UNIVERSITÉ DE MONTRÉAL

# ÉTUDE DE LA STRATÉGIE D'EXTRACTION IN-SITU SUR LA PRODUCTION DE MÉTABOLITES SECONDAIRES DANS DES CULTURES DE CELLULES VÉGÉTALES

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Ce mémoire intitulé :

# ÉTUDE DE LA STRATÉGIE D'EXTRACTION IN-SITU SUR LA PRODUCTION DE MÉTABOLITES SECONDAIRES DANS DES CULTURES DE CELLULES VÉGÉTALES

**présenté par KLVANA Maya**

**en vue de l'obtention du diplôme de : Maitrise ès sciences appliquées**

**a été dûment accepté par le jury d'examen constitué de :**

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À G, T-B, G-P, M

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## RÉSUMÉ

La culture in vitro de cellules de plantes attire beaucoup d'intérêt en tant que moyen de production de certains composés chimiques qui sont recherchés pour leurs propriétés thérapeutiques. Malheureusement, ce moyen reste, à ce jour, peu rentable puisque le procédé de production est limité par des rendements très faibles. L'ajout d'une phase extractive, liquide ou solide, permet une augmentation de la production des ces métabolites secondaires et améliore leur récupération. Dans la perspective d'un procédé industriel, il est important de comprendre si le contact est nécessaire entre les cellules et la phase extractive puisque cela permettrait d'optimiser l'extraction et la purification des métabolites secondaires. En effet, dans le cas des phases solides, la récolte des produits recherchés est difficile puisque les résines ont une densité comparable aux cellules donc la séparation n'est pas simple, et nécessite la terminaison de la culture.

L'objectif principal de cette étude consiste à simplifier le procédé de production des alcaloïdes benzophénanthridiques dans des cultures en suspension d'*Eschscholtzia californica* avec des résines XAD-7. Pour ce faire, il faut tout d'abord comprendre comment le contact entre la phase extractive et les cellules agit sur la production totale des métabolites secondaires mais aussi comment il agit sur la dynamique des flux métaboliques.

Pour pouvoir effectuer une étude poussée sur le métabolisme secondaire, une méthode par chromatographie liquide haute performance (HPLC) a été développée pour mesurer de façon rapide et précise les principaux alcaloïdes benzophénanthridiques

produits dans les cultures cellulaires d'*E. californica*, soit la sanguinarine, la chelirubine, la macarpine, la chelerythrine ainsi que la chelilutine. Cette méthode permet de séparer sur colonne chromatographique $C_{18}$ les composés recherchés en 35 minutes à l'aide d'un gradient d'acétonitrile et d'acide phosphorique 50 mM. La quantification des métabolites se fait de façon sélective et précise à l'aide d'une détection par fluorescence ($\lambda_{ex}$ 330 nm; $\lambda_{em}$ 570 nm) et par UV. Étant donné que seules la sanguinarine et la chelerythrine sont disponibles commercialement, il a été nécessaire de purifier des alcaloïdes additionnels par chromatographie semi-préparative et de les identifier par spectrométrie de masse et résonance magnétique nucléaire. Ainsi, des standards de chelirubine, de chelilutine et de macarpine ont été purifiés de sorte à pouvoir quantifier de façon précise les cinq alcaloïdes produits dans les cultures in-vitro d'*E. californica*.

Afin d'étudier l'effet du contact entre la phase extractive et les cellules, un système de culture a été développé de façon à pouvoir extraire les alcaloïdes benzophénanthridiques de la culture sans qu'il n'y ai de contact entre les cellules et les résines extractives XAD-7: seul le milieu de culture était recirculé à travers une colonne remplie de la phase extractive. Cette méthode a été comparée avec la méthode traditionelle où les résines sont directement mélangées à la suspension cellulaire. L'extraction des produits par recirculation a engendré une hausse importante de la production, soit 20 fois plus de sanguinarine et 10 fois plus de chelerythrine. Cette méthode de recirculation a grandement simplifié le procédé de production étant donné que les résines sont faciles à récupérer de la culture et cette stratégie permet la récolte des composés recherchés sans l'arrêt de la culture. Malgré cela, les conditions

expérimentales ont limité le flux de recirculation. La méthode de recirculation s'est avérée moins efficace que la stratégie du rajout direct des résines dans la suspension. En plus d'augmenter la production de métabolites secondaires pas rapport à une culture témoin sans particules extractives, l'extraction des métabolites secondaires du milieu par les colonnes externes a causé un changement de la distribution des flux métaboliques, ce qui semble témoigner d'un mécanisme complexe du contrôle de production.

## ABSTRACT

In vitro plant cell culture has garnered a lot of interest as a source of secondary metabolites, several of which have been shown to have great therapeutic value. Unfortunately, due to low yields, this production process remains unprofitable. Important increases in cell production of the desired chemicals can be achieved by the addition of solid or liquid extractive phases, which have the added benefit of facilitating harvest. Considering a large scale industrial production process, it is important to understand whether contact is necessary between the cells and the extractive phase since this could enable optimization of the extraction and purification of the secondary metabolites. Indeed, in the case of solid phases, harvest of the compounds of interest from in vitro cultures can be difficult since resins often have very similar densities as the plant cells therefore separation of the two phases can be arduous and requires termination of the culture.

The main objective of this research project is to simplify the production process of benzophenanthridines alkaloids in suspension cultures of *Eschscholtzia californica* with XAD-7 adsorptive resins. In order to be able to improve the process, it is necessary to first understand whether contact between cells and resins affects production of secondary metabolites and how it influences metabolic flux distribution within secondary metabolism.

In order to perform an in depth analysis of secondary metabolism, a high performance liquid chromatography method was developed to perform rapid and reliable measurements of the main benzophenanthridine alkaloids produced in *E. californica* cell

cultures, namely sanguinarine, chelirubine, macarpine, chelerythrine and chelilutine. Separation is achieved on a $C_{18}$ reversed phase column with gradient elution using acetonitrile and 50 mM phosphoric acid as a buffer. Detection is performed by both fluorescence ($\lambda_{ex}$ 330 nm; $\lambda_{em}$ 570 nm) and diode array detection, leading to good selectivity and precision in determining peak purity. In turn, this permits simple and quick sample preparation involving a methanolic extraction for the measurement of intracellular concentrations and a solid phase extraction for their quantification in the culture medium. Lack of commercially available standards is addressed by the development of a method to purify chelirubine, macarpine and chelilutine by semi-preparative HPLC. Coupled together, the isolation method and analytical method proved reliable for screening of the alkaloids of interest produced in *E. californica*.

The effect of contact between cells and extractive phase on secondary metabolite production was investigated in two-phase suspension cultures of *E. californica*. A system was designed to extract benzophenanthridine alkaloids from the cell culture, without contact between XAD-7 resins and the cells: only medium was recirculated through a column packed with the extractive phase. This strategy was compared to the classic method of addition of resins directly into the cell suspension. Removal of the product directly from the medium enabled important increases in production of alkaloids, namely a 20-fold increase in sanguinarine production and a 10-fold increase in chelerythrine, with high recovery in the resin. The recirculation strategy greatly simplified the production process since the resins are easily recovered from the cell culture and enable harvest of product without termination of culture. However, due to

limited flow rate, the recirculation strategy appeared slightly less effective than direct addition of resins into the cell suspension. In addition to enabling increased production, removal of secondary metabolites from the medium changed metabolic flux distribution, testifying to a complex control mechanism of production.

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## LISTE DES SIGLES ET ABRÉVIATIONS

| | |
|---|---|
| 2,4-D | acide 2,4-dichlorophenoxyacétique |
| ACN | acétonitrile |
| $C_i$ | concentration d'alcaloïde dans l'échantillon avant ajout |
| $C_a$ | concentration d'alcaloïde ajouté |
| $C_{i+a}$ | concentration d'alcaloïde dans l'échantillon après ajout |
| *C. roseus* | *Catharanthus roseus* |
| DMSO | dimethylsulfoxide |
| *E. californica* | *Eschscholtzia californica* |
| FPLC | chromatographie liquide à performance rapide |
| HCl | acide chlorhydrique |
| HPLC | chromatographie liquide haute performance |
| $H_3PO_4$ | acide phosphorique |
| KOH | hydroxide de potassium |
| MeOH | méthanol |
| PTFE | polytetrafluoroethylene (Teflon) |
| *P. somniferum* | *Papaver somniferum* |
| rpm | tours par minute |
| TFA | acide trifluoroacétique |
| TMS | tetramethyl silane |
| UV | ultraviolet |
| % v/v | pourcentage du volume |

| | |
|---|---|
| $\lambda_{ex}$ | longueur d'onde d'excitation |
| $\lambda_{em}$ | longueur d'onde d'émission |

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# Chapitre 1 : Introduction

Depuis longtemps, les plantes ont été une source privilégiée de produits thérapeutiques. Ainsi, parmi les produits les plus prescrits se retrouvent des molécules complexes issues de plantes dont des stéroïdes, des analgésiques, des molécules agissant sur le système cardio-vasculaire ainsi que des produits antibactériens et anticancéreux.

Plusieurs méthodes de production industrielle ont été envisagées pour obtenir de grandes quantités de ces molécules complexes qui sont produites au sein du métabolisme secondaire végétal. La culture traditionelle de plantes entières ou la récolte de cultures sauvages s'avère une option peu renouvelable puisque de nombreuses plantes recherchées sont rares et ne bénéficient pas d'une croissance rapide. En outre, la purification des produits à partir de plantes entières requiert de nombreuses étapes et n'est pas simple d'un point de vue industriel. Une autre option, la synthèse chimique, s'avère difficile, voir impossible, puisque les molécules désirées sont parfois très complexes et leur synthèse nécessite trop d'étapes pour que le procédé soit rentable. La culture de cellules in vitro a ainsi attiré beaucoup d'intérêt comme moyen de production de ces composés recherchés. Néanmoins, cette option reste à ce jour peu rentable puisque la production est limitée par des rendements très faibles et donc une productivité faible. Une des solutions utilisées pour augmenter la production de métabolites secondaires in vitro est le rajout d'une phase extractive à la suspension cellulaire. Non seulement ceci augmente la production, mais les composés recherchés se retrouvent adsorbés sur la phase extractive, qui peut être soit solide, soit liquide.

## 1.1 Problématique

Dans la perspective d'un procédé de production industriel, il est important de comprendre si le contact est nécessaire entre les cellules et les phases extractives puisque cela permettrait d'optimiser l'extraction et la purification des métabolites secondaires. Dans le cas des phases extractives solides, la récupération des résines ajoutées à une suspension cellulaire rend le procédé de production plus complexe. En effet, la séparation de ces deux phases se fait difficilement parce que leurs densités sont comparables. Par conséquent, pour récupérer les métabolites secondaires adsorbés sur résines, il faut arrêter la culture puis décanter le mélange de façon très délicate et incertaine, une étape qui deviendrait complexe à l'échelle industrielle.

## 1.2 Objectifs

L' objectif principal du projet de recherche consistait à simplifier le procédé de production des métabolites secondaires pour des cultures d'*E. californica* en séparant la phase extractive solide de la suspension cellulaire. Ainsi, à l'échelle industrielle, la récupération des produits recherchés serait simple et pourrait se faire en continu, sans devoir mettre un terme à la culture. Pour pouvoir atteindre cet objectif, deux sous-objectifs ont dû être atteints:

1. Le premier sous-objectif consistait à développer une méthode chromatographique permettant d'identifier et de quantifier les alcaloïdes principaux, soit la sanguinarine, la chelerythrine, la macarpine, la chelirubine ainsi que la chelilutine dans les différents échantillons de cultures de cellules

d'*E. californica*. De plus, une méthode a dû être dévelopée afin de purifier les alcaloïdes qui ne sont pas disponibles sur le marché, soit la chelirubine, la chelilutine et la macarpine.

2. Le deuxième sous-objectif consistait à comprendre comment le contact entre la phase extractive et les cellules agit sur la production de métabolites secondaires. Plus spécifiquement, les travaux ont portés sur:
    - Le dévelopement d'un sytème de culture qui permet de retirer les métabolites secondaires du milieu sans qu'il n'y ait de contact entre les cellules et les résines extractives
    - L'effet du contact entre les cellules et la phase extractive sur la production totale d'alcaloïdes
    - L'influence du mode d'extraction sur la dynamique des flux métaboliques

# Chapitre 2 : Revue de littérature

## 2.1: Produits thérapeutiques issus de plantes

Quelques examples concrets de molécules utilisées comme médicaments sont présentés au tableau 2-1.

**Tableau 2-1: Médicaments dérivés de plantes**

| Type de médicaments | Molécules chimiques et provenance |
|---|---|
| anticancéreux | taxol (*Taxus brevifolia*) |
| | vincristine, vinblastine (*Catharanthus roseus*) |
| | camptothecine (*Camptotheca acuminata* ) |
| | podophyllotoxin (*Podophyllum peltatum*) |
| agent cardio-vasculaire | digitoxin (*Digitalis purpurea*) |
| | reserpine, deserpine (*Rauvolfia serpentina*) |
| | atropine, scopalamine (*Atropa belladonna*) |
| antipyretique | quinine (*Cinchona*) |
| analgésiques | codeine, morphine, papaverine (*Papaver somniferum*) |
| antibactériens | sanguinarine (*Sanguinaria Canadensis, Eschscholtzia californica*) |

La plante utilisée comme modèle pour nos expériences, *E. califonica*, produit des alcaloïdes benzophénanthridiques, dont la sanguinarine et la chelerythrine. En plus de son utilisation dans des produits dentaires pour son effet antibactérien (Grenby 1995; Eley, 1999), la sanguinarine a récemment attiré l'attention du milieu médical par son

effet potentiel contre le cancer, notament la leucémie (Ahmad et al. 2000; Slaninova et al. 2001; Weerasinghe et al. 2001). De facon similaire, la chelerythrine a attiré l'attention comme médicaments potentiel pour combattre certaines tumeurs, grâce à son effet inhibiteur sur la kinase C de protéines (Chmura et al. 2000).

## *2.2: Production de métabolites secondaires in vitro*

### 2.2.1: Optimisation

La production des métabolites secondaires est très étroitement contrôlée par la plante et il est alors important de bien comprendre la dynamique du métabolisme secondaire de sorte à pouvoir améliorer la productivité en conditions in vitro. Plusieures avenues ont été étudiées pour augmenter les niveaux production. D'une part, les conditions de cultures ont été optimisées: il est possible de moduler la production en maintenant des conditions nutritionelles spécifiques et en induisant la production durant la croissance exponentielle (Lecky et al. 1992; Archambault et al. 1996, Brodelius et al. 1993). De plus, il est possible d'activer la production des métabolites secondaires en élicitant les cellules. En exposant les cellules à certains composés chimiques dont les extraits de levures et la chitine, les cellules répondent à ce stress en activant le métabolisme secondaire, qui est une réaction défensive (Schumacher et al. 1987; Collinge et al. 1989).

### 2.2.2: Phases extractives

De nombreuses études ont démontré que l'ajout d'une phase extractive dans les cultures in vitro engendraient une augmentation significative de la production de

métabolites secondaires, en plus de simplifier le procédé de production en améliorant l'éfficacité de la récupération des produits. Ainsi, pendant la période de production, une phase pour laquelle les produits recherchés ont une affinité elevée est rajoutée dans la culture pour extraire les métabolites secondaires. Qu'elles soient liquides ou solides, les phases extractives doivent avoir un coefficient de partition élevé pour pouvoir assurer une bonne récupération du produit, tout en n'ayant aucun effet sur la culture, tel une oxygénation limitée ou un retrait de nutriments essentiels. Plusieurs examples de phases extractives sont résumés dans le tableau 1-2 qui suit.

**Tableau 2-2: Phases extractives utilisées dans les cultures de plantes en suspension**

| **Phase extractive** | **Plante** | **Composés chimiques** | **Auteurs** |
|---|---|---|---|
| Résines XAD-7 | *Catharanthus roseus* | Indoles | Asada et al. (1989) |
| Resines XAD-7 | *Papaver somniferum* | Alcaloïdes benzophénanthridiques | Williams et al. (1992) |
| Resines XAD-4 | *Nicotiana rustica* | Phénols | Maisch et al (1986) |
| Resines XAD-7 | *Catharanthus roseus* | Indoles | Lee-parsons et al (2002) |
| Resines XAD-7 | *Cinchona ledgeriana* | Anthraquinones | Southern et al. (1987) |
| Tricapryline | *Eschscholtzia californica* | Alcaloïdes benzophénanthridiques | Dutta et al. (1994) |
| Huile de silicone | *Eschscholtzia californica* | Alcaloïdes benzophénanthridiques | Byun et al. (1990 et 1994) |
| Huile de silicone | *Catharanthus roseus* | Indoles | Thikomiroff et al. (2002) |
| Hexadecane | *Lithospermum erythrorhizon* | Shikonin | Gurrieri et al. (1990) |
| Myglyol | *Matricaria chamomilla* | Huiles essentielles | Orbach et al. (1988) |

En ajoutant des résines XAD-7 à des cultures de cellules de *Catharanthus roseus*, Asada et Schuler (1989) sont parvenus à augmenter la production d'ajmalicine de 67%, tout en récupérant 63% du composé dans les résines. De façon similaire, Williams et al. (1992) ont obtenu une augmentation de l'ordre de 50 à 85 % dans la production de sanguinarine avec des cellules de *Papaver somniferum*, en récupérant 75% de l'alcaloïde dans les résines. Plus récemment, Lee-Parsons et al. (2002) ont démontré que l'augmentation de la production ainsi que le taux de récupération d'indoles étaient dépendants du moment auquel les résines XAD-7 étaient ajoutées à la culture de *Catharanthus roseus*. Plusieurs phases liquides ont aussi été utilisées pour améliorer la production de métabolites secondaires. Ainsi, en ajoutant de l'huile de silicone dans des cultures d'*E. californica*, Byun et al. (1990, 1994) ont pu augmenter la production par un facteur de 3,4 dans les cultures en flacons agités et par un facteur de 3 en réacteur de type gazo-syphon. De façon similaire, l'ajout de tricapryline (une huile de silicone) dans des cultures d'*E. californica* a engendré une production 4 fois plus importante d'alcaloïdes benzophénanthridiques. Dans des cultures de racines de *Catharanthus roseus*, l'ajout d'huile de silicone a permis d'augmenter le rendement de la lochnericine ainsi que la tabersonine, des indoles qui font partie de la voie métabolique produisant des anticancéreux dont la vincristine et la vinblastine (Tikhomiroff et al. 2002).

### 2.2.3: Contrôle de la production et du transport des métabolites secondaires dans les plantes

De nombreux travaux ont démontrés que l'utilisation de phases extractives permet d'augmenter la production de métabolites secondaires, mais il reste que le mécanisme précis par lequel elles engendrent cette hausse n'est pas entièrement compris. Les voies métaboliques sont controlées de façon très étroite dans les cellules et il semblerait que le transport des produits finaux et des précurseurs dans la cellule et vers l'extérieur de celle-ci joue un rôle important dans ce contrôle. Ainsi, il a été proposé que la capacité de production est dépendante du transport des métabolites secondaires loin du lieu de production, où les produits peuvent causer une inhibition négative des voies de synthèse de ces molécules (Brodelius et Pedersen, 1993; Lee-Parsons et Schuler, 2002). Trois mécanismes de transport sont connus pour les métabolites secondaires tel qu'illustré dans la figure 2-1.

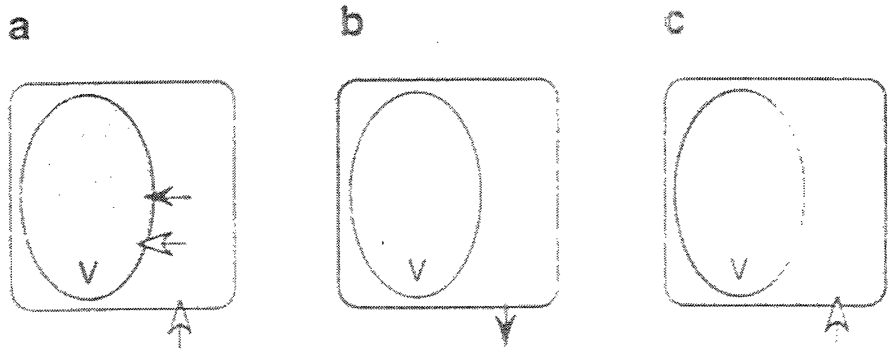



**Figure 2-1: Transport des métabolites secondaires dans les cellules de plantes.**

**a: stockage vers la vacuole; b: sécrétion vers l'extérieur de la cellule; c: absorption vers l'intérieur de la cellule à partir de sources externes. V: vacuole, flèches: transport des alcaloïdes (tiré de Brodelius et Pedersen, 1993)**

Tout d'abord, il est proposé que les produits puissent être transportés vers la vacuole où ils sont stockés. Dans ce cas, la capacité de production de la cellule est limitée par la capacité de stockage des vacuoles. D'autre part, les produits peuvent être voués au transport vers l'extérieur des cellules, dans quel cas le taux de production est limité par les propriétés du mileu ainsi que les mécanismes de sécrétion. Enfin, les métabolites et les précurseurs provenant des autres cellules dans la culture peuvent être absorbés et stockés.

Dans les trois cas, le transport est controlé par des mécanismes actifs et passifs. A ce jour, ces mécanismes ne sont pas clairement élucidés mais plusieures hypothèses ont été suggérées. D'après Brodelius et Pedersen (1993), le transport passif permet aux molécules neutres de diffuser librement à travers les membranes, par contre les composés protonés ne peuvent diffuser. Par conséquent, les métabolites secondaires peuvent être accumulés dans les compartiments plus acides que le cytosol (pH 7-7.5) tel la vacuole (pH 3-6) et jusqu'à un certain niveau le mileu de culture (pH 4.5-6), dépendement du pKa des alcaloïdes. Les gradients de pH sont eux-mêmes générés par transport actif par des ATPases. Outre le transport actif qui génère les gradients pH, il a été proposé que certains métabolites sont transportés à travers les membranes par des transporteurs spécifiques. Ainsi, dans des cellules de *Thalictrum minus*, des inhibiteurs d' ATPase ont limité l'excretion des alcaloïdes, indiquant un transport actif (Renaudin et Guern 1982). De façon similaire, Villegas et al. (2000) ont conclu que les alcaloïdes benzophénanthridiques étaient transporté par des mécanismes actifs dans des cultures d'*E. californica*. Ainsi, la production de métabolites secondaires dépend, du moins en

partie, de mécanisme de transport passifs et actifs qui permettent de déplacer les produits des lieux de production. En ce qui concerne les phases extractives, l'hypothèse la plus commune propose que les phases extractives réduisent la retro-inhibition en retirant les produits de la culture en devenant une source externe de stockage pour les alcaloïdes. Le transport des métabolites est alors redirigé vers l'extérieur des cellules au lieu de la vacuole, dont la capacité limite la production. Les résultats de Williams et al. (1992) semblent confirmer cette hypothèse puisqu'en parallèle à une production accrue, l'ajout de résines a réduit les concentrations de sanguinarine dans les cellules de même que le milieu de culture. Par contre, dans le cas de Byun et al. (1990, 1994), les concentrations en alcaloïdes n'ont pas été significativement changées par l'ajout de résines. Dans certaines cultures de *Taxus*, l'ajout de trycapryline a engendré une bonne récupération à partir du milieu, sans une augmentation de la production totale, ce qui a permis aux auteurs de proposer que l'ajout d'une phase extractive n'agissait pas sur la rétro-inhibition (Collins-Pavao et al. 1996). Dans des cultures de *Catharanthus roseus*, Tikhomiroff et al. (2002) ont pu observer des alcaloïdes dans l'huile de silicone, qui jusqu'à présent n'avait pas été détecté en dehors des cellules. Wang et al (2001) ont proposé que l'augmentation de la production en métabolites secondaires pourrait être liée à l'interaction physique entre la phase extractive et la membrane cellulaire. Peut-être les phases extractives influencent-elles la sécretion des métabolites secondaires en modulant le transport actif à travers la membrane externe des cellules ?

En somme, il semble que l'effet des phases extractives dépend de l'espèce, de même que de la lignée cellulaire et le mécanisme d'action reste mal compris, notamment

si le contact entre les cellules et la phase extractive est nécessaire pour obtenir une production plus élevée. Le dernier point représente un paramètre de procédé très important car il pourrait permettre de simplifier l'étape de récolte des cultures, étape essentiellement destructive actuellement et complexe compte-tenu de la difficulté à séparer les cellules des résines.

# Chapitre 3 : Méthodologie et organisation du projet

Pour répondre à nos objectifs, le projet a été réalisé avec des cellules *d'E. californica*. Non seulement cette plante produit des métabolites secondaires d'intérêt, mais son métabolisme est bien connu puisque elle a fait l'objet de plusieurs études scientifiques. De plus, une lignée cellulaire stable et bien charactérisée existe au laboratoire Bio-$P^2$. Ainsi, la production d'alcaloïdes benzophenantridiques dans des cultures d'*E. californica* avec une phase extractive solide (résines XAD-7) a été choisie comme modèle de culture.

## *3.1 Méthode analytique*

Etant donné que les molécules recherchées d'*E. californica* font partie de voies métaboliques complexes, il est important de bien comprendre la dynamique des flux métaboliques. Ainsi, il est nécessaire de pouvoir mesurer les concentrations de plusieurs alcaloïdes dans les voies métaboliques impliquées (illustrées en détail à l'annexe 1), et non seulement les produits les plus recherchés commercialement, soit la sanguinarine et la chelerythrine.

Pour répondre à ce premier sous objectif du projet, une recherche bibliographique a tout d'abord été effectué. Un résumé complet des nombreuses méthodes publiées est présenté en annexe 2. La plupart de ces méthodes chromatographiques demandent un temps important et nécessitent une préparation d'échantillon longue et compliquée. De plus, la plupart des méthodes publiées ne sont pas suffisamment sélectives pour permettre une quantification précise.

Additionellement, seul des standards purs de deux des multiples alcaloïdes benzophénanthridiques sont disponibles sur le marché, soit la sanguinarine et la chelerythrine. Certaines publications proposent des méthodes de purification pour quelques rares alcaloïdes, mais aucune de ces méthodes ne s'est avérée satisfaisante. Apres avoir testé plusieurs des méthodes proposées dans la littérature sans résultats satisfaisants, nous avons du élaborer une méthode semi-préparative pour purifier des standards ainsi qu'une méthode analytique pour la quantification des composés recherchés. Le développement de ces méthodes est présenté dans le chapitre 4, sous la forme d'un article soumis pour publication dans la revue *Journal of Chromatography A*.

## *3.2 Conception du système de culture*

Pour pouvoir déterminer comment le contact entre la phase extractive et les cellules agit sur la production de métabolites secondaires, il a fallu tout d'abord élaborer un système de culture qui permet de retirer les métabolites secondaires du milieu à l'aide de résines extractives sans qu'il n'y ait de contact entre celles-ci et les cellules. Un premier essai a été fait en renfermant les résines dans des poches de nylon (75 μm) comme décrit par Lee-Parsons et Shuler (2002). Malheureusement, cette option a du être abandonnée puisque le nylon agissait comme phase extractive donc il aurait été impossible de déterminer si le contact avec une phase extractive était nécessaire. Par la suite, des poches en grillage fin d'acier inoxydable (45 μm) on été testées. Malheureusement, le grillage en acier inoxydable s'est avéré dommageable pour les cellules de plantes qui sont sensibles au cisaillement. Ainsi, l'idée de poche a été

abandonnée en faveur d'une colonne de recirculation qui empêche le passage des cellules de plantes. Après de nombreux essais un modèle fonctionnel a été élaboré avec un flux de recirculation maximal de 0.5 ml/min. Avant les expériences principales, le système de recirculation a été testé pour comparer les cinétiques d'adsorption avec le système classique où les résines sont mélangées à la suspension cellulaire. Une fois le système de culture avec recirculation bien caractérisé, nous avons pu le comparer au système de culture classique en terme de production de métabolites secondaires lors d'une culture élicitée. Ces résultats sont présentés au chapitre 5 sous la forme d'un article soumis à la revue *Biotechnology and Bioengineering*. Les données détaillées de ce chapitre sont présentées à l'annexe 3 et les résultats complets de l'analyse statistique par test de Student sont présentés à l'annexe 4.

La discussion générale des résultats obtenus est présentée au chapitre 6. Le chapitre 7 présente brièvement les conclusions ainsi que les recommendations pour les futurs travaux.

# Chapitre 4 : Criblage des métabolites secondaires d'*Eschscholtzia californica* par chromatographie liquide haute performance

## *4.1 Présentation de l'article*

Ce chapitre présente un article soumis à la revue Journal of Chromatography A. Il fait l'objet d'une méthode par chromatographie liquide haute performance (HPLC) pour mesurer de façon rapide et précise les principaux alcaloïdes benzophénanthridiques produits dans les cultures cellulaires d'*E. californica*, soit la sanguinarine, la chelirubine, la macarpine, la chelerythrine ainsi que la chelilutine. La séparation des composés a été effectuée sur une colonne $C_{18}$ à phase inverse à l'aide d'un gradient d'acétonitrile et d'acide phosphorique 50 mM. Les molécules recherchées ont été détectées par fluorescence ($\lambda_{ex}$ 330 nm; $\lambda_{em}$ 570 nm) et UV, ce qui a permis une sélectivité adéquate ainsi qu'une bonne précision pour déterminer la pureté des pics. Ainsi, cela a permis une préparation simple et rapide des échantillons par extraction méthanolique pour les mesures intracellulaires et par extraction sur phase solide pour les analyses du milieu de culture. Le manque d'une source commerciale de standards a nécessité le développement d'une méthode semi-préparative pour purifier la chelirubine, la macarpine et la chelilutine. Ensemble, la méthode d'isolation et la méthode analytique ont permis de cribler les métabolites secondaires produits par *Eschscholtzia californica*.

## *4.2 Screening of Eschscholtzia californica secondary metabolites by high-performance liquid chromatography*

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### 4.2.1: Abstract

A rapid and precise HPLC analytic method has been developed for the screening of the major benzophenanthridine alkaloids produced in cell cultures of *E. californica*, namely sanguinarine, chelirubine, macarpine, chelerythrine and chelilutine. Separation is achieved on a $C_{18}$ reversed phase column with gradient elution using acetonitrile and 50 mM phosphoric acid as a buffer. Detection is performed by both fluorescence ($\lambda_{ex}$ 330 nm; $\lambda_{em}$ 570 nm) and diode array detection, leading to good selectivity and precision in determining peak purity. In turn, this permits simple and quick sample preparation involving a methanolic extraction for the measurement of intracellular concentrations and a solid phase extraction for their quantification in the culture medium. Lack of commercially available standards is addressed by the development of a method to purify

chelirubine, macarpine and chelilutine by semi-preparative HPLC. Coupled together, the isolation method and analytical method proved reliable for screening of the alkaloids of interest produced in *E. californica.*

### 4.2.2 Introduction

Benzophenanthridine alkaloids, especially sanguinarine and chelerythrine have attracted a lot of interest for their medical properties. In addition to being used in dental products due to its antibacterial effect (Grenby 1995; Eley 1999), sanguinarine has recently been shown to be promising as a form of cancer treatment (Ahmad et al. 2000; Slaninova et al. 2001; Weerasinghe et al. 2001a; Weerasinghe et al. 2001b). Similarly, due to its inhibition of protein kinase C, chelerythrine is seen as a potential antitumor drug (Chmura et al. 2000). Both of these compounds are produced by *E. californica* plant cells within the metabolic pathway illustrated in Figure 4-1.

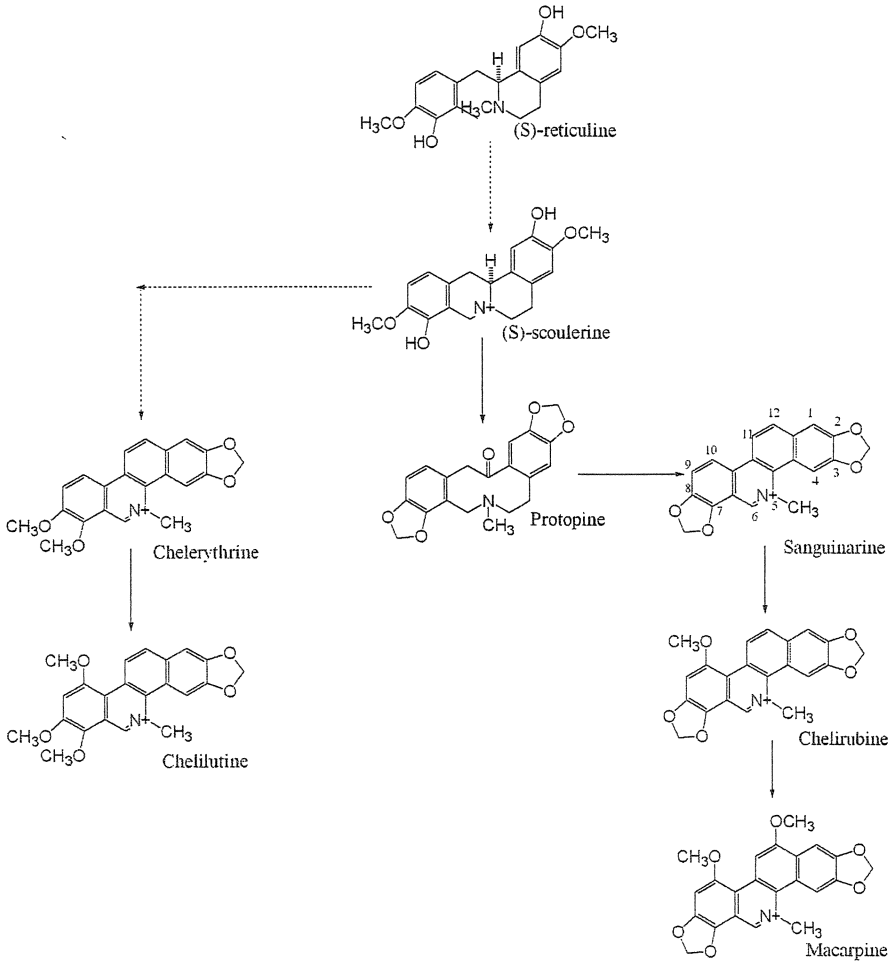



**Figure 4-1: Metabolic pathways of major benzophenanthridine alkaloids found in *E. californica* cell cultures**

**Dotted lines: simplified pathways; Solid lines: known pathways**

The major alkaloids most commonly reported in this plant include sanguinarine, chelirubine, macarpine, chelerythrine, chelilutine and sanguirubine, as well as dihydro intermediates of all of the above. Most of the alkaloids found in *E. californica* can be produced aseptically and under controlled environmental conditions using cell culture. Research related to in vitro production of these secondary metabolites has succeeded in improving production through elicitation (Schumacher et al. 1987; Brodelius et al. 1989;

Byun et al. 1992), in-situ product recovery (Byun et al. 1991; Williams et al. 1992; Dutta 1996) and more recently, metabolic engineering of enzymes involved in the alkaloid metabolic pathway (Facchini et al. 1996; Haider et al. 1997; Park et al. 2002). However, because only some of the constituents of the metabolic pathway are sought after, it is necessary to properly understand the dynamics of the metabolic flux. Thus, an accurate and precise analytical method is needed to measure levels of the main benzophenanthridine alkaloids.

Many different high-performance liquid chromatography methods have been used to evaluate benzophenanthridine alkaloid production. Most of these methods are lengthy at more than 45 min (Schumacher et al. 1987; Tanahashi and Zenk 1990; Tome et al. 1999; Fabre et al. 2000; Park et al. 2002), and require tedious sample preparation due to poorly selective detection. Indeed, a vast majority of methods use UV detection at wavelengths around 280 nm, leading to poorly defined and sometimes overlapping peaks making quantification unreliable (Schumacher et al. 1987; Collinge et al. 1989; Park et al. 2002). To increase selectivity, Chauret et al. (1990, 1991) took advantage of the alkaloids' fluorescence properties, to propose a simple sample preparation involving only an extraction with acidified methanol. Unfortunately, results could not be replicated due to low concentrations of metabolites in cell and especially in medium samples, which made the alkaloids virtually undetectable due to the important dilution during the extraction step. As such, several methods allow quantification of the most important products of *E. californica* secondary metabolism, but most are complicated and long, or imprecise.

In addition, accurate screening and quantification of metabolic changes is complicated to achieve because only sanguinarine and chelerythrine standards are commercially available. In their quantification of alkaloids, Roos et al. (1998) converted fluorescence intensities of all their alkaloids assayed into concentrations via a calibration curve obtained with sanguinarine, rendering the results imprecise. In contrast, two reports describe alkaloid isolation to solve this problem. Byun et al (1990) proposed a method to isolate macarpine that included several tedious extraction steps, followed by FPLC and a final purification with dichloromethane. Schumacher et al. (1987) briefly describe an isolation by semi-preparative HPLC, but no details are provided on the solvents used, the elution profile, or fraction collection. Therefore, to our knowledge, no simple, accurate and detailed method has been proposed for standard purification.

This work proposes an accurate and fast screening method for the secondary metabolism of *E. californica*, based on both fluorescence and UV detection, optimized with a simple sample preparation. In addition, it addresses the need to isolate pure alkaloids that are commercially unavailable.

## 4.2.3 Experimental

### *4.2.3.1 Chemicals*

Sanguinarine and chelerythrine were purchased from Sigma Aldrich (Oakville, Canada) in addition to phosphoric acid, trifluoroacetic acid (TFA), potassium hydroxide (KOH) and glucose. Methanol (MeOH), acetonitrile (ACN) and hydrochloric acid (HCl), all HPLC grade, were obtained from Fisher Scientific (Nepean, Canada).

### 4.2.3.2 Fluorescence spectra

Using a Jasco model 821-FP fluorescence detector, emission spectra were obtained for sanguinarine, chelerythrine and a high-phenolic cell extract at excitation wavelengths of 220, 330 and 460 nm.

### 4.2.3.3 Semi-preparative method to obtain standards

*4.2.3.3.1 Sample preparation*

Previous in vitro experiments with *E. californica* suspension cells have yielded large quantities of a very concentrated alkaloid extract maintained in acidified MeOH (0.5% HCl, v/v). Before injection into the HPLC system, the extract was mixed (1:4 ratio) with a solution containing 0.1% TFA and 20% ACN in water and filtered through a 0.45 μm PTFE membrane syringe filter (Acrodisc, Fisher Scientific)

*4.2.3.3.2 Chromatographic equipment and conditions*

The HPLC apparatus consists of a Beckman Coulter pump module 126 and a model 508 Beckman Coulter auto-sampler, coupled with a Jasco model 821-FP fluorescence detector, a Beckman Coulter model 168 photo diode array absorbance detector as well as a Beckman Coulter Sc-100 fraction collector.

Chromatographic separation of the concentrated extract (injection volume: 0.5 ml) was performed on a semi-preparative Zorbax Eclipse XDB-$C_{18}$ column (250 mm x 11.2 mm I.D.) at a flow rate of 4ml/min using solvent A: 0.1% (v/v) TFA in water and solvent B: ACN. The elution profile was: 0-3 min: 20% B; 3-40 min: linear gradient to 35.2 % B; 40-55 min: linear gradient to 33.25% B; 55-65 min: 33.25% B; 65-95 min: linear gradient to 80% B; 95-120 min: 80% B; 120-125 min: linear gradient to 20% B,

125-130 min: 20% B. Peaks were collected according to peak detection by fluorescence (ex 330 nm, em 570nm) following these parameters: peak detector = 10 mV; peak width = 1 min; peak threshold= 0; flow delay = 15 seconds.

*4.2.3.3.3 Mass spectrometry*

Samples collected from the semi-preparative method were analysed individually using a Micromass Quattro II triple-quadrupole mass spectrometer in positive electrospray ionization. The system parameters were: capillary voltage: 3.2 kV; cone voltage: 32 V; extractor: 5V nebulizing gas flow-rate: 50 l/min; source temperature: 120°C; desolvation gas temperature: 150°C and scanning mass range: 130-750 Da. Samples dissolved in a mixture containing 90 % water, 10 % ACN and 0.1 % TFA were injected into the system at 5 µl/min.

*4.2.3.3.4 NMR analysis*

To obtain $^{1}$H-NMR spectra of the alkaloids, a Varian 400 MHz NMR was used with the following settings: 5 mm probe, T = 25°C, TMS as internal standard, 80 degree pulse (1 sec for H NMR), delay time 1 sec. The data was collected using the Varian software. The samples were dried under nitrogen and dissolved in DMSO before a reading was done.

*4.2.3.3.5 Purification of compounds and standard solution*

The compounds that were identified by MS and NMR were further purified using solid phase extraction to get rid of residual mobile phase. Strata SPE $C_{18}$-E columns (Phenomenex) were conditioned with 3 ml acidified MeOH (0.5% HCl; v/v) followed by 3 ml water. Samples that had been diluted in water (ratio of 1:50) were then loaded,

followed by a 10 ml wash (10% acidified MeOH in water), before three successive elutions with 1 ml acidified MeOH (0.5% HCl; v/v). Methanolic extracts were dried under vacuum at 35°C in pre-weighed glass vials. After 24 hours in a desiccant chamber, glass vials containing pure alkaloids were weighed again on a Sartorius model 1712 precision balance (1 x $10^{-5}$ g precision). The purified compounds were dissolved in known amounts of MeOH to obtain stock solutions with concentrations ranging from 0.1 to 0.5 mg/ml; the solutions were stored at –20°C.

### 4.2.3.4 Analytical method

*4.2.3.4.1 Sample preparation*

*Intracellular alkaloid concentration.* Cell suspensions (10 ml) were filtered and washed twice with 20 ml water under vacuum through a Whatman GF/D 47mm filter (Fisher Scientific). The samples were assayed for fresh and dry cell weight. Filtered cells were extracted overnight in 5 ml acidified MeOH, followed by a 30 min sonication. After a 10 min centrifugation (15 000 RPM), 4 ml of the supernatant was evaporated to dryness under vacuum and re-suspended in 400 µL acidified MeOH (0.5% HCl, v/v) and filtered through a PTFE 0.45 µm filter.

*Extracellular alkaloid concentration.* Medium samples (10ml) obtained by the previously mentioned filtration were subjected to solid phase extraction on a Phenomenex Strata C18-E column (3 ml capacity, 300 mg packing). The column was conditioned with 3 ml acidified MeOH (0.5% HCl; v/v) followed by 3 ml distilled water. 10 ml of sample medium was then loaded and a 3 ml wash (10% acidified MeOH in water) was applied before the final elution with 1 ml of acidified MeOH (0.5% HCl;

v/v). Before injection, samples were filtered through a PTFE 0.45 μm filter.

*Standard solution.* A standard mix was prepared from the stock solutions of sanguinarine, chelirubine, macarpine, chelerythrine and chelilutine, obtaining known individual concentrations ranging from 14.29 to 119.05 mg/l in acidified MeOH (0.5% HCl; v/v). Six dilutions of the standard mix were used to obtain a calibration curve that covers concentrations from 0.22-1.86 mg/l to 7.14-59.52 mg/l.

*4.2.3.4.2 Chromatographic conditions*

The HPLC system used is the same as previously described. Chromatographic separation was performed using a Zorbax Eclipse XDB-$C_{18}$ column (250 mm x 4.6 mm I.D.; 5 μm) coupled with a Securiguard $C_{18}$ guard column maintained at 35°C, with a flow rate of 1.5 ml/min and 20 μl injection volume. The mobile phase consisted of solvent A: 50mM $H_3PO_4$, pH adjusted to 3 with KOH and solvent B: ACN. The elution profile was: 0-2 min: 25% B; 2-12 min: linear gradient to 35% B; 12-14min: 35%B; 14-22 min: linear gradient to 80% B; 22-29 min: 80% B; 29-31min: linear gradient to 25% B, 31-33 min: 25%. Fluorescence signal (ex. 330 nm, em. 570 nm) as well as UV scan from 250 to 450 nm were collected. Sanguinarine, chelerythrine and chelirubine were quantified by fluorescence (ex. 330 nm, em. 570 nm) while macarpine and chelilutine were quantified using the photo-diode array detector at a wavelength of 341 nm. Peak purity was verified by UV spectra symmetry.

*4.2.3.4.3 Extraction yields*

Medium and cell samples were prepared as previously described and spiked with either a known amount of standard mix dissolved in acidified MeOH (0.5% HCl, v/v) or

the same quantity of pure acidified MeOH (0.5% HCl, v/v). The extraction yield was calculated as:

extraction yield = $(C_{i+a} - C_i) / C_a$

where $C_{i+a}$ is the concentration of alkaloid in the spiked sample, $C_i$ is the concentration of alkaloid in the non-spiked sample and $C_a$ is the concentration of alkaloid added to spiked samples.

#### *4.2.3.5 Eschscholtzia californica cell culture*

Suspension cell cultures of *E. californica* were grown in 500 ml Erlenmeyer flasks on B5 medium (Gamborg et al. 1968) supplemented with 30 g/l glucose (HPLC grade, Sigma-Aldrich) as well as 0.2mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin. Cultures were transferred to fresh medium every 10 days and cultivated at 120 rpm, in ambient light at a temperature of 23˚C ± 3 ˚C.

### 4.2.4 Results and Discussion

#### *4.2.4.1 Fluorescence detection*

To determine optimal detection parameters that would improve selectivity and enable method simplification, fluorescence spectra were obtained for the two benzophenanthridine alkaloids available on the market: sanguinarine and chelerythrine and compared to cell extracts containing high levels of phenolic compounds. Based on previous studies, emission spectra were measured at three excitation wavelengths: 220 nm (Chauret et al. 1991), 330 nm (Chauret et al. 1990; Das et al. 1996) and 460 nm (Roos et al. 1998). Maximum signal intensities for sanguinarine and chelerythrine were

obtained at an excitation wavelength of 330 nm. As shown in Figure 4-2, two emission bands appear at this wavelength: a very strong one around 570 nm and a weak one around 440 nm.

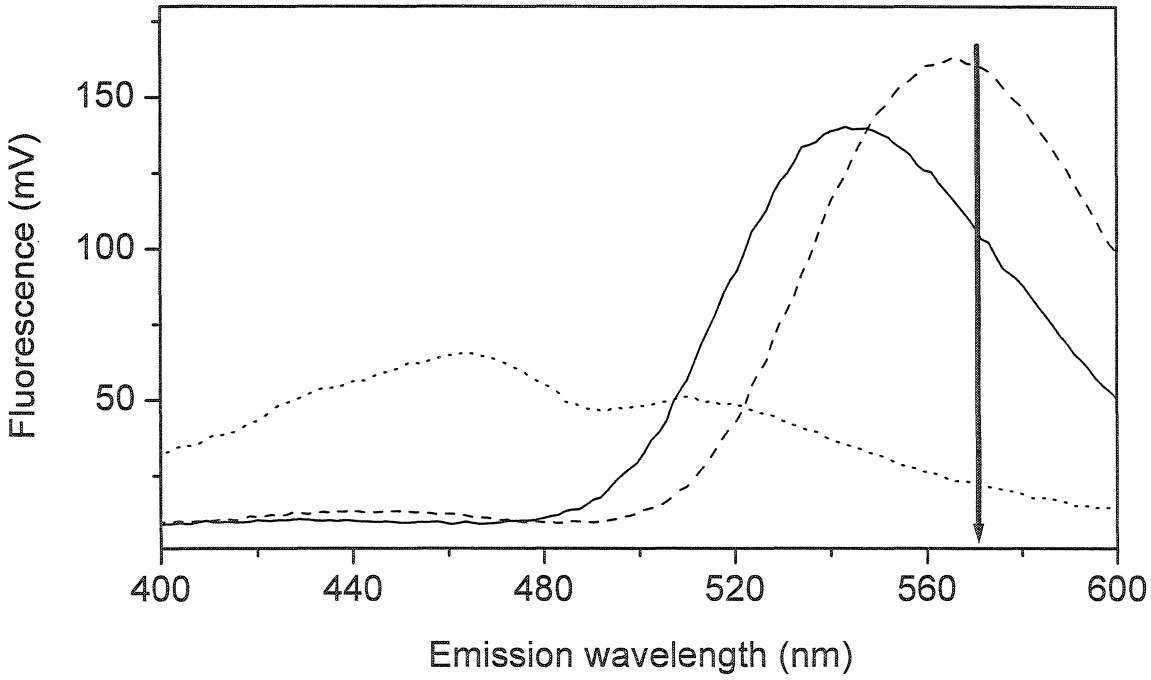



**Figure 4-2: Emission spectra of alkaloids in acidified MeOH (0.5% HCl, v/v) at an excitation wavelength of 330 nm**

**Solid line: Sanguinarine, Dashed line: chelerythrine, dotted line: cell extract with high phenolic compound content**

These fluorescence profiles confirm results by Das et al. (1996) that showed a very strong signal at 580 nm in acidic solvent, attributed to the iminium form of the alkaloid. In addition to a very strong signal at an emission wavelength of 570 nm for sanguinarine and chelerythrine, the signal for the cell extract containing high levels of phenolic compounds was very weak, improving selectivity. Thus, to maximize

selectivity and signal intensity for the compounds of interest fluorescence wavelength of $\lambda_{ex}$ 330 nm and $\lambda_{em}$ 570 nm were chosen.

### 4.2.4.2 Isolation of alkaloids by semi-preparative separation

To facilitate subsequent mass spectrometry analysis, a semi-preparative separation was developed using water, ACN and TFA, based on an LC / MS method by Lépine et al. (2002). Figure 4-3 illustrates the separation that was obtained.

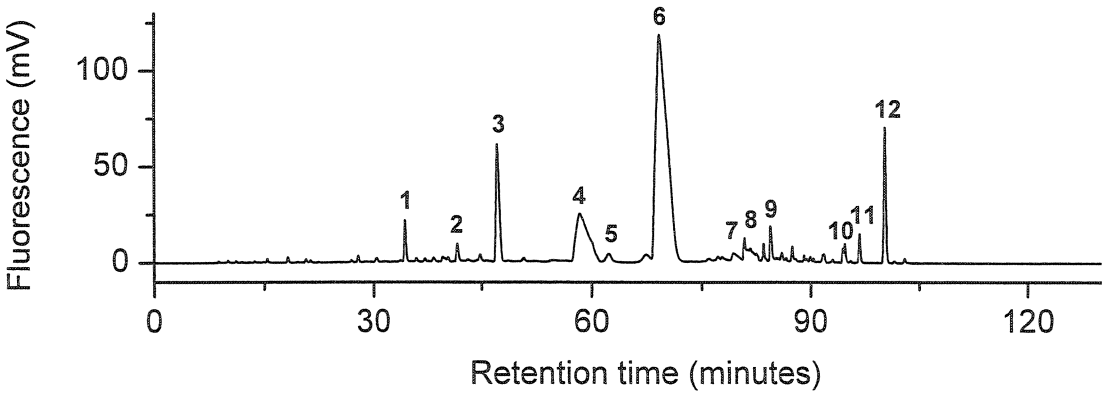



**Figure 4-3: HPLC-fluorescence chromatogram of semi-preparative separation of E. californica extract.**

**Peaks 1 to 12 are those collected by the fraction collector according to the parameters previously described.**

To improve peak separation after 40 minutes, the gradient was reversed. However, no adjustment in gradient allowed better separation after 80 minutes. Twelve peaks were collected with the fraction collector parameters selected. Peaks 1, 2, 3, 5, 7, 9, 10, 11 and 12 all were contained in a single fraction, but three broader peaks were separated into several minute-long fractions, namely peak 4, in 3 fractions, peak 6 in 4 fractions and peak 8 in 2 fractions. To assess the purity of the fractions, samples of each

were analysed by HPLC, using the analytical method described above. Peak UV spectra symmetry was used to determine purity. The fractions corresponding to peak 9, 10 and 11 contained several compounds, while HPLC data were inconclusive for those of peaks 1, 2, 7 and 12. Interestingly, white crystals appeared in the fraction containing peak 12. By comparison with standard, the fraction containing peak 3 was shown to contain mainly sanguinarine and traces of other compounds. Only the middle fraction of peak 4 contained a single compound, identified as chelerythrine by comparison with an authentic standard. The latter fraction contained traces of the compound found in peak 5, while the first fraction contained another compound in addition to chelerythrine. All the fractions of peak 6 were shown to contain the same compound. The second fraction of peak 8 appeared to be pure, while the first contained traces of the compound found in the fraction corresponding to peak 7.

To identify unknown peaks and verify purity, the fractions were further analysed by mass spectrometry. Results are shown in Table 4-1.

**Table 4-1: Mass spectrometry results**

| Peak | Theory $[M+H]^+$ | Observed ions |
|---|---|---|
| chelerythrine | 348 | 348, 332, 318, 304, 290 |
| 4 | 348 | 348, 332, 318, 304, 290 |
| 5 | 362 | 362, 347, 332, 318 |
| 6 | 362 | 362, 348, 334 |
| 7 | 378 | 378, 362, 348 |
| 8 | 392 | 392, 377, 362, 348, 334 |

Only the fractions corresponding to peak 4 to 8 appeared to contain a single chemical, all the others showing multiple compounds. No results were obtained for fraction 12 due to the crystallization of the sample, and our subsequent inability to dissolve it in any of

the several possible common solvents. As expected from previous results, the middle fraction containing peak 4 was confirmed to be chelerythrine by comparison to a standard. The fractions containing peak 5 and 6 presented the same mass spectrum, but according to fractionation and expected abundance, it was hypothesized that peak 6 was chelirubine. Peak 5 could not be identified, as no other common alkaloid known to be present in *E. californica* could present an ion at m/z of 362. Two isomers are possible for peak 7: chelilutine and sanguirubine, thus requiring further investigation. The mass spectrum and chromatographic behaviour of peak 8 are indicative of macarpine.

To discern between possible isomers, and to confirm hypotheses made on the basis of MS results, $^{1}$H-NMR spectra were obtained for compounds that were identified by MS. The chemical shifts of the compounds of interest are shown in Table 4-2.

**Table 4-2: 1H NMR chemical shifts of benzophenanthridine alkaloids**

| Atom | Chelerythrine | Compound 7 | Compound 6 | Compound 8 |
|---|---|---|---|---|
| 1 | 7.77 | 7.73 | 7.72 | 7.73 |
| 4 | 8.28 | 8.19 | 8.13 | 8.15 |
| 6 | 10.07 | 10.00 | 9.97 | 9.83 |
| 9 | 8.81 | 7.75 | 7.80 | 7.91 |
| 10 | 7.51 | - | - | - |
| 11 | 8.84 | 9.41 | 9.42 | 8.87 |
| 12 | 8.31 | 8.23 | 8.26 | - |
| 2 ($OCH_2O$) | 6.33 | 6.32 | 6.33 | 6.32 |
| 5 ($NCH_3$) | 4.96 | 4.90 | 4.89 | 4.81 |
| 7 ($OCH_2O$) | - | - | 6.57 | 6.52 |
| 7 ($OCH_3$) | 4.16 | 4.06 | - | - |
| 8 ($OCH_3$) | 4.02 | 4.14 | - | - |
| 10 ($OCH_3$) | - | 4.24 | 4.24 | 4.16 |
| 12 ($OCH_3$) | - | - | - | 4.18 |

Results for compounds 6 and 8 correspond to the structure of chelirubine and macarpine and closely resemble those found by Tanahashi and Zenk (1990), thus

confirming our previous hypothesis. Chemical shifts obtained for peak 7 indicate that it is chelilutine and not its isomer sanguirubine. The identity of peak 5 was not determined, as the NMR spectra showed traces of another compound.

The semi-preparative method we developed allowed us to isolate pure samples of chelirubine, macarpine and chelilutine, for the study of the main secondary metabolites in *E. californica*.

### *4.2.4.3 Analytical method*

Based on literature, several organic solvents and buffers were tested to separate a crude methanolic extract of *E. californica*. A relatively good separation was obtained using a simple gradient elution similar to that used by Tanahashi and Zenk (1990) and Schumacher et al. (1987), with acetonitrile and 50 mM phosphoric acid buffer adjusted to pH 2.5. However, some peaks remained poorly separated and the method was too lengthy. Therefore, a more complex method, with several gradient steps, was devised leading to a shorter method and good separation of the main alkaloids. Figures 4-4 and 4-5 illustrate the separation of intracellular and medium samples.

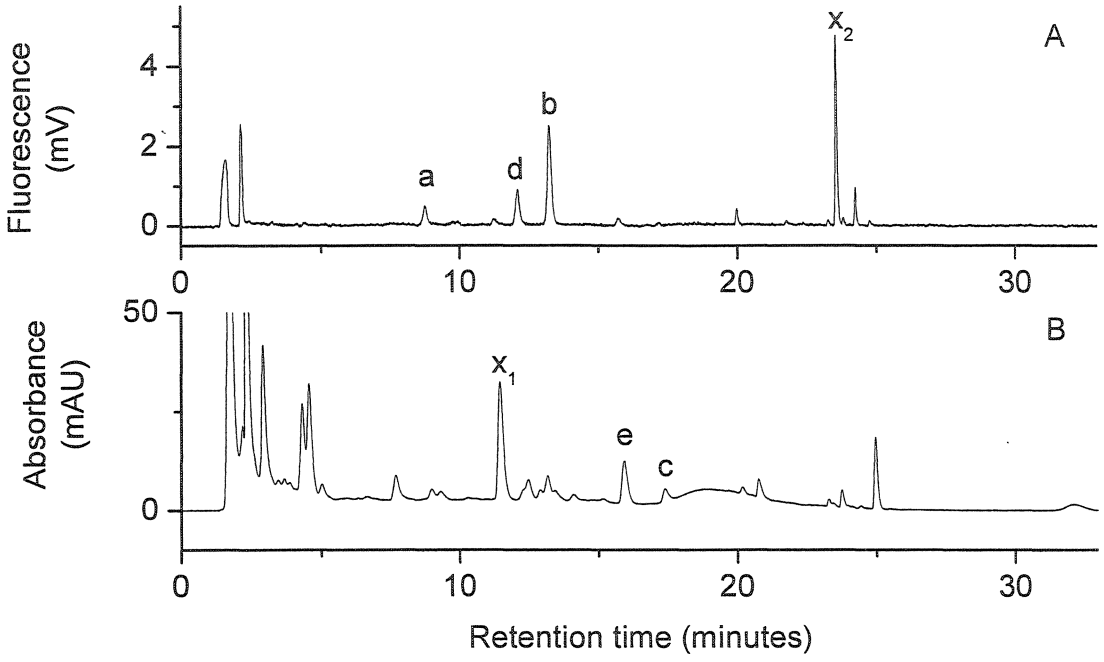



**Figure 4-4: HPLC chromatogram of analytical separation of *E. californica* cell extract.**

**(A): Fluorescence (ex 330 nm; em 570 nm), (B): Absorbance at 343 nm. a: sanguinarine, b: chelirubine, c: macarpine, d: chelerythrine, e: chelilutine, x1 and x2: unknowns 1 and 2**

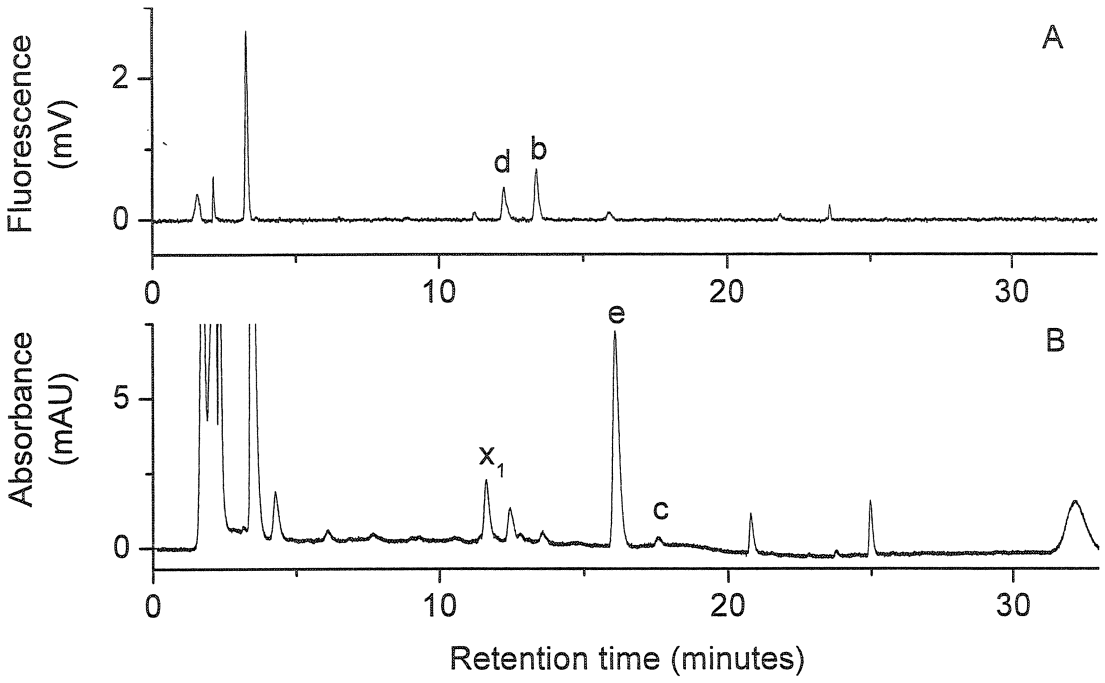



**Figure 4-5: HPLC chromatogram of analytical separation of *E. californica* cell culture medium extract.**

**(A): Fluorescence (ex 330 nm; em 570 nm), (B): Absorbance at 343 nm. a: sanguinarine, b: chelirubine, c: macarpine, d: chelerythrine, e: chelilutine, x1: unknown 1**

In addition to fluorescence detection, an absorbance scan was performed from 250 to 450 nm and the UV spectra were recorded (Fig 4-6.) for standards.

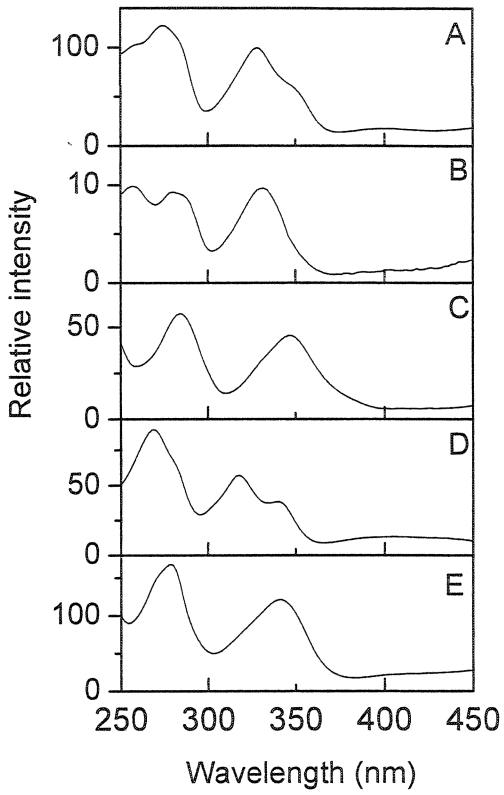



**Figure 4-6: Absorption spectra of benzophenanthridine alkaloids.**
**A: sanguinarine, B: chelirubine, C: macarpine, D: chelerythrine, E: chelilutine**

In the case of macarpine and chelilutine, the fluorescence signal was low, so the main detection was performed by absorbance. Both of these alkaloids had two peaks in their UV spectra, at approximately 285 nm and 343 nm. Even though the maximum absorbance wavelength was 285 nm, 343 nm was chosen for detection since the selectivity was increased, as less interfering peaks appeared in the chromatograms of intracellular plant extracts and cell medium extracts (results not shown). The detection of chelerythrine and chelirubine by fluorescence compared to UV clearly improved selectivity, since many interfering peaks appear in the absorbance chromatogram, while

well-defined peaks allow precise quantification with fluorescence. Optimized detection parameters for each alkaloid are summarized in Table 4-3.

**Table 4-3: Detection parameters and extraction yield of each alkaloid**

| **Chemical** | Retention time (min) | detection | Extraction yield (cells)[a] | Extraction yield (medium)[a] |
|---|---|---|---|---|
| Sanguinarine | 8.3 | Fluorescence, ex 330 nm, em 570 | 91 ± 24 | 91 ± 3 |
| Chelerythrine | 12.4 | Fluorescence, ex 330 nm, em 570 | 99 ± 10 | 92 ± 5 |
| Chelirubine | 13.2 | Fluorescence, ex 330 nm, em 570 | 81 ± 16 | 90 ± 4 |
| Macarpine | 17.3 | PDA, 343 nm | 81 ± 27 | 94 ± 5 |
| Chelilutine | 19.5 | PDA, 343 nm | 84 ± 25 | 91 ± 4 |

[a] Intervals are standard deviations (n=3)

Sample preparation was based on methods proposed by Chauret et al. (1990, 1991), but optimized to take into account low sample concentrations. Indeed, for intracellular assay, only one extraction with methanol was performed, followed by an evaporation step to concentrate the sample by a factor of 10. In the case of culture medium samples, which contain very low concentrations of alkaloids, solid phase extraction was performed, concentrating the alkaloids contained in 10 ml of medium into 1 ml of acidified MeOH (0.5% HCl; v/v). The accuracy of our sample preparation techniques was tested by measuring extraction yield. The results are shown in Table 4-3. All yields are above 80 % for both intracellular and culture medium extraction, testifying to the reliability of our method.

Aside from the main alkaloids assayed, two large unknown peaks appeared in the chromatograms (Figures 4-4 and 4-5). According to its UV spectrum and retention time,

unknown 1 appears to be the additional compound that was found by semi-preparative isolation in the early fraction containing peak 4, while unknown 2 could be the compound that crystallized in fraction 12. Further investigation will be required to properly purify and identify these two additional peaks. Nevertheless, the current method is adequate to study major metabolic fluxes in *E. californica* secondary metabolism.

### 4.2.5 Conclusion

An analytical HPLC method was developed that allows accurate and rapid quantification of sanguinarine, chelirubine, macarpine, chelerythrine and chelilutine. Detection by fluorescence and absorbance led to improvement in selectivity and precision compared to previous methods reported. In addition, due to higher selectivity, a simplified sample preparation proved to be adequate and reliable. In parallel, a simple, automated semi-preparative method was developed to purify the alkaloids unavailable commercially, namely macarpine, chelirubine and chelilutine. As a whole, this report presents a technique that will facilitate future metabolic engineering studies requiring measurements of fluxes in benzophenanthridine alkaloid metabolism.

### 4.2.6 Acknowledgments

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# Chapitre 5 : L'effet de la stratégie d'extraction in situ sur la production de métabolites secondaires dans des cultures en suspension d'*Eschscholtzia californica*.

## *5.1: Présentation de l'article*

Ce chapitre présente un article soumis à la revue Biotechnology and Bioengineering. L'effet du contact entre la phase extractive et les cellules a été étudié dans des cultures bi-phasiques de cellules d'*E. californica* en suspension. Un système de culture a été développé de façon à pouvoir extraire les alcaloïdes benzophénanthridiques de la culture sans qu'il n'y ait de contact entre les cellules et les résines extractives XAD-7: seul le milieu de culture était recirculé à travers une colonne remplie de la phase extractive. Cette méthode a été comparée avec la méthode traditionnelle où les résines sont directement mélangées à la suspension cellulaire. L'extraction des produits par recirculation a engendré une hausse importante de la production, soit 20 fois plus de sanguinarine et 10 fois plus de chelerythrine. Ce système a grandement simplifié le procédé de production étant donné que les résines sont faciles à récupérer de la culture et cette stratégie permet la récolte des composés recherchés sans l'arrêt de la culture. Malgré cela, les conditions expérimentales ont limité le flux de recirculation. En plus d'augmenter la production de métabolites secondaires par rapport à une culture témoin sans particules extractives, l'extraction des métabolites secondaires du milieu par les colonnes externes a causé un changement de la distribution des flux métaboliques, ce qui semble témoigner d'un mécanisme complexe du contrôle de production.

## *5.2: In situ extraction strategy affects benzophenanthridine alkaloid production fluxes in suspension cultures of Eschscholtzia californica*

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### 5.2.1: Abstract

The effect of contact between cells and extractive phase on secondary metabolite production was investigated in two-phase suspension cultures of *E. californica*. A system was designed to extract benzophenanthridine alkaloids from the cell culture, without contact between XAD-7 resins and the cells: only medium was recirculated through a column packed with the extractive phase. This strategy was compared to the classic method of addition of resins directly into the cell suspension. Removal of the product directly from the medium enabled important increases in production of alkaloids, namely a 20-fold increase in sanguinarine production and a 10-fold increase in chelerythrine, with high recovery in the resin. The recirculation strategy greatly

simplified the production process since the resins are easily recovered from the cell culture and enable harvest of product without termination of culture. However, due to limited flow rate, the recirculation strategy was slightly less effective than direct addition of resins into the cell suspension. In addition to enabling increased production, removal of secondary metabolites from the medium changed metabolic flux distribution, testifying to a complex control mechanism of production.

### 5.2.2: Introduction

As part of their secondary metabolism, plants produce a broad array of complex molecules, some of which have very potent pharmaceutical activity. Benzophenanthridine alkaloids, especially sanguinarine and chelerythrine have attracted a lot of interest for their medicinal properties. In addition to being used in dental products due to its antibacterial effect (Grenby 1995; Eley 1999) sanguinarine has recently shown promise as a form of cancer treatment, namely for leukemia (Ahmad et al. 2000; Slaninova et al. 2001; Weerasinghe et al. 2001; Weerasinghe et al. 2001). Similarly, due to its inhibition of protein kinase C, chelerythrine is seen as a potential antitumor drug (Chmura et al. 2000). Both of these compounds are produced as part of the metabolic pathway illustrated in Figure 5-1. As interest has grown for these fine chemicals, different large-scale production methods have been investigated. Traditional whole plant culture and harvest is not sustainable due to plant scarcity and unpractical purification. Chemical synthesis of these very complex molecules is difficult and involves too many steps to be financially viable. *In vitro* suspension cell culture has thus

garnered a lot of attention, but for most plants, it remains a non-competitive option due to low and non-reproducible metabolite yields and thus, poor productivity. Several methods have been used to improve yield, namely elicitation to activate secondary metabolism (Schumacher et al. 1987; Collinge and Brodelius 1989), medium optimization to maximize metabolite production (Lecky et al. 1992; Archambault et al. 1996) and *in-situ* extraction to recover chemicals from culture and increase production. The addition of extractive phases, either solid or liquid, into plant cell cultures has been shown to improve secondary metabolite production in several plant species, in addition to simplifying bioprocesses by improving efficiency of product recovery. For example, Asada and Shuler (1989) reported a 67% increase in ajmalicine production with the addition of XAD-7 resins to *Catharanthus roseus* cultures, with 63% recovery on resins. Williams et al. (1992) found that the addition of XAD-7 resins to *Papaver somniferum* cell cultures improved total sanguinarine production by 50 to 85 %, with about 75 % of the alkaloid recovered in the resin. A more recent report on the addition of XAD-7 to *C. roseus* showed that increase in production and the recovery of products were both dependant on the timing of the addition (Lee-Parsons and Shuler 2002). Several types of liquid extractive phases were also investigated. Byun et al. (1990) found that the addition of silicon oil to *E. californica* cell cultures increased alkaloid production 3.4 fold in shake flask cultures, and 3 fold in two-phase airlift fermentor cultures (Byun and Pedersen 1994). Similarly, Dutta et al. (1994) reported a 4 fold increase in alkaloid production with the addition of tricaprylin to *E. californica* cell cultures. In hairy roots

cultures of *C. roseus*, Tikhomiroff et al. (2002) found that the addition of silicon oil increased specific yields of lochnericine and tabersonine.

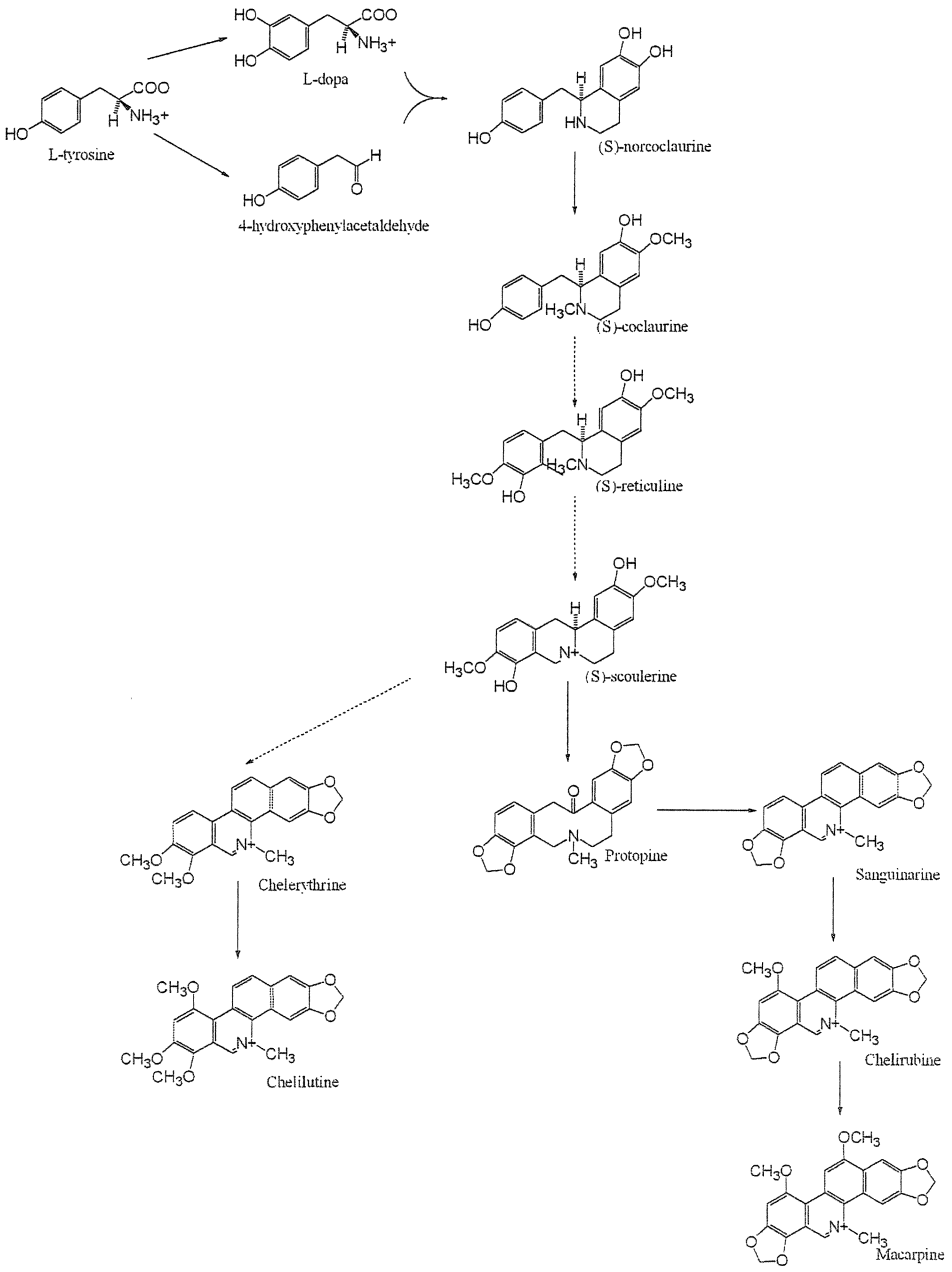



**Figure 5-1: Metabolic pathway in *Eschscholzia californica* leading to benzophenanthridine alkaloids**

**Dotted lines indicate the simplification of several steps**

It remains unclear exactly how extractive phases increase secondary metabolite production. It has been suggested that intracellular levels of alkaloids are tightly controlled: production depends on storage capacity as well as secretion mechanisms that both purportedly enable removal of the product from the site of synthesis, thereby alleviating negative feedback (Lee-Parsons and Shuler 2002). Thus, the most common hypothesis is that extractive phases reduce negative feedback by removing the metabolites from the cell culture and providing an external storage site for the alkaloids. As described by Brodelius and Pedersen (1993), transport of the secondary metabolites is redirected towards the extracellular space instead of being directed towards the vacuole, whose storage capacity limits alkaloid production. Results reported by Williams et al. (1992) seem to support this hypothesis since the addition of extractive resins into cell suspensions reduced sanguinarine concentrations in both cells and medium by about 50%. However, in other cultures, the addition of an adsorptive phase did not substantially decrease cellular concentrations of alkaloids, despite increases in total production (Byun et al. 1990; Byun and Pedersen 1994). In some *Taxus* cultures, the addition of tricaprylin resulted in high recovery from medium, but did not significantly increase total taxol production, leading the authors to propose that feedback inhibition was not a limiting factor in the cell cultures that were investigated (Collins-Pavao et al. 1996). In *C. roseus* hairy roots cultures, Tikhomiroff et al. (2002) showed that alkaloids that were normally observed to remain intracellular were recovered extracellularly using silicon oil. Wang et al. (2001) suggested that the increase in secondary metabolite production may be due to the interaction of the extractive phase, in

this case liquid solvents, with the cell membrane. Perhaps the extractive phase can influence the secretion of metabolites by modulating the transport across the external membrane, which has been shown to be active (Villegas et al. 2000). In sum, it appears that the effect of extractive phases are species specific, as well as cell line specific and the mechanism of action remains unclear, namely whether the contact between cells and extractive phase is necessary for increased secondary metabolite production.

In this study, we investigated whether the contact between XAD-7 resins and *E. californica* cells is necessary for increased alkaloid production and whether it has an effect on the metabolic fluxes of the secondary metabolism. Shake flask cultures of *E. californica* were performed and only culture medium was recirculated into an external tube containing a packed bed of XAD-7 resins. This study aims at improving the understanding of the mechanism by which the presence of an extractive phase increases alkaloid production. Furthermore, it may offer potential improvement of production processes using suspension plant cells by simplifying the harvesting procedure.

## 5.2.3: Material and methods

### *5.2.3.1 Plant cell cultures*

To obtain plant cell calluses, sterile hypocotyls sections of *E. californica* were placed on B5 medium (Gamborg et al. 1968) containing 30 $g.L^{-1}$ of glucose (Sigma) as well as 0.2 $mg.L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma) and 0.1 $mg.L^{-1}$ kinetin (Sigma) adjusted to pH 5.6 using KOH and solidified with 8 $g.L^{-1}$ agar (Fisher Scientific). Cell suspension cultures were initiated by adding calli to 500 mL Erlenmeyer

flasks with B5 liquid medium (same composition as described above) maintained at 130 rpm. Once established several years ago, the suspension cultures were subcultured every 10-13 days, when the sedimented cell volume (after 5 minutes) attained 70-80% of the total volume: 80 g of cell suspension was added to 170 g fresh medium. Cultures were maintained at 130 rpm, 24°C ± 2°C, under normal continuous indoor light.

### 5.2.3.2: Adsorbent resin preparation

Polymeric XAD-7 Amberlite resins (Sigma) were washed overnight in excess methanol at 130 rpm. The resins were subsequently rinsed four times in deionised water during 2 hours to remove all traces of methanol (500 mL $H_20$ per 100 mL resins). After separation on a nylon 400 µm mesh, only larger resins were kept for experiments. Prior to addition into culture, resins were autoclaved for 30 min.

### 5.2.3.3: Elicitor preparation

A chitin extract was used to elicit cell cultures. Crude chitin (from crab shells, Sigma) was crushed in a mortar with deionised water and subsequently, enough water was added to obtain a mixture containing 2 $g.L^{-1}$ crude chitin. The mixture was then autoclaved for 30 minutes, stirred during cool down and filtered through two layers of Miracloth (Calbiochem, La Jolla, CA)

### 5.2.3.4: Cell culture system with recirculation of medium and culture conditions

To investigate whether contact between cells and extractive phase was necessary, a system was devised to recirculate medium through a column containing 7 g of polymeric XAD-7 resins. Figure 4-2 illustrates the system used. To separate medium

from cells, which are about 75-200 μm and form clusters, a 45 μm stainless steel mesh (Spectrum Laboratories) was fitted to the end of a 4.5 mm internal diameter glass column using a glass tube of a slightly larger diameter. The outside glass tube, protruding by 2.5 mm, also provided protection from cell lysis by preventing mechanical shear between the stainless steel mesh and the bottom of the glass Erlenmeyer. Medium flow through the column was obtained by running the tubing attached to the column through a peristaltic pump (Masterflex, Labcor, Anjou, Québec, Canada) set to 0.5 $mL.min^{-1}$. The void volume of the column and tubing was 5 mL, not a significant amount compared to the total working volume of 145 mL.

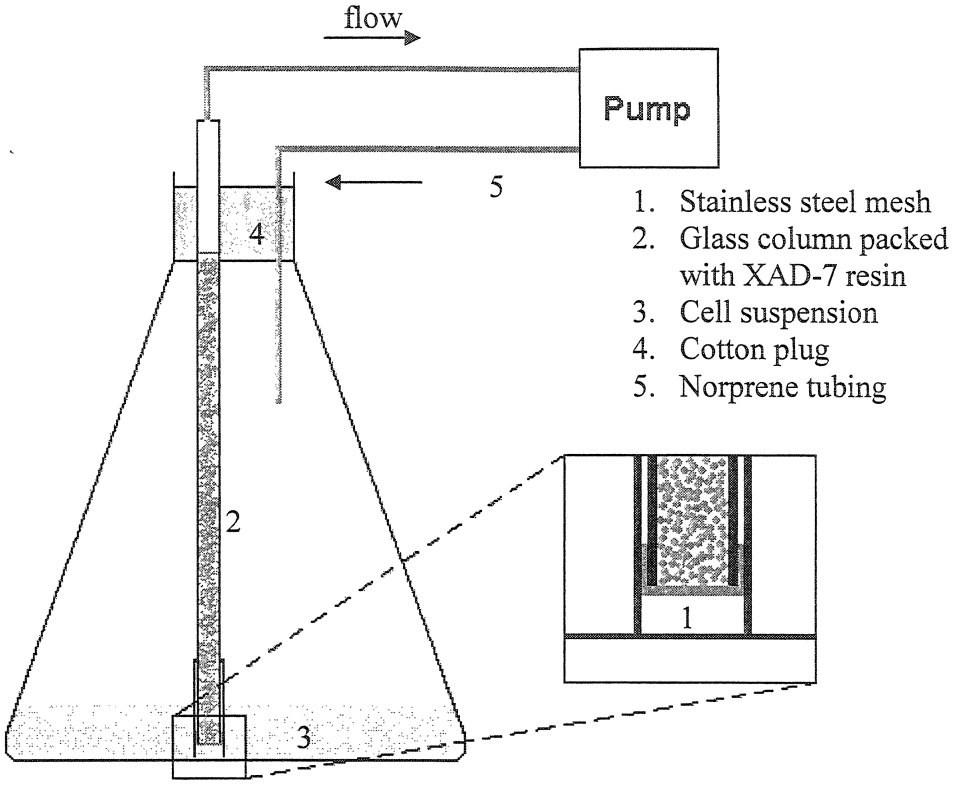



**Figure 5-2: Culture system configuration with recirculation of medium through column filled with XAD-7 resins**

**A 4.5 mm internal diameter glass column (2) was fitted with a 45 µm stainless steel mesh (1) as well as a second section of glass column with a 4.7 mm internal diameter, protruding 2.5 mm from the end of the thinner column. A 1.6 mm internal diameter norprene tubing (5) was used to recirculate medium at a flow rate of 0.5 ml.min$^{-1}$ with a peristaltic pump. The column was placed against the bottom of a 500 mL glass Erlenmeyer flask filled with 145 mL cell suspension, closed of with a cotton plug permeable to air.**

Experiments on alkaloid production were performed on elicited cell cultures in 500 mL glass Erlenmeyer flasks. Four day old cells were decanted on a doubled 210 µm nylon mesh (Spectrum Laboratories) and 30 g of the obtained wet biomass was weighted into flasks containing 20 mL elicitor extract in addition to 125 mL MS (Murashige and Skoog 1962) medium modified to contain no phosphate ions, no vitamins and no added hormones. Previous studies performed on the same cell line (results not shown) to

determine optimal ion, vitamin, hormone and elicitor concentrations as well as elicitation timing had shown that these conditions were best for high secondary metabolite production in this cell line. The cultures, which were maintained at 130 rpm and 24°C ± 2°C under continuous indoor light, were subjected to either of the four following conditions. A: control condition without any extractive phase; B: 7 g (wet weight) XAD-7 resins added to cell suspension; C: medium recirculation through column containing 7 g XAD-7 resins; D: medium recirculation through column containing 7 g XAD-7 resins, removal of column at day 6 and addition of 7 g resins into cell suspension. Cultures were harvested 7 days after elicitation and assayed for pH, fresh and dry cell weights as well as alkaloid content in cells, medium and resins.

### *5.2.3.5: Adsorption kinetics*

Kinetics of alkaloid removal from the medium with the recirculation system were compared to those with resins in suspension. A crude concentrated mixture of alkaloids obtained from previous cultures was dissolved into MS medium without glucose, adjusted to pH 6 to mimic conditions found in cell culture. Initial alkaloid concentration were within the range to those found in cell culture, at 0.12 $mg.L^{-1}$ sanguinarine, 0.70 $mg.L^{-1}$ chelirubine, 0.66 $mg.L^{-1}$ macarpine, 0.27 $mg.L^{-1}$ chelerythrine and 0.90 $mg.L^{-1}$ chelilutine. 5 mL samples were collected from test mixtures (initial volume = 150 mL) where either 7 g of resins were added directly to the medium or medium was recirculated through a column with 7 g resins at 0.5 $mL.min^{-1}$.

### *5.2.3.6: Alkaloid extraction and analysis*

Cell suspension (10 mL) was filtered and washed twice with 20 mL distilled water under light vacuum through a Whatman GF/D 47 mm filter (Fisher Scientific). The sample thus obtained was assayed for fresh cell weight and dry cell weight after 24 hours in a 60°C oven. Biomass, obtained by filtration of 10 mL cell suspension as previously described, was extracted overnight in 5 mL acidified MeOH (0.5% HCl, v/v), followed by a 30 min sonication. To concentrate the extract, after a 10 min centrifugation, 4 mL of the supernatant was evaporated to dryness under vacuum and re-suspended in 400 µL acidified MeOH (0.5% HCl, v/v). Extracellular medium alkaloid extract was obtained by solid phase extraction on a Phenomenex Strata $C_{18}$-E column (3 mL capacity, 300 mg packing). The column was conditioned with 3 mL acidified MeOH (0.5% HCl; v/v) followed by 3 mL distilled water. 10 mL of sample medium was then loaded and a 3 mL wash (10% acidified MeOH in water; v/v) was applied before the final elution with 1 mL of acidified MeOH (0.5% HCl; v/v). Resins were separated from biomass by decantation and several rinses in distilled water. No significant amounts of alkaloids were lost in the rinses. Total resin content (7g wet weight) was extracted overnight under agitation in 10 mL acidified MeOH (0.5% HCl; v/v). After removal of saturated MeOH, the extraction was repeated 9 more times and all 10 fractions were combined.

Extracts from cells, medium and resins were analysed for alkaloid content using the following chromatographic method. The HPLC apparatus used consists of a model 126 Beckman Coulter pump module and a model 508 Beckman Coulter auto-sampler,

coupled with a model 821-FP Jasco fluorescence detector and a model 168 Beckman Coulter photo diode array absorbance detector. Chromatographic separation was obtained using a Zorbax Eclipse XDB-$C_{18}$ column (250 mm x 4.6 mm I.D.; 5 µm) coupled with a Securiguard $C_{18}$ guard column maintained at 35°C, with a flow rate of 1.5 $mL.min^{-1}$ and 20 µl injection volume. The mobile phase consisted of solvent A: 50mM $H_3PO_4$, pH adjusted to 3 with KOH and solvent B: acetonitrile. The elution profile was: 0-2 min: 25% B; 2-12 min: linear gradient to 35% B; 12-14 min: 35%B; 14-22 min: linear gradient to 80% B; 22-29 min: 80% B; 29-31 min: linear gradient to 25% B, 31-33 min: 25%. Sanguinarine, chelerythrine and chelirubine were quantified by fluorescence (ex. 330 nm, em. 570 nm) while macarpine and chelilutine were quantified by absorbance at a wavelength of 341 nm. Peak purity was verified by UV spectra symmetry. Calibration curves were obtained with standards that were purchased: sanguinarine and chelerythrine (Sigma) and standards obtained from a semi-preparative method.

#### *5.2.3.6: Statistical analysis*

All relevant experimental data was analyzed for statistical significance by t-test considering a degree of probability of 5%.

### 5.2.4: Results

To determine whether there was a contact effect between extractive phase and cells, a culture system was designed to allow extraction of alkaloids from medium without cell contact with XAD-7 resins and without modification of culture parameters.

Different extraction strategies were assessed. A first attempt was made by enclosing resins in nylon pouches (75 μm, Spectrum Laboratories) similarly to what was described by Lee-Parsons and Shuler (2002), but results (not shown) showed that the nylon itself adsorbed some alkaloids, therefore we were not able to determine whether contact was necessary. Pouches made of very fine stainless steel mesh were thus tested to separate cells from resins because they do not adsorb alkaloids or affect medium composition. However, due to its mesh size (45 μm), the stainless steel damaged the shear-sensitive plant cells when used to enclose resins in pouches. It is thought that the shear stress generated from the contact of the nylon and the stainless steel meshes elicited and damaged the cells. For this reason, a column with a stainless steel mesh cap was designed to limit shear between cells and mesh by the addition of a second glass tube around the inside tube in order to create an area with reduced cell mixing near the mesh (see enlargement in Figure 5-2). To establish the proper flow rate, preliminary cell cultures were run at different rates while culture parameters were followed. Results (not shown) revealed that the maximal flow rate possible was 0.5 $mL.min^{-1}$. At higher flow rates, cells would plug the mesh covering the inlet of the recirculation column and thus recirculation would be terminated.

The ability of the recirculation system to remove alkaloids was compared to that of XAD-7 resins mixed in with the medium (Figure 5-3). At concentrations comparable or higher to those found in cell culture, the resins mixed into the medium removed more than 95 % of the alkaloids from the medium within 15 minutes. There was no apparent difference in adsorption kinetics between the different alkaloids. In contrast, due to the

slow flow rate, it took 10 hours for the recirculation system to remove 90% of alkaloids from the medium.

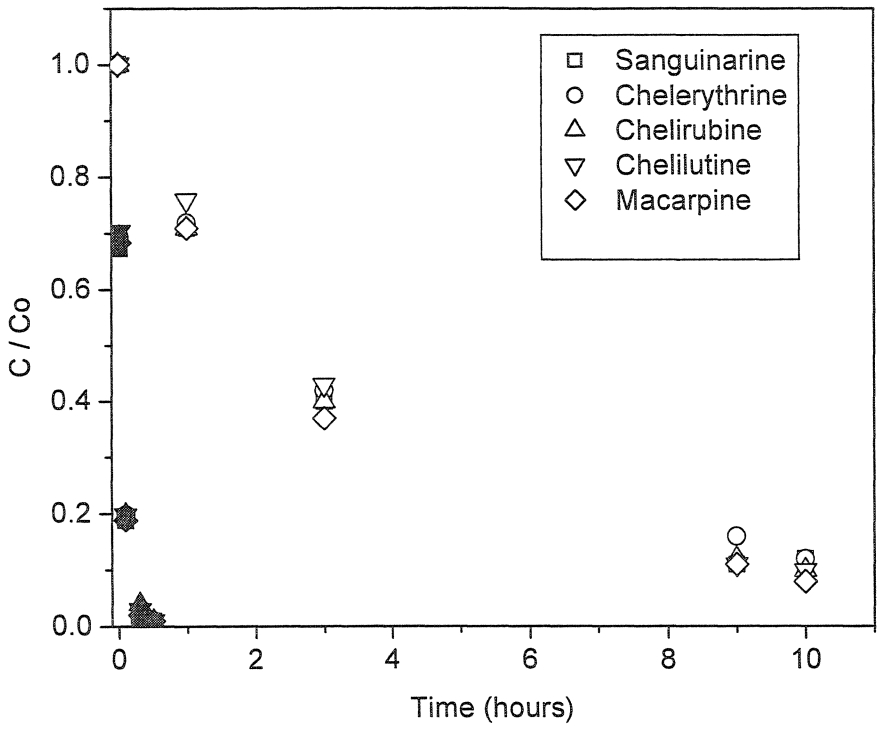



**Figure 5-3: Removal rate of alkaloids**

**Kinetics of adsorption of alkaloids from MS medium (no glucose), at pH 6, with initial concentrations of 0.12 mg.L$^{-1}$sanguinarine (■, □), 0.70 mg.L$^{-1}$ chelirubine (▲, △), 0.66 mg.L$^{-1}$ macarpine (◆, ◇), 0.27 mg.L$^{-1}$ chelerythrine (●, ○) and 0.90 mg.L$^{-1}$ chelilutine (▼, ▽). C: concentration of alkaloid in the medium, Co: initial concentration of alkaloid in the medium. Solid symbols: removal of alkaloids by resins in suspension; Empty symbols: removal of alkaloids by recirculation of medium through column with XAD-7 resin at 0.5 mL.min$^{-1}$ (n=3)**

### *5.2.4.1: Effect of extractive strategy on alkaloid production*

To verify whether addition of extractive phase to cell cultures had an effect on cell growth and cell viability, fresh and dry weights were assayed at harvest. Results shown in Figure 5-4 demonstrate that neither addition of resins into cell suspension nor recirculation of medium through a column at 0.5 mL.min$^{-1}$ had any negative effect on

cell growth or water content. In addition, extractive phase, whether in contact with cells or not, does not appear to have had an important impact on the pH in the culture medium (Figure. 5-5). Thus, the adsorption capacities relative to pH were the same in all experimental conditions (Williams et al. 1992).

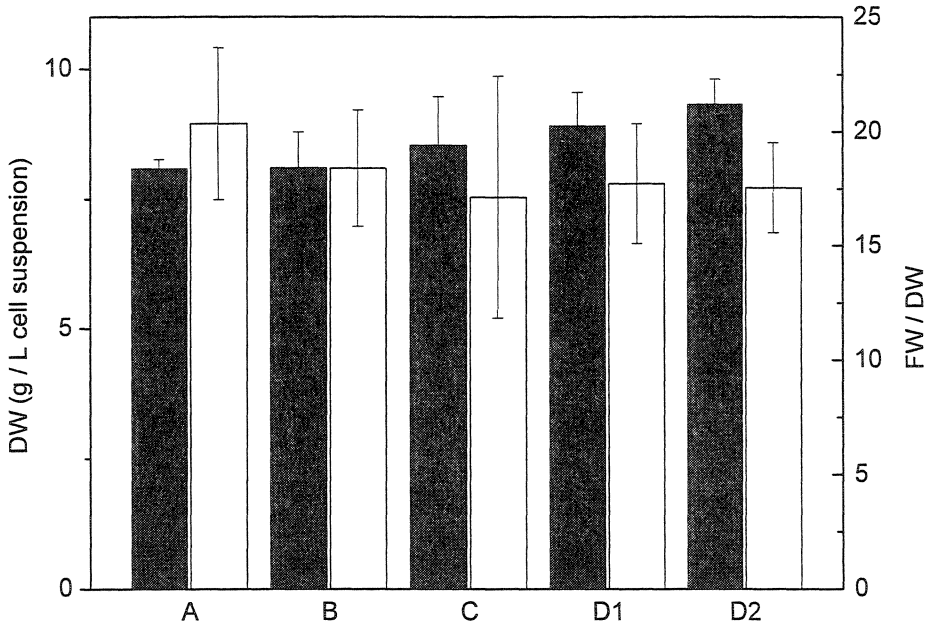



**Figure 5-4: Growth of plant cells for the different extraction strategies**

**Dry weight: DW (Solid bars) and ratio fresh weight to dry weight: FW/DW (Empty bars) values at harvest in different culture conditions. A: control, no extractive phase (harvest after 7 days); B: XAD-7 resins in suspension with plant cells (harvest after 7 days); C: recirculation of medium through column with XAD-7 resins (harvest after 7 days); D1: recirculation of medium through column with XAD-7 resins (harvest after 6 days); D2: removal of column 6 days after elicitation, and addition of resins into suspension (harvest 1 day later, at day 7). Error bars are standard errors (n=4)**

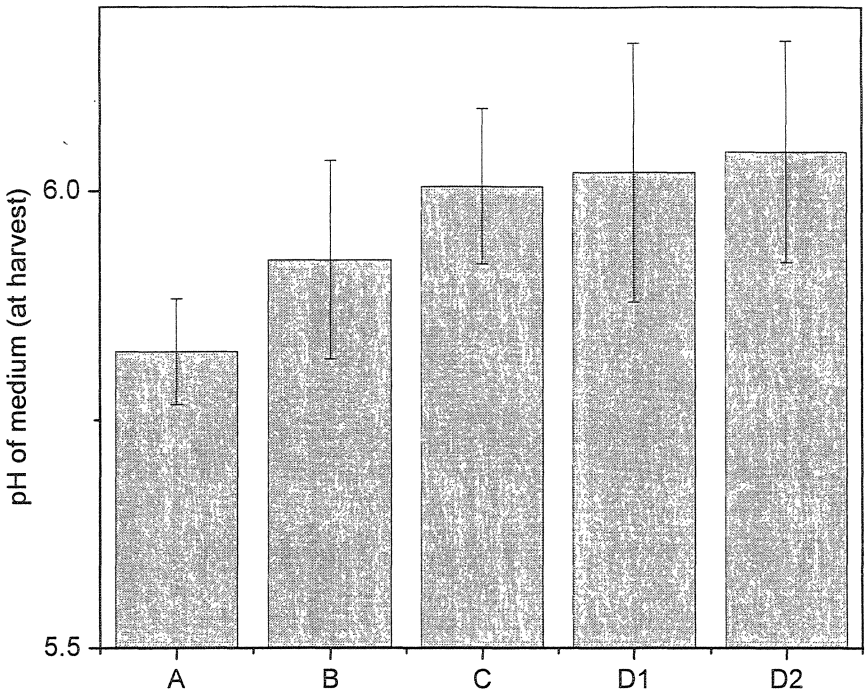



**Figure 5-5: pH of suspension culture for the different culture conditions Refer to Figure 5-4 for legend. Error bars are standard errors, n=4.**

The effect of contact between cells and extractive phase was investigated by measuring the known alkaloids produced in elicited cultures where, either XAD-7 was added directly to the cell suspension, or only medium was recirculated through a packed bed of the extractive phase. Since the recirculation flow rate through the column limited adsorption rate of alkaloids from the medium as previously discussed, a fourth treatment was designed to determine whether the high production capacity derived from the recirculation could be harnessed by increasing the adsorption rate. Thus, a culture was run where the medium was recirculated during 6 days, then the column was removed, and resins were added to the cell suspension. Figure 5-6 illustrates the total production of each alkaloid in the four different culture conditions. In general, an important increase

in alkaloid production was observed when alkaloids were adsorbed from the medium, either by addition of resins directly into the cell suspension or recirculation of medium through a column with resins (Fig. 5-6: conditions B, C and D versus A). However, the influence on the production was not the same for each alkaloid.

In the case of sanguinarine, the addition of XAD-7 resins into the cell suspension resulted in a 20 to 40 fold increase in total production, 96% of which was recovered in the resins (Fig. 5-6: B versus A). Recirculation of the medium through a column with XAD-7 also brought about an important rise in production, at about 10 to 30 times the sanguinarine in the control (Fig. 5-6: C versus A), but less recovery in the resins at 83%. Although total sanguinarine production seems lower with recirculation compared to direct contact, the difference is not statistically significant. The addition of resins into the cell suspension after 6 days of recirculating the medium greatly enhanced the total production; the amount of sanguinarine recovered from production during the $7^{th}$ day was about the same as the amount recovered in 7 days with the XAD-7 resins in the cell suspension (Fig. 5-6: D versus A). Interestingly, no significant difference was observed in intracellular levels of sanguinarine between control and all other culture conditions, even though the intracellular level with recirculation appears higher. Unfortunately, sanguinarine was not detected in the medium under any of the conditions, most likely because the very low levels were below the detection limits of our analytical methods, despite concentrating samples before HPLC analysis. Therefore, the impact of each culture condition on sanguinarine medium concentrations was not evaluated.

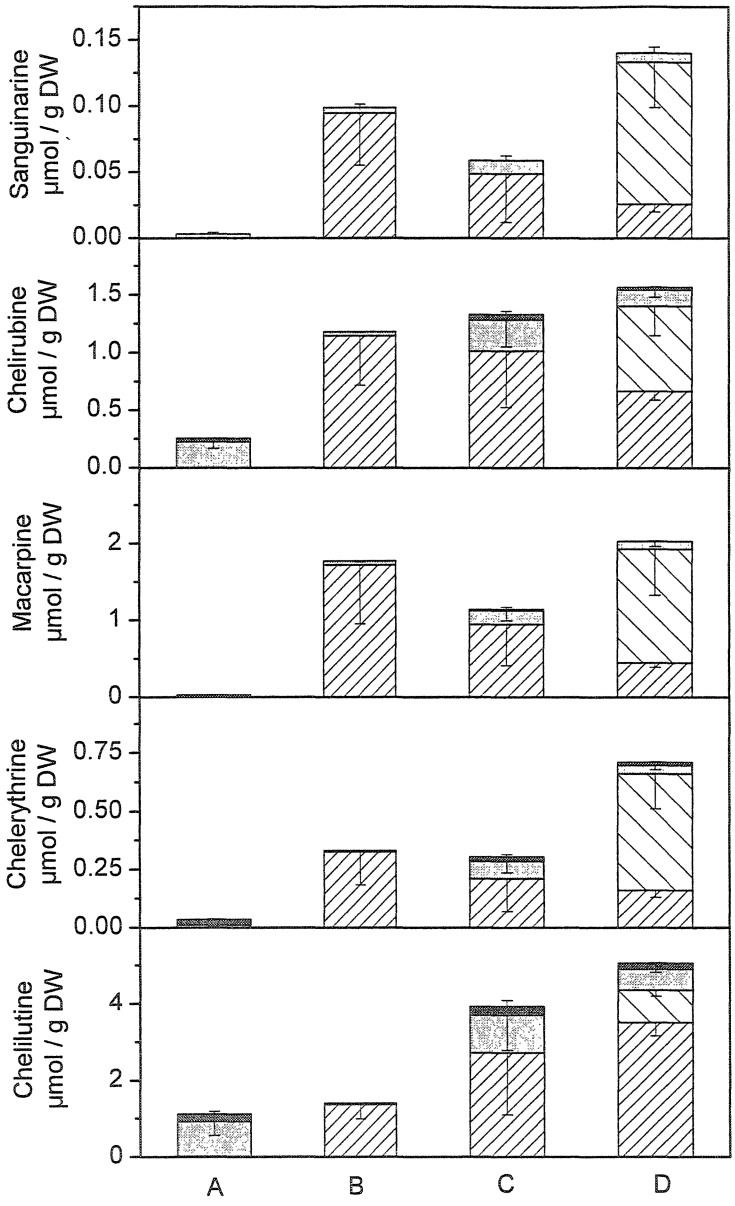



**Figure 5-6: Effect of extraction strategy on total alkaloid production**

**light grey: intracellular alkaloids; dark grey: alkaloids in medium; Tight right slanted dash: alkaloids in resins (initial addition); Sparse left slanted dash: alkaloids in resin added after 6 days (harvest at day 7) A: control, no extractive phase (harvest after 7 days); B: XAD-7 resins in suspension with plant cells (harvest after 7 days); C: recirculation of medium through column with XAD-7 resins (harvest after 7 days); D: recirculation of medium through column with XAD-7 resins, harvest 6 days after elicitation and addition of resins into suspension (harvest 1 day later, at day 7). Error bars are standard errors (n=4).**

The production of the next alkaloid in the metabolic pathway, (see fig. 5-1) chelirubine, was also enhanced by the addition of extractive phase, but to a much lesser extent than with sanguinarine. As a matter of fact, in both contact and no contact conditions, the total production was about 3 to 8 times that in the control, (Fig. 5-6: B and C versus A) but when in contact with cells, the resins collected about 97% of the alkaloids, compared to about 76 % for the recirculation. Indeed, when in contact, the resins clearly appeared to drain chelirubine, significantly reducing the cellular and medium concentrations of the alkaloid. This was not the case when the medium was recirculated, as levels of the alkaloid were comparable to those found in control cells.

In the case of the end product macarpine, the results were similar to those found with sanguinarine. The addition of XAD-7 resins into the cell suspension led to a 30 to 90-fold increase in the total production (Fig. 5-6: B versus A), while recirculation resulted in a 15 to 55-fold gain (Fig. 5-6: C versus A). Despite appearing slightly lower, total production was not significantly different with recirculation compared to direct contact. The average value for the culture subjected to recirculation seems to indicate that intracellular levels are higher than in the control or when the cells are in contact with the resins. However, due to large experimental error, it is impossible to confirm a statistically significant difference between these experimental conditions. The addition of resins after six days did not significantly depress the intracellular levels. Nonetheless, with the additional alkaloids recovered during the $7^{th}$ day, the increase was about the same as for cells in contact with resins for a full 7 days (Fig.6: D versus B).

The production of chelerythrine was also positively affected by the addition of resins into the culture. Total production was about 5 to 15 times as important, with about 99% recovery in resins (Fig. 5-6: B versus A). Recirculation did not result in significantly lower levels (Fig. 5-6: C versus B), but only about 69 % of the alkaloid was recovered in the resin. However, the combination of both methods permitted a 5-35 fold increase, with a satisfactory 93 % recovery (Fig. 5-6: D versus A). The total production in this was significantly higher than with recirculation only. Interestingly, cellular concentrations of chelerythrine were significantly higher with medium recirculation than in the control while they were depleted when resins were in contact with cells. As expected, due to a high adsorption rate, medium concentrations were exhausted when resin was directly added to the cell suspension, while they weren't significantly changed by the recirculation.

In contrast to all the other alkaloids investigated, the addition of resins into the cell suspension without recirculation barely increased the production of chelilutine. In fact, the addition of resins to the cell suspension appears to have resulted in the removal of all the chelilutine from the cells and medium. However, there was no increase in production as observed with other alkaloids (Fig. 5-6: B versus A). In the case of recirculation, the cells and medium had concentrations comparable to those found in the control, while the amount recovered in the resins appeared to be higher than with cell contact, although the difference was not statistically significant. The addition of resins after 6 days only resulted in a small increase (Fig. 5-6: D versus C), corresponding to the amount of alkaloids that were found in the cells and medium at day 6. In this

experimental condition, the amount recovered in resins was significantly higher than with contact throughout the culture.

To further look into the possible equilibrium that cells establish between alkaloid concentrations inside and outside the cells, we investigated whether the proportion of alkaloids found inside cells remained the same for different culture conditions. As shown in Table 5-1, under control conditions (A), most benzophenanthridine alkaloids remain primarily inside the cells, except for chelerythrine, which appears mostly in the medium. The addition of resins into the cell suspension completely depletes medium concentrations at a very rapid rate (Fig. 5-3), which is why none of the alkaloids are found in the medium as the adsorption rate onto the resins is much faster than the production and extracellular transport rate. In contrast, when medium is recirculated through an adsorptive column, the ratio between cells and medium is not significantly different from control (with the exception of chelerythrine). This would seem to indicate that cells work to re-establish a stable equilibrium between intracellular and extracellular alkaloid levels.

#### *5.2.4.2: Effect of extractive strategy on metabolic pools*

To determine whether the addition of XAD-7 resin to suspension cultures or the recirculation of medium through an extractive column had an effect on the metabolic pools, we investigated the change in the proportion of each alkaloid relative to the total of the five alkaloids we measured (see Fig. 5-1). The results obtained for both cell and resin contents are presented in Table 5-2.

**Table 5-1: Effect of extraction strategy on alkaloid compartmenting**

| Culture conditions | Proportion of alkaloids inside cells compared to the total of intracellular and medium contents | | | | |
|---|---|---|---|---|---|
| | Sanguinarine | Chelirubine | Macarpine | Chelerythrine | Chelilutine |
| A | 100 % | 91 % ± 6 | 100 % | 28 %± 12 | 81 % ± 3 |
| B | 100 % | 100 % | 100 % | 100 % | 100 % |
| C | 100 % | 86 % ± 3 | 99 % ± 2 | 73 %± 4 | 78 % ± 3 |
| $D_1$ | 100 % | 90 % ± 7 | 97 % ± 4 | 75 % ± 8 | 81 % ± 5 |
| $D_2$ | 100 % | 97 % ± 3 | 100 % | 88 % ± 1 | 97 % ± 7 |

[a] data results from cultures with n = 4, expressed as mean ± standard error
Refer to Figure 5-4 for legend.

**Table 5-2: Effect of extraction strategies on metabolic pool**

| Culture conditions | | Proportion of each alkaloid in total[a,b] | | | | |
|---|---|---|---|---|---|---|
| | | Sanguinarine | Chelirubine | Macarpine | Chelerythrine | Chelilutine |
| A | cells | < 1% | 20 % ± 3 | 3 % ± 2 | 1 % ± 0 | 76 % ± 1 |
| | resin | --- | --- | --- | --- | --- |
| B | cells | 3 % ± 2 | 29 % ± 5 | 44 % ± 2 | 3 % ± 3 | 21 % ± 4 |
| | resin | 2 % ± 0 | 24 % ± 1 | 37 % ± 3 | 7 % ± 1 | 29 % ± 3 |
| C | cells | 1 % ± 0 | 17 % ± 2 | 12 % ± 4 | 4 % ± 1 | 67 % ± 8 |
| | resin | 1 % ± 0 | 14 % ± 1 | 10 % ± 0 | 3 % ± 0 | 72 % ± 1 |
| $D_1$ | cells | 1 % ± 1 | 28 % ± 24 | 9 % ± 6 | 5 % ± 2 | 58 % ± 21 |
| | resin | 1 % ± 1 | 21 %± 6 | 21 % ± 11 | 4 % ± 2 | 53 % ± 18 |
| $D_2$ | cells | 4 % ± 0 | 23 % ± 1 | 51 % ± 4 | 11 % ± 1 | 11 % ± 4 |
| | resin | 3 % ± 0 | 20 % ± 1 | 41% ± 5 | 14 % ± 0 | 23 % ± 6 |

[a] data results from cultures with n = 4, expressed as mean ± standard error
[b] total: addition of sanguinarine, chelirubine, macarpine, chelerythrine and chelilutine
Refer to Figure 5-4 for legend

In the control culture, without any extractive phase, the main alkaloid produced was chelilutine at 76%, while both chelerythrine and sanguinarine, the alkaloids that are sought after, represented only 1% and less than 1% of the total production. Addition of resins into the cell suspension shifted the metabolic pools away from chelilutine towards the end-product macarpine at approximately 40 %, with increased proportion of both sanguinarine and chelerythrine, with about 2% and 5% respectively. The proportions inside the cells and in the resins were only slightly different, with an apparent increased preference towards the chelerythrine-chelilutine pathway in the resins. As for the culture in which the medium was recirculated through a column with XAD-7 resin, the pools were shifted towards chelilutine at about 70% of total alkaloids, but unlike the control culture, higher sanguinarine and chelerythrine proportion were obtained, at 1% and 4% respectively. In the case of the culture with recirculation and addition of resins at day 6, the recirculation part of the culture, harvested at day 6, showed proportions similar to those found in the recirculation culture harvested after day 7 (considering large standard error intervals). The addition of resins into the cell suspension at day 6 shifted the metabolic pools towards the end product macarpine, with proportions similar to those found when resins were in contact with the cells for 7 days, with the highest proportions found for sanguinarine and chelerythrine, at 3% and 12% respectively.

## 5.2.5: Discussion

### *5.2.5.1: Medium recirculation enabled continuous alkaloid extraction*

The culture system that was designed to extract alkaloids from the medium without interaction with cells was shown not to have any detrimental effect on cell growth or viability. In addition, this system greatly simplifies harvest since the resin column, which contains most of the alkaloids, can easily be removed from the cell culture, without disruption or termination of the cell culture. In contrast, when the resins are mixed in with the cell suspension, harvest of the resins is cumbersome since the cells and resins have very similar densities. Thus harvest is impossible without terminating the production process. Nevertheless, although the recirculation system is a vast improvement to the production process, the low flow rate limits extraction of alkaloids from the medium, as will be discussed in later sections. Technical improvements are being looked at presently to allow higher throughput of recirculation without plugging and thus create a system where the adsorption rates would be competitive to the production rates.

### *5.2.5.2: Alkaloid production and release do not require the adsorbent phase to be in contact with the cells*

The experimental results have clearly shown that contact between cells and extractive phase is not necessary to achieve important increases in the production of benzophenanthridine alkaloids. Indeed, it appears that removing alkaloids from the medium alone induces higher production, while interaction between cells and extractive phase does not play a crucial role as was suggested by Wang et al. (2001). With the

recirculation of medium through an adsorptive column, production rates of sanguinarine and chelerythrine were increased 10-30 fold and 5-15 fold respectively. This compares favorably to studies where liquid extractive phases, which are easy to separate from cells, were used. In fact, with silicon oil, Byun et al. (1990) achieved a 3.4 fold increase of total alkaloid production in elicited *E. californica* cell cultures, while Dutta et al. (1994) achieved a 4 fold increase in total alkaloid production with the addition of tricaprylin to elicited *E. californica* cell cultures. Nevertheless, even though the difference is not statistically significant, recirculation appeared to be slightly less successful than the process of adding resins to the cell suspension, probably due to the limiting flow rate that did not enable significant reduction of alkaloid concentrations in the medium. Indeed, the production rate of the cells is thought to be faster than the removal rate by recirculation. As a result of the limiting flow rate, recovery of the product onto the resin was significantly lowered. Addition of resins after six days of recirculation enabled extra production, most likely due to removal of accumulated alkaloids in the cells and medium and additional relief of feedback inhibition.

It still remains unclear exactly how the adsorption of used medium components increases biosynthesis. Indeed, the main hypothesis reported in previous studies is that the addition of an extractive phase relieves some inhibition by acting as an alkaloid sink: lowering extracellular levels, increasing extracellular transport and thus removing some intracellular alkaloids that might cause negative feedback. This appeared to be the case in the report by Williams et al. (1992) where the addition of XAD-7 resins into suspension cultures of *P. somniferum* significantly decreased extracellular and

intracellular levels of sanguinarine. In contrast, studies by Byun et al. (1990, 1994) have shown that the addition of an extractive phase does not significantly decrease intracellular levels of alkaloids. In the case of our experiments, it seems unlikely that only relief of direct feedback inhibition increased biosynthesis of each alkaloid. Indeed, with medium recirculation, intracellular levels of products were either similar, or significantly higher than in the control, where the product concentrations were at levels that are suspected to cause negative feedback. Despite these concentrations that are considered inhibitive, production was greatly enhanced with recirculation. A possible explanation is that the feedback control is indirect and more complex, as it might involve precursors to the alkaloids that were measured. Although some feedback inhibition might come into play, it appears that there is also some level of control to maintain a set equilibrium between intracellular and medium concentrations.

#### *5.2.5.3: Alkaloid extraction strategy clearly affected the flux distribution of the secondary metabolism*

The extraction of alkaloids by resins in contact with cells or by medium recirculation clearly had an impact on metabolic fluxes. Indeed, compared to control without extractive phase, the production was strongly shifted towards the end product macarpine when extractive phase was in contact with cells, which coincided with low alkaloid concentrations in the cells and medium concentrations below detection limits (Figure. 5-6). In the culture with medium recirculation through an extractive column, the alkaloid concentrations in cells were similar or higher than in the control culture and the alkaloid proportions resembled those found in the control culture. Nonetheless,

sanguinarine, chelerythrine and macarpine were found in higher proportions than in the control, indicating that the entire secondary metabolism was up-regulated. The change in metabolic pools might be due to the fact that specific enzymes in the secondary metabolic pathway are controlled differently. In their investigation of benzophenanthridine alkaloid induction by barbiturates, Haider et al. (1997) noted that 8 of the 19 enzymes that convert L-tyrosine to the end product macarpine were elicitor-inducible. In addition, their experiments showed that different elicitors did not induce the same enzymes, some led to macarpine accumulation, while others led to chelirubine accumulation, with little or no macarpine production. In our experiments, when products were rapidly drained from the medium and cells by the addition of resins into the suspension, the metabolic fluxes were shifted away from chelilutine and towards macarpine. This could indicate that the pathway leading to chelilutine is saturated at a lower flux rate than the one leading to macarpine, and thus cannot respond as much to the continuous removal of feedback inhibition. Regulation of the metabolic fluxes of the secondary metabolism still has to be further investigated.

### 5.2.6: Conclusion

Our experiments with elicited *E. californica* cell cultures have shown that contact between cells and extractive phase is not necessary to achieve important alkaloid production increases. Although the exact mechanism remains unclear, we have shown that removal of alkaloids from the culture medium induces increased production and modifies metabolic flux distribution. The system that was developed to recirculate

medium through a column with XAD-7 resin greatly simplifies the production process, but it remains to be optimized. This work opens the way to the development of a production process based on repeatable productions on the same cell suspensions.

### 5.2.7: Acknowledgments

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# Chapitre 6 : Discussion générale

Pour pouvoir réaliser une étude approfondie de l'effet de phases extractives sur la production d'alcaloïdes dans des cultures d' *E. californica*, il a été important, en premier lieu, d'élaborer une technique pour quantifier les produits majeurs du métabolisme secondaire. La méthode développée pour l'identification des alcaloïdes produits par *E. californica* est constituée de deux volets importants : une méthode semi-préparative pour purifier les alcaloïdes non-disponibles sur le marché et une méthode analytique HPLC pour la quantification des alcaloïdes importants dans les échantillons de milieu, de cellules et de résines.

Grace à une détection par fluorescence, les solutés correspondant aux 12 pics importants ont été collectés pendant la séparation semi-préparative sur une colonne chromatographique $C_{18}$ à l'aide d'un gradient acétonitrile, eau et TFA. Malheureusement, seulement six de ces pics se sont avérés contenir des fractions pures. L'identification des composés par spéctrométrie de masse puis par résonance magnétique nucléaire n'a permis de confirmer que l'identité de trois alcaloïdes additionnels: la macarpine, la chelirubine et la chelilutine. Des travaux supplémentaires seront nécessaires pour pouvoir purifier et identifier quelques pics supplémentaires, mais comme telle, la méthode semi-preparative permet une purification de trois alcaloïdes additionels non disponibles sur le marché. La quantification précise à l'aide de standards est donc possible pour cinq alcaloïdes benzophénanthridiques importants produits dans les cultures d' *E. californica*.

En se basant sur les méthodes de Tanahashi et al. (1990) et Schumacher et al. (1987), une méthode analytique a été développée pour pouvoir quantifier de façon rapide et précise la sanguinarine, la chelerythrine, la chelirubine, la chelilutine ainsi que la macarpine. Un gradient d'acétonitrile et d'acide phosphorique (pH 2.5) permet de séparer tous les composés en question en moins de 35 minutes. La détection des composés est réalisée par fluorescence ($\lambda_{ex}$ 330 nm; $\lambda_{em}$ 570 nm) et par UV. Ceci permet une sélectivité élevée donc une préparation d'échantillons rapide par extraction méthanolique. En somme, cette méthode analytique est plus rapide que la plupart des méthodes publiées qui durent au moins 45 minutes (Schumacher et al. 1987; Tanahashi et al. 1990; Tome et al. 1999; Park et al. 2000 et 2002), et offre une précision élevée en plus d'une préparation d'échantillon rapide. Ainsi, cette méthode constitue un outil efficace pour une étude poussée sur l'effet d'une phase extractive solide dans les cultures d' *E. californica*.

Le système de culture développé pour permettre l'extraction des métabolites secondaires du mileu sans qu'il n'y ait contact entre les cellules et les résines s'est avéré efficace pour simplifier le procédé de production sans qu'il n'y ait aucun effets néfastes sur la croissance des cellules ou leur viabilité. En effet, le système de recirculation permet d'adsorber les métabolites secondaires sur des résines XAD-7 contenues dans une colonne qui peux être facilement retirée ou changée sans devoir pertuber ou mettre fin à la culture. Ceci est une amélioration nette comparativement à la méthode habituelle où les résines sont mélangées aux cellules et en conséquent, sont difficiles à récolter puisque les cellules et les résines ont des densités comparables. Malgré cette

amélioration du procédé, la vitesse de recirculation lente limite l'extraction des alcaloïdes à partir du milieu. En effet, la vitesse de recirculation n'est pas assez rapide pour réduire de façon significative les concentrations de métabolites secondaires dans le milieu.

Les résultats obtenus avec le système de recirculation démontrent clairement que le contact entre les cellules et les résines n'est pas nécessaire pour augmenter la production de métabolites secondaires. En effet, il semblerait que ce soit uniquement le retrait des métabolites du milieu qui engendre une production plus importante, contrairement à la suggestion de Wang et al. (2001). Ces derniers pensent que l'interaction entre les cellules et la phase extractive joue un rôle important. Nous avons constaté qu'en recirculant le milieu de culture à travers une colonne contenant des résines XAD-7, la production de sanguinarine et de chelerythrine a été 10-30 fois et 5-15 fois plus importante que dans la culture témoin sans extraction. Cette amélioration est intéressante comparativement aux augmentations de production obtenues avec des phases extractives liquides, qui sont, comme la colone, faciles à séparer des cellules. En effet, grâce à l'huile de silicone, Byun et al. (1990) ont obtenu une production d'alcaloïdes totaux 3,4 fois plus importante avec des cultures d' *E. californica*, alors que Dutta et al. (1994) ont obtenu une production 4 fois plus importante grâce à l'ajout de tricapryline dans des cultures élicitées d' *E. californica*. Malgré les résultats très positifs avec le sytème de recirculation, il semblerait que l'ajout de résines directement dans la culture augmente légèrement plus la production, même si la différence n'est pas significative du point de vue statistique. Cette différence est sans doute causé par la

vitesse de recirculation limitée, qui n'a pas permis de réduire substantiellement les concentrations d'alcaloïdes dans le milieu. En effet, il semblerait que le taux de production des métabolites est plus rapide que le taux d'extraction par recirculation. Par conséquent, le taux de récupération des alcaloïdes sur résines a été nettement moins important que lorsque les résines sont en contact directe avec les cellules. En rajoutant, après 6 jours, des résines aux suspensions cellulaires soumise jusque là au système de recirculation, il a été possible d'obtenir une production encore plus importante, sans doute due à l'extraction des alcaloïdes contenus dans le milieu et à l'interieur des cellules en plus de la réduction de leur effet inhibiteur.

Il n'est toujours pas entièrement clair comment la récupération des métabolites secondaires contenus dans le milieu réussit à augmenter la production de ces composés. En effet, l'hypothèse la plus commune évoquée dans les travaux précédents est que l'addition des phases extractives permet de réduire l'inhibition négative sur les voies métaboliques en drainant les alcaloïdes. Par ce fait on réduit les concentrations dans le milieu, augmentant ainsi le transport vers l'extérieur des cellules. Cette évacuation diminue la teneur des alcaloïdes intracellulaires qui sont la source d'une rétroaction négative. Cette hypothèse semble être confirmée par les travaux de Williams et al. (1992) où l'addition de résines XAD-7 dans des cultures de *P. somniferum* coincidait avec une diminution nette des concentrations de sanguinarine dans le milieu et les cellules. Par contre, dans les travaux de Byun et al. (1990, 1994), l'ajout d'une phase extractive ne coincide pas avec une réduction importante des niveaux intracellulaires d'alklaloides. D'après les résultats présentés au chapitre 4, il ne semble pas plausible que

ce soit seulement la réduction de la rétroaction négative qui engendre une augmentation de la production de chaque alcaloïde. En effet, avec le système de recirculation du milieu, les concentrations intracellulaires des métabolites secondaires étaient similaires ou nettement plus élevées que dans la culture témoin. En somme, les concentrations étaient à des niveaux qui causeraient une inhibition. Néanmoins, l'inhibition négative joue peut-être un rôle indirect et plus complexe puisque le contrôle de certaines réactions pourrait se faire au niveau des précurseurs des alcaloïdes mesurés. Il faut remarquer que même s'il semble qu'une certaine forme de rétroaction négative pourrait jouer un rôle dans le contrôle de la production, aucune de nos données ne le confirme.

Le type de stratégie d'extraction a clairement eu un impact sur les flux métaboliques. Ainsi, lorsque les résines ont été rajoutées directement à la suspension cellulaire, la production a été redirigée vers la macarpine, un produit en fin de voie métabolique. Cette modulation des flux métaboliques a coincidé avec des concentrations très faibles d'alcaloïdes dans les cellules et le milieu. Par contre, en ce qui concerne le système avec recirculation, les proportions d'alcaloïdes étaient semblables à celles retrouvées dans la culture témoin. De plus, les concentrations d'alcaloïdes dans les cellules et le mileu étaient comparables à celles retrouvées dans la culture témoin.

Les changements dans les flux métaboliques pourraient être reliés au fait que les différents enzymes qui constituent les voies métaboliqures secondaires sont contrôlés différemment. En effet, Haider et al. (1997) ont noté que 8 des 19 enzymes impliqués dans le métabolisme de la macarpine à partir de la L-tyrosine sont inductibles par différents éliciteurs. De plus, leurs résultats ont démontré que chacun des éliciteurs

agissait de façon différente sur chacun des enzymes. En effet, alors que certains éliciteurs engendraient une accumulation de macarpine, d'autres engendraient une accumulation de chelirubine, sans aucune production de macarpine. Les résultats de nos expériences semblent indiquer que lorsque les produits sont rapidement retirés du milieu et des cellules par l'ajout de résines dans la suspension cellulaire, les flux métaboliques sont redirigés vers la macarpine alors que la voie vers la chelilutine est négligée. Ce phénomène pourrait s'expliquer si la voie vers la chelilutine devient saturée plus rapidement que celle vers la macarpine et ainsi est incacapable de réagir autant à l'atténuation de l'inhibition. En somme, il a été fort intéressant de constater que la vitesse à laquelle les alcaloïdes sont retirés du milieu influencait la répartition des flux métaboliques.

# Chapitre 7 : Conclusion et recommandations

Grâce à la méthode analytique développée, il a été possible d'effectuer une étude détaillée de l'effet du contact entre des résines extractives XAD-7 et des cellules d'*E. californica* sur la production des alcaloïdes benzophénanthridiques. La méthode chromatographique a permis une quantification exacte, précise et rapide des principaux alcaloïdes produit dans les cultures d'*E. californica* : la sanguinarine, la chelerythrine, la chelilutine, la chelirubine ainsi que la macarpine. La détection par fluorescence et UV a amélioré la précision et la sélectivité par rapport aux méthodes publiées précédemment. Ainsi, grâce à une sélectivité élevée, la préparation des échantillons a pu être simplifiée, réduisant ainsi le temps d'analyse requis. Une méthode semi-préparative a été développée afin de pouvoir obtenir des standards de macarpine, chelerythrine et chelirubine qui ne sont pas disponible sur le marché.

Un système de culture a été développé permettant de recirculer le milieu de culture à travers une colonne contenant de la résine XAD-7, de sorte à pouvoir retirer les métabolites secondaires de la culture sans qu'il n'y ait de contact entre la phase extractive et les cellules. Ce système simplifie beaucoup le procédé de récolte des métabolites secondaires puisque les résines contenant les composés recherchés peuvent être récupérées facilement sans nécessiter l'arrêt de la culture. Néanmoins, le flux de recirculation a été limité par les conditions expérimentales de culture à tel point que les concentrations d'alcaloïdes dans le milieu n'étaient pas significativement réduites. De plus, les résultats obtenus avec ce système ont pu démontrer que le contact entre les

cellules et la phase extractive n'est pas nécessaire pour obtenir une augmentation importante de la production d'alcaloïdes benzophénanthridiques. Bien que le mécanisme exact reste inconnu, il semble que le retrait des métabolites secondaires du milieu engendre une augmentation de la production, mais aussi un changement de la distribution des flux métaboliques. En effet, l'augmentation de la production n'est pas similaire pour les cinq alcaloïdes mesurés, les flux étant redirigés vers certaines voies spécifiques du métabolisme secondaire, au détriment d'autres.

Étant donné les questions qui restent à élucider, voici les recommandations pour les travaux futurs :

- Confirmer le procédé de production sans contact à l'échelle de production en bioréacteur de 20 L.
- Améliorer le système de culture de sorte à pouvoir augmenter la vitesse de recirculation dans la boucle extractive pour pouvoir retirer une quantité plus importante d'alcaloïdes et ainsi « vider » le milieu des métabolites secondaires. Par example, dans un plus gros bioréacteur (20 L), il faudrait augmenter la surface des embouchures de recirculation et augmenter le nombre de ces embouchures de sorte à pouvoir augmenter de façon significative le débit de recirculation tout en limitant la pression au niveau des embouchures (cette pression cause l'obstruction des embouchures). De plus, étant donné que la

question de contact semble désormais résolue, un grillage de nylon pourrait être utilisé pour limiter le cisaillement.

- Identifier les inconnus dans la méthode analytique de sorte à pouvoir pousser plus loin l'analyse de la distribution des voies métaboliques. Similairement, il serait intéressant d'étudier l'activité de certains enzymes clé dans les voies métaboliques afin de mieux comprendre le contrôle de la production.

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# Annexes

## *Annexe 1: Voie métabolique complète des alcaloïdes benzophénanthridiques*

Dans cet annexe est présentée la voie métabolique complète menant à la biosynthèse des alcaloïdes benzophénanthridiques à partir de la tyrosine. Les voies exactes menant à la biosynthèse de la chelerythrine ainsi que la chelilutine ne sont pas à présent élucidées. Le tableau A.1.1 explique la nomenclature utilisée dans la figure A.1.1 pour décrire les enzymes intervenant dans la voie métabolique. Cette nomenclature a été proposée par le comité de l' International Union of Biochemistry and Molecular Biology (NC-IUBMB).

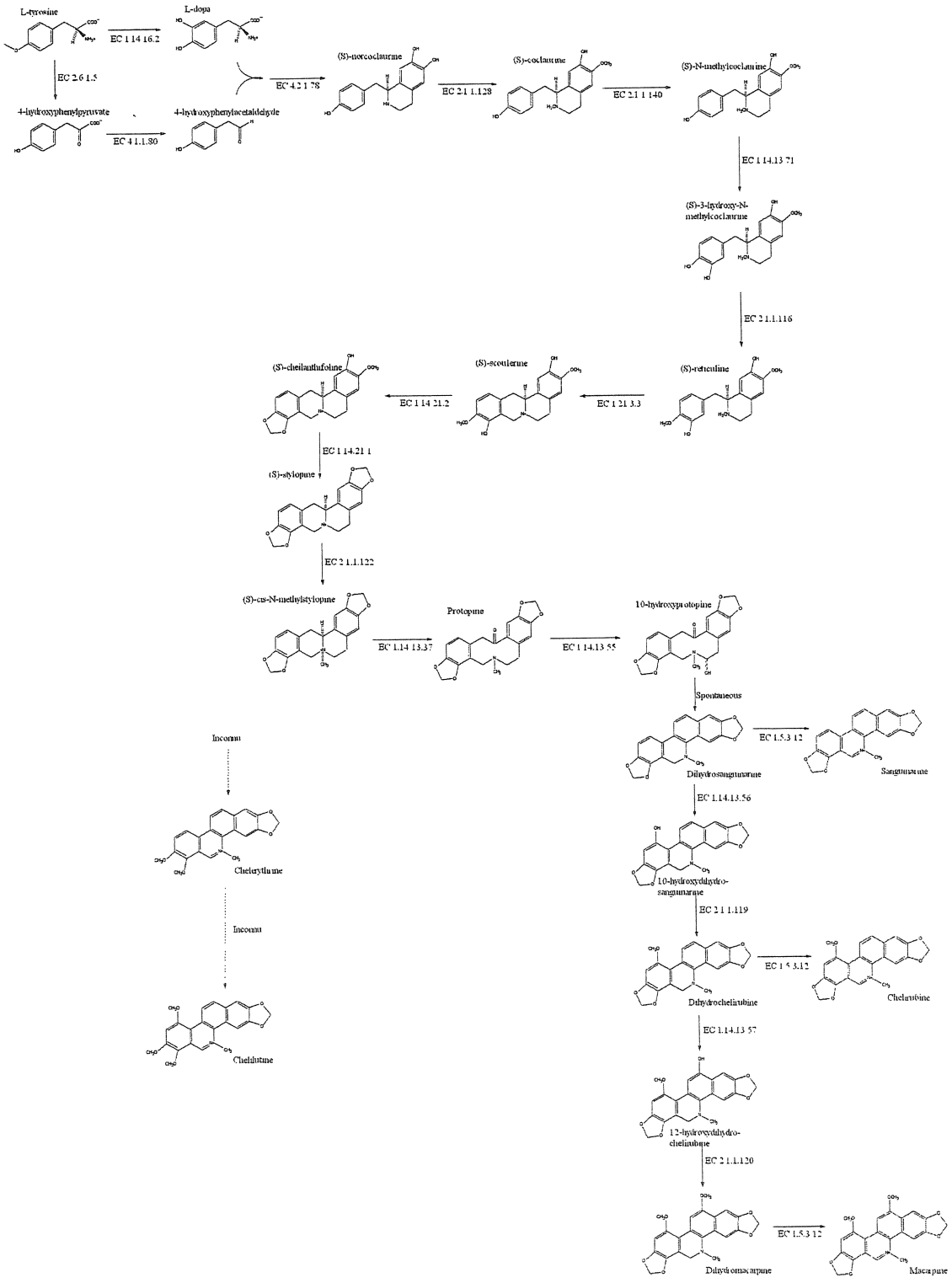



**Figure A1-1: Voie métabolique des alcaloïdes benzophénanthridiques**

**Tableau A1-1: Nomenclature des enzymes de la voie métabolique des alcaloïdes benzophénanthridiques**

| Code de l'enzyme | Nom complet de l'enzyme | Type d'enzyme |
|---|---|---|
| EC 1.14.16.2 | tyrosine 3-monooxygenase | |
| EC 2.6.1.5 | tyrosine transaminase | Enzyme mitochondriale, A pyridoxal-phosphate protein |
| EC 4.1.1.28 | | |
| EC 4.1.1.80 | 4-hydroxyphenylpyruvate decarboxylase | |
| EC 4.2.1.78 | (*S*)-norcoclaurine synthase | |
| EC 2.1.1.128 | (*RS*)-norcoclaurine 6-*O*-methyltransferase | |
| EC 2.1.1.140 | (*S*)-coclaurine-*N*-methyltransferase | |
| EC 1.14.13.71 | *N*-methylcoclaurine 3'-monooxygenase | Protéine heme-thiolate (*P*-450) |
| EC 2.1.1.116 | 3'-hydroxy-*N*-methyl-(*S*)-coclaurine 4'-*O*-methyltransferase | |
| EC 1.21.3.3 | Berberine bridge enzyme, reticuline oxidase, | Berberine bridge enzyme |
| EC 1.14.21.2 | *S*-cheilanthifoline synthase | Protéine heme-thiolate (*P*-450) |
| EC 1.14.21.1 | (*S*)-stylopine synthase | Protéine heme-thiolate (*P*-450) |
| EC 2.1.1.122 | (*S*)-tetrahydroprotoberberine *N*-methyltransferase | |
| EC 1.14.13.37 | methyltetrahydroprotoberberine 14-monooxygenase | Protéine heme-thiolate (*P*-450) |
| EC 1.14.13.55 | protopine 6-monooxygenase | Protéine heme-thiolate (*P*-450) |
| EC 1.5.3.12 | dihydrobenzophenanthridine oxidase | Enzyme $Cu^{II}$ |
| EC 1.14.13.56 | dihydrosanguinarine 10-monooxygenase | Protéine heme-thiolate (*P*-450) |
| EC 2.1.1.119 | 10-hydroxydihydrosanguinarine 10-*O*-methyltransferase | |
| EC 1.14.13.57 | dihydrochelirubine 12-monooxygenase | Protéine heme-thiolate (*P*-450) |
| EC 2.1.1.120 | 12-hydroxydihydrochelirubine 12-*O*-methyltransferase | |

# *Annexe 2: Méthodes analytiques pour alcaloïdes publiées*

**Tableau A2-1: méthodes analytiques publiées**

| Reference | Alkaloids | Sample preparation | SPE | Column + detector | Separation conditions |
|---|---|---|---|---|---|
| **(Chauret et al. 1990)** | Chelirubine<br>Sanguirubine<br>Sanguilutine<br>Chelerythrine<br>Chelilutine<br>Sanguinarine | Cells : 5 g FW, 30s homogenizer with 20 mL MeOH (0.5%HCl), 15min at 12000g, cells debris: 2nd extraction, combine extracts 10 mL extract + 30 mL HS (0.05M)<br>Medium: filtered at 0.45um, 30 mL medium + 10 MeOH + 1.5mL HS (1 M) | $C_{18}$ cartridge (400mg), preparation: 5ml ACN; 5 ml MeOH with 5 % HS (0.05M); 5 ml HS (0.05M)<br>sample loading, wash: 10 ml MeOH with 25 % HS (0.05M)<br>recovery: 2ml MeOH with 5 % HS (0.05M).<br><br>Sample (10 ul) acidified with 2 ul acid MeOH (33% $H_3PO_4$ ) | Column: Guard column (15 mm x 3.2 mm I.D., 7 um) + 2 **spheri-5 cyano** columns (100 mm x 4.6 mm I.D., 5 um)<br>detection: UV: 280 nm<br>fluorescence 338 nm ex, 425 nm em | 86:14 mixture of MeOH and solution with triethylamine (5 mM) and $H_3PO_4$ (5 mM), pH 5.6 (with NaOH)<br>flow rate: 1ml/min, 40°C<br><br>>35 min |
| | Fluorescence is selective, so no need for SPE<br>suspension cultures of California poppy | | | | |
| **(Williams et al. 1992; Archambault et al. 1996)** | | Extraction with MeOH (0.5% HCl), 2h, 80°C<br>Cells (1-2 g FW) and resins (0.5g): 2x 20ml + 1x10ml<br>medium (5ml): 1x10ml | | Same as Chauret et al. (1990) | Same as Chauret et al. (1990)<br><br>>35 min |
| | Suspension cultures of P. somniferum, Tissue maceration doesn't yield more alkaloids | | | | |
| **(Chauret et al. 1991)** | Chelirubine<br>Sanguirubine<br>Sanguilutine<br>Chelerythrine<br>Chelilutine<br>Sanguinarine<br>Norsanguinarine | Cells : 5 g FW, 30s homogenizer with 20 mL MeOH (0.5%HCl), 15min at 12000g, cells debris: 2nd extraction, combine extracts, filter (0.45um)<br>medium: filter (0.45um) | None<br><br>Sample (10 ul) acidified with 2 ul acid MeOH (33% $H_3PO_4$ ) | Column: Guard column (15 mm x 3.2 mm I.D., 7 um) + 2 **spheri-5 cyano** columns (100 mm x 4.6 mm I.D., 5 um)<br>detection: UV: 280 nm<br>fluorescence 225 nm ex, 410 nm em | 86:14 mixture of MeOH and solution with triethylamine (5 mM) and $H_3PO_4$ (5 mM), pH 5.6 (with NaOH)<br>flow rate: 1ml/min, 40°C<br><br>>35 min |
| | Pb: coelution; no SPE needed with fluorescence detection<br>suspension cultures of California poppy | | | | |

| Reference | Alkaloids | Sample preparation | SPE | Column + detector | Separation conditions |
|---|---|---|---|---|---|
| (Roos et al. 1998) | | Cells: 1 ml cell suspension + 1 ml 96% EtOH (1% HCl), 30 min at 40°C, 10 min at 5000 rpm | | fluorescence 460 nm ex, 570 nm em total alkaloids with sanguinarine as standard<br><br>Column : $C_{18}$ | Gradient elution with ACN: water: $H_3PO_4$ |
| | Macarpine 55%; chelirubine 25% suspension cultures of California poppy | | | | |
| (Park et al. 2000) | Sanguinarine morphine noscapine | 0.5 g FW extracted by grinding with 95 % MeOH, extract dried under vacuum, redissolved in 50 ul MeOH | none | Column: $C_{18}$ reversed-phase (4.6 x 250 mm) detector: PDA | Isocratic gradient MeOH: water (8:2) with 0.1% triethylamine, flow rate 0.75 ml/min |
| | Transformed root cultures of California poppy | | | | |
| (Park et al. 2002) (Park et al. 2003) | Macarpine<br>Dihydro-macarpine<br>Chelerythrine<br>Dihydro-chelerythrine<br>Chelirubine/ Chelilutine<br>Dihydro-chelilutine<br>Sanguinarine<br>Dihydro-sanguinarine<br>m/z = 338 | FW frozen with liquid $N_2$, ground, extracted in MeOH in boiling water bath (15 min)<br>Extracts dried under vacuum, dissolved in 0.1M $NaCO_3$/ $Na(CO_3)_2$ (3:2), pH 10, extracted 3 times in ethyl acetate. Pooled fractions dried and dissolved in 1 ml MeOH | | Column: $C_{18}$ reversed-phase (4.6 x 250 mm) detector: PDA (UV spectra)<br><br>Initial Identification: LC-MS | Isocratic gradient MeOH: water (3:1) with 0.1% triethylamine, flow rate 0.75 ml/min<br><br>>45 min |
| | Cell cultures of California poppy and root cultures<br>Most abundant: m/z=338, dihydro-macarpine | | | | |

| Reference | Alkaloids | Sample preparation | SPE | Column + detector | Separation conditions |
|---|---|---|---|---|---|
| **(Villegas et al. 1999)**<br><br>**(Villegas et al. 2000) *** | Chelerythrine<br>Sanguinarine<br>Macarpine | Cells: 1-2 g FW extracted in MeOH (refluxing), dried at 40°C under low pressure. Dissolved in 1 ml MeOH<br>Medium: pH adjusted to 9, extraction with EtOAc (3x10ml). Dried with anhydrous $Na_2SO_4$ and evaporated at 40°C under low pressure. Dissolved in 1 ml MeOH<br>* 1-2 g FW in MeOH, homogenized and (4ml/g ) sonicated 10min, 10m at 10000g | | Column: Nucleosil $C_8$ pre-column (20 x 4.6mm, 5 um) Nucleosil $C_8$ column (250 x 4.6mm, 7 um)<br><br>Detection: UV at 254 and 280 nm | Mobile phase A: 5 mM 1-pentanesulfonic acid, 10 mM octanesulfonic acid sodium salt, pH 2.8 ($H_3PO_4$)<br>B: ACN<br>1ml/min:<br>0-5 min: 40%B<br>5-6 min: 40-50% B<br>6-11 min: 50 % B<br>11-20min: 50-67%<br>20-24 min: 67-80% B<br>24-26 min: 80% B<br>26-27 min: 80-40% B<br>27-30 min: 40% B |
| | Cell suspension of California poppy<br>Cell/ medium proportions<br>No graph of separation | | | | |
| **(Salmore et al. 2001)** | Sanguinarine<br>Chelerythrine<br>Berberine<br>*Protopine*<br>*Allocryptonine*<br>*Chelirubine*<br>*Sanguirubine*<br>*Chelilutine*<br>*Sanguilutine* | 1 g FW of rhizome reduced to small pieces with scalpel and extracted in 10 ml MeOH | | Column: Phenomenex $C_{18}$ silica (4.6 x 150 mm, 5um)<br><br>Detection: PDA, UV 284 nm | Mobile phase: 0.1N tartaric acid (0.125% SDS), ACN (1:1)<br>0.5 ml/min |
| | Rhizomes of Sanguinaria Canadensis<br>Most calculated as sanguinarine equivalents | | | | |
| **(Reinhart et al. 1991)** | Sanguinarine<br>Chelerythrine | 1 ml saliva + 10 ml 0.5% HCl in $H_2O$. Sonicated 2 min | Conditioning: 2 ml MeOH then 2 ml $H_2O$ Sample drawn by vacuum. Wash: 2 x 5 ml $H_2O$ Elution: 2 ml MeOH (0.5% HCl) | Column: Apex I ethyl (4.6 x 250 mm, 5um)<br><br>Detection: PDA, UV 280 and 328 nm | Mobile phase: $H_2O$ with 2.75mM HSA and 2.25 mM HTAP; ACN (60:40 v/v) pH 2.7<br>3 ml/min |
| | Saliva | | | | |

| Reference | Alkaloids | Sample preparation | SPE | Column + detector | Separation conditions |
|---|---|---|---|---|---|
| **(Dostal et al. 2000)** | Sanguinarine<br>Chelerythrine | Plants extracted with MeOH (heat).<br>Concentrated extract dissolved in $H_2SO_4$. Solution is alkalinized with $NaCO_3$<br>Precipitate is extracted with ether. Organic phase is separated, evaporated and crystallized with dilute HCl. | | Column separation on acid $Al_2O_3$. (see Dostal et al (1992))<br><br>Prep column separation (see Tanahashi et al. 1990 ) | |
| **(Byun et al. 1990)** | Sanguinarine<br>Chelerythrine<br>macarpine (chelirubine) | Cells :<br>1 g FW extracted in 10 ml MeOH, 10 min 125 W sonication<br>Medium: filtered accumulation phase:<br>1 ml stirred overnight with 20ml MeOH | Preparation of macarpine std: 500 g FW stirred overnight with 1.5 l MeOH, then cells washed with 1 l MeOH.<br>Extract dried under low pressure; 200 ml $H_2O$-HOAc (1:1) added, filtered.<br>Extraction with 200 ml petrol.<br>Aq. layer made alkaline with 15% $NH_4OH$, extracted with 400 ml CHCl2.<br>After evaporation, extract dissolved in MeOH and separated by FPLC, gradient of 30% B to 65% B in 20 min.<br>identification by MS | Column:<br>Supelcosil LC-18-DB (150 x 4.6mm)<br><br>Detection:<br>UV at 280 nm | Mobile phase<br>A: water with 1mM tetrabutylammonium phosphate, pH 2 ($H_3PO_4$)<br>B: ACN<br>1.5 ml/min<br>gradient: 65% A, 25% B |
| | Two-phase cell suspension of California poppy (silicone oil)<br>Total alkaloids = sanguinarine +chelerythrine + macarpine + chelirubine (macarpine eq.)<br>Sanguinarine partition is pH dependant<br>Total: 50% macarpine<br>in silicone: mostly not macarpine | | | | |

| Reference | Alkaloids | Sample preparation | SPE | Column + detector | Separation conditions |
|---|---|---|---|---|---|
| (Collinge et al. 1989) | Sanguinarine<br>Chelerythrine<br>Macarpine | Cells: 1-2 g FW extracted in MeOH (30-50 ml) by refluxing, dried at 40°C under low pressure. Dissolved in 1 ml MeOH<br>Medium: pH adjusted to 9, extraction with EtOAc (3x10ml). Dried with anhydrous $Na_2SO_4$ and evaporated at 50°C under low pressure. Dissolved in 1 ml MeOH | | System A: Column: Nucleosil $C_8$ pre-column (20 x 4.6mm, 5 um) Nucleosil $C_8$ column (250 x 4.6mm, 7 um)<br><br>System B: Hypersil silica pre-column (20 x 4.6mm, 5 um) Hypersil silica column (250 x 4.6mm, 5 um)<br><br>Detection: UV at 280 nm<br><br>Identification of macarpine by UV spectrum and $^1$H-NMR | System A: Mobile phase A: 5 mM 1-pentanesulfonic acid, 10 mM octanesulfonic acid sodium salt, pH 2.8 ($H_3PO_4$)<br>B: ACN<br>1ml/min:<br>0-5 min: 40%B<br>5-6 min: 40-50% B<br>6-11 min: 50 % B<br>11-20min: 50-67%<br>20-24 min: 67-80% B<br>24-26 min: 80% B<br>26-27 min: 80-40% B<br>27-30 min: 40% B<br><br>System B: hexane-toluene-MeOH (93:6:1) 1ml/min |
| | Suspension cultures of California poppy<br>End product = macarpine<br>mostly extracellular at end of culture, most abundant | | | | |
| (Tome et al. 1999) | Sanguinarine<br>chelerythrine<br>californidine<br>eschscholtzine<br>protopine<br>O-methyl-cariachine<br>α-allocryptopine | 4-5 g FW or 3g DW extracted in 70% ethanol with Soxhlet and alkaloid fraction separated by ion-pair (see Bugatti et al., 1991) | | Column: LiChrospher $C_8$ (250 x 4mm, 5 um)<br><br>Detection UV 280 nm PDA for identification | A: ACN, $H_2O$ (20:80) with 10 mM octylsulphonic acid, 0.15 M triethylamine, pH 3 ($H_3PO_4$)<br>B: ACN, $H_2O$ (40:60) with 10 mM octylsulphonic acid, 0.15 M triethylamine, pH 3 ($H_3PO_4$)<br>1ml/min:<br>0-5 min: 0%B<br>5-25 min: 0-100% B<br>25-35 min: 100 % B (then 15 min at 0% B to stabilize) |
| | Whole plant samples (california poppy) | | | | |

| Reference | Alkaloids | Sample preparation | SPE | Column + detector | Separation conditions |
|---|---|---|---|---|---|
| **(Fabre et al. 2000)** | | 5 g DW extracted in 100 ml MeOH (15 min, 60°C), dried under low pressure at 35-40°C, dissolved in 50 ml $H_2O$ at pH 1 (HCl with 0.2% dodecylsulfate sodium salt). Extraction with $CHCl_3$ (3 x 50ml), dried over anhydrous $Na_2SO_4$ at 40°C, under low pressure. Residue diluted in 10 ml MeOH | | Column: Hypersil $C_8$ (150 x 4.6mm, 5 um)<br><br>Detection: UV 280 nm<br><br>HPLC-electrospray ionization MS-MS | Mobile phase A: ACN<br>B: $H_2O$ with 1 mM sodium dodecylsulfate, 10 mM triethylamine, pH 2.5 ($H_3PO_4$)<br>1ml/min:<br>0-40 min: 20-40%B<br>40-45 min: 40% B<br>45-50 min: 40-100% B<br>50-55min: 100% B<br>55-60 min: 100-20% B<br>+ 10 min stabilization |
| | Whole plant samples | | | | |
| **(Husain et al. 1999)** | sanguinarine | Oil sample (1 ml) dissolved in 10 ml mobile phase | | Column: $C_{18}$ reversed-phase (4.6 x 250 mm, 5um)<br><br>detection: PDA, UV 280 nm | Mobile phase: MeOH-ACN-THF-$H_2O$ (21:55:4:20) 1 ml/min |
| | Edible oils | | | | |
| **(Haider et al. 2000)** | Total alkaloids | Cells: 1 ml cell suspension centrifuged, 1ml EtOH (0.1% HCl) added. Extracted 2h at 60°C. Centrifuged to remove insolubles | No SPE | No separation<br><br>Detection: UV at 490 nm | None |
| | Cell suspensions of California poppy<br>No quantitative data given, only activity score | | | | |
| **(Jones et al. 1986)** | Sanguinarine | No cell extraction. Tests from standard | No SPE | Column: Radial-Pak 5 CN (10um)<br><br>Detection : UV at 280 nm<br><br>H-NMR | Mobile phase: MeOH-$H_2O$ (84:16) with 5 mM triethyl-amine, pH 5.4 ($H_3PO_4$) |
| | In $H_2O$-EtOH (80:20), below pH 5.4, iminium form. Above pH 7.0, alkanolamine form: precipitate(insoluble in $H_2O$ or EtOH). Identified by NMR in DMSO as possibly 6-hydroxy-sanguinarine<br>Recovery of iminium when pH 3.5 | | | | |

| Reference | Alkaloids | Sample preparation | SPE | Column + detector | Separation conditions |
|---|---|---|---|---|---|
| (Das et al. 1996) | Sanguinarine | No cell extraction. Tests from standard | No SPE | No separation<br><br>Detection:<br>-UV spectra<br>-fluorescence spectra<br>- H-NMR | None |
| | UV peaks at 273, 327 and 468 nm, and 235 nm with high pH fluorescence at ex 329 nm, peaks at 418 nm and 580 nm alkanol-amine at 418 nm intense at high pH or in pure MeOH. Iminium form at 580 nm with low pH | | | | |
| (Seckarova et al. 2002) | Sanguinarine<br>Sanguirubine<br>Sanguilutine<br>Chelerythrine<br>Chelirubine<br>Chelilutine | No cell extraction. Tests from standard | No SPE | No separation<br><br>- H-NMR | None |
| | NMR spectroscopy | | | | |
| (Mahady et al. 1998) | Sanguinarine<br>Chelerythrine | | Cyano prep Sep column eluated with MeOH, filtered with Gelman Acro LC13 HPLC filter | Column:<br>$C_{18}$ reversed-phase (4.6 x 250 mm)<br><br>Detection:<br>PDA, UV 284, MS | See Hashimoto at al , 1986<br>2 ml/min |
| (Tanahashi et al. 1990) | Dihydro-sanguinarine<br>dihydro-chelirubine<br>dihydro-chelerythrine<br>dihydro-chelilutine<br>dihydro-macarpine<br>10-hydroxy-dihydro-sanguinarine<br>12-hydroxy-dihydro-chelirubine<br>10-hydroxy-dihydro-chelerythrine | Medium: acidified medium subjected to a servachrome XAD cc (6x16 cm) eluted with MeOH-$H_2O$-HCl (different fractions from 0:1000:1 to 1000:0:1)<br>cells: extracted in hot MeOH. Evaporated in vacuo to 1/10 of volume. Filtered, residue extracted with $C_6H_6$-MeOH (9:1). | Eluted on Sephadex LH-20 (5x52cm) with MeOH-$H_2O$-HCl (500:500:1 or 800:200:1 for medium extract, 1000:0:1 for cell extract) | Column:<br>Nucleosil $C_{18}$ column (250 x 4.6mm, 10 um) with Vydac SC-201 RP pre-column (40 x 4.6mm, 30-40 um)<br><br>Detection:<br>UV 280 nm<br><br>UV and IR spectra<br>H-NMR<br>EiMS | Mobile phase A: $H_2O$-ACN-$H_3PO_4$ (98:2:0.01)<br>B: $H_2O$-ACN-$H_3PO_4$ (90:10:0.01)<br>1.5ml/min:<br>0-20 min: 35-75%B<br>20-40 min: 75-100% B |

## *Annexe 3: Résultats du chapitre 5*

**Tableau A3-1: Paramètres de culture**

| | poids humide (g/10 mL) | | poids sec (g/10 mL) | | pH | |
|---|---|---|---|---|---|---|
| | moyenne | erreur type | moyenne | erreur type | moyenne | erreur type |
| A | 1,6451 | 0,2314 | 0,0809 | 0,0018 | 5,83 | 0,06 |
| B | 1,4909 | 0,0778 | 0,0810 | 0,0069 | 5,93 | 0,11 |
| C | 1,4624 | 0,2879 | 0,0854 | 0,0093 | 6,01 | 0,08 |
| D1 | 1,5797 | 0,1189 | 0,0891 | 0,0064 | 6,02 | 0,14 |
| D2 | 1,6369 | 0,0993 | 0,0933 | 0,0048 | 6,04 | 0,12 |

**Tableau A3-2: Concentrations en sanguinarine (µmol / gDW)**

| | résines | | cellules | | Milieu | |
|---|---|---|---|---|---|---|
| | moyenne | erreur type | moyenne | erreur type | moyenne | erreur type |
| A | --- | --- | 0,00316 | 0,00113 | 0 | 0 |
| B | 0,09479 | 0,03961 | 0,00398 | 0,00269 | 0 | 0 |
| C | 0,0487 | 0,03678 | 0,01003 | 0,0037 | 0 | 0 |
| D1 | 0,02593 | 0,00599 | 0,00669 | 0,00473 | 0 | 0 |
| D2 | 0,03437 | 0,03437 | 0,01377 | 0,00404 | 0 | 0 |

**Tableau A3-3: Concentrations en chelirubine (µmol / gDW)**

| | résines | | cellules | | Milieu | |
|---|---|---|---|---|---|---|
| | moyenne | erreur type | moyenne | erreur type | moyenne | erreur type |
| A | --- | --- | 0,22569 | 0,05583 | 0,02881 | 0,00414 |
| B | 1,1479 | 0,42975 | 0,03566 | 0,00434 | 0 | 0 |
| C | 1,01503 | 0,48901 | 0,26831 | 0,22978 | 0,04727 | 0,02928 |
| D1 | 0,66511 | 0,07724 | 0,14041 | 0,06367 | 0,02182 | 0,00663 |
| D2 | 0,25406 | 0,25406 | 0,07707 | 0,02354 | 0,00121 | 0,00242 |

**Tableau A3-4: Concentrations en macarpine (µmol / gDW)**

| | résines | | cellules | | milieu | |
|---|---|---|---|---|---|---|
| | moyenne | erreur type | moyenne | erreur type | moyenne | erreur type |
| A | --- | --- | 0,03191 | 0,02656 | 0 | 0 |
| B | 1,72158 | 0,76321 | 0,0525 | 0,00954 | 0 | 0 |
| C | 0,94969 | 0,53648 | 0,17401 | 0,12765 | 0,02171 | 0,02804 |
| D1 | 0,45043 | 0,05911 | 0,10092 | 0,06684 | 0,00239 | 0,00477 |
| D2 | 0,60156 | 0,60156 | 0,16977 | 0,06372 | 0 | 0 |

**Tableau A3-5: Concentrations en chelerythrine (µmol / gDW)**

| | résines | | cellules | | milieu | |
|---|---|---|---|---|---|---|
| | moyenne | erreur type | moyenne | erreur type | moyenne | erreur type |
| A | --- | --- | 0,03191 | 0,02656 | 0 | 0 |
| B | 1,72158 | 0,76321 | 0,0525 | 0,00954 | 0 | 0 |
| C | 0,94969 | 0,53648 | 0,17401 | 0,12765 | 0,02171 | 0,02804 |
| D1 | 0,45043 | 0,05911 | 0,10092 | 0,06684 | 0,00239 | 0,00477 |
| D2 | 0,60156 | 0,60156 | 0,16977 | 0,06372 | 0 | 0 |

**Tableau A3-6: Concentrations en chelilutine (µmol / gDW)**

| | résines | | cellules | | milieu | |
|---|---|---|---|---|---|---|
| | moyenne | erreur type | moyenne | erreur type | moyenne | erreur type |
| A | --- | --- | 0,91233 | 0,34836 | 0,20934 | 0,06489 |
| B | 1,37079 | 0,37603 | 0,02762 | 0,00907 | 0 | 0 |
| C | 2,71341 | 1,61079 | 1,00016 | 0,92357 | 0,21529 | 0,16651 |
| D1 | 3,5137 | 0,34696 | 0,55534 | 0,09058 | 0,15291 | 0,01524 |
| D2 | 0,16222 | 0,16222 | 0,03661 | 0,01003 | 0,00127 | 0,00254 |

## *Annexe 4: Analyses statistiques du chapitre 5*

Les données présentées au chapitre 5 ont été analysées par test de Student pour déterminer s'il y avait des différences significatives entre les conditions de cultures :

- A: contrôle, aucune phase extractive (récolte après 7 jours)
- B: résines XAD-7 en suspension avec les cellules (récolte après 7 jours)
- C: recirculation du milieu à travers une colonne contenant de la résine XAD-7 (récolte après 7 jours)
- D1: recirculation du milieu à travers une colonne contenant de la résine XAD-7 (récolte après 6 jours) ; D2: retrait de la colonne 6 jours après l'élicitation, et rajout de résines à la suspension cellulaire (récolte un jour plus tard, au jour 7)

En considérant un degré de liberté de 6 (4 réplicats, deux fois, moins 2) et une probabilité de 0.05, les valeurs de t supérieures à 2,447 indiquent une différence significative entre les conditions de cultures comparées.

**Tableau A4-1: valeurs t des paramètres de culture par analyse statistique t-test**

| | poids sec | poids humide | FW/DW | pH |
|---|---|---|---|---|
| A.vs.B | 0,049 | 1,263 | 1,200 | 1,633 |
| A.vs.D1 | ***2,472*** | 0,502 | 1,655 | ***2,562*** |
| A.vs.C | 0,956 | 0,989 | 1,875 | ***3,519*** |
| A.vs.D2 | ***4,836*** | 0,065 | 1,771 | ***3,255*** |
| B.vs.D1 | 1,714 | 1,250 | 2,111 | 1,072 |
| B.vs.C | 0,752 | 0,191 | 1,613 | 1,169 |
| B.vs.D2 | ***2,911*** | 2,316 | ***2,765*** | 1,454 |
| D1.vs.C | 0,663 | 0,753 | 0,843 | 0,183 |
| D1.vs.D2 | 1,034 | 0,738 | 0,784 | 0,243 |
| C.vs.D2 | 1,510 | 1,146 | 0,638 | 0,510 |

**Tableau A4-2 : valeurs t pour la sanguinarine par analyse statistique t-test**

| | cellules | milieu | résines | total | % intracellulaire |
|---|---|---|---|---|---|
| A.vs.B | 0,572 | -- | -- | ***4,574*** | -- |
| A.vs.D1 | 1,451 | -- | -- | ***3,430*** | -- |
| A.vs.C | 1,826 | -- | -- | ***4,479*** | -- |
| A.vs.D2 | ***5,078*** | -- | -- | ***6,630*** | -- |
| B.vs.D1 | 0,990 | -- | ***3,438*** | ***2,895*** | -- |
| B.vs.C | 1,337 | -- | 1,705 | 1,812 | -- |
| B.vs.D2 | ***4,043*** | -- | 0,486 | 0,680 | -- |
| D1.vs.C | 0,298 | -- | 1,223 | 1,525 | -- |
| D1.vs.D2 | 2,287 | -- | ***4,680*** | ***4,356*** | -- |
| C.vs.D2 | 1,938 | -- | 2,337 | ***2,975*** | -- |

**Tableau A4-3: valeurs t pour la chelirubine par analyse statistique t-test**

| | cellules | milieu | résines | total | % intracellulaire |
|---|---|---|---|---|---|
| A.vs.B | ***6,786*** | ***13,941*** | -- | ***3,543*** | ***9,799*** |
| A.vs.D1 | 2,014 | 1,790 | -- | ***2,747*** | 1,225 |
| A.vs.C | 0,360 | 1,249 | -- | ***4,813*** | 0,490 |
| A.vs.D2 | ***4,906*** | ***11,523*** | -- | ***5,980*** | ***5,425*** |
| B.vs.D1 | ***3,282*** | ***6,580*** | 2,212 | 1,067 | ***9,532*** |
| B.vs.C | 2,025 | ***3,228*** | 0,408 | 0,473 | ***2,917*** |
| B.vs.D2 | ***3,458*** | 1,000 | 1,637 | 0,006 | 1,000 |
| D1.vs.C | 1,073 | 1,696 | 1,414 | 1,702 | 1,102 |
| D1.vs.D2 | 1,866 | ***5,838*** | 0,560 | 1,410 | ***6,058*** |
| C.vs.D2 | 1,656 | ***3,135*** | 1,000 | 0,603 | 2,286 |

**Tableau A4-4: valeurs t pour la macarpine par analyse statistique t-test**

| | cellules | milieu | résines | total | % intracellulaire |
|---|---|---|---|---|---|
| A.vs.B | 1,459 | -- | -- | ***4,828*** | -- |
| A.vs.D1 | 1,919 | 1,000 | -- | ***3,935*** | 1,000 |
| A.vs.C | 2,180 | 1,548 | -- | ***6,585*** | 1,671 |
| A.vs.D2 | ***3,993*** | -- | -- | ***5,744*** | -- |
| B.vs.D1 | 1,435 | 1,000 | ***3,321*** | ***3,139*** | 1,000 |
| B.vs.C | 1,898 | 1,548 | 1,655 | 1,586 | 1,671 |
| B.vs.D2 | ***3,640*** | -- | 0,496 | 0,208 | -- |
| D1.vs.C | 1,014 | 1,358 | 1,850 | ***2,675*** | 0,899 |
| D1.vs.D2 | 1,491 | 1,000 | ***3,408*** | ***3,515*** | 1,000 |
| C.vs.D2 | 0,059 | 1,548 | 1,317 | 1,612 | 1,671 |

**Tableau A4-5: valeurs t pour la chelerythrine par analyse statistique t-test**

| | cellules | milieu | résines | total | % intracellulaire |
|---|---|---|---|---|---|
| A.vs.B | 1,711 | 13,521 | -- | ***4,585*** | ***11,873*** |
| A.vs.D1 | ***2,652*** | ***4,422*** | -- | ***3,407*** | ***6,933*** |
| A.vs.C | ***2,615*** | 0,839 | -- | ***4,794*** | ***6,414*** |
| A.vs.D2 | ***4,470*** | ***9,714*** | -- | ***6,858*** | ***9,815*** |
| B.vs.D1 | ***3,265*** | 6,153 | 2,286 | 1,453 | ***13,241*** |
| B.vs.C | ***2,847*** | ***4,165*** | 1,150 | 0,174 | ***6,106*** |
| B.vs.D2 | ***5,485*** | ***6,449*** | 1,691 | 2,341 | ***16,391*** |
| D1.vs.C | 1,463 | 1,465 | 0,703 | 1,346 | 0,544 |
| D1.vs.D2 | 0,316 | ***3,323*** | ***4,459*** | ***3,825*** | ***7,027*** |
| C.vs.D2 | 1,381 | ***3,044*** | ***2,816*** | ***2,588*** | ***3,121*** |

**Tableau A4-6: valeurs t pour la chelilutine par analyse statistique t-test**

| | cellules | milieu | résines | total | % intracellulaire |
|---|---|---|---|---|---|
| A.vs.B | ***5,078*** | ***6,453*** | -- | 0,415 | ***11,965*** |
| A.vs.D1 | 1,984 | 1,694 | -- | ***3,010*** | 1,135 |
| A.vs.C | 0,178 | 0,067 | -- | 2,267 | 0,728 |
| A.vs.D2 | ***5,026*** | ***6,409*** | -- | ***6,688*** | ***4,329*** |
| B.vs.D1 | ***11,593*** | ***20,066*** | ***8,377*** | ***2,855*** | ***12,579*** |
| B.vs.C | 2,106 | ***2,586*** | 1,623 | 2,149 | ***2,870*** |
| B.vs.D2 | 1,332 | 1,000 | ***2,575*** | ***6,424*** | 1,000 |
| D1.vs.C | 0,959 | 0,746 | 0,971 | 0,062 | 1,205 |
| D1.vs.D2 | ***11,383*** | ***19,629*** | ***13,943*** | 0,749 | ***4,978*** |
| C.vs.D2 | 2,086 | ***2,570*** | 2,310 | 0,613 | 1,905 |

## *Annexe 5: Cinétiques d'adsorption - simulation*

Pour vérifier si la capacité de la colonne adsorptive n'est pas limitée, nous avons simulé la cinétique d'adsorption d'alcaloïdes en considérant qu'à la sortie de la colonne, la concentration est nulle. Voir la section 5.2.3.5 pour les détails du montage.

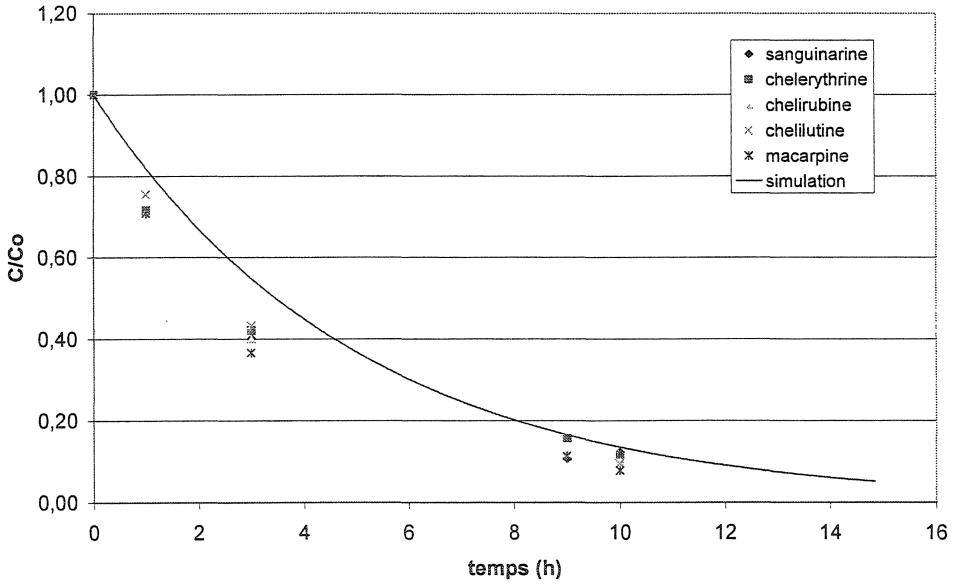



**Figure A5-1: Simulation de la cinétique d'adsorption avec le recirculation et colonne**