5 (v/v/v). The column was filled at 200 rpm with the organic stationary phase containing the extractant Al336 at a molar ratio of n_{Al336}/n_{SNB} = 4.3. The column rotation speed was then adjusted to 1000 rpm for the purification process. The crude glucosinolate extract was dissolved in a 95:5 (v/v) mixture of KI-free aqueous mobile phase and Al336-free organic phase and loaded into the column. The mobile phase was gradually pumped from 0 to operating flow rate value in the descending mode. This flow rate gradient was used to minimize the hydrodynamic equilibrium disturbance inside the column during the loading step. After the mobile phase front signal, one column volume of the KI-free aqueous mobile phase was pumped to ensure the elution of unretained compounds. Potassium iodide (KI) was then added to the mobile phase for the back-extraction step at a molar ratio n_{Al336}/n_{KI} = 1. Analytes were expelled from the stationary phase by the displacer, resulting in a series of adjacent bands eluting ahead of the displacer front. Analytes with the lowest affinity for Al336 were displaced first. The extraction profiles were monitored with an UV detector at 275 nm. Fractions of 20 mL were collected during the experiments.

III.2.6. Flow rate optimization strategy

Experiments were performed at 20, 30, 40 and 50 mL/min. For each experiment, 25 g of the crude extract of white mustard seeds were loaded into the column. The Al336 and KI concentrations were fixed at 160 mM in the stationary phase and mobile phase, respectively. The operating conditions are summarized in Table III.1.

Assay	Flow rate (mL)	Sample mass (g)	Sample volume (mL)	$C_{AI336} = C_{KI}$ (mM)	Sf_i (%)	Sf_f (%)	Recovery (%)	Process run time (min	t_{KI} (min)	Productivity (g/h/V_c)	Mean purity (%)
1	20	25	60	160	66.1	43.7	96	45	24	21.08	97.1
2	30	25	60	160	64.7	33.7	92	32	18.5	28.33	94.7
3	40	25	60	160	59.4	31.1	89	23	12	31.30	87.7
4	50	25	160	160	57.3	19.5	37	19	12.4	19.11	74.0

Table III. 1: Operating conditions of the SIX-CPE methodological study. SP=stationary phase; Solvent system: EtOAc/n-BuOH/water 3:2:5 v/v; Sample: crude extract of white mustard seeds; descending mode; rotation speed = 1000 rpm

(f)

III.2.7. TLC, HPLC analyses

All fractions were checked by TLC on Merck 60 F_{254} silica gel plates, developed with *n*-BuOH/acetic acid/water (60:15:25, v/v/v) and revealed by using a spray reagent of ammoniacal silver nitrate [10]. After heating the TLC plate at 120 °C for 2–3 min, the presence of sinalbin gave rise to a dark-brown stain. Quantitative analyses were performed on a Waters HPLC system (Saint-Quentin, France) equipped with a 600E pump, a 717plus autosampler and a Jasco CO965 column oven. The chromatographic column (Luna, 250 × 4.6 mm, 5 µm, Phenomenex, Le Pecq, France) was maintained at 22 °C. The mobile phase was 10 mM of Al336 in CH_3CN/water (50:50, v/v) and eluted isocratically at 1 mL/min. UV detection was fixed at λ = 226 nm. Calibration curves were established by serial dilution of three independent stock solutions of pure sinalbin (0.1, 0.5, 1, 1.5, and 2 g/L) and by plotting the peak area recorded from HPLC chromatograms as a function of sinalbin concentration. The identity of sinalbin in the crude extract of white mustard seeds and in the collected fractions was confirmed on the basis of the retention time of the corresponding pure standard and by comparison to NMR chemical shifts. NMR experiments were performed on a Bruker (Wissembourg, France) Avance DRX 500 MHz spectrometer. ^{1}H and ^{13}C spectra were recorded in D_2O.

III.3. Results

III.3.1. Choice of the model separation

The model used for this study was sinalbin, a glucosinolate extracted from white mustard seeds. White mustard (*Sinapis alba* L., Brassicaceae) is an easily available and cheap glucosinolate-rich plant material. Sinalbin accounts for over 90% of the total glucosinolate content of white mustard seeds [13] and breaks down under myrosinase hydrolysis to produce the characteristic taste of mustard [14] and [15]. The purification process of sinalbin by strong ion-exchange CPC (SIX-CPC) using the lipophilic quaternary ammonium salt Al336 as an anion-exchanger and iodides as displacer was previously investigated with both a laboratory-scale and a pilot-scale CPC [10] and [11]. Thus it constitutes a good comparison point for this study.

III.3.2. Purification process optimization on the FCPE300® instrument

III.3.2.1. Influence of the ionized compounds on the separation process and of the flow rate on the hydrodynamics of the biphasic solvent system

The major constraint limiting the use of high flow rates in solid support-free liquid–liquid separation techniques arises from the risk of losing a high volume of the stationary phase during the experiment, thus decreasing the chromatographic resolution and capacity. The flow pattern in a given column geometry depends not only on the physico-chemical properties of the biphasic solvent system but also on the mobile phase flow rate and on the nature and quantity of the sample loaded into the column. In the present work, the mass of the loaded sample was limited to 25 g to ensure at least 50% of initial stationary phase retention volume at a flow rate of 50 mL/min. Beyond this mass, the amount of extractant required to extract all sinalbin and the influence of the sample itself (presence of polysaccharides, etc.) led to a poor stationary phase retention volume [16] and [17]. Different pictures in Figure III.1, obtained using the Visual-CPC device, show the flow patterns of the biphasic system EtOAc/*n*-BuOH/water (3:2:5, v/v/v) in the descending mode, before and after adding the the extractant (Al336) and the analyte (sinalbin).

The observed flow pattern for the blank system (Figure III.1.A) was a wavy film for the aqueous phase in each part of the twin-cell, deviated from the radius by the *Coriolis* acceleration. At the outlet of the cells, there was an efficient separation of the two phases, corresponding to a good stationary phase retention. Figure III.1.B illustrates the effect of adding Al336 (under its chloride form) to the organic stationary phase. The flow patterns were modified, mainly where both phases have to be settled (dotted circles). This was probably due to the amphiphilic nature of Al336 which could reduce the surface tension between the two phases. In addition, the deviation of the mobile phase was enhanced when the coalescence zone increased in size, illustrating a loss of stationary phase. In particular the wave-shaped contour of these areas of emulsion reflects the appearance of recirculation zones. In Figure III.1.C, the ion pair [$Al336^+$, $sinalbin^-$] seemed less disturbing than the ion pair [$Al336^+$, Cl^-]. The back-extraction process, corresponding to the substitution of sinalbin by iodide generates an intermediate situation (Figure III.1.D). Finally, the ion pair [$Al336^+$, I^-] leads to a flow pattern similar to that observed with the blank system (Figure III.1.E).

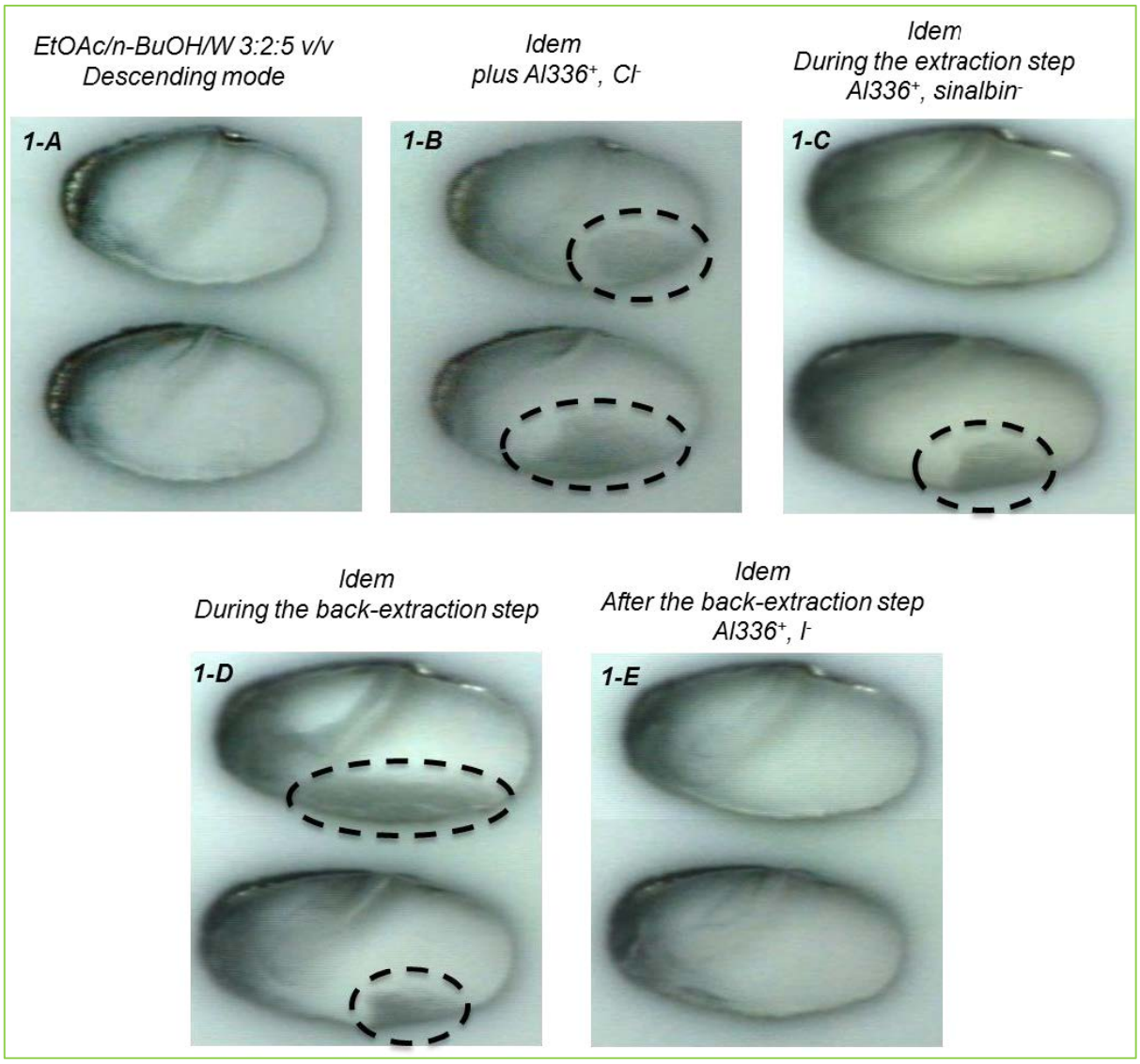


Figure III. 1: *Raw images of the Visual-CPC experiments showing the flow patterns of the biphasic solvent system EtOAc/n-BuOH/water (3:2:5, v/v/v). (A), with extractant Al336 (B) and at different steps of the SIX-CPE process (from C to E). CAl336 = CKI = 160 mM; descending mode; rotation speed = 1000 rpm; sample mass loading (pure sinalbin): 5 g. (C) Flow pattern during the extraction step, (D) during the back-extraction step and (E) after the back-extraction of sinalbin; dash line: recirculation zones and wave-shape formation*

In independent assays, 25 g of the crude extract from white mustard seeds were loaded and the flow rate was set at 20, 30, 40 and 50 mL/min. The chromatograms corresponding to the extraction and the back-extraction profiles are presented in Figure III.2.

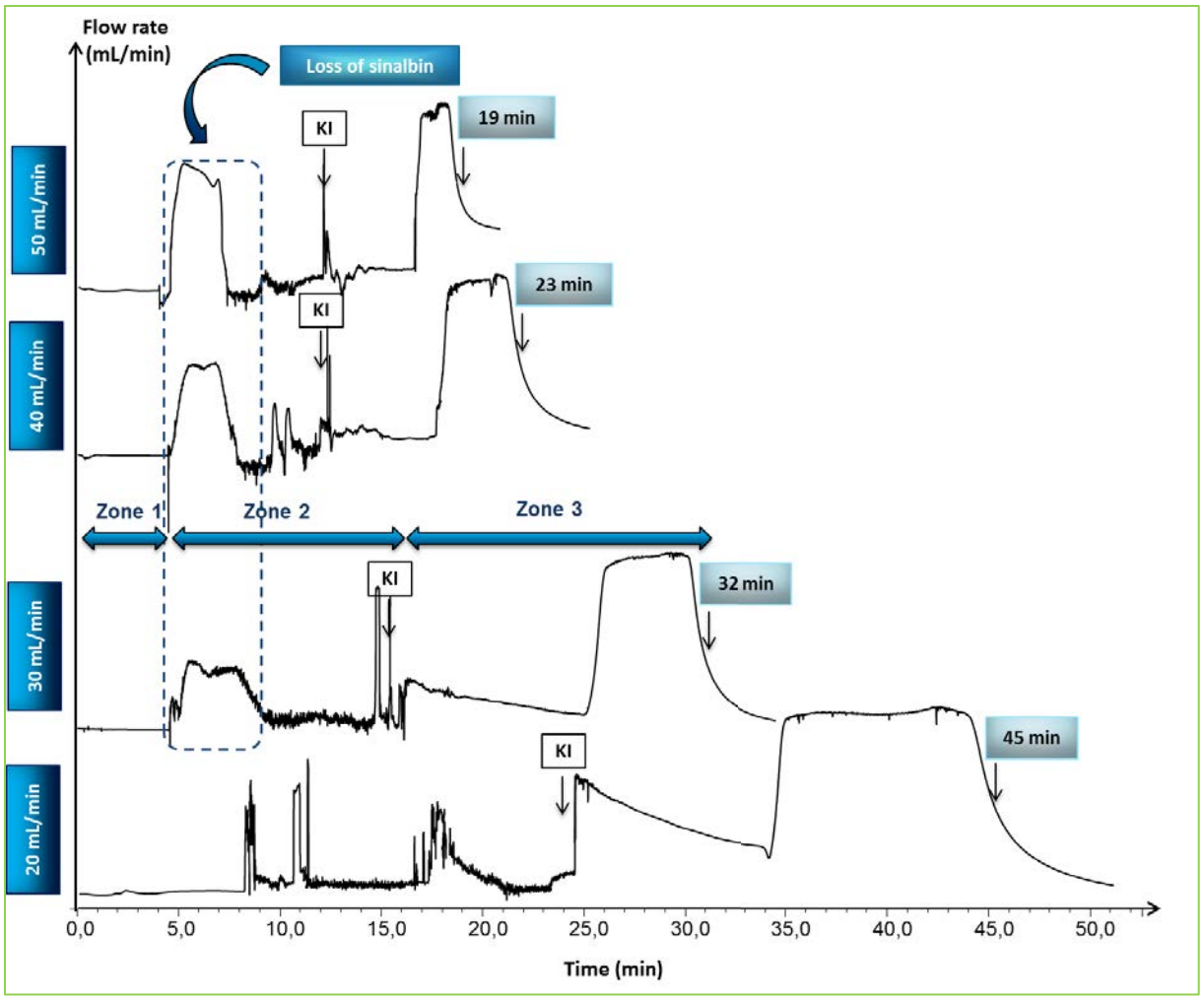
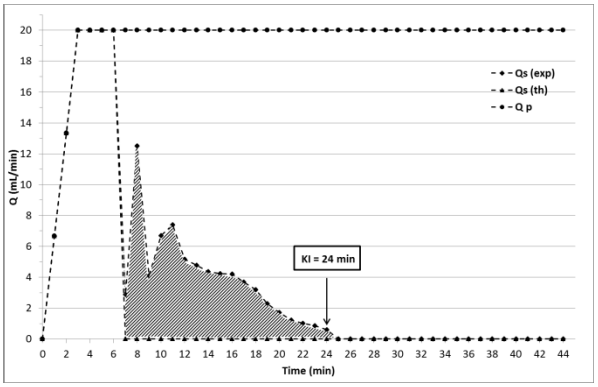


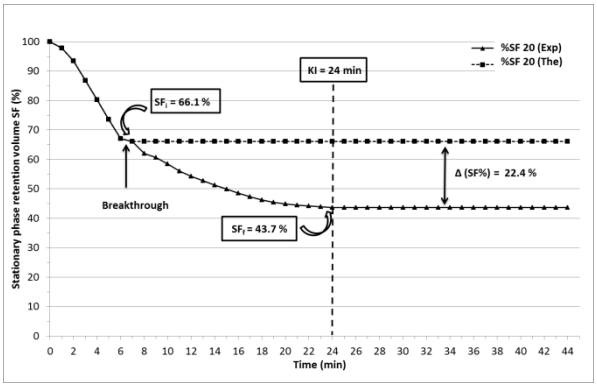
FIGURE III. 2: *Influence of the flow rate on the extraction profile of sinalbin from crude extract of mustard seeds at 20, 30, 40 and 50 mL/min. Solvent system: EtOAc/n-BuOH/water (3:2:5, v/v/v). CAI336 = CKI = 160 mM; descending mode; rotation speed = 1000 rpm; sample mass loading: 25 g. UV absorbance monitored at 275 nm. Zone 1: loading and extraction step; Zone 2: washing step; Zone 3: back-extraction step; dash line: zone of sinalbin loss*

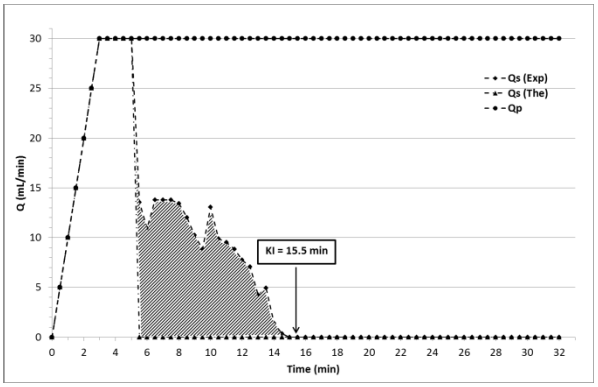
The hydrodynamics and stability of the two immiscible phases inside the column were evaluated during each experiment by measuring the initial (Sf_i) and final (Sf_f) stationary phase retention. Sf_i was measured just after the release of the mobile phase front signal (breakthrough). Sf_f was measured as the total remaining stationary phase at the end of the process. The difference between Sf_i and Sf_f reflects the disturbances of the hydrodynamic steady state in the cells during the separation process. As illustrated in Figure III.3.A (right hand graphs), we observed a decrease of Sf_i from 66.1% to 57.3% and Sf_f from 43.7% to 19.8% when increasing the flow rate from 20 to 50 mL/min. The stationary phase loss when increasing the flow rate is well-known in CPC [18], [19] and [20]. The solutes and their mass transfer during processing amplified the stationary phase loss

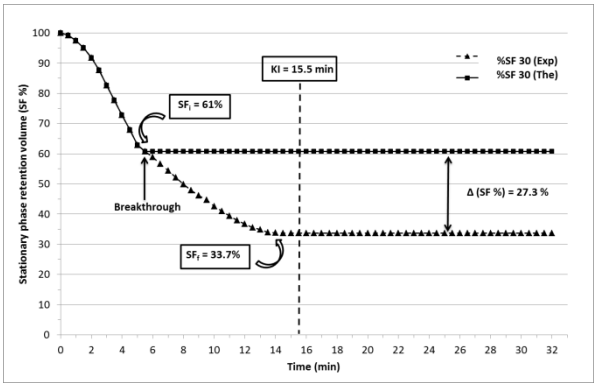
when increasing the mobile phase flow rate, i.e. when increasing the interfacial area while decreasing the residence time in each cell.

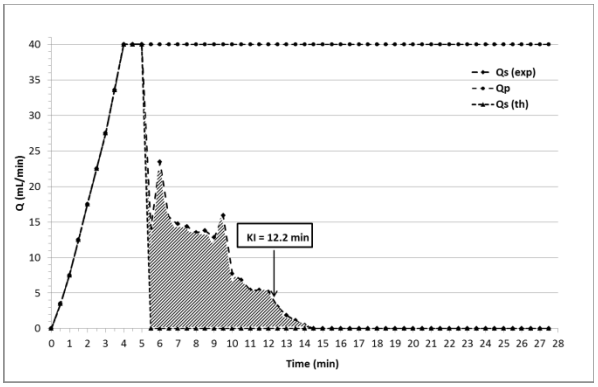
A) SIX-CPE Experiments made on crude extract of white mustard seed at 20, 30, 40 and 50 mL/min

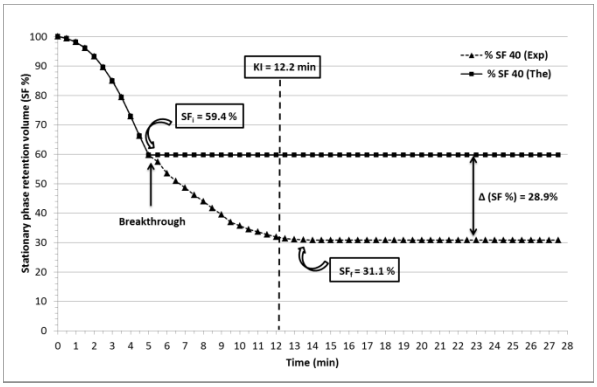


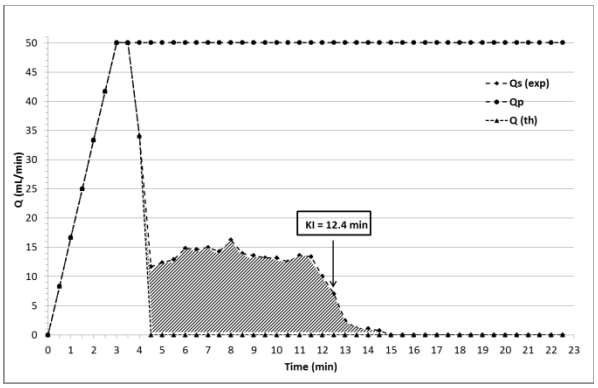


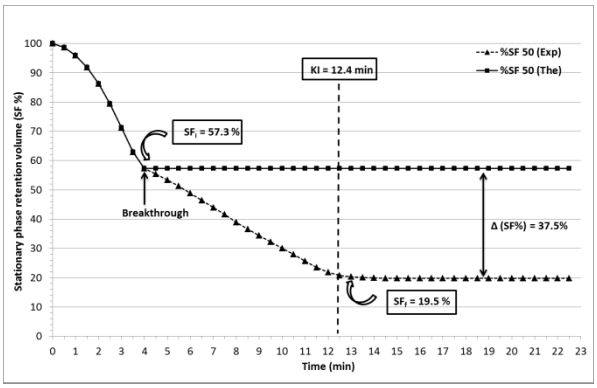












B) SIX-CPE Experiment made on pure sinalbin at 40 mL/min

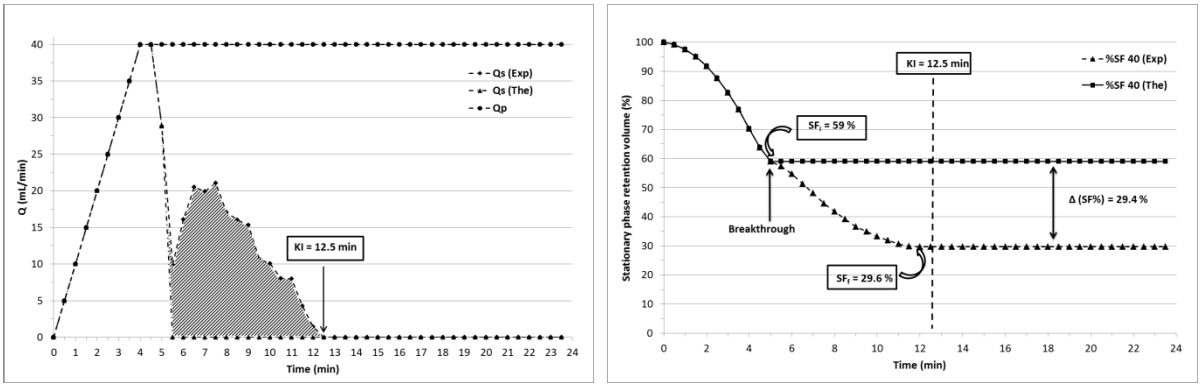


FIGURE III. 3: *Influence of the flow rate on the hydrodynamic steady state during the SIX-CPE experiments. (A) 25 g of a crude extract of white mustard seeds were purified at 20, 30, 40 and 50 mL/min; (B) 5 g of pure sinalbin at 40 mL/min. Solvent system: EtOAc/n-BuOH/water (3:2:5, v/v/v). CAl336 = CKI = 160 mM; descending mode; rotation speed = 1000 rpm; Qs(exp) and Qs(th): experimental and theoretical stationary phase flow rate; Qp: mobile phase flow rate; Sf (%): stationary phase retention volume; Zone 1: loading and extraction step; Zone 2: washing step; Zone 3: back-extraction step; dash line: beginning of the back-extraction step*

The graphs in Figure III.3.A show the flow rate delivered by the pump (Qp) at the column inlet, the theoretical stationary phase flow rate ($Qs_{(th)}$), when the hydrodynamic equilibrium is reached and the observed stationary phase flow rate ($Qs_{(exp)}$) at the column outlet. These data allowed quantifying the stationary phase loss due to the separation process after column equilibration. The differences between theoretical and experimental curves indicate at which step of the process the loss of stationary phase is the most important. Three distinct zones were clearly observed. The first zone from t = 0 to about 5 min corresponded to the replacement of a part of the stationary phase by the mobile phase in the ducts and in a part of each cell. During this period the volume of the stationary phase inside the column was strongly reduced in all experiments until the initial retention value Sf_i was reached. The second zone corresponded to a transition state where a decrease of the stationary phase retention occurred after the release of the mobile phase front signal. This zone reflects the extraction step, *i.e*. the formation of specific ion pairs between the extractant Al336 and all ionic analytes (including sinalbin) present in the crude extract of white mustard seeds. At this stage the mobile phase was still free of displacer and allowed the non-retained neutral or highly hydrophilic compounds (polysaccharides, etc.) to exit from the column. The loss of stationary phase was materialized by a very noisy chromatogram (Figure III.2), the UV detection being disturbed by the biphasic nature of the effluent. The third period corresponded to a stable hydrodynamic state with

no more loss of stationary phase. Visual CPC experiments carried out with pure sinalbin showed that the surfactant character of Al336 associated with chloride or sinalbin anions during the extraction step disturbed the system, the loss of the stationary phase stopping with the introduction of iodides as the displacer (Figure III.1). This was confirmed by an experiment carried out by injecting 5 g of pure sinalbin (corresponding to the amount contained in 25 g of extract) at 40 mL/min. As shown in Figure III.3.B, the stationary phase loss after the equilibration process of the column was similar to that obtained during the injection of the *Sinapis alba* extract, thus showing the absence of deleterious effect of the polar non-retained compounds.

III.3.2.2. Influence of the flow rate on sinalbin recovery, purity and on process productivity

As illustrated in Figure III.4, the recovery of sinalbin in experiments performed at 20, 30 and 40 mL/min were 96%, 92% and 73%, respectively. At 50 mL/min, a significant loss of sinalbin was observed with only 37% recovery. Sinalbin recovery was clearly proportional to the remaining stationary phase volume.

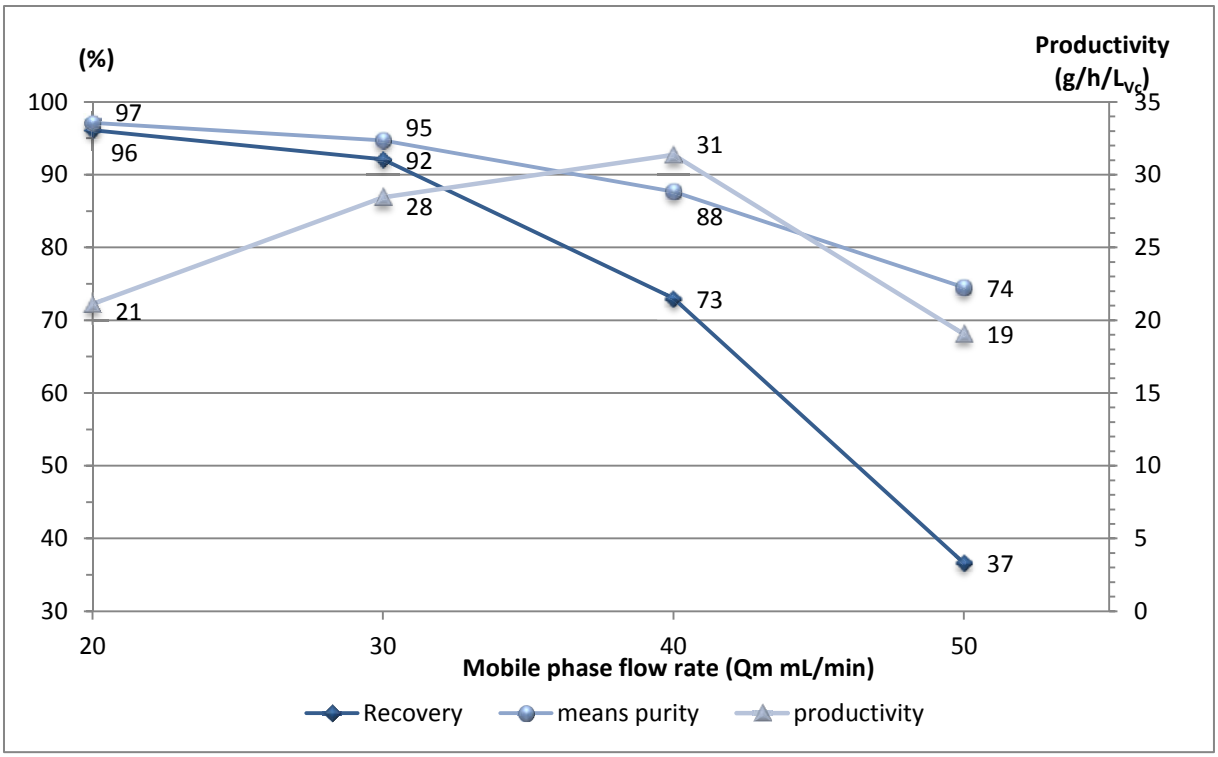


FIGURE III. 4: Influence of the flow rate on the sinalbin recovery, purity and process productivity at 20, 30, 40 and 50 mL/min. Experimental conditions: solvent system: EtOAc/n-BuOH/water (3:2:5 v/v/v); $C_{Al336} = C_{KI} = 160$ mM; descending mode; rotation speed = 1000 rpm; sample mass loading: 25 g (see Table III.1 for more details on experimental conditions)

The decrease in sinalbin recovery with increasing flow rates was due to the reduction of interaction sites between the extractant Al336 and sinalbin inside the column. More precisely at flow rates higher than 30 mL/min, the loss of stationary phase was so important that a part of sinalbin was not captured by the extractant due to insufficient available exchange sites, resulting in poor recovery values. This sinalbin loss was observed at the solvent front release in the separation profiles (Figure III.2). Another hypothesis is that this loss of sinalbin could be due to a kinetic limitation, as increasing the flow rate reduces the contact time between the phases during the process. In this case, the recovery should decrease faster than stationary phase loss, in a non-linear way. It would also affect the compressive character of the shock layers at the beginning and at the end of the isotachic train during the back-extraction step, but Figure III.5 clearly demonstrates that it is not the case. It even seems that the flow rate slightly improves the compression aspect of the profile, probably through a currently observed better dispersion of the mobile phase [18], thus increasing the interface and the mass transfer between the two phases.

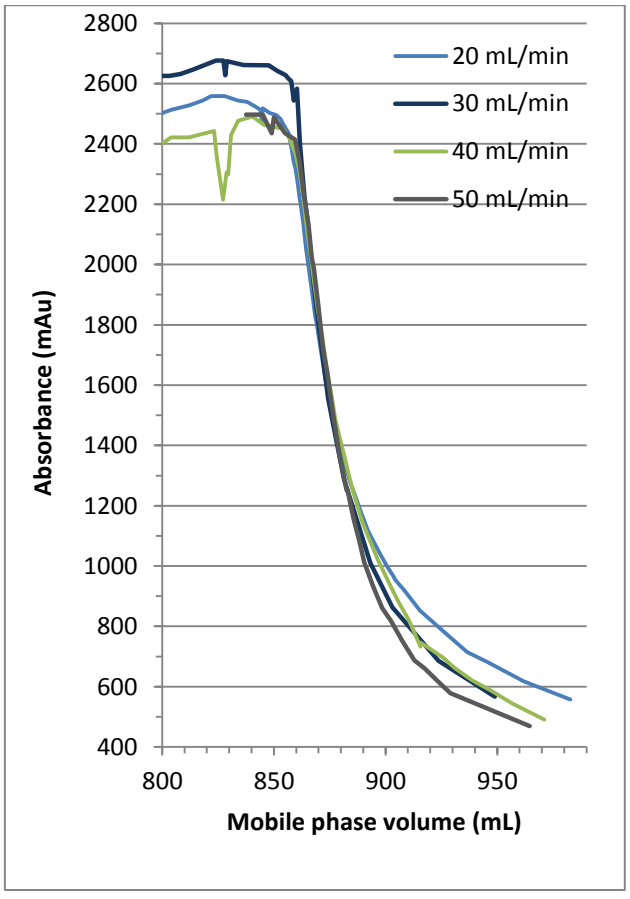


Figure III. 5: Comparison of the ends of the isotachic train (corresponding to the back-extraction step) at 20, 30, 40 and 50 mL/min. Solvent system: EtOAc/n-BuOH/water (3:2:5 v/v/v); CAl336 = CKI = 160 mM; descending mode; rotation speed = 1000 rpm; sample mass loading: 25 g; UV absorbance monitored at 275 nm

Sinalbin purity, calculated from HPLC analyses, decreased from 97% to 75% when the flow rate increased from 20 to 50 mL/min (Figure III.4). As mentioned above, when applying flow rates higher than 30 mL/min, the number of ion exchange sites inside the column was dramatically reduced due to stationary phase loss. This resulted not only in a significant loss of sinalbin but also in a decrease of selectivity. A range of other ionic compounds is naturally present with sinalbin in the crude extract of white mustard seeds. These compounds are competitively retained by the extractant in the stationary phase. A limitation in the available exchange sites negatively affects the organization of analytes as a well-structured isotachic train, thus leading to poor purity of the analytes at the column outlet.

The performance of an industrial separation process is usually evaluated as a compromise between the productivity and the target compound purity. The productivity of the present SIX-CPE process was defined as the amount of sinalbin isolated per unit of time and per liter of column volume (L_{Vc}). For the calculation, the total amount of sinalbin present initially in the crude extract (5 g) was multiplied by the recovery value obtained in each experiment.

As illustrated in Figure III.4, the productivity increased almost linearly from 21 to 31 g/h/L_{Vc} when increasing the flow rate from 20 to 40 mL/min. Above 40 mL/min, the productivity decreased significantly to reach 19 g/h/L_{Vc} at 50 mL/min. This is mainly due to the low sinalbin recovery obtained in these conditions, despite the significant gain of time (19 min). The maximum productivity was obtained at 40 mL/min, but was accompanied by only a fairly good sinalbin purity of 88%. For this reason, we considered it preferable not to exceed 30 mL/min, this flow rate yielding the best compromise between sinalbin recovery (92%), sinalbin purity (95%) and total process duration (32 min), resulting in a productivity of 28 g/h/L_{Vc}. In these optimum operating conditions, the SIX-CPE process allowed sufficient stationary phase retention at high flow rate and for high sample injected mass. It led to the recovery of 4.6 g of 94.7% pure sinalbin from 25 g of crude white mustard seed extract. The TLC-guided fractogram and HPLC analyses of the collected fractions showed that sinalbin was displaced between 26 and 32 min. The identity of sinalbin was confirmed by 1H and 13C NMR spectroscopy [11].

III.3.3. A comparative study between FCPC® and FCPE® instruments: when a modification of partition cell geometry provides an increase of productivity

In our laboratory, several glucosinolates including sinalbin were previously purified by strong ion-exchange centrifugal partition chromatography (SIX-CPC) using a 200 mL capacity laboratory-scale CPC column [10]. On this system, experiments were limited to a maximum flow rate of 2 mL/min to maintain a sufficient retention of the stationary phase inside the column. From 12 g of a crude extract of white mustard seeds, 2.4 g of 97% pure sinalbin were isolated (98% recovery) in about 170 min, corresponding to a productivity of 3.3 g/h/L_{Vc} (Table III.2). This method was then transposed at larger scale by using a 5.7 L pilot CPC column [11]. Experiments performed at 50 mL/min resulted in a one-step purification of 70.3 g of sinalbin in about 160 min from 341 g of white mustard crude extract, corresponding to a productivity of 4.3 g/h/L_{Vc}.

These previous studies clearly demonstrated that the ion-exchange mode combined to centrifugal partition chromatography showed a strong potential for the industrial production of glucosinolates. In the present work, we have expanded the scope of this technique on a CPE instrument by demonstrating that a decrease in the number of partition cells and a modification of their geometry (1260 rectangular single cells for the 200 mL CPC column and 231 oval twin cells for the 300 mL CPE column) does not affect the efficiency of the separation. It can be hypothesized that, even if the CPE column contains less partition cells than the CPC column, the ion-exchange chemical reaction sites can also play a role of transfer units regardless of the number of theoretical plates. In addition, the twin cells in the CPE column are of larger volume, allowing higher flow rates, better stationary phase retention and a much more efficient interfacial mass transfer (2 mL/min in CPC and 30 mL/min in CPE). As a result, a 8.5-fold increase of productivity was observed between the laboratory-scale SIX-CPC (3.3 g/h/L_{Vc}) and the SIX-CPE (about 28.3 g/h/L_{Vc}) processes.

In solid support free liquid–liquid separation technologies, the transition from laboratory-scale to pilot-scale instruments can generally be achieved by a simple linear calculation of the scale-up factor (F), which refers to the ratio between the rotor volumes [21]. Thus, we could predict that about 5 kg of crude extract of white mustard seeds could be processed per day using a SIX-CPE method (45 min per run including column conditioning, 12 runs per day), and that a daily production of 1 kg pure sinalbin could be achieved by using a CPE apparatus with a capacity of 5.7 L (Table III.2).

	Laboratory scale CPE	*Predicted Pilot scale CPE*	*Laboratory scale CPC*	*Pilot-scale CPC*
Column capacity (mL)	303.5 mL	5700 mL	200 mL	5700 mL
Scale up factor		≈ 19		≈ 28
Flow rate (mL/min)	30 mL/min	570 mL/min	2 mL/min	50 mL/min
Loading per injection (g)	25 g	475 g	12 g	341 g
Sample volume (mL)	60 mL	1140 mL	20 mL	1300 mL
Total process duration (min)	32 min	≈ 32 min	160 min	170 min
Aqueous phase consumption per run (mL)	960 mL	18.5 L	320 mL	8.5 L
Organic phase consumption per run (mL)	600 mL	11.5 L	400 mL	11.5 L
Productivity (g/h/L_{vc})	28.3	28.7	3.3	4.3

Table III. 2 : Comparison of the productivity obtained from different experimental laboratory-scale CPE experiments, predicted pilot-scale CPE, and experimental pilot-scale CPC experiments. Solvent system: EtOAc/n-BuOH/water 3:2:5 v/v; sample: crude extract of white mustard seeds. Descending mode; rotation speed = 1000 rpm

III.4. Conclusion

The design of new CPC devices opens new perspectives in terms of industrial development in solid support-free liquid-liquid chromatography. The FCPE® device initially developed for applications in the field of extraction, shows real potential for the large scale purification of high added value ionized compounds when the ion-exchange mode is used. The decrease of the partition cell number does not appear to significantly affect the quality of the separation in our purification model of sinalbin obtained from a crude extract of *Sinapis alba*. CPE column design allows the application of higher mobile phase flow rates and therefore is beneficial in terms of productivity. A sufficient stationary phase retention volume was obtained to purify 4.6 g of sinalbin in high recovery (92%) and with good purity (94.7%). The productivity of the SIX-CPE process presented here was mainly governed by the mobile phase flow rate and by the mass sample loading conditions. The logical perspective of this work will be to provide tools for predicting the process engineering design (column capacity and partition cell number) for CPC (or CPE) devices, based on the target productivity and the type of chromatographic mode used for the separation (elution or displacement mode).

III.5. References

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CHAPITRE 2 - PARTIE 3

EXTRACTION DE PARTAGE CENTRIFUGE EN MODE ÉCHANGE D'IONS FORT : APPLICATION À L'EXTRACTION ET À LA CONCENTRATION SIMULTANÉES DE L'ACIDE ITACONIQUE

CHAPTRE 2 - PART 3

SIMULTANEOUS EXTRACTION AND CONCENTRATION OF ITACONIC ACID FROM AQUEOUS SOLUTIONS USING STRONG ION-EXCHANGE CENTRIFUGAL PARTITION EXTRACTION

IV. Extraction de partage centrifuge en mode échange d'ions fort : Application à l'extraction et à la concentration simultanées de l'acide itaconique

Introduction du Chapitre 2 – Partie 3

Cette troisième partie du chapitre 2 présente une application de l'extracteur de partage centrifuge dans le domaine des biotechnologies blanches. Le but principal de cette étude était d'étudier l'extraction de l'acide itaconique, un diacide à 5 carbones produit par le champignon *Aspergillus Terreus*, via un procédé de fermentation. À l'heure actuelle, la production industrielle d'acide itaconique est caractérisée par des rendements modérés voir faibles, principalement parce qu'il reste difficile de l'extraire à partir de milieux fermentaire fortement dilués. L'enjeu était donc de développer un procédé par extraction de partage centrifuge en échange d'ions fort permettant d'extraire et de concentrer l'acide itaconique à partir d'une solution aqueuse initialement très diluée La méthode développée ici est présentée comme un procédé a multi-finalités permettant de réaliser plusieurs opérations telles que : la clarification, la concentration, l'extraction et la récupération de l'acide itaconique produit par fermentation. Cette étude s'appuie sur la possibilité de traiter des volumes important de phase aqueuse mobile injectée, contenant de faibles quantités d'acide itaconique (C = 50 mg/L) tout en procédant avec un débit relativement élevé (30 mL/min).

Simultaneous extraction and concentration of itaconic acid from aqueous solutions using strong ion-exchange centrifugal partition extraction

Mahmoud Hamzaoui[a], Jane Hubert[a], Romain Reynaud[b] and Jean-Hugues Renault[a]*

[a]*UMR CNRS 6229, Université de Reims Champagne-Ardenne, Bât. 18, Moulin de la Housse, BP 1039, 51687 Reims, Cedex 2, France*

[b]*Soliance, Route de Bazancourt - 51110 Pomacle France*

Abstract

The simultaneous extraction and concentration of a highly diluted itaconic acid solution (IA, 50 mg/L, 0.384 mM) were investigated using strong ion-exchange centrifugal partition extraction (SIX-CPE). The method was optimized on the basis of an extraction/back-extraction scheme using trioctylmethylammonium chloride (Al336) as an anion-extractant and potassium iodide (KI) as a displacer. The process development was initiated by studying the extractant to itaconic acid molar ratio for two different solvent systems. The SIX-CPE procedure was then performed in the descending mode at a flow rate of 30 mL/min with the solvent system ethyl acetate/water (1:1, v/v) requiring the lowest theoretical molar ratio (nAl336/nIA = 4.75). In only 53 min, 94% of the itaconic acid initially present in the aqueous solution was recovered and its concentration was 7-fold increased. The SIX-CPE method proposed in this study represents an interesting alternative for the rapid extraction and concentration of organic acids starting from highly diluted liquid mixtures. In particular it offers promising perspectives for a significant reduction of the production costs invested in the production of microbial ionic bioproducts, and thus could take part in the industrialization of new white biotechnological processes.

Keywords: Centrifugal partition extraction, centrifugal partition chromatography, ion-exchange, itaconic acid, downstream processes, white biotechnology.

IV.1. Introduction

"White biotechnologies" are based on the use of fungi, yeasts, bacteria and/or enzymes for the production of high added value biomolecules as a replacement for petroleum-derived chemicals [1, 2]. Today, white biotechnologies are mainly used for the industrial production of bioethanol [3-5] and biodiesel [6-9], amino acids (L-lysine, L-threonine, L-tryptophan) [10-12], lipids (stearic acid, linolenic acid, arachidonic acid) [13-17], glycolipids (trehalose lipids, rhamnolipids, sophorolipids) [18-21], glycerol [22, 23], vitamins (riboflavin, folate, cobalamin) [24-26] and carboxylic acids (citric, lactic, gluconic, succinic) [1, 27-29].

Microbial carboxylic acids such as succinic, fumaric, malic and itaconic acid are currently used as food additives [30] or chemical synthons for the synthesis of polymers and biodegradable materials [1, 28, 30-32]. However, despite this diversity of potential applications, only a few microbial carboxylic acids are produced industrially via large-scale fermentation processes. Itaconic acid (Figure IV.1), also known as methylenebutanedioic acid or methylenesuccinic acid, is an unsaturated C_5 dicarboxylic acid whose bio-production is usually performed by the filamentous fungi Aspergillus terreus in the presence of sucrose, glucose or xylose as substrates [33-36]. Recent advances in metabolic and genetic engineering have led to the increase of itaconic acid productivity by using other *Aspergillus species*, new strains or yeasts such as *Pseudozynma Antractica* [37, 38]. Today, itaconic acid is exclusively used in non-food applications. Due to the two carboxyl groups and carbon-carbon double bond, it can be easily incorporated into polymers, for instance as a substitute for petrochemical-based acrylic or methacrylic acids [34, 39]. Itaconic acid is also used as a co-monomer for the production of resins, synthetic fibers, coatings, adhesives, thickeners, and binders [40-42].

Itaconic acid production costs are strongly dependent on the performance of the extraction, concentration and purification procedures. In most cases, the crude fermentation medium is treated by calcium hydroxide for the precipitation of itaconic acid which is then separated by filtration [27, 37]. Itaconic acid calcium salts are then washed to remove some impurities and dissolved in sulfuric acid. The limiting step of this process relies on the elimination of the high amount of calcium sulfates [27, 37]. Liquid-liquid extraction processes using organic/aqueous solvent systems are also frequently used to recover itaconic acid from aqueous solutions [37, 43, 44]. More particularly reactive extraction processes, using alkylamines (trioctylamine, trioctylmethylammonium chloride) or organophosphorus extractants (tributylphosphate) in the organic phase have proven to be efficient strategies for the extraction of itaconic acid [45-48].

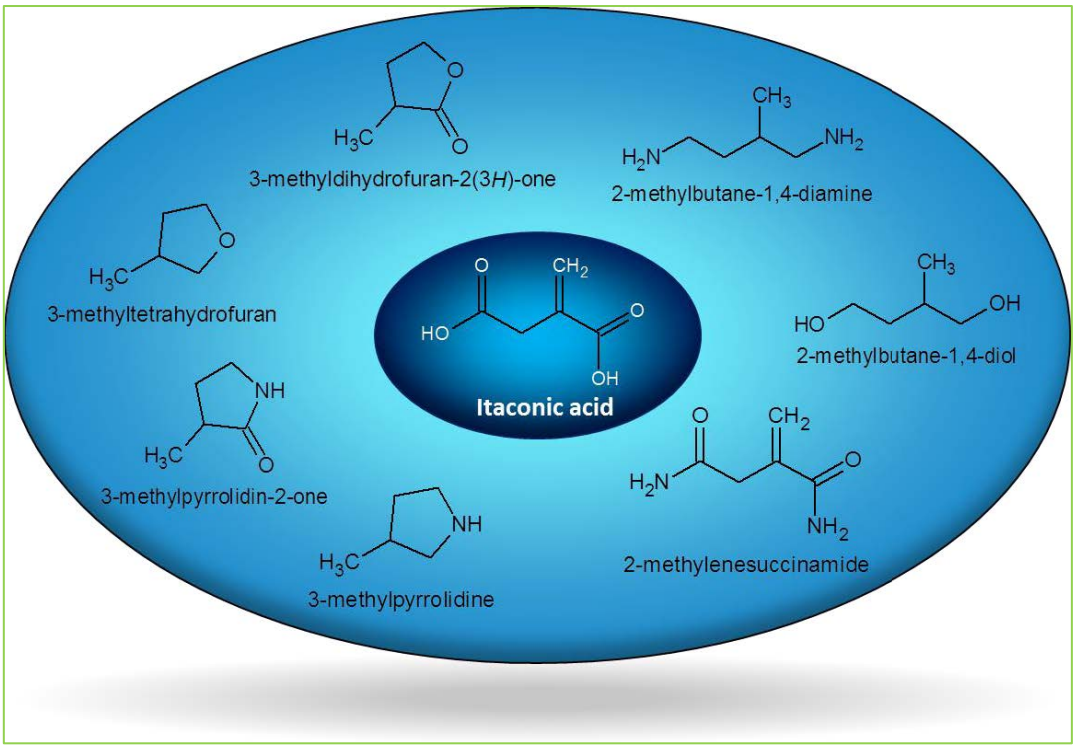

Figure IV. 1 : Itaconic acid and its derivatives

In the present work, centrifugal partition extraction (CPE) combined to the strong ion-exchange mode (SIX) is proposed as a new alternative for the simultaneous extraction and concentration of itaconic acid from aqueous media. CPE is an original solid support-free liquid-liquid separation system based on the distribution of solutes between immiscible liquid phases according to their partition coefficient. At the laboratory-scale, a CPE column design allows the application of mobile phase flow rates ranging from 10 to 100 mL/min and the loading of crude extract on the multi-ten-gram scale. It was demonstrated recently that CPE combined to the strong-ion exchange mode is very efficient for the purification of ionic metabolites from complex plant extracts [49, 50]. In the present study, we intended to evaluate the potentialities of this SIX-CPE strategy for the simultaneous extraction and concentration of itaconic acid when starting from highly diluted aqueous solutions.

IV.2. Experiment

IV.2.1. Reagents

Aliquat 336® (trioctylmethylammonium chloride, Al336) was purchased from Sigma-Aldrich (Saint-Quentin, France) as a mixture of C_8 and C_{10} chains with C_8 predominating. Potassium iodide (KI)

and sodium hydroxide (NaOH) were obtained from Prolabo (Fontenay, France). Ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and sulphuric acid (H_2SO_4) were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Itaconic acid standard (IA) was obtained from TCI EUROPE N.V. (Zwijndrecht, Belgium) with a purity > 95%. Deionized water was used to prepare all aqueous solutions.

IV.2.2. Instrumentation

The extraction process was developed on a laboratory-scale Fast Centrifugal Partition Extractor (FCPE300®, Kromaton Technology, Angers, France) with a total column capacity of 303.5 mL. The column is made of 7 circular partition disks engraved with a total of 231 oval and symmetric twin-cells (33 twin-cell by disk, 0.986 mL per twin-cell) arranged circumferentially and connected together by ducts representing 24% of the total column volume. A liquid phase can be maintained as stationary phase inside the column by a centrifugal force field generated by the rotor around a single central axis. The rotation speed can be adjusted from 200 to 2000 rpm, producing a relative centrifugal acceleration in the partition cells up to 437 *g*. Another non-miscible liquid phase is pumped through the stationary phase from cell to cell either in the descending mode (in the centrifugal direction) or in the ascending mode (in the centripetal direction) through a KNAUER Preparative Pump 1800® V7115 (Berlin, Germany). The system was coupled to a UVD 170S detector (Dionex, Sunnivale, CA, USA) equipped with a preparative flow cell (6 µL internal volume, 2 mm path length). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). All experiments were carried out at room temperature (20 ± 2 °C).

IV.2.3. Solvent system selection and optimization of the extractant concentration

A binary biphasic solvent system composed of EtOAc/water (1:1, v/v) (system 1) and a ternary biphasic solvent system composed of EtOAc/*n*-BuOH/water (5:5:10, v/v) (system 2) were investigated. In pill-box experiments and for both systems, the strong anion-extractant Al336 was added to six independent organic phases of 2 mL at different concentrations. These organic phases were then mixed with their corresponding aqueous solutions containing a fixed amount of itaconic acid. The molar ratios obtained between Al336 and itaconic acid for each assay are given in Table IV.1. The amount of itaconic acid extracted in the organic phase (IA_{org}) was obtained by quantifying the difference between the initial and final itaconic acid concentrations in the aqueous phase: IA_{org} = IA_{aq} (initial) – IA_{aq} (final). In order to transpose the results to the CPE system, the method of the tangent at zero was used to determine the optimal n_{Al336}/n_{IA} ratio required to extract theoretically

100% of itaconic acid into the organic phase inside the CPE column. As illustrated in Figure IV.2 for solvent systems 1 and 2, the optimum molar ratios n_{Al336}/n_{IA} correspond to the abscissa projection of the intersection between the tangents at zero (τ_1 and τ_2) of the extraction curves obtained from the pill-box experiments and the extraction percentage y = 100% at the points I and J, respectively.

Solvent system	Sample	n_{Al336}/n_{IA}	IA_{org} (%)
EtOAc/water 1:1, v/v	1	1.3	12
	2	2.5	24
	3	5.0	37
	4	8.7	49
	5	10.0	52
	6	12.3	54
EtOAc/n-BuOH/water 5:5:10, v/v	1	0.3	6
	2	1.2	20
	3	2.3	37
	4	3.5	43
	5	4.3	47
	6	5.2	49

Table IV. 1: Influence of Al336 concentration on the extraction percentage of itaconic acid into the organic phase for the biphasic solvent systems EtOAc/water (1:1, v/v) and EtOAc/n-BuOH/water (5:5:10, v/v)

IV.2.4. SIX-CPE procedure for the simultaneous extraction and concentration of itaconic acid

The biphasic solvent system EtOAc/ water (1:1, v/v, total of 2 L) was prepared in a separatory funnel. After liquid phase separation, Al336 was added to the organic stationary phase at a molar ratio n_{Al336}/n_{IA} = 5. The column was filled at 200 rpm with this organic solution and then the rotation speed was increased to 1200 rpm. To investigate the ability of our SIX-CPE method to treat high sample volume at low itaconic acid concentration, 50 mg of pure itaconic acid were dissolved in 950 mL of the aqueous phase of the biphasic solvent system. The pH was adjusted to 6-7 using NaOH (1 M) to ensure the ionization of itaconic acid (pK_{a1} = 3.85 and pK_{a2} = 5.45). This diluted sample solution was directly pumped into the CPE column in the descending mode at a flow rate gradually increased from 0 to 30 mL/min in 3 minutes. This ramp was used to minimize the hydrodynamic equilibrium disturbance inside the column during the loading step. After 38 minutes (corresponding to the total

loading step duration), potassium iodide (KI) was added to the aqueous mobile phase at a molar ratio n_{Al336}/n_{KI} = 1 for the back-extraction of itaconic acid. Fractions of 15 mL were collected over the whole experiment and analyzed by UHPLC.

IV.2.5. UHPLC analysis

Analyses were performed on an UltiMate 3000® UHPLC system (Dionex, Sunnyvale, USA) equipped with a LPG-3400SD" pump, a WPS-3000 (T) autosampler with an integrated column oven. The chromatographic column (Rezex ROA-organic acid H^+ (8%), 250 x 4.6 mm, Phenomenex) was used at a constant temperature of 40 °C. A H_2SO_4 solution (2.5 mM) was used as the mobile phase and isocratically pumped at a flow rate of 0.17 mL/min for 22 minutes. The injection volume was 10 µL. The system was coupled to a Dionex DAD-3000RS photodiode array detector monitored at 254 nm. Data acquisition was controlled by the Chromeleon® version 6.8 software. Calibration curves were established by serial dilution of three independent stock solutions of pure itaconic acid (0.01, 0.05, 0.1, 0.5, 1, 1.5, and 2 g/L) and by plotting the peak area recorded at 254 nm from UHPLC chromatograms as a function of itaconic acid concentration.

IV.3. Results and discussion

IV.3.1. Solvent system selection and optimization of the extractant concentration

In strong ion-exchange centrifugal partition extraction processes, the biphasic solvent system is mainly selected on the basis of three main criteria. First the organic phase must fully solubilize the lipophilic extractant, and the aqueous phase alone (free of displacer), should not allow the elution of the target analytes captured in the organic phase. Secondly, the displacer must provide a stronger affinity for the extractant than the target analytes. Thirdly, the liquid phase equilibrium inside the rotating CPE column must be preserved even in the presence of the extracting agent which often exhibits surfactant properties.

Here, two solvent systems were investigated on the basis of previous studies. It was demonstrated that itaconic acid can be efficiently extracted in EtOAc by using the quaternary amine Al336 as an extractant [47]. The low solubility of ionized itaconic acid in pure EtOAc free of extractant is another interesting property [44, 51] which would favor the transfer of itaconic acid into the aqueous phase when adding the displacer (KI) in the mobile phase. In another study dedicated to the extraction and purification of strongly hydrophilic natural compounds (glucosinolates) by strong ion

exchange centrifugal partition chromatography, it was shown that the addition of n-BuOH as a third solvent to the mixture EtOAc/water could enhance the transfer of the analytes from the aqueous to the organic phase [52]. Therefore we have investigated the two solvent systems EtOAc/water (1:1, v/v) and EtOAc/n-BuOH/water (5:5:10, v/v).

The nature of the ion-pairs formed between the extractant Al336 and itaconic acid is another important feature to be considered when optimizing the extraction step. It was suggested previously that a simple 2:1 stoichiometric molar ratio between Al336 and dicarboxylic acids was theoretically sufficient to promote their complete extraction in the organic phase [53-56]. However, in practice higher ratios like 3:1 or 4:1 are required [47, 57, 58]. In the present work, the Langmuir isotherms of both solvent systems were constructed by plotting the itaconic acid extraction percentage as a function of n_{Al336}/n_{IA} molar ratio. As illustrated in Figure IV.2, the extraction percentage of itaconic acid was higher in the solvent system EtOAc/water (1:1, v/v) than in the solvent system EtOAc/*n*-BuOH/water (5:5:10, v/v). We were however expecting that the addition of *n*-BuOH to the mixture of EtOAc/water would increase the organic phase polarity and consequently stabilize the Al336 - itaconic acid ion-pairs in the organic phase via solvatation effects. The extraction percentage of itaconic acid increased linearly when the molar ratio n_{Al336}/n_{IA} increased to reach a maximum of 49% and 54% for system 1 and system 2, respectively. Beyond these values, no evolution of the extraction percentage of itaconic acid was observed, indicating a saturation of the organic phase. This saturation phenomenon was directly due to the small volumes of organic phase (2 mL) and to the liquid phase volume ratios which were used to perform these pill-box assays. Therefore, the theoretical molar ratio n_{Al336}/n_{IA} required to achieve the complete extraction of itaconic acid inside the CPE column was extrapolated by only considering the linear zone of the *Langmuir* isotherms and applying the method of tangents at zero (Figure IV.2). As a result, n_{Al336}/n_{IA} ratios of 4.7 and 9.8 were obtained for system 1 and 2, respectively. As the extractant Al336 exhibits surfactant properties, it was preferable to minimize its concentration to maintain the hydrodynamic equilibrium of the biphasic solvent system. For this reason, the solvent system EtOAc/water (1:1, v/v) was selected for the SIX-CPE procedure optimization.

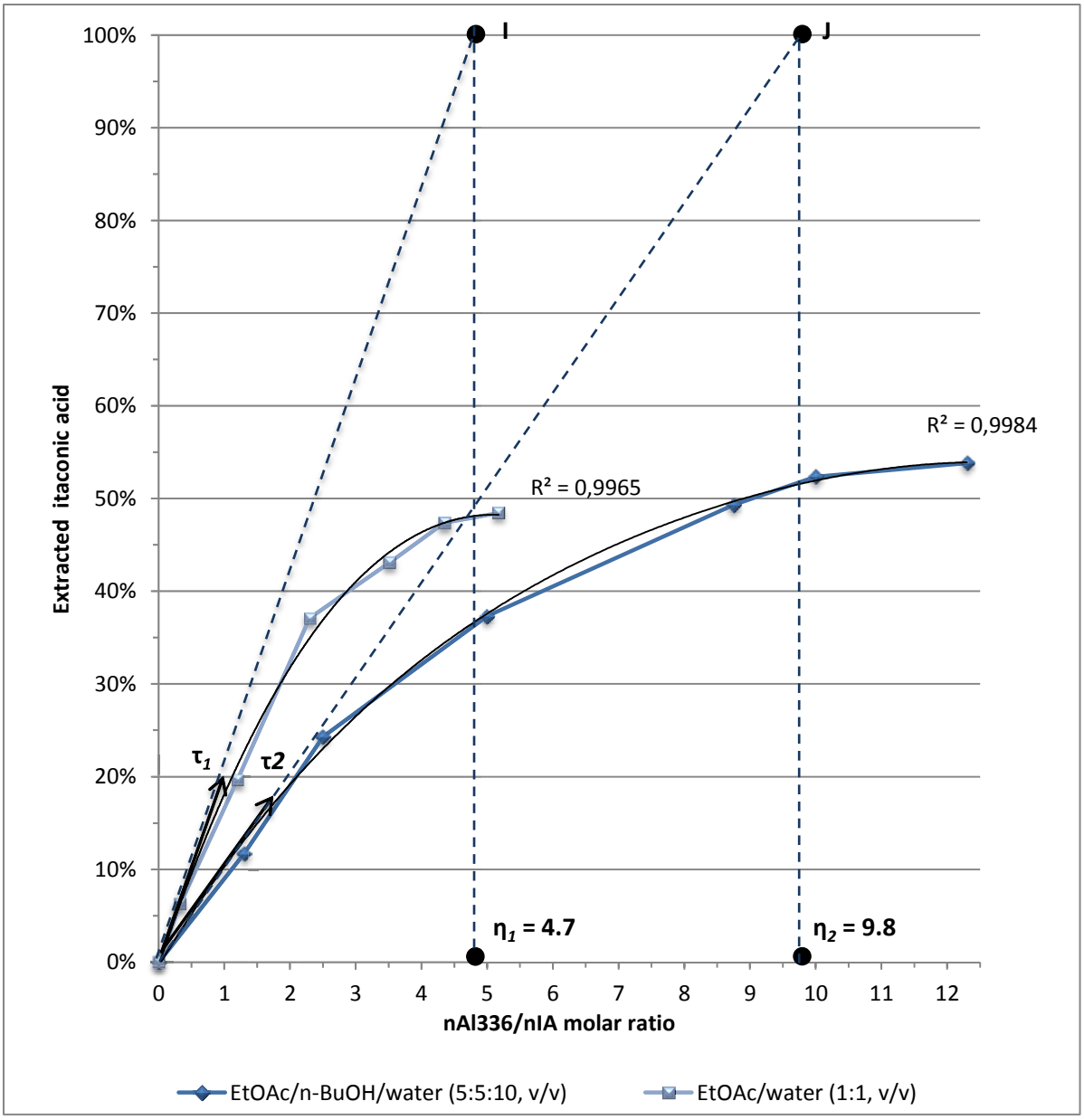

Figure IV. 2: Extraction percentage of itaconic acid as a function of the molar ratio nAl336/nIAf or the biphasic solvent systems EtOAc/water (1:1, v/v) and EtOAc/n-BuOH/ water (5:5:10, v/v). IA: itaconic acid. Dashed lines: Determination of the theoretical molar ratios (η1 and η2) required for a complete extraction of itaconic acid. I and J: Intersection between the tangents (τ1 and τ2) at zero with y = 100%

IV.3.2. Hydrodynamic behavior of the solvent system during the process

The first part of the process consisted in the hydrodynamic equilibration of the two liquid phases inside the CPE column until the release of the mobile phase front at t_0 = 4.9 min. The initial stationary phase retention volume was 66%. Then a slight decrease in the stationary phase retention

volume (bleeding of the stationary phase) was measured until the complete hydrodynamic steady state was established at about 12 minutes, resulting in final stationary phase retention of 48.2%. These values indicate that both liquid phases were stable enough to pump the mobile phase at 30 mL/min.

IV.3.3. Recovery and concentration of itaconic acid from high diluted aqueous phase by SIXCPE

Liquid-liquid extraction processes using lipophilic amines as extractants have been applied to numerous mono and dicarboxylic acids produced by microbial fermentation [53, 56, 59, 60]. However, only a few studies have been dedicated to the extraction of itaconic acid [43, 47, 48, 51, 57, 61]. At the industrial scale, the main difficulty to obtain high recovery of organic acids from high volumes of fermentation broths arises from the high dilution of the target analytes. This requires the use of several steps to concentrate the crude liquid mixture before starting the extraction and purification procedures. Here, 50 mg of itaconic acid standard (50 mg) diluted in 1 L of aqueous mobile phase were loaded into the CPE column, corresponding to an initial itaconic acid concentration of 0.384 mM. The extraction/back-extraction process was performed in only 53 minutes. During the extraction step (38 min), itaconic acid was directly trapped into the organic stationary phase through the formation of stable IA/Al336 ion-pairs. The back-extraction step was then initiated at t = 38 min by pumping the aqueous mobile phase containing the displacer agent KI. Figure IV.3 shows the UHPLC concentration profile of the back-extracted itaconic acid. These analyses indicated that itaconic acid was released from the CPE column between 45 and 49.5 minutes. The non-compressive and compressive behaviors of the front and back parts of the concentration profile, respectively, were probably linked to the differences in selectivity between the cationic exchanger ($Al336^+$) and the anionic species (Cl^-), (IA^-) and (I^-) involved in the ion-exchange mechanisms. If the difference between the association constants Cl^-/Al336+ and $IA^-/Al336^+$ is not significant, the back-extraction concentration profile is non-compressive. In contrast, the strong affinity of the iodides for the exchanger allowed a rapid dissociation of the $Al336^+/IA^-$ to form stronger ion-pairs ($Al336^+$, I^-), resulting in a compressive concentration profile behavior. In total, 47 mg of itaconic acid were obtained, corresponding to a recovery of 94%. Itaconic acid was detected in only 9 fractions of 15 mL, corresponding to a final itaconic acid concentration of 348 mg/L. Thus the itaconic acid sample volume was reduced from 1L to 135 mL and its concentration was 7-fold increased. As shown in Figure IV.3, the maximum itaconic acid concentration (5 mM) was reached at 48 min.

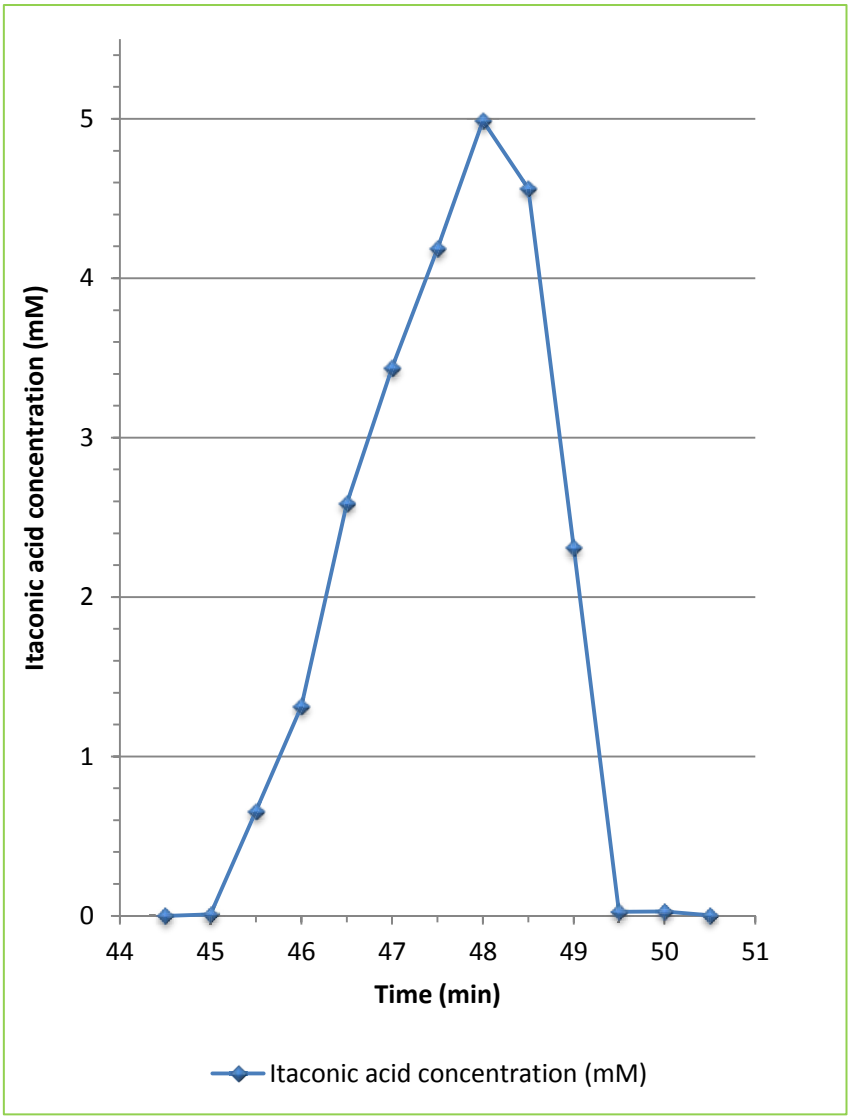

Figure IV. 3 : Concentration profile of itaconic acid during the back-extraction step

IV.4. Conclusion

A new efficient method was developed for the simultaneous extraction and concentration of itaconic acid initially presents at very low concentration (50 mg/L) in an aqueous solution. The process was achieved in in only 53 min by using the solvent system EtOAc/water (1:1, v/v) and an extractant to itaconic acid molar ratio n_{Al336}/n_{IA} = 5. A high itaconic acid recovery of 94% was obtained and its concentration was 7-folds increased. This process must now be applied to a real fermentation broth containing itaconic acid in order to evaluate the potential of this strategy to purify ionic bioproducts (microbial carboxylic acids) produced through white biotechnological processes.

IV.5. References

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CHAPITRE 3:

EXTRACTION DE PARTAGE CENTRIFUGE POUR L'EXTRACTION ET LA SIMPLIFICATION D'EXTRAITS NATURELS COMPLEXES

CHAPTER 3:

CENTRIFUGAL PARTITION EXTRACTION: A NEW STRATEGY FOR THE EFFICIENT FRACTIONNATION OF NATURAL PLANT EXTRACTS

Chapitre 3 : Extraction de partage centrifuge pour l'extraction et la simplification d'extraits naturels complexes

Introduction du chapitre 3

Les extraits naturels issus des plantes ou des micro-organismes sont souvent très complexes car ils renferment de nombreuses familles de molécules (lipides, phénols et polyphénols, acides organiques, alcaloïdes, triterpenes et saponines, tanins, peptides) structures chimiques (aliphatiques, aromatiques, longue chaines, cycliques et hétérocycliques, groupements fonctionnels) et de caractéristiques physico-chimiques différentes (hydrophilie/ hydrophobicité, amphiphilie, caractère ionique et ionisables).

Parmi ces molécules, certaines sont actives, d'autres non. Afin d'isoler de nouvelles substances actives, l'une des méthodes envisageable aujourd'hui est le fractionnement en une seule étape des extraits bruts afin de simplifier l'extrait initial et de passer par la suite au criblage de l'activité biologique des fractions obtenues. La difficulté réside en particulier dans la longue durée (quelques semaines) que demandent les techniques usuelles chromatographiques (CLHP, Chromatographie-flash, colonne classique de silice). L'autre problématique, concerne les faibles rendements obtenus principalement liés aux faibles masses d'extrait traités. De ce fait, les mises au point de méthodes de fractionnement efficaces et de simplification des matrices naturelles complexes sont souvent longues, couteuses et peu productives.

D'autre part, les procédés de fractionnement et de purification des substances naturelles doivent capables de fournir rapidement et en quantité suffisante des fractions en vue de l'évaluation de leur activité biologique et ensuite pour une montée en échelle pour l'industrialisation des procédés. Dans ce contexte, deux méthodes de fractionnement ont été développées en extraction de partage centrifuge à partir d'un extrait éthanolique (99%) de l'écorce d'un arbre sub-saharien *Anogeissus leiocarpus* Guill. & Perr., (Combrétacée). Le choix de l'extrait de départ a été défini avec notre partenaire industriel (Soliance) et l'objectif était de développer des procédés de permettant de fractionner et de simplifier d'une manière rapide et efficace l'extrait brut initial afin d'accéder directement et en un temps court à des fractions enrichies par des composés ou familles de composés ciblés.

Deux méthodes EPC basées sur deux mécanismes de séparations différents ont ainsi été développées et feront l'objet des deux parties de ce chapitre. La première méthode a été développée en combinant l'EPC à un mode original d'élution tri-phasique séquentiel balayant un large spectre de polarité. Les résultats obtenus ont déjà conduit à la publication d'un article dans le journal « *Phytochemical Analysis* »[1]. La seconde méthode de fractionnement a été développée par pH-zone-refining, basée sur le changement du coefficient de distribution (K_D) des composés ciblés (polyphénols et dérivés de l'acide ellagique notamment) par une simple variation du pH (protonation et déprotonation des métabolites). Les résultats de cette méthode ont conduit à la rédaction d'un article qui vient d'être soumis dans « *Journal of Chromatography B* ».

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CHAPITRE 3 – PARTIE 1

DÉVELOPPEMENT DE L'ÉLUTION TRIPHASIQUE SÉQUENTIELLE EN « EPC » POUR LE FRCATIONNEMENT ET LA SIMPLIFICATION D'UN EXTRAIT D'ÉCORCE D'ANOGEISSUS LEIOCARPUS GUILL. & PERR.

CHAPTER 3 – PART 1

STEPWISE ELUTION OF A THREE-PHASE SOLVENT SYSTEM IN CENTRIFUGAL PARTITION EXTRACTION: A NEW STRATEGY FOR THE FRACTIONATION AND PHYTOCHEMICAL SCREENING OF A CRUDE BARK EXTRACT

V. Développement de l'élution tri-phasique séquentielle en « EPC » pour le fractionnement et la simplification d'un extrait d'écorce *d'Aogeissus leiocarpus* Guill. & Perr.

Introduction du chapitre 3 – Partie 1:

Le processus général de criblage chimique et de caractérisation de nouvelles molécules bioactives à partir de matrices naturelles complexes, telles que les plantes, fait intervenir différentes étapes, dont les trois principales sont classiquement l'extraction solide-liquide permettant d'obtenir un extrait brut, le fractionnement-liquide-liquide et l'identification des composés d'intérêt, toutes ces étapes étant guidées par des analyses phytochimiques et des tests biologiques. Le fractionnement liquide-liquide, qui a pour but de simplifier un extrait brut en séparant les molécules en fonction de leurs propriétés physico-chimiques, est l'une des étapes clées de ce processus car elle va permettre de réduire la complexité du matériel de départ et donc faciliter, à posteriori, l'identification des molécules actives. Quelque soit la méthodologie utilisée, il est nécessaire d'analyser les fractions collectées sur des systèmes chromatographiques (HPLC, TLC) et par des systèmes de détection et d'élucidation structurale (UV, MS, RMN) afin d'analyser la composition des fractions et de déterminer la pureté et la nature des composés isolés. Ainsi, le développement de méthodologies rapides qui permettent l'analyse et l'identification des molécules séparées par fractionnement liquide-liquide est actuellement l'un des défis majeurs en phytochimie.

Dans ce contexte général et dans le cas d'*Anogeissus leiocarpus*, l'extrait éthanolique de l'écorce obtenu est très riche en molécules présentant des propriétés physico-chimiques variées (acide éllagique et ses dérivés, catechine et ses dérivés, sericoside, sericic acide, tannins, etc.), et plus particulièrement, des composés ayant un large spectre d'hydrophobicité, ce qui rend le fractionnement par les techniques classiques (CLHP, colonne de silice, etc.) souvent long et fastidieux.

Nous avons donc développé une méthode EPC de fractionnement des différentes familles moléculaires de cet extrait (acide éllagique et ses dérivés, catechine et ses dérivés, tannins, tritérpènes et saponines : séricoside, sericic acide, etc.) par un mode d'élution original tri-phasique séquentiel balayant un large spectre de polarité. La mise au point de ce procédé a été réalisée en premier temps par la caractérisation et l'étude hydrodynamique du système de solvant conduisant à obtenir trois phases thermodynamiquement stable ayant des polarités différentes et résultant d'un

mélange de quatre solvants; heptane, méthyl *ter*-butyle éther, acétonitrile et eau. Ce système de solvant a été ensuite utilisé pour un fractionnement ciblé afin d'obtenir des fractions riches en acide éllagique et ses dérivés. En une seule étape, 5 g de l'extrait brut initial de l'écorce *d'Anogeissus leiocarpus* ont été fractionnés en fonction des différences d'hydrophobicité et de l'affinité de chaque famille de composés pour chacune des phases du système tri-phasique.

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Stepwise Elution of a Three-phase Solvent System in Centrifugal Partition Extraction: A New Strategy for the Fractionation and Phytochemical Screening of a Crude Bark Extract

Mahmoud Hamzaoui,[a] Jean-Hugues Renault,[a] Jean-Marc Nuzillard,[a] Romain Reynaud[b] and Jane Hubert[a]*

ABSTRACT:

Introduction – Tree bark represents an interesting source of bioactive molecules for the discovery of new pharmaceutical agents. However, the detailed screening of secondary metabolites in crude bark extracts is often hampered by the presence of tannins, which are difficult to separate from other plant constituents.

Objective – In the present study, a new centrifugal partition extraction (CPE) method was developed in order to fractionate a crude bark extract of *Anogeissus leiocarpus* Guill. & Perr. (Combretaceae).

Methods – A three-phase solvent system composed of *n*-heptane, methyl *tert*-butyl ether, acetonitrile and water was optimised for the stepwise elution at 20 mL/min of different phytochemical classes according to their hydrophobicity. One-dimensional and two-dimensional NMR analyses of the simplified fractions were then performed in order to characterise potentially interesting metabolites.

Results – In one step, 5 g of the initial crude extract were efficiently fractionated to yield highly simplified fractions that contained triterpenes, ellagic acid derivatives, flavonoids and phenolic compounds. All undesired compounds, that is, the highly abundant water-soluble tannins (78.8%), were totally removed and each run was rapidly achieved in 90 min on a the multi-gram scale and with low solvent volumes.

Conclusion – Centrifugal partition extraction in the elution mode using a three-phase solvent system can thus be proposed as an efficient and cost-effective alternative for a rapid fractionation of crude bark extracts and for an effective screening of potentially active secondary metabolites.

Keywords: Centrifugal partition extraction; counter-current chromatography; three-phase solvent system; ellagic acid derivatives; stem bark; *Anogeissus leiocarpus*

V.1. Introduction

Fast Centrifugal Partition Extraction (CPE) is an original solid support-free liquid-liquid extraction technique that involves the distribution and the transfer of solutes between two immiscible liquid phases according to their partition coefficient. This technique is similar to Centrifugal Partition Chromatography (CPC), but with a significant difference in the partition cell design. For an equivalent column volume, the CPE rotor contains less partition cells of larger volume than a CPC column, with an oval twin-cell design and interconnected by larger ducts. These particular features make it possible to pump the mobile phase at higher flow rates, ranging from 10 to 50 mL/min, while maintaining a good hydrodynamic stability of the liquid phases inside the column.

Until now, separation procedures using CPE have only been investigated for the purification of ionic compounds when combined to the ion-exchange displacement mode [1]. Two recent articles dedicated to the purification of glycyrrhizin from licorice root and of sinalbin from white mustard seeds have demonstrated that the introduction of chemical exchange sites in the liquid stationary phase results in the recovery of highly pure ionic compounds (> 95%) [2]. In other development modes, such as elution, it is possible that the small number of partition cells in the CPE rotor could decrease the number of unit transfers and thus drastically reduce the process efficiency and thus the separation resolution. Nevertheless, a higher flow rate could favor the rapid isolation of high added-value biomolecules by fractionation of complex crude extracts.

Anogeissus leiocarpus Guill. & Perr. (Combretaceae) is a sub-Saharian African tree whose bark and leaves are traditionally used as an antimalarial remedy. Several recent studies have confirmed these effects *in vitro* on different *Plasmodium falciparum*, *Leishmania donovani* and *Trypanosoma brucei* strains [3-9]. In European countries, the ethanol-soluble fraction of the bark of *A. leiocarpus* has found applications in the cosmetic industry as a skin protective ingredient [10, 11]. A range of hydrolysable tannins, ellagic acid derivatives, flavonoids, triterpenes and saponosides have been detected in *A. leiocarpus* bark and leaves [4, 12, 13]. However, it is still unclear whether the individual components are involved in the biological effects of *A. leiocarpus* or if certain molecular associations are required to produce a biological effect. As in many plant extracts, hydrolysable and condensed tannins are largely predominant in the chemical composition of *A. leiocarpus* bark and their selective separation from other potentially interesting bioactive compounds remains difficult. In addition, tannins and other polyphenolic compounds can interfere in a non-specific way in many enzymatic tests [14]. This represents a major source of error in the evaluation of the biological activity of crude bark extract components.

The primary objective of this work was to develop a reliable method for the fractionation of the ethanolic bark extract of *A. leiocarpus*. In an attempt to capture all the compounds in the widest possible range of polarity, an original stepwise elution mode using a three-phase solvent system was proposed. This approach has never been reported in the framework of CPC or CPE separations. Only a few CCC studies have dealt with the solvent combinations that provide stable three-phase solvent systems and with the distribution pattern of solutes between the resulting immiscible phases [15-18]. Two studies have also demonstrated that the use of a three-phase solvent system in CCC enables the separation of compounds exhibiting a large polarity range, but these studies were carried out

either on a simple mixture of standard compounds [19] or on very small quantities of natural plant extracts [20].

The present work demonstrates that the high injection capacity of the CPE instrument combined to the use of a three-phase solvent system offers promising perspectives for the rapid and efficient fractionation of high quantities of crude extracts and could greatly facilitate the characterization of complex mixtures of natural products.

V.2. Experimental

V.2.1. Chemicals and reagents

Methyl-tert-butyl ether (MTBE), ethyl acetate (EtOAc), *n*-heptane, acetonitrile (CH_3CN), toluene (CH_3Ph) and sulphuric acid (H_2SO_4) were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Acetic acid (AA) was purchased from Acros Organics (New Jersey, USA) and formic acid (FA) was purchased from Prolabo (Paris, France). Deionized water was used to prepare all aqueous solutions.

V.2.2. Instrumentation

The extraction process was developed on a lab-scale Fast Centrifugal Partition Extractor of 303.5 mL capacity (FCPE300®, Kromaton Technology, Angers, France) whose rotor contains 7 circular partition disks, engraved with a total of 231 oval partition twin-cells (≈1 mL per twin-cell). The stationary phase was maintained inside the column by application of a constant centrifugal force field generated by the column rotation around a single central axis. The mobile phase was pumped by a KNAUER Preparative Pump 1800® V7115 (Berlin, Germany). The system was coupled to a UVD 170S detector (Dionex, Sunnivale, CA, USA). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). All experiments were carried out at room temperature (20 ± 2 °C).

V.2.3. Three-phase solvent system preparation and characterization

Preparation of the three-phase solvent system is described in Figure V.1. A total volume of 2.8 L was prepared by mixing *n*-heptane (700 mL), MTBE (700 mL), CH_3CN (700 mL) and water (700 mL). The solvent mixture was thoroughly equilibrated in a separatory funnel. The volumes of the

upper, middle and lower phases were measured (1288 mL, 476 mL and 1036 mL, respectively). After separation of the *n*-heptane rich upper phase (UP_0), one equivalent volume of MTBE (700 mL) was added to the mixture of middle (MP_0) and lower (LP_0) phases in order to slightly reduce the polarity of the remaining two-phase solvent system. After decantation, the final middle and lower phases (MP and LP) were separated and their respective volume was measured (1371 mL and 841 mL, respectively). The exact composition of each liquid phase at each step of this procedure was determined by ^{1}H NMR. A 200 µL aliquot of each phase was mixed with 300 µL of chloroform-*d* for the (UP_0) and DMSO-*d6* for the (MP_0, MP) and (LP_0, LP) respectively. Analyses were achieved on a Bruker Avance 500 spectrometer (^{1}H at 500.13 MHz) (Bruker, Germany). Spectra were acquired at 298 K with 1 scan (no dummy scan) over a spectral width of 12 ppm using the standard zg pulse program and a 20 s relaxation delay. The ^{1}H NMR signals of *n*-heptane, MTBE, CH_3CN and water, identified by comparison with previously published chemical shifts (Gottlieb et al., 1958), were integrated in all spectra and the volume percentage (V %) of each solvent was calculated as V % = (A/n) × (MW/d), with A = signal area, n = number of proton(s), MW = molecular weight of the solvent, d = density of the solvent at 298K.

V.2.4. Sample preparation

The crude extract (ethanol 99%) of *A. leiocarpus* bark was kindly provided by the Soliance (Pomacle (51), France) company. For each CPE run, 5 g of the crude extract was dissolved in a mixture of LP/MP/UP_0 (45:10:5 v/v) to ensure that the three liquid phases are also in equilibrium in the sample solution. The pH of the sample solution was adjusted to 4-5 with sodium hydroxide.

V.2.5. FCPE fractionation procedure

The column was filled with the LP at the minimal rotation speed of 200 rpm. The rotation speed was then increased to 1000 rpm. The sample solution was loaded into the column by progressively pumping the less polar UP_0 from 0 to 20 mL/min in 3 minutes in the ascending mode. This ramp was used to minimize the hydrodynamic perturbations during the loading step. The flow rate of the mobile phase was then maintained at 20 mL/min until the end of the experiment. The UP_0 was pumped for 50 minutes after the release of the dead volume to ensure the elution of all hydrophobic compounds. The moderately polar MP was then pumped for 33 minutes to elute compounds with a medium hydrophobicity. Finally, in order to recover the most hydrophilic compounds retained at the head of the column, the role of the two liquid phases was switched by

pumping the aqueous LP as mobile phase in the descending mode at 20 mL/min. Fractions of 20 mL were collected. The separation was monitored by UV at 210, 254, 280 and 366 nm.

V.2.6. TLC and NMR analyses of the collected fractions

All fractions were checked by Thin Layer Chromatography (TLC) on Merck 60 F_{254} pre-coated silica gel plates and developed with EtOAc/toluene/acetic acid/formic acid (70:30:11:11 v/v). UV light was used to detect ellagic acid and its derivatives at 254 and 365 nm. TLC plates were then sprayed with a vanillin solution (5% w/v in EtOH), with a H_2SO_4 solution (50% v/v in MeOH), and finally revealed by heating at 100-120°C for 2-3 min. Some fractions were combined on the basis of TLC composition similarities. About 20 mg of each pooled fraction were dissolved in 600 µL DMSO-*d6* and analyzed by NMR spectroscopy at 298 K on a Bruker Avance AVIII-600 spectrometer. ^{1}H and ^{13}C NMR spectra were acquired at 600.15 MHz and 150.91 MHz, respectively. Additional HSQC, HMBC, and COSY 2D-NMR experiments were performed using standard Bruker microprograms.

V.3. Results and discussion

V.3.1. Three-phase solvent system modification and characterization

Due to the high chemical diversity within the *A. leiocarpus* crude bark extract, it was difficult to find an efficient, rapid and direct fractionation method by means of a classical biphasic solvent system. This was mainly due to the large polarity range that was covered by the different *A. leiocarpus* components (triterpenes, ellagic acid and derivatives, saponosides, flavonoids, phenolic acids …). The use of MTBE in the solvent system was essential to guarantee good solubility and partition of the ellagic and phenolic acid derivatives in the liquid phases. Because the initial crude extract was composed of 80% (w/w) water-soluble tannins, a highly polar stationary phase, rich in water and CH_3CN, was selected in order to trap them in the CPE column. On this basis, we first selected a biphasic solvent system made of MTBE/CH_3CN/water (4/1/5, v/v/v). However, the fractionation of the less polar compounds, including triterpenes and the non-glycosylated ellagic acid derivatives was not selective. This problem was circumvented by adding a fourth apolar solvent (*n*-heptane) to the solvent system.

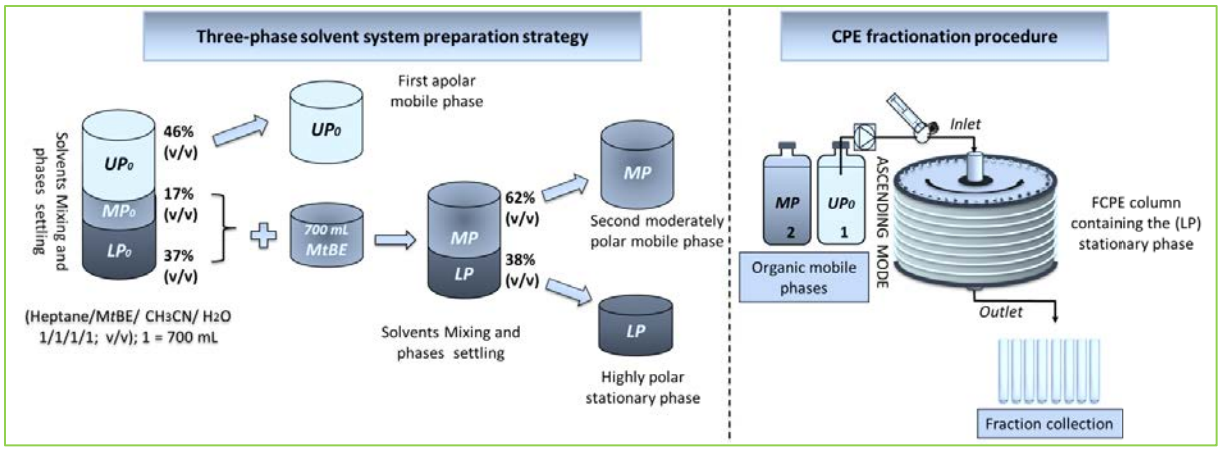

Figure V. 1 : Three-phase solvent system preparation (left) and CPE-fractionation procedure for the extraction and polyphenol enrichment of a crude bark extract of Anogeissus leiocarpus

Very few liquid-liquid extraction and separation methods use a three-phase solvent system, mainly because of the limited solvent combinations that lead to stable and volume-equilibrated immiscible phases. When working with a complex natural extract, the solvent system must also be selected for its ability to completely dissolve the sample, to distribute components between the immiscible phases (UP_0, MP and LP) and to keep a high hydrodynamic stability during the fractionation process. Ternary solvent systems composed of *n*-hexane, CH_3CN and water in combination with a fourth solvent like methyl acetate, EtOAc or MTBE have previously been described [15, 19, 22]. In particular, the system suggested by Shibusawa et *al*. [19], composed of *n*-hexane/MTBE/CH_3CN/water in the proportions 2:2:3:2 (v/v) resulted in stable and well-equilibrated volume percentages between the three phases (UP_0 35%; MP 35%; LP 30%).

	System A (n-heptane/MTBE/CH_3CN/water, 1:1:1:1, v/v)			System A' (MP_0/LP_0 + 1 volume of MTBE)		Phase composition before and after contacting UP_0 with LP			
	UP_0 ($\%_V$)	MP_0 ($\%_V$)	LP_0 ($\%_V$)	MP ($\%_V$)	LP ($\%_V$)	$UP_{0\ (before)}$ ($\%_V$)	$UP_{0\ (after)}$ ($\%_V$)	$LP_{(before)}$ ($\%_V$)	$LP_{(after)}$ ($\%_V$)
***n*-heptane**	52.3	5.3	0	3.1	0.3	52.3	53.2	0.3	0
MTBE	37.9	29.9	5.8	60.3	5.3	37.9	39.8	5.3	2.4
CH_3CN	9.9	54.0	30.9	30.4	18.7	9.9	6.4	18.7	21.4
Water	0.02	11.5	63.3	6.1	75.8	0.02	0.5	75.8	76.2

Table V. 1: ^{1}H-NMR characterization of the initial upper (UP_0), middle (MP_0) and lower (LP_0) phases of the three-phase solvent system n-heptane/ MTBE/CH_3CN/ water (1:1:1:1, v/v) (system A) and after modification by adding one supplementary volume of MTBE to the mixture of MP_0/LP_0, resulting in the middle phase MP and lower phase LP (system A'). Phase composition before and after contacting UP_0 and LP at the first step of the elution process

(i)

In the present study, *n*-hexane was replaced by *n*-heptane which is much less toxic [23], and presents similar physico-chemical properties (density, viscosity and polarity). However, the use of this system in the 2:2:3:2 (v/v) proportions in our CPE process resulted in a total loss of the stationary phase when switching the first to the second mobile phase. These strong hydrodynamic perturbations were probably linked to the higher flow rate (20 mL/min) of the CPE method, compared to the previously published CCC method (2 mL/min), and also to the complexity of the natural extract that was loaded into the CPE column. With a 1:1:1:1 (v/v) composition, the *n*-heptane/MTBE/CH_3CN/water solvent system was more stable in our operating conditions but resulted in volume percentages of 46%, 17% and 37% for the upper phase (UP_0), middle phase (MP_0) and lower phase (LP_0), respectively. To circumvent this volume disparity, their composition was determined by ^{1}H NMR (Table V.1). In this way, each phase could be prepared independently in the required volume. These ^{1}H NMR phase data were extremely useful for the preparation of each phase independently in the desired volume and thus significantly helped to reduce the solvent needed in each CPE experiment. In addition, the high content of CH_3CN (54%, see Table V.1) in MP0 induced a partial solubility of the tannins in this phase and their simultaneous elution with other moderately hydrophobic compounds (flavonoids and glycosilated ellagic acid derivatives) when the second mobile phase was pumped. For these reasons we decided to reduce the polarity of the middle phase MP_0 by adding one supplementary volume of MTBE to the MP_0/LP_0 system after removing UP_0.

The exact composition of each phase before and after MTBE volume adjustment was determined by ^{1}H NMR. The resulting phase compositions are reported in Table V.1. The apolar UP_0 was made up of a majority of *n*-heptane (52.3%) and MTBE (37.9%), and the initial middle phase MP_0 of CH_3CN (54.0%) and MTBE (29.9%). The high percentage of CH_3CN in the initial MP_0 used as a second mobile phase (54.0%) explains the partial solubility of the highly polar tannins during the process. After adjustment of the solvent system polarity with MTBE, the CH_3CN volume percentage represented only 30.4%, MTBE 60.3% and water 6.1% of the final middle phase (MP) composition, indicating a significant reduction of the phase polarity. In these conditions all the highly polar tannins were retained inside the stationary phase during the overall process duration. As the first step of the fractionation process requires a contact between the upper phase (UP_0) and the modified lower phase (LP), it was interesting to examine the variation of both phases after contact and thermodynamic equilibrium were established. As indicated in Table V.1, no significant variation in their composition was observed. These data confirmed that even if UP_0 and LP are no longer in equilibrium after modification of the three-phase solvent system by additional MTBE, these phases are anyhow so different that their contact exerts a limited alteration of their initial composition.

V.3.2. Hydrodynamic behavior and evolution of the three phase solvent system during the CPE fractionation process

In CCC and CPC separation techniques, the volume of the stationary phase remaining in the column (*i.e*. the relative retention volume Sf %) is an essential parameter which directly influences the separation quality [24]. For instance, in the elution mode, this retention volume plays a key role on the number of theoretical plates and resolution [25]. A significant loss of stationary phase can result from mutual solvent solubility if mobile and stationary phases are not in equilibrium, from the application of high flow rates and also from the nature, composition and mass of the loaded sample. When the sample mass loading is too high, the hydrodynamic equilibrium between the immiscible phases can be strongly affected, for example through emulsion formation, especially when amphiphilic substances are present in the loaded mixture or by affecting the density difference between the phases [26]. This generally results in a drastic loss of stationary phase and thus in a significant decrease in process capacity. In our operating conditions, the initial stationary phase retention was 68 % when working at 20 mL/min after loading 5 g of the crude bark extract of *A. leiocarpus*. This value was certainly good with respect to the maximum (76%) which can be obtained with this instrument (the interconnecting ducts involved in the transport of the mobile phase between partition cells represent 24% of the total CPE column volume) [1].

During the first step of the process, the n-heptane rich UP_0 was pumped at 20 mL/min through the lower stationary phase in order to elute the most hydrophobic compounds. After the release of the solvent front (10 min after t_0), no bleeding was observed, indicating that the stationary phase (LP) was well retained inside the CPE column. In the second step (59 min after t_0), the mobile phase was changed to the moderately polar MP while maintaining the same flow rate at 20 mL/min. At this stage, all the UP_0 of the three-phase solvent system left the column and was replaced by the MP. Stationary phase bleeding was observed until a new hydrodynamic equilibrium was reached but did not exceed 26 mL (8.6% of the column volume). This data indicates a good hydrodynamic stability of the solvent system over the whole experiment. From 59 to 93 min, all compounds of medium hydrophobicity were fractionated. Finally, the role of LP as stationary phase was switched, *i.e*. LP was used as mobile phase and pumped in the descending mode in order to recover the most hydrophilic compounds which were retained in the stationary phase. The two first fractions collected from this step represented 78.8% of the crude bark extract mass and were mainly composed of tannins, suggesting that these highly polar compounds were strongly retained at the head of the CPE column.

V.3.3. Phytochemical composition of the collected fractions

In an attempt to obtain a sufficient mass of each collected fraction for further structural investigations in a single run, 5 g of the ethanolic bark extract of *A. leiocarpus* was fractionated. Using our method, all of the tannins (78.8%) were fully retained inside the stationary phase over the whole experiment. As a result, the 96 collected fractions were highly simplified in comparison with the initial mixture. Moreover, the order in which the compounds emerged was closely related to their hydrophobic character. Some fractions were combined on the basis of composition similarities using TLC fractogram analysis to yield 13 pools, denoted as F_I to F_{XIII}. Each pool was analyzed by NMR in order to characterize the class of compounds in each pool and to identify at least one major member of each class. As illustrated in Figure V.2, all of the most hydrophobic compounds were eluted by the *n*-heptane-rich mobile phase during the first step of the process (from t0 to 58 min). In total, 340mg (from F_I to F_{III}) were recovered, corresponding to 6.8% (w/w) of the initial crude extract.

The pool F_I was composed of a complex mixture of minor lipid compounds, which were eluted just after the release of the mobile phase solvent front. The fraction pool F_{II}, eluted from 15 to 36 min contained a major triterpene, identified as sericic acid (TTP1), and two ellagic acid derivatives, identified as 3,4,3'-tri-*O*-methylflavellagic acid (EAd2) and 3,3'-di-*O*-methylellagic acid (EAd3) on the basis of ^{1}Hand ^{13}C NMR spectral data. Their structures (Figure V.3) were validated by further 2DNMR analyses (HSQC and HMBC) and by comparison with reference NMR data from the literature [27, 28]. The elution of EAd2 and EAd3 by the *n*-heptane-rich UP_0 was principally linked to their relatively high hydrophobicity, due to the absence of glycoside substituent. During the second step of the CPE process, other compounds progressively migrated from the stationary phase to the MTBE-rich mobile phase. This elution resulted from their moderate hydrophobic character, solubility and strong affinity for MTBE. A total of 719mg were recovered from 59 to 92min (from F_{IV} to F_{XII}), representing 14.4 % (w/w) of the initial crude bark extract. One major triterpene (TTP2) was detected in F_{IV} and F_V. The ^{1}H, ^{13}C, HSQC and HMBC NMR signals detected in these fractions were consistent with the structure of trachelosperogenin E.

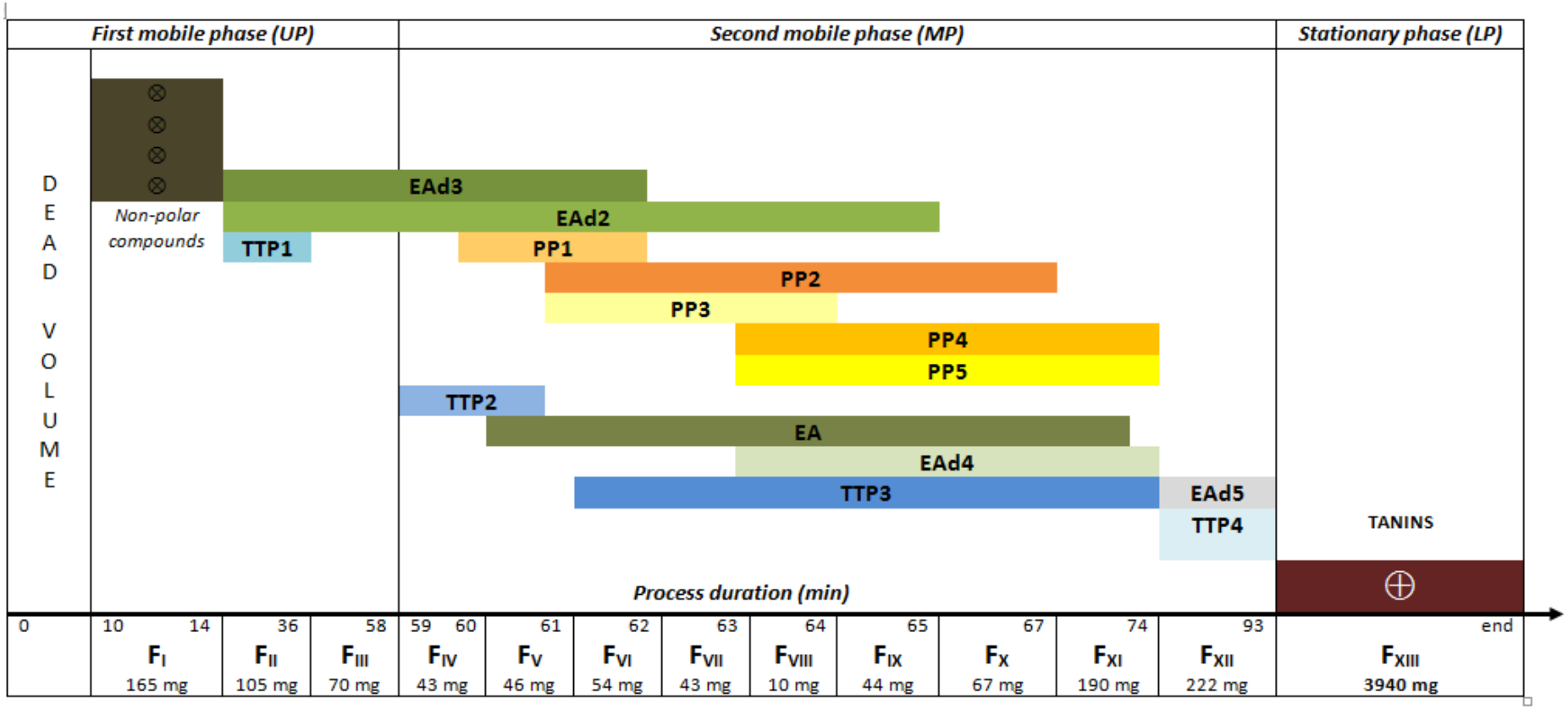

Figure V. 2: Elution scheme obtained after the CPE fractionation of a crude bark extract of Anogeissus leiocarpus using a three-phase solvent system

EA: *Ellagic acid*
EAd2: *3,4,3'-tri-O-methylflavellagic acid*
EAd3 : *3,3'-di-O-methylellagic acid*
EAd4: *3,3'-di-O-methylellagic acid 4-O-xylopyranoside*
EAd5: *3,4,3'-tri-O-methylflavellagic acid 4-O-glucopyranoside*

TTP1 : *Sericic acid*
TTP2: *Trachelosperogenin E*
TTP3: *Sericoside*
TTP4: *non-identified*

PP1: *Protocatechuic acid*
PP2: *Gallic acid*
PP3: *(+)-Catechin*
PP4: *(+)-Gallocatechin*
PP5: *(-)-Epigallocatechin*

(k)

Another major saponin (TTP3) identified as sericoside was detected in fractions eluting from 61 to 74 min (F_{VI} to F_{XI}). This data indicated that the present CPE process enables the separation of closely related triterpenes and saponins (sericic acid, trachelosperogenin E and sericoside) by order of their polarity. Analyses of F_{IV}-F_{XII} also revealed the presence of ellagic acid (EA), 3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside (EAd4) and 3,4,3'-tri-*O*-methylflavellagic acid 4-*O*-glucopyranoside (EAd5) [12, 29, 30]. It can also be noted that EAd2 and EAd5, both derived from a flavellagic skeleton, were isolated as pure compounds after spontaneous crystallization in the fraction pools F_{III} and F_{XII}, respectively. Two phenolic acids (PP1 and PP2), as well as three proanthocyanidins (PP3, PP4 and PP5), were eluted between 61 and 74 min in an increasing order of polarity (Figure V.2). After comparing their chemical shifts with previously published NMR spectral assignments, PP1 was identified as protocatechuic acid, PP2 as gallic acid, PP3 as catechin, PP4 as gallocatechin and PP5 as epigallocatechin [31, 32].

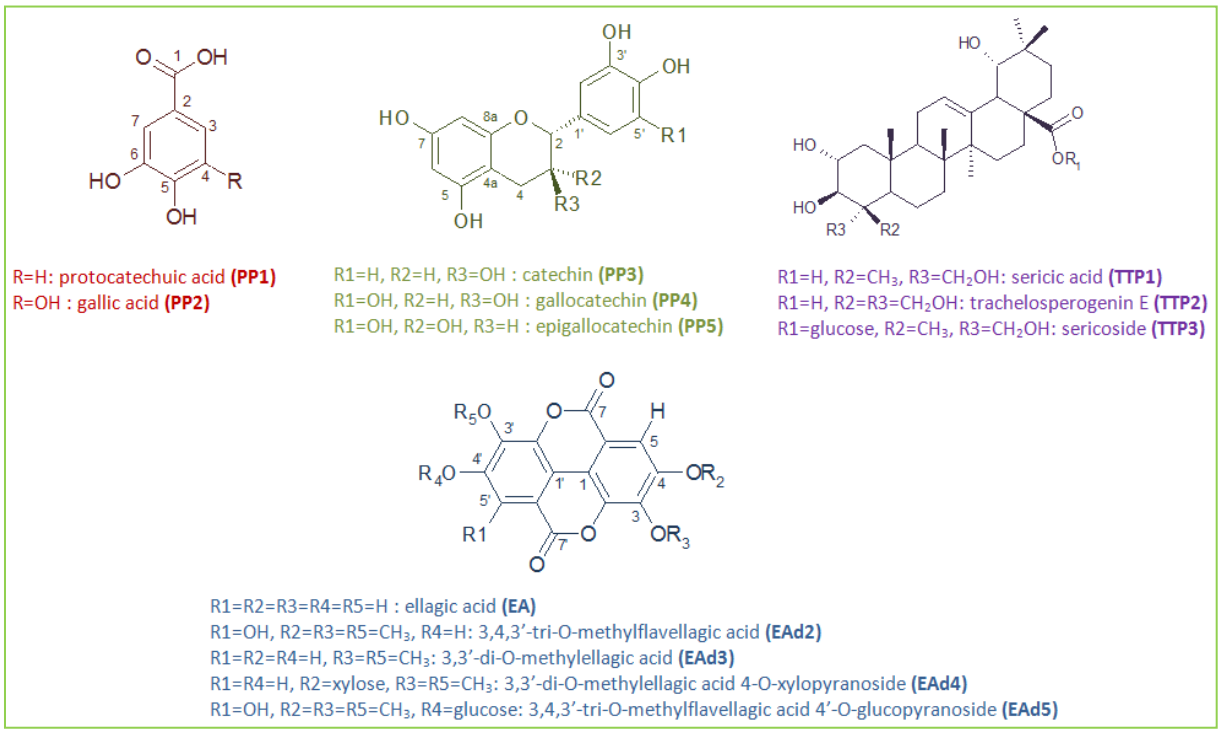

Figure V. 3: Chemical structures of the major phytochemicals recovered from the crude ethanolic extract of the bark of Anogeissus leiocarpus

V.4. Conclusion

Centrifugal Partition Extraction in the elution mode with a three-phase solvent system led to a rapid and effective fractionation of the crude bark extract of *A. leiocarpus*. The total removal of undesired compounds, i.e. the highly abundant water-soluble tannins (78.8%), was accomplished while enriching simplified fractions in different ellagic acid derivatives, flavonoids and triterpenoids. This CPE process thus provides an interesting alternative for the fractionation of complex natural products, as compared to conventional chromatographic or sequential solvent extraction methods. Each run was rapidly achieved at a multi-gram scale, in a single step, and with low solvent volumes. Thus, starting from 5 g of a crude bark extract, the quantity of recovered fraction pools were sufficient to achieve the structural characterization of potentially interesting metabolites while leaving ample material for future biological evaluation of these compounds.

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CHAPITRE 3 – PARTIE 2:

EXTRACTION DE PARTAGE CENTRIFUGE EN MODE DE DÉPLACEMENT PAR pH-ZONE-REFINING : UNE APPROCHE EFFICACE POUR LE FRACTIONNEMENT BIO-GUIDÉ ET L'ISOLEMENT DE COMPOSÉS PHÉNOLIQUES NATURELS

CHAPTER 3 – PART 2:

CENTRIFUGAL PARTITION EXTRACTION IN THE pH-ZONE-REFINING DISPLACEMENT MODE: AN EFFICIENT STRATEGY FOR THE SCREENING AND ISOLATION OF BIOLOGICALLY ACTIVE PHENOLIC COMPOUNDS

VI. Extraction de partage centrifuge en mode de déplacement par pH-zone-refining : Une approche efficace pour le fractionnement bio-guidé et l'isolement de composés phénoliques naturels

Introduction du chapitre 3 – Partie 2

Ce chapitre est consacré à l'étude des potentialités de l'extracteur de partage centrifuge en tant qu'instrument permettant de simplifier des extraits naturels complexes par extraction fractionnée sélective et enrichissement des fractions en composés phénoliques. Dans ce contexte, l'extrait éthanolique de l'écorce d'*Anogeissus leiocarpus* Guill. & Perr. (Combretacée) a été pris comme support méthodologique. La méthode de fractionnement a été développée par pH-zone-refining, basée sur le changement du coefficient de distribution (K_D) des composés ciblés (dérivés de l'acide éllagique notamment) par simple variation du pH (protonation et déprotonation des métabolites). Les fractions enrichies ont été rassemblées en se basant sur leurs profils CCM avant d'être analysées par RMN 1D et 2D. Cette caractérisation avait pour but de déterminer la composition de chaque fraction et l'identification des constituants majeurs.

Les fractions générées à l'issue de ce procédé ont été évaluées pour leur potentiel antioxydant par analyse chimique (piégeage du radical DPPH$^{\bullet}$). Une relation structure/activité a été ensuite proposée, mettant en évidence l'influence du degré de substitution du squelette acide éllagique sur le potentiel antioxydant.

Centrifugal partition extraction in the pH-zone-refining displacement mode: An efficient strategy for the screening and isolation of biologically active phenolic compounds

*Mahmoud Hamzaoui[a], Jean-Hugues Renault[a], Romain Reynaud[b] and Jane Hubert[a]**

[a]UMR CNRS 7312, Université de Reims Champagne-Ardenne, Bât. 18, Moulin de la Housse, BP 1039, 51687 Reims, Cedex 2, France

[b]Soliance, Route de Bazancourt - 51110 Pomacle France

Abstract

Centrifugal partition extraction (CPE) was developed for the first time in the pH-zone-refining mode to fractionate a crude ethanolic extract of the bark of the African tree *Anogeissus leiocarpus* Guill. & Perr. (Combretaceae). The fractionation process was performed at a flow rate of 20 mL/min using a biphasic solvent system composed of methyl *tert*-butyl ether/acetonitrile/water (4:1:5, v/v/v) in the ascending mode. Sodium hydroxide (40 mM) and trifluoroacetic acid (30 mM) were used as retainer and displacer agents, respectively. In a single run of 67 min, 3 g of the initial crude extract were successfully separated into fractions selectively enriched in ionizable triterpenes, ellagic acid derivatives and flavonoids. The antioxidant potential of the initial crude extract, isolated compounds and fraction pools was also evaluated by using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) stable free radical scavenging assay, providing an interesting view about the effect of the degree of substitution of ellagic acid derivatives on their radical scavenging activity. Centrifugal partition extraction used in the pH-zone-refining mode can thus be proposed as an efficient strategy for the rapid screening of antioxidant phenolic compounds from complex natural extracts.

Keywords: Centrifugal partition extraction, centrifugal partition chromatography, pH-zone-refining, *Anogeissus leiocarpus*, ellagic acid, DPPH.

VI.1. Introduction

Plant-derived secondary metabolites represent a valuable source of bioactive molecules to be used in the cosmetic or pharmaceutical industries [1]. However, the main challenge for their isolation arises from the complex nature of plant extracts (leaves, stems, barks, roots…) which are composed of highly diverse chemical structures with distinct physico-chemical properties. Efficient and versatile fractionation methods are thus required to facilitate the screening and purification of bioactive molecules from complex natural mixtures.

Centrifugal Partition Extraction (CPE) is a solid support-free liquid-liquid extraction technique involving the distribution and the transfer of solutes between at least two immiscible liquid phases according to their partition coefficient. This technique is similar to centrifugal partition chromatography (CPC), but for an equivalent capacity, a CPE column contains less partition cells of larger volume and interconnected in series by larger ducts. These characteristics make it possible to pump the mobile phase at higher flow rates, to handle higher sample quantities and thus to enhance the productivity. It was previously demonstrated that CPE combined to the strong ion-exchange (SIX) displacement mode can result in the recovery of highly pure ionic molecules (purity >95%). For instance, 2.2 g of the biologically active ionic saponin glycyrrhizin were isolated by SIX-CPE in a single run of 90 min starting from 20 g of a licorice root extract [2]. In another work, 4.6 g of the glucosinolate sinalbin were isolated from 25 g of a crude extract of white mustard seeds in only 32 min [3]. More recently, it was demonstrated that CPE combined to the use of a three-phase solvent system in a sequential elution mode enables the selective fractionation of different chemical classes present in a crude bark ethanolic extract of the African tree *Anogeissus leiocarpus* on a multi-gram scale (5 g) [4]. In this previous study, the undesired water-soluble tannins representing more than 80% (w/w) of the crude extract were totally removed while fractions highly enriched in triterpenes, ellagic acid derivatives, flavonoids and phenolic acids were obtained in only 90 min.

Considering the possibility to protonate or deprotonate phenolic compounds under pH modifications, it could be expected that the pH-zone-refining mode, another displacement mode commonly used in solid support-free liquid-liquid chromatography, would be very interesting when combined to CPE for the separation of phenolic compounds present in the crude bark extract of *Anogeissus leiocarpus*. The pH-zone-refining mode is basically dedicated to the separation of compounds whose electric charge depends on the pH-value [5-7]. It has been widely used for the separation of alkaloids [7-10], amino-acids, peptides [11-13], and dyes [14-17], but a very few studies were devoted to the separation of polyphenols [18-21]. When working in the ascending mode, the

ph-zone-refining method is generally performed by adding a base used as a “retainer” to the aqueous stationary phase to solubilize and capture the target analytes inside the column. The pK_a of the retainer must be higher than that of the target analytes. Then an acid is added to the organic mobile phase as a “displacer” in order to recover the analytes in the decreasing order of pK_a and hydrophobicity [19-22]. Since the analytes are organized inside the column according to their respective acidity constants and hydrophobicity, the pH-zone-refining mode results generally in highly selective separations.

In the present study, the pH-zone-refining mode was investigated for the first time in combination to centrifugal partition extraction (CPE) for the separation of phenolic compounds. The crude bark extract of *Anogeissus leiocarpus* previously fractionated by CPE using a three-phase solvent system in the sequential elution mode [4], was selected as separation model. Briefly, *Anogeissus leiocarpus* Guill. & Perr. (Combretaceae) is a sub-Saharian African tree whose bark and leaves are traditionally used as an antimalarial remedy [23-29]. In European countries, the ethanol-soluble fraction of *Anogeissus leiocarpus* bark has found applications in the cosmetic industry as a skin protective ingredient [30-32]. A range of water-soluble tannins, ellagic acid derivatives, flavonoids, triterpenes and saponosides are present in this crude ethanolic extract. However, it is still unclear whether the individual components are involved in the skin protective effects of *Anogeissus leiocarpus* or if certain molecular associations are required to produce a beneficial activity.

Here, the fractions obtained from the pH-zone-refining CPE fractionation process were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay in order to identify the compounds having the highest activity. Even if a simple DPPH spectrophotometric assay does not exactly reflect a real biological effect, it can rapidly give an idea about the antioxidant potential of natural crude extracts, fraction pools or isolated molecules. The strategy presented in this paper shows that CPE used in the pH-zone-refining mode enables a rapid screening and isolation of antioxidant compounds from crude natural mixtures.

VI.2. Experimental

VI.2.1. Materials

Methyl *tert*-butyl ether (MTBE), ethyl acetate (EtOAc), acetonitrile (CH_3CN), trifluoroacetic acid (TFA) and toluene were purchased from Carlo ErbaReactifs SDS (Val de Reuil, France). Both sodium hydroxide (NaOH) and formic acid were purchased from PROLABO (Paris, France) and acetic

acid was purchased from ACROS ORGANICS (New Jersey, USA). Deuterated dimethyl sulfoxide (DMSO-*d*6) was obtained from Sigma-Aldrich (Saint-Quentin, France). Deionized water was used to prepare all aqueous solutions. The crude bark extract of *Anogeissus leiocarpus* (99% in ethanol) was kindly provided by the Soliance society (Pomacle, France). DPPH (1,1-diphenyl, 2-picrylhydrazyl) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

VI.2.2. Instrumentation

The laboratory-scale Fast Centrifugal Partition Extractor FCPE300® (Kromaton Technology, Angers, France) was equipped with a rotor made of 7 circular partition disks containing a total of 231 partition twin cells connected together by ducts of 0.8 mm internal width. The column capacity is 303.5 mL. The mobile phase can be pumped either in the ascending or descending mode with low residual pulsation through a KNAUER Preparative Pump 1800® V7115 (Berlin, Germany). The CPE system was coupled to a UVD 170S detector (Dionex, Sunnivale, CA, USA) equipped with a preparative flow cell (6 µL internal volume, 2 mm path length) and monitored at 210 nm and 254 nm. Fractions of 20 mL were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). Samples were injected through a 40 mL sample loop. All experiments were conducted at room temperature (20 ± 2 °C).

VI.2.3. pH-zone-refining CPE fractionation procedure

A biphasic solvent system of 2 L was prepared by mixing into a separatory funnel MTBE, CH_3CN and water in the proportions 4/1/5 (v/v). After solvent system equilibration, the two liquid phases were separated. NaOH was used as retainer in the aqueous stationary phase (40 mM, pH ≈ 10) and TFA was used as displacer in the organic mobile phase (30 mM, pH ≈ 2). The column was filled at 200 rpm with the basic aqueous stationary phase and the rotation speed was then adjusted to 1000 rpm. The crude ethanolic extract of the bark of *Anogeissus leiocarpus* (3 g) was dissolved in a 40mL mixture of aqueous phase/TFA-free organic phase in the proportions 8/2 (v/v). The sample solution was then adjusted to pH ≈ 10 with NaOH and loaded into the column. The fractionation procedure was initiated by pumping progressively the acid mobile phase in the ascending mode from 0 to 20 mL/min in 5 min. This flow rate ramp was used to minimize the hydrodynamic disturbance which can occur inside the partition cells of the CPE column during the loading of the highly concentrated sample solution (75 g/L). The flow rate was then maintained at 20 mL/min. The pH of the collected fractions was checked over the whole experiment using a pH indicator paper.

VI.2.4. TLC and NMR analyses of the CPE fractions

The collected fractions were analyzed by thin-layer chromatography (TLC) on Merck 60 F254 pre-coated silica gel plates and developed with toluene/EtOAc/acetic acid/water (30:70:11:11, v/v). Detection was performed under UV light (254 and 366 nm) and by spraying a 5% (w/v) vanillin solution in EtOH 95% followed by a 50% (v/v) sulfuric acid solution in MeOH and heating the plate at 100-120°C for 2-3 min. Fractions were then pooled on the basis of their TLC profile similarities, resulting in 8 pools noted from P_I to P_{VIII}. Each pool was dissolved in 600 µL of DMSO-*d6* and analyzed by NMR spectroscopy at 298 K on a Bruker Avance AVIII-600 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 600.15 MHz and 150.04 MHz, respectively to determine the global composition and the major compound(s) present in each pool. More details about the mass and global composition of each fraction pool from P_I to P_{VIII} are given in Table VI.1.

Fraction Pool	*Mass (mg)*	*Global composition*	*CPE output*
P_I	200.1	Complex mixture of minor hydrophobic compounds	**Zone 1 = elution** from t_0 = 8.7 to 45 min
P_{II}	21.4	Pure sericoside	
P_{III}	155.4	Mixture of triterpenes among which trachelosperogenin E and sericic acid were predominant	**Zone 2 = displacement** from 45.1 to 64 min
P_{IV}	22.7	Ellagic acid + mixture of minor phenolic acids	
P_V	24	Mixture of flavonoids among which catechin, gallocatechin and epigallocatechin were predominant	
P_{VI}	9.3	3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside + minor ellagic acid derivatives + minor flavonoids	
P_{VII}	74	3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside + minor ellagic acid derivatives	
P_{VIII}	2265.1	Water-soluble tannins	**Zone 3 = dual-mode** from 64.1 to 67 min

Table VI. 1: Description of the fraction pools from P_I to P_{VIII} obtained after the fractionation of the crude bark extract of Anogeissus leiocarpus by CPE combined to the pH-zone-refining mode

VI.2.5. DPPH free radical scavenging assays

The free radical scavenging activity of the crude extract, fraction pools P_{III}, P_{IV}, P_{VI}, and P_{VIII}, and of pure compounds ellagic acid, 3,3'-di-*O*-methylellagic acid, 3,3'-di-*O*-methylellagic acid 4-*O*-

xylopyranoside was evaluated by using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Briefly, a stock solution of DPPH in EtOH/water (1:1, v/v) was prepared at a concentration of 133.2 µg/mL. Each sample was then dissolve in DMSO and serially diluted as indicated in Table VI.2. Each solution from S_1 to S_4 (0.5 mL) was added to the DPPH stock solution (0.5 mL). these solutions were prepared in triplicate. The evolution of the $DPPH^{\bullet}$ absorbance in each reaction mixture was monitored at 515 nm by using a UV-Vis Spectrophotometer Micro-plate reader (FLUOstar Omega, BMG-LABTECH). Absorbance measurements were performed each 1.5 min for 70 min at room temperature for all samples. The EtOH/water (1:1, v/v) solution was used as a blank and the free DPPH solutions were used as a control. The absorbance of S_4, allowing the best visualization of the reaction kinetics, was then plotted as a function of reaction time.

Samples	*Concentration (g/L)*			
	S_1	S_2	S_3	S_4
DPPH	0.1	0.1	0.1	0.1
Crude extract	5.3	2.6	1.3	0.6
P_{III}	5.1	2.5	1.3	0.6
P_{IV}	5.7	2.8	1.4	0.7
P_{VI}	4.7	2.3	1.2	0.6
P_{VIII}	5.1	2.5	1.3	0.6
P_{II} (Sericoside)	5.2	2.6	1.3	0.6
Ellagic acid	5.2	2.6	1.3	0.6
3,3'-di-*O*-methylellagic acid	5.5	2.8	1.4	0.7
3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside	4	2	1	0.5

Table VI. 2: *Sample preparation for the DPPH free radical scavenging assays*

VI.3. Results and discussion

VI.3.1. Hydrodynamic behavior of the biphasic solvent system during the CPE fractionation process

Solvent system selection strongly influences the performance of solid support-free liquid-liquid separation processes. Biphasic solvent systems composed of MTBE and water are commonly used when applying the pH-zone-refining mode in countercurrent chromatography (CCC) or centrifugal partition chromatography (CPC) [5, 33-35]. In this study, a solvent system composed of

MTBE/CH_3CN/water in the proportions 4/1/5 (v/v) was used. CH_3CN was used as "best solvent" to guarantee the solubility of the phenolic compounds present in the initial extract and to increase the polarity of the organic mobile phase. Starting from 3 g of the crude bark extract of *Anogeissus leiocarpus,* the CPE fractionation procedure was performed at a flow rate of 20 mL/min and at a rotation speed of 1000 rpm. Under these operating conditions, the pressure at the head of the column increased up to≈ 9 bars at the mobile phase front output (t_0 = 8.7 min) and then was stabilized at 6 bars until the end of the experiment. The initial (Sf_i) and final (Sf_f) stationary phase retention volumes, defined as the volume percentage of stationary phase remaining inside the column at t_0 and at the end of the experiment, were 59.1% and 45.3%, respectively. These values were satisfying regarding the complex phytochemical composition and the high amount of the crude extract (3 g) loaded into a column of only 303.5 mL. A significant loss of stationary phase (28 mL) was observed from t_0 = 8.7 min to 14.7 min, probably induced by the progression of a highly concentrated mixture of hydrophobic compounds through the CPE partition cells. An additional stationary phase loss of 14 mL was also measured from 45 min to 51 min, indicating the beginning of the displacement step of the pH-zone-refining CPE fractionation process. Such stationary phase loss during the beginning of the displacement step has already been observed when performing CPE in the ion-exchange mode [2, 3]. It can be attributed to the high concentration of the displaced compounds which probably disturbed the hydrodynamic behavior of the liquid phases inside the CPE column and thus induced a slight loss of stationary phase.

VI.3.2. Fractionation of the crude bark extract by CPE in the pH-zone-refining mode

The pH-zone-refining CPE fractionation process was performed by supplementing the aqueous stationary phase with the retainer NaOH (40 mM) to ensure the deprotonation of all ionizable compounds and therefore to promote their trapping into the aqueous stationary phase at the beginning of the experiment. At the same time, all the non-ionizable hydrophobic compounds were eluted through the CPE column according to their distribution coefficient. The role of the acidic displacer TFA added to the organic mobile phase (30 mM) was to neutralize progressively the retainer (OH^-) and to displace the ionizable compounds from the stationary to the mobile phase according to their electric charge. The whole fractionation process was achieved in 65 min. In total, 66 fractions were collected, checked by TLC and then gathered together into 8 fraction pools, noted from P_I to P_{VIII}, according to their TLC profile similarities (Table VI.1). As illustrated in Figure VI.1, after the mobile phase solvent front (t_0 = 8.7 min), three zones can be distinguished. The first zone (from t_0 to 45 min) corresponded to the elution of a complex mixture of minor hydrophobic compounds

which were not trapped into the aqueous stationary phase. These compounds, corresponding to the pool P_I (200.1 mg, 7.2 % of the crude extract mass), were expelled from the column from t_0 to 35 min due to their high affinity for the organic mobile phase rich in MTBE. The elution of pure sericoside (*C1*) (Figure VI.2) was then observed from 35 to 45 min, corresponding to the pool P_{II} (21.4 mg, 0.8 % of the crude extract mass). The identity of sericoside was confirmed by 1D and 2D NMR analyses (Table VI.3). The second zone from 45 min to 64 min corresponded to the displacement step of the pH-zone-refining fractionation process. This zone consisted in the release and selective transfer from the aqueous stationary phase to the organic mobile phase of all pH-sensitive compounds under the effect of the displacer agent TFA. The fraction pools from P_{III} to P_{VII} obtained during this displacement step corresponded to a total mass of 285.4 mg (10.3 % of the crude extract mass). TLC and NMR analyses of the pool P_{III} revealed a mixture of ionizable triterpenes among which trachelosperogenin E (*C2*) and sericic acid (*C3*) (Figure VI.2) were largely predominant. The ^{1}H and ^{13}C NMR spectral characteristics of both compounds are given in Table VI.3.

The displacement mechanism of these two compounds was directly linked to the presence of a carboxylic acid group in their triterpene skeleton. The pool P_{IV} (22.7 mg, 0.8 % of the crude extract mass), consisted in a single fraction composed of a mixture of phenolic acids (gallic acid (*C4*) and protocatechuic acid (*C5*)), flavonoids and ellagic acid derivatives among which ellagic acid (*C6*) was predominant. Afterwards a mixture of flavonoids was displaced and the resulting pool P_V (24 mg, 0.9% w/w) was mainly composed of catechin (*C7*), gallocatechin (*C8*) and epigallocatechin (*C9*) (Figure VI.2) on the basis of ^{1}H and ^{13}C NMR analyses (Table VI.3). Their structures were also confirmed by comparison with reference data found in the literature. The next pool P_{VI} (9.3 mg, 0.3 % w/w) was composed of 3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside (*C10*) (Figure VI.2) as major constituent mixed with the same catechin-derived flavonoids as detected in the pool P_V. The fraction pool P_{VII} was only composed of substituted ellagic acid derivatives including 3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside (*C10*), 3,3'-di-*O*-methylellagic acid (*C11*) and 3,4,3'-tri-*O*-methylflavellagic acid (*C12*) as major constituents (Figure VI.2). The recovered mass of P_{VII} was 74 mg (2.7 % of the crude extract mass).

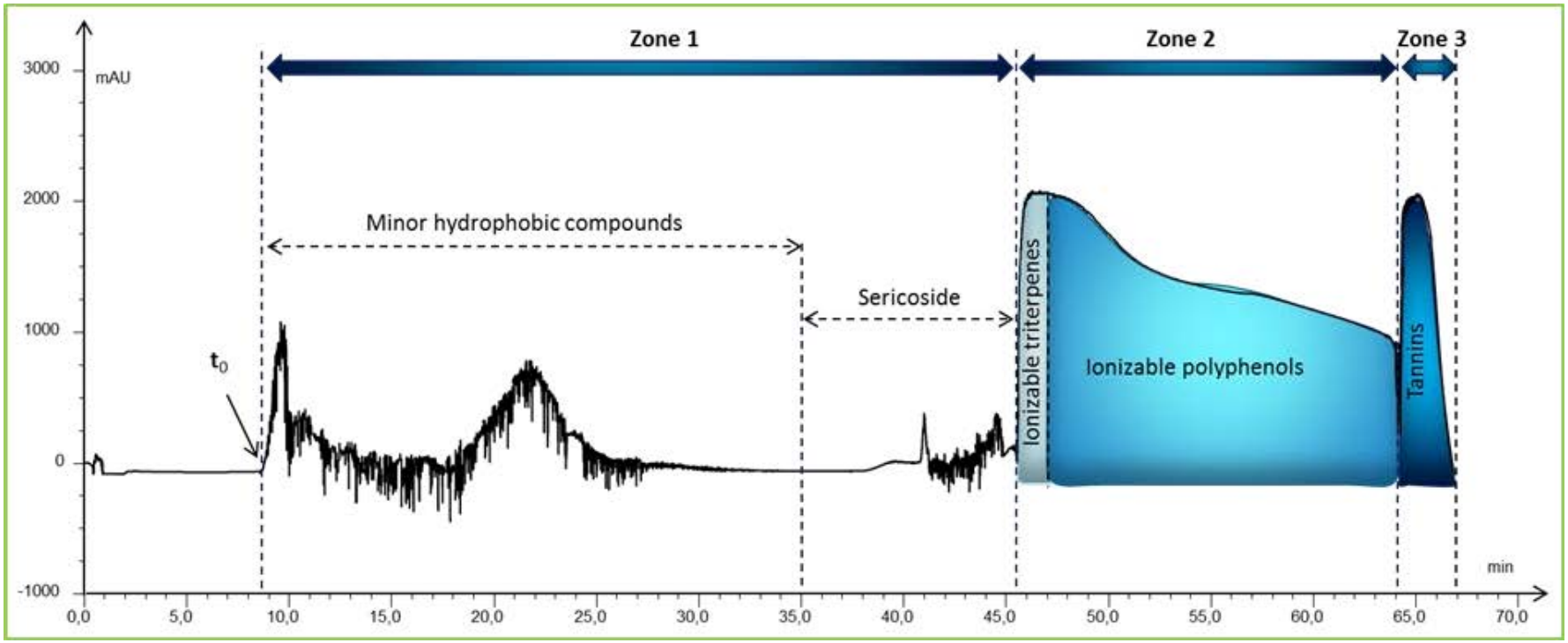

Figure VI. 1: pH-zone-refining CPE fractionation process profile monitored at 254 nm. Zone 1: non-ionizable eluted compounds; Zone 2: Displaced ionizable compounds; Zone 3: tannins obtained by dual-mode elution operation

Finally, the third zone of the pH-zone-refining CPE fractionation process corresponded to a dual-mode elution step [36-38]. It was performed by switching the role of the liquid phases, the aqueous phase becoming the mobile phase and by reversing the pumping mode (from ascending to descending mode). As a result, the abundant water-soluble tannins which were so far retained in the stationary phase at the head of the column were rapidly eluted out of the column from 64 min to 66.5 min. The collected fractions were pooled (P_{VIII}) and evaporated to dryness, resulting in 2265.1 mg (81.7 % of the crude extract mass).

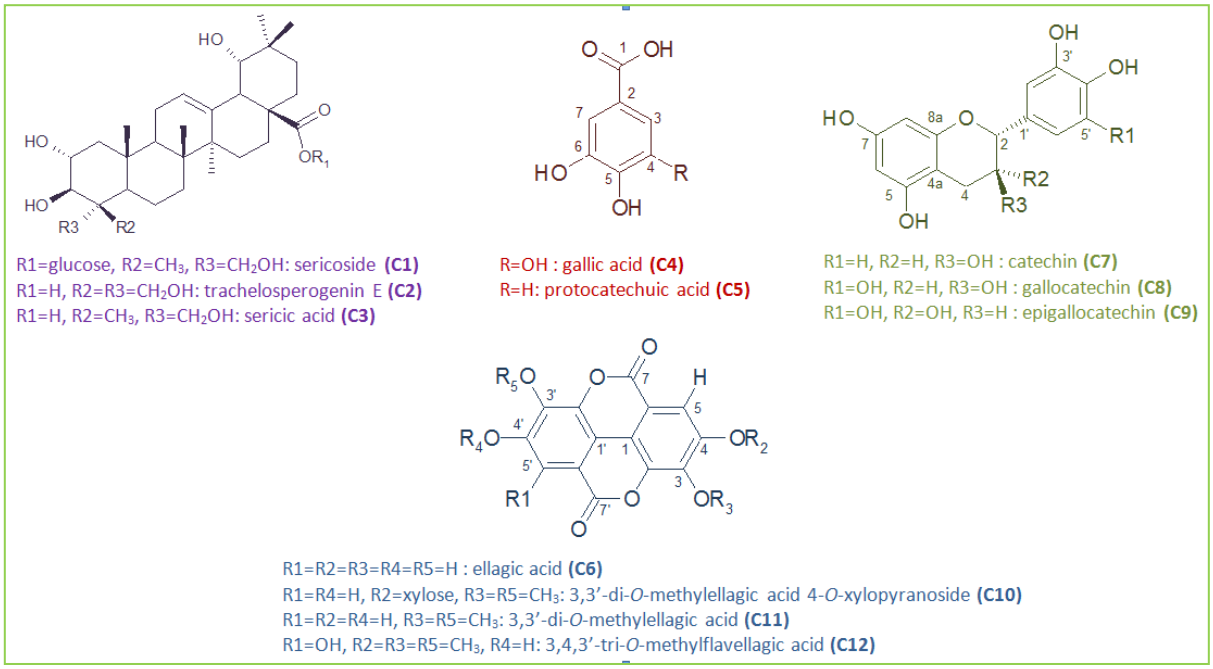

Figure VI. 2: Chemical structures of the major phytochemicals recovered from the crude ethanolic extract of the bark of Anogeissus leiocarpus

Triterpenes				Phenolic acids			Flavanols				Ellagic acid derivatives				
Carbon n°	C1	C2	C3	Carbon n°	C4	C5	Carbon n°	C7	C8	C9	Carbon n°	C6	C10	C11	C12
C1	47.1	47.1	47.2	C1	167.6	167.8	C2	81.3	81.3	78.4	C1	112.6	111.5	112.0	111,4*
C2	67.3	67.6	67.4	C2	122.1	120.8	C3	66.6	66.6	65.3	C2	136.7	141.1	141.6	140.4*
C3	84.2	77.4	84.2	C3	121.9	109.1	C4	28.2	27.3	27.7	C3	139.9	140.5	140.6	152.0
C4	43.3	39.3	43.3	C4	115.4	145.8	C4a	99.4	99.3	98.9	C4	148.4	151.6	152.6	133.7
C5	55.8	46.7	55.9	C5	145.3	138.3	C8a	155.7	156.7	156.8	C5	110.5	111.9	111.8	147.3
C6	19.2	18.8	19.2	C6	150.3	145.8	C8	94.1	95.5	95.4	C6	107.9	112.2	112.5	97.7
C7	32.6	32.6	32.2	C7	116.8	109.1	C7	156.5	156.5	156.5	C7	159.5	158.7	158.8	161.2
C8	39.2	40.3	39.5				C6	95.4	94.1	94.4	C1'	112.6	114.6	112.0	113.2*
C9	47.7	47.8	47.8				C5	156.8	155.6	156.1	C2'	136.7	142.0	141.6	140.9*
C10	37.9	37.6	37.9				C1'	130.9	130.1	130.0	C3'	139.9	142.2	140.6	141.5
C11	23.7	23.7	23.8				C2'	114.8	106.3	106.4	C4'	148.4	153.2	152.6	153.4
C12	122.4	122.5	122.5				C3'	145.1	145.9	145.7	C5'	110.5	112.2	111.8	111.9
C13	143.8	143.8	143.6				C4'	145.1	132.8	132.4	C6'	107.9	113.1	112.5	113.6
C14	41.4	41.4	41.3				C5'	115.4	145.9	145.7	C7'	159.5	158.8	158.8	158.6
C15	28.7	28.2	28.3				C6'	118.7	106.3	106.4	OCH_3		62.0	61.3	62.0
C16	27.5	28.7	32.9								OCH_3		61.4	61.3	61.5
C17	45.0	45.0	45.6								OCH_3				61.3
C18	43.5	43.5	43.4								C1 xyl		102.1		
C19	80.4	80.4	80.4								C2 xyl		73.4		
C20	35.2	35.2	35.1								C3 xyl		76.4		
C21	28.4	27.5	28.6								C4 xyl		69.6		
C22	33.1	32.8	27.6								C5 xyl		66.1		
C23	22.4	60.8	23.7								C1 Glc				
C24	64.2	62.8	64.2								C2 Glc				
C25	16.7	16.7	16.8								C3 Glc				
C26	16.9	16.9	16.9								C4 Glc				
C27	24.8	24.4	24.5								C5 Glc				
C28	179.5	179.5	176.2								C6 Glc				
C29	28.2	28.4	28.4												
C30	24.4	24.8	24.8												
C1 Glc			94.4												
C2 Glc			72.7												
C3 Glc			77.0												
C4 Glc			69.8												
C5 Glc			78.1												
C6 Glc			61.0												

Table VI. 3: ^{13}C NMR spectral assignments (ppm) of A. leiocarpus compounds. Samples were dissolved in 500 µL DMSO-d6 and analyzed at 600MHz;

(*): Values in the same column can be interchanged

VI.3.3. Antioxidant Activity evaluation by the DPPH scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) free radical exhibits a characteristic absorbance at 515 nm which decreases significantly on exposure to proton radical scavengers. The $DPPH^{\bullet}$ assay is widely used to measure the free radical scavenging activity of a compound and estimate its antioxidant potential. The free radical scavenging activity of the initial ethanolic (99%) crude bark extract of *Anogeissus leiocarpus* was compared to that of different fraction pools (P_{III}, P_{IV}, P_{VI}, and P_{VIII}) enriched in particular phytochemical classes obtained from the pH-zone-refining CPE process. The free radical scavenging activities of three pure compounds present in the initial crude extract (sericoside, ellagic acid, 3,3'-di-*O*-methylellagic acid and 3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside) were also measured.

The evolution of the absorbance of the $DPPH^{\bullet}$ free radical as a function of reaction time with each sample is given in Figure VI.3. As indicated by the significant decrease of the $DPPH^{\bullet}$ free radical absorbance, it appears clearly that the crude bark extract of *Anogeissus leiocarpus* exhibits a free radical scavenging activity. However, some compounds present in the crude extract were not active. In Figure VI.3, two $DPPH^{\bullet}$ reactivity behaviors were clearly distinguished. For the fraction pool P_{III} containing a mixture of triterpenes with trachelosperogenin E and sericic acid predominating, for pure sericoside, 3,3'-di-*O*-methylellagic acid and 3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside, only a slight decrease of the $DPPH^{\bullet}$ absorbance was observed, indicating that these compounds do not possess a significant antioxidant potential.

By contrast, the fraction pools P_{IV} and P_{VI} enriched in ellagic acid or flavonoids, the water-soluble tannins (P_{VIII}), and pure ellagic acid were found to exhibit a strong scavenging effect. It should be noted that the activity observed for the fraction pool P_{VI} was more linked to the presence of flavonoids (with catechin, gallocatechin and epigallocatechin predominating) than to the presence of substituted ellagic acid derivatives since these latter compounds were revealed inactive. In addition, the antioxidant activity of catechin derivatives is today well-recognized and has been described in numerous studies [39-41].

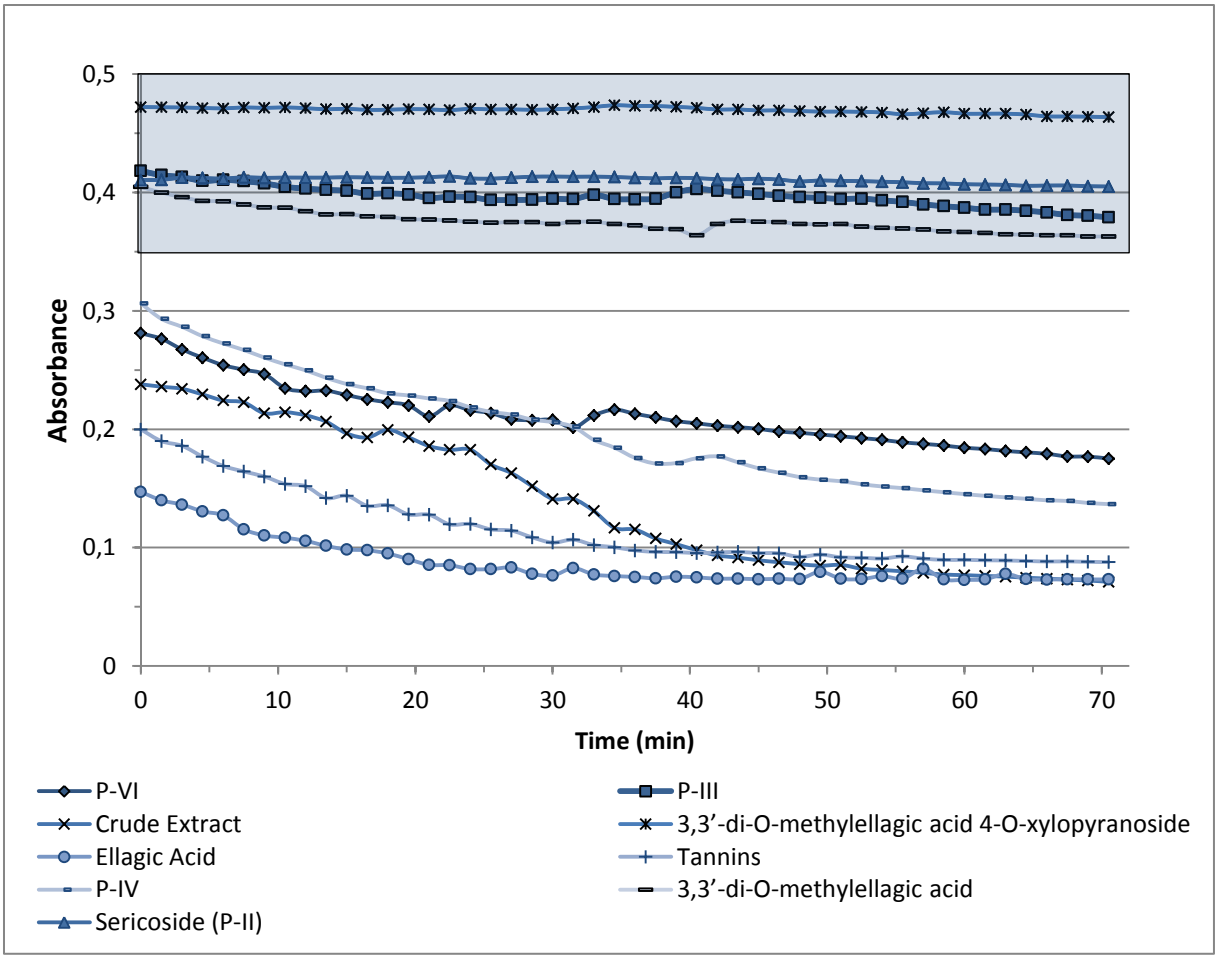

Figure VI. 3: Evolution of the DPPH• free radical absorbance during the reaction time with the crude extract, fraction pools and pure compounds from the bark of Anogeissus. leiocarpus

By comparing the free radical scavenging activities of pure ellagic acid and its derivatives, it appears clearly that the higher the degree of substitution on the ellagic acid skeleton, the lower the free radical scavenging activity. The structure of ellagic acid showed the highest scavenging activity probably due to the presence of four free hydroxyl groups which can give four active oxygen radicals. The di-substituted derivative 3,3'-di-*O*-methylellagic acid was less active than ellagic acid due to the presence of two methyl groups. The activity was even worse in the case of the tri-substituted 3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside due to the presence of only one hydroxyl radical available on the ellagic acid skeleton.

VI.4. Conclusion

In this work, centrifugal partition extraction used in the pH-zone-refining displacement mode has demonstrated its efficiency for the fractionation of phenolic compounds present in a complex bark extract. The fractionation process was performed after injecting 3 g of the crude extract and

successfully achieved in a single run of only 67 min with a recovery of 92.4 %. This process allowed firstly obtaining a mixture of minor hydrophobic compounds followed by the isolation of sericoside as pure saponin by elution. The displacement part of the process allowed obtaining fraction enriched in ionizable triterpenes (a mixture of trachelosperogenin E and sericic acid), ellagic acid and phenolic acids (gallic acid and protocatechuic acid), flavonoids (catechin, gallocatechin and epigallocatechin) and finally substituted ellagic acid derivatives (3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside, 3,3'-di-*O*-methylellagic acid and 3,4,3'-tri-*O*-methylflavellagic acid). At the end of this process, a simple dual-mode elution operation allowed the recovery of the abundant tannins (81.7%, w/w) in only two concentrated fractions. The antioxidant activities of the initial ethanolic (99 %) crude bark extract of *Anogeissus leiocarpus*, was compared to the activity of several pools, and isolated pure compounds using the DPPH$^{\bullet}$ free radical scavenging assays. The fraction pools enriched in ellagic acid or flavonoids, the water-soluble tannins, and pure ellagic acid were found to exhibit a strong scavenging effect on the DPPH$^{\bullet}$ free radical. Triterpenoids, sericoside, 3,3'-di-*O*-methylellagic acid and 3,3'-di-*O*-methylellagic acid 4-*O*-xylopyranoside were found to be inactive. By comparing the free radical scavenging potential of ellagic acid and its two substituted derivatives, we found that the higher the degree of substitution on the ellagic acid skeleton, the lower the free radical scavenging activity. These results indicated that the CPE apparatus combined with the pH-zone-refining mode opens highly interstting perspectives for the rapid fractionation and biological screening of natural products.

VI.5. References

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CONCLUSION GÉNÉRALE

Conclusion générale

L'objectif de ce travail de thèse était la caractérisation puis l'étude des applications potentielles dans le domaine des extraits naturels végétaux ou d'origine fermentaire d'un nouvel Extracteur de Partage Centrifuge (EPC) prototype, réalisé par la Société Rousselet-Robatel Kromaton (Annonay, France). Ce but originel a été sensiblement infléchit et a conduit, au fil de l'avancement des études menées, à des développements différents et/ou complémentaires à ceux initialement imaginés. Par ailleurs, ce travail de thèse a nécessité la mise en œuvre d'outils développés au laboratoire, tels les protocoles de mise au point de méthodologies de purification par CPC, les méthodes d'analyses de substances naturelles, notamment en CLHP, RMN, ESI-MS. J'ai pu ainsi m'enrichir de l'expérience des chercheurs et des doctorants du laboratoire (Pr. Catherine Lavaud, Dr. Jean-Marc Nuzillard, Mme Charlotte Sayagh,…), ainsi que de l'expertise et de l'implication de la Société Soliance (Romain Reynaud, Arnaud Guilleret). De plus, l'interaction forte de notre équipe avec les Docteurs Luc Marchal et Alain Foucault (UMR CNRS 6144, Saint-Nazaire) nous a été d'une grande aide pour ce qui est de la partie « génie des procédés » de ce travail. C'est donc tout cet environnement qui m'a permis de mener à bien et de faire avancer mon projet de thèse dans les meilleures conditions.

Cette thèse s'inscrit initialement dans un contexte socio-économique dynamique et une volonté politique affirmée (cette thèse a été entièrement financée par le Conseil Général de la Marne) visant à développer une pôle d'excellence dans le domaine des bioraffineries et des biotechnologies (principalement blanche) en Champagne-Ardenne - plus précisément sur le site de Pomacle (51). Les contraintes industrielles liées à la mise à disposition de milieux fermentaires industriels contenant les molécules d'intérêt tels que des 1,4-diacides nous ont conduit à réorienter le champ d'applications potentielles de cette nouvelle technique vers les besoins de la cosmétique. Par ailleurs, les préoccupations et les questionnements soulevés par les premiers résultats obtenus nous ont conduit à travailler en synergie avec l'équipementier (Rousselet-Robatel Kromaton) et l'équipe de Saint-Nazaire afin de réfléchir aux implications que les performances inattendues en terme de pouvoir séparatif de ce prototype pouvaient induire dans la conception et le dimensionnement des colonnes d'EPC ou de CPC. C'est ainsi que ce travail se poursuivra dans le cadre d'un projet ANR (appel d'offres CDII, thèse Alexis Kotland) incluant l'équipementier, Saint-Nazaire (porteur du projet), le Laboratoire de Génie Chimique de Toulouse et les Laboratoires Pierre Fabre (Plantes&Industrie, Gaillac).

Plus précisément, ce travail portait au départ sur l'extraction et la purification des métabolites issus principalement des biotechnologies blanches (acides succinique, itaconique,…), mais très rapidement, nous avons été amené à recentrer nos recherches sur d'autres métabolites naturels issus d'extraits de plantes (*Glycyrrhiza glabra*, *Sinapis alba*, et *Anogeissus leiocarpus*) et une seule cible fermentaire (l'acide itaconique). Ces cibles ont été définies en collaboration avec la société Soliance qui nous a fourni les extraits végétaux et d'autres produits, tel que des solvants agro-sourcés. Les travaux directement liés à la partie « cosmétique » trouveront des prolongements dans le cadre de la thèse de Sylvain Purson, financée par Soliance.

La livraison du prototype d'extraction « l'Extracteur de Partage Centrifuge FCPE300® » par la société Kromaton, qui en 2009 ne faisait pas partie du groupe Rousselet-Robatel, a pris du retard. Nous avons donc fait le choix de travailler, en début de thèse, sur d'autres projets qui portaient essentiellement sur des développements méthodologiques pour la séparation des biomolécules (lignines, peptides) par CPC. Ces travaux périphériques au sujet de thèse m'ont permis d'acquérir rapidement les bases pratiques de la CPC, de me familiariser avec les systèmes biphasiques de solvants, l'extraction solide-liquide et liquide-liquide de biomolécules.

Janvier 2010 fût marqué par l'arrivée de notre prototype d'extracteur : le FCPE300®. Comme toute nouvelle machine, il fallut tout d'abord entreprendre sa caractérisation afin d'en définir les conditions de fonctionnement optimales. Ainsi, cette caractérisation a porté sur la détermination du volume exact de son rotor, le volume des conduits reliant les cellules partage, ainsi que le volume des-dites cellules, celles-ci consistant en la partie la plus intéressante de l'EPC puisqu'étant le siège des phénomènes de transfert de matière et donc des processus séparatifs. L'hydrodynamique des phases liquides de plusieurs systèmes de solvants biphasiques a été aussi étudiée afin d'évaluer l'effet du débit de la phase mobile à différentes vitesses de rotation sur la rétention de la phase stationnaire. Cela nous a permis de bien connaitre les différentes caractéristiques de l'EPC par rapport aux systèmes existants de CPC.

Avec un nombre de cellules réduit mais un volume intra-cellule plus important par rapport à la CPC, l'EPC a été conçu au départ pour intensifier l'extraction liquide-liquide de métabolites d'intérêts (cellules de large volume tolérant des débits de phase mobiles importants) ou pour des opérations d'extraction en continu lorsque le mode co-courant pouvait être appliqué. Afin de positionner cette technologie par rapport aux appareils de CPC et également afin de trouver d'autres développements aux travaux initiés au laboratoire sur le mode « échange d'ions » en liquide/liquide, la combinaison de l'EPC et du mode par échange d'ions fort a été initialement explorée. L'intérêt principal de cette étude fut de voir dans quelle mesure la diminution du nombre de plateaux

physiques (231 cellules) en EPC par rapport aux chromatographes CPC (aux alentours de 1000 cellules) allait impacter la perte d'efficacité du procédé séparatif. Ce travail s'est appuyé sur la purification de la glycyrrhizine, une saponine ionique présente en quantité significative dans les racines de réglisse comme support méthodologique. D'une façon très intéressante, l'EPC utilisé en mode de déplacement par échange d'ions fort nous a permis d'obtenir des taux de recouvrement et puretés équivalents à ceux obtenus en CPC, et ce avec une bien meilleure productivité du fait notamment des débits de phase stationnaire applicables. C'est ainsi que la possibilité d'utiliser l'EPC comme un équipement permettant d'intensifier la purification de métabolites naturels ioniques a été étudié de manière plus approfondie. Le mode de développement par échange d'ions fort a alors été évalué en EPC mais cette fois d'un point de vue hydrodynamique, i.e. au niveau des écoulements des phases liquides à l'intérieur des cellules en cours d'expérience. Pour comparer les résultats obtenus en EPC et en CPC, nous avons utilisé pour cette partie du travail un support méthodologique déjà bien connu au laboratoire, la sinalbine, un glucosinolate ionique présent en quantité importante dans les graines de moutarde blanche (*Sinapis alba*. L) et dont la purification avait déjà été réalisée par CPC au laboratoire et au stade pilote. Les résultats obtenus ont validé la possibilité d'appliquer des débits importants de phase mobile (20-30 mL/min sur l'appareil de laboratoire) en EPC tout en traitant des quantités relativement importantes d'extrait (pouvant atteindre 20 g par injection toujours sur l'appareil de laboratoire). Par conséquent, des productivités élevées (28 g/h/L_{VC}) et des produits purs (> 95%) ont été obtenus. Ces résultats intéressants et inattendus sur la purification de métabolites naturels ioniques sont aujourd'hui directement utilisés en routine à l'échelle du laboratoire, mais peuvent aussi ouvrir des perspectives prometteuses à l'échelle industrielle. A noter aussi que ces travaux ont clairement posé la problématique de l'optimisation du dimensionnement des colonnes type CPC/EPC en fonction de l'application visée et du procédé qui y est associé. Comme signalé précédemment, ces résultats ont significativement contribué au démarrage du projet ANR CDII « xPC » 2012-2015 en collaboration avec différents partenaires académiques et industriels (UMR CNRS 6144, UMR CNRS 5503, Laboratoires Pierre Fabre, Rousselet-Robatel Kromaton).

Par la suite, et dans une optique de simplification d'extraits naturels bruts, le FCPE® a été exploité en tant qu'appareil permettant le fractionnement d'échantillons complexes et l'enrichissement des fractions obtenues par des familles de métabolites secondaires d'intérêt. Dans ce volet, le support méthodologique utilisé est un extrait éthanolique de l'écorce de l'arbre Africain *Anogeissus leiocarpus*. Cet extrait, fourni par la société Soliance, est actuellement utilisé en cosmétique comme ingrédient actif baptisé "*Anogelline*" dans la gamme corps "Bikini" commercialisée par Christian Dior en France. Le fractionnement, utile dans une stratégie d'objectivation de l'activité biologique de cet extrait, a été réalisé par deux méthodes différentes. La

première méthode développée consiste en un processus d'élution séquentiel utilisant un système de solvant tri-phasique et a permis de fractionner 5 g de l'extrait initial en familles de métabolites selon leur « polarité » en 90 min. Le résultat direct est l'efficacité de cette méthode pour éliminer les tanins - très majoritaires - du reste des métabolites de l'extrait. Dans la seconde méthode, l'extracteur de partage centrifuge a été combiné au mode de déplacement par pH-zone-refining, aboutissant à un fractionnement rapide et ciblé de 3 g de l'extrait brut de l'écorce tout en enrichissant des fractions par des composés phénoliques d'intérêt (dérivés de l'acide éllagique). L'évaluation du potentiel antioxydant de l'extrait de départ, des pools de fractions obtenues ainsi que de produits purs isolés a permis de rattacher cette activité à certains métabolites tels que l'acide éllagique ou les tannins. L'activité antiparasitaire de l'extrait brut, des pools de fractions et des produits purs isolés a été également évaluée, en collaboration avec l'équipe du Pr. Frédéric Bringaud du Centre de résonance magnétique des systèmes biologiques (UMR 5536, Université de Bordeaux Segalen), sur les formes sanguines de *Trypanosoma brucei*, parasite responsable de la trypanosomiase africaine (maladie du sommeil). Bien que les analyses soient toujours en cours, des résultats préliminaires très intéressants ont été obtenus.

Cette partie de la thèse qui a porté sur le développement de méthodologies de fractionnement par EPC, et plus particulièrement la méthode originale de fractionnement EPC par élution séquentielle tri-phasique a ouvert les portes sur d'autres idées en collaboration avec la société Soliance, et trouvera également directement des prolongements dans le cadre du projet européen Natprotec (FP7, appel à projets IAPP) porté par l'Université d'Athènes.

Durant la dernière année de thèse, nous nous sommes également intéressés aux applications de l'EPC dans le domaine des biotechnologies blanches. Après de nombreuses discussions avec mes encadrants, nous avons choisi de prendre pour modèle l'acide itaconique, 1,4 diacide identifié parmi les 12 « building blocks » stratégiques par le « Department of Energy » aux USA. Etant donné la nature ionisable de l'acide itaconique, le mode de développement par échange d'ions fort a été appliqué dans le but d'extraire et de concentrer ce diacide à partir de son milieu de départ (phase aqueuse) très dilué (50 mg/L). Ces travaux sur l'acide itaconique standard mériteront d'être poursuivis sur un extrait fermentaire réel, même si, à ce jour, les aspects stratégiques et les enjeux économiques de premier ordre rendent extrêmement difficile l'accès à des milieux fermentaires industriels.

Enfin, il ressortait de la partie dite « bibliographique » de ce manuscrit qu'un « vide technologique » restait à combler pour obtenir un continuum d'équipement entre des extracteurs liquide-liquide permettant au mieux d'accéder à la dizaine d'unités de transfert et la

chromatographie. Il apparaît clairement que l'Extraction de Partage Centrifuge se positionne dans ce vide technologique et que, suivant la finalité de l'opération unitaire réalisée et donc du procédé mis en place, cette technique peut remplir le rôle d'extracteur ou de chromatographe. Il conviendra donc de bien distinguer le nom de l'appareil, donné par l'équipementier (FCPE®) et sur lequel l'utilisateur n'a pas à agir et l'utilisation que l'on va en faire en y associant la terminologie adéquate : nombre d'unité de transfert *vs* plateau théoriques, mode déplacement et/ou échange d'ions *vs* extraction et back-extraction, *etc*…

Développements méthodologiques en Extraction de Partage Centrifuge (EPC). Application au fractionnement et à la purification de substances naturelles végétales et issues des biotechnologies blanches.

Institut de Chimie Moléculaire de Reims (UMR CNRS 7312, Groupe « Isolement & Structures »)

Résumé : Les travaux présentés dans ce manuscrit s'inscrivent dans une optique d'étude et de caractérisation d'un prototype Extracteur de Partage Centrifuge (FCPE) son application au fractionnement et à la purification de produits naturels à partir des extraits végétaux ou fermentaires. La première partie des travaux de thèse consiste aux développements méthodologiques réalisés en EPC en mode de déplacement par échange d'ions fort (SIX-CPE) pour l'intensification de l'extraction et de la purification de composés ioniques naturels (glycyrrhizine, sinalbine, acide itaconique). Les développements méthodologiques étaient focalisés en particulier sur l'étude hydrodynamique des phases en écoulement à l'intérieur de la colonne EPC durant les différentes étapes du processus de séparation, sur la détermination des facteurs limitants ainsi que sur les conditions opératoires optimales conduisant à des taux de recouvrement, productivité et pureté satisfaisants. La deuxième partie des travaux est consacrée à l'investigation des potentialités de l'extracteur FCPE en tant qu'instrument permettant de simplifier des extraits naturels complexes par extraction fractionnée sélective et enrichissement des fractions en composés ciblés. Deux méthodes sont en particulier présentées, utilisant comme support méthodologique un extrait éthanolique de l'écorce d'un arbre sub-saharien, *Anogeissus leiocarpus* Guill. & Perr. (Combretaceae). La première a été développée en combinant l'EPC à un mode original d'élution tri-phasique séquentiel balayant un large spectre de polarité. La seconde a également été développée par pH-zone-refining, basée sur le changement du coefficient de distribution (K_D) des composés ciblés (polyphénols et dérivés de l'acide ellagique notamment) par une variation du pH. Les fractions générées à l'issue de ce procédé ont été analysées pour leur potentiel antioxydant par analyse chimique (piégeage du radical DPPH).

Mots clés : Extraction de partage centrifuge, intensification, glycyrrhizin, sinalbine, acide itaconique, polyphénols, acide éllagique, DPPH, echange d'ion, three-phase solvent systems, pH-Zone refining.

Methodological developments of Centrifugal Partition Extraction (CPE). Application to fractionation and purification of the natural substances derived from plants and white biotechnology.

(Institute of Molecular Chemistry Reims (UMR CNRS 7312), Groupe: "Isolation & Structures")

Abstract: This work presented in this manuscript deal with the study and the characterization of the new Centrifgal Partition Extractor (FCPE) and its application to the fractionation and the purification of natural products from plant and microbial extracts. In the first part of this work, the potential of the extractor CPE combined with the Ion-Exchange (SIX-CPE) mode to simultaneously extract and purify natural ionized compounds (glycyrrhizin, sinalbine and itaconic acid). Methodological developments were focused in particular on the study of the hydrodynamic behavior of the liquid phases inside the CPE partition cells during the different stages of the separation process, the determination of the limiting factors, as well as, the optimal operating conditions leading to optimal product recovery, productivity and purity. The second part of the work is devoted to the investigation of the potentialities of the FCPE extractor to simplify complex natural extracts by fractional extraction and selective enrichment of fractions in target compounds. Two methods are developed using an ethanolic extract of the bark of a sub- Saharan tree, Anogeissus leiocarpus Guill. & Perr. (Combretaceae) as methodological support. The first was developed by combining the EPC to an original sequential three-phase elution method with a wide range of polarity. The second was also developed in displacement pH-zone refining mode, based on the change of the distribution coefficient (KD) of the target compounds (polyphenols and ellagic acid derivavtives) by a changing the pH. Fractions generated at the end of this process were analyzed for their antioxidant potential by chemical analysis (DPPH radical scavenging).

Key words: Centrifugal partition extraction, intensification, glycyrrhizin, sinalbin, itaconic acid, polyphenols, ellagic acid, DPPH, ion-exchange, three-phase solvent systems, pH-zone refining.