Development of experimental methods to determine high lipophilicity of new chemical compounds : applications to cyclosporin A and bioconcentration

Guillot, Amandine

UNIVERSITÉ DE GENÈVE **FACULTÉ DES SCIENCES**

Section des sciences pharmaceutiques Professeur Pierre-Alain CARRUPT

Development of experimental methods to determine high lipophilicity of new chemical compounds. Applications to cyclosporin A and bioconcentration.

THÈSE

présentée à la Faculté des sciences de l'Université de Genève

pour obtenir le grade de Docteur ès sciences, mention interdisciplinaire

par

Amandine Guillot

de

Domessin (France)

Thèse n° 4250

Genève

Atelier ReproMail

2010

UNIVERSITÉ DE GENÈVE **FACULTÉ DES SCIENCES**

Section des sciences pharmaceutiques Professeur Pierre-Alain CARRUPT

Development of experimental methods to determine high lipophilicity of new chemical compounds. Applications to cyclosporin A and bioconcentration.

THÈSE

présentée à la Faculté des sciences de l'Université de Genève

pour obtenir le grade de Docteur ès sciences, mention interdisciplinaire

par

Amandine Guillot

de

Domessin (France)

Thèse n° 4250

Genève

Atelier ReproMail

2010

UNIVERSITÉ DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès sciences
Mention interdisciplinaire

Thèse de *Madame Amandine GUILLOT*

intitulée :

" Development of Experimental Methods to Determine High Lipophilicity of New Chemical Compounds. Applications to Cyclosporin A and Bioconcentration "

La Faculté des sciences, sur le préavis de Messieurs P.-A. CARRUPT, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), J.-L. VEUTHEY, professeur ordinaire (Section des sciences pharmaceutiques), Madame S. MARTEL, docteure (Section des sciences pharmaceutiques), Messieurs C. ALTOMARE, professeur (Università degli Studi di Bari – Dipartimento Farmaco-chimico – Bari, Italia), et M. NEUWELS, docteur (L'Oréal Recherche et Innovation – Asnières-sur-Seine, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 27 septembre 2010

Thèse - 4250 -

Le Doyen, Jean-Marc TRISCONE

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Guillot,A., Henchoz,Y., Moccand,C., Guillarme,D., Veuthey,J.L., Carrupt,P.A., & Martel,S. (2009) Lipophilicity determination of highly lipophilic compounds by liquid chromatography. *Chem. Biodivers.*, 1828-1836.

La phrase...
Voici quelques proverbes à méditer.

"L'erreur n'annule pas la valeur de l'effort accompli."

Proverbe Africain

"Que celui qui n'a pas traversé ne se moque pas de celui qui s'est noyé."

Proverbe Africain

"Celui qui pose une question risque cinq minutes d'avoir l'air bête. Celui qui ne pose pas de question restera bête toute sa vie."

Proverbe Chinois

"Point n'est besoin d'élever la voix quand on a raison."

Proverbe Chinois

Remerciements

Je tiens à adresser ma plus sincère reconnaissance au Professeur Pierre—Alain Carrupt pour m'avoir accordé sa confiance et permis d'effectuer cette thèse de doctorat au sein de son laboratoire. Merci pour son soutien, pour les ressources exceptionnelles mises à disposition et pour la grande liberté de travail.

J'aimerais remercier le Dr Sophie Martel pour son aide et son soutien lors de la rédaction du manuscrit, des abstracts et de la publication ainsi que lors des préparations des présentations orales.

Je suis reconnaissante envers le Professeur Jean—Luc Veuthey de m'avoir permis de bénéficier de leurs instruments analytiques de haute qualité avant l'acquisition de l'UPLC par notre laboratoire.

Un grand merci au Dr Davy Guillarme pour sa disponibilité, sa bonne humeur mais surtout pour ses précieux conseils chromatographiques et ses compétences analytiques hors normes.

Merci aux membres du jury de thèse, les Professeurs Jean—Luc Veuthey, Cosimo Altomare et le Dr Michel Neuwels pour leur lecture attentive, leur regard critique et leurs précieuses recommandations.

Je tiens à remercier Bénédicte Gross—Valloton et Laurent Starrenberger de m'avoir initiée au monde de la chromatographie liquide. Mais également Fabrice Gillerat pour son assistance scientifique, technique et psychologique.

Des remerciements également aux collègues qui ont dû me supporter dans le bureau 420, en particulier le Dr Bruno Bard, Philippe Eugster, Karine Vuignier, Laurent Carrez et Alessandra Zizzari.

Merci au Dr Antoine Daïna et à Elisabeth Favre pour leur collaboration sur le projet cyclosporine, et notamment pour l'étude conformationnelle.

Un grand merci aux Dr Hansjorg Eder et Rivara–Minten Elisabeth pour leur aide et leur perpétuelle bonne humeur lors des Travaux pratiques de chimie organique pharmaceutiques, ainsi qu'en dehors du cadre professionnel. Et je remercie Jessica Ortelli pour son soutien technique et psychologique lors de ces TP.

J'aimerais également remercier tous mes collègues de Pharmacochimie, en particulier Delphine Campos–Ruiz–Cressend pour son optimisme, ses blagues excessivement drôles et son amitié tout au long de cette thèse, ainsi qu'après, je l'espère. Merci aussi à Céline Le Bourdonnec, Bénédicte Gross–Valloton, Kevin Nadin et au Dr Liliana Sintra Grilo pour leur amitié. Et je remercie les Dr Yveline Henchoz, Marianne Reist–Oechslin, Vincent Gasparik, Juan Bravo, Julien Boccard, Alessandra Nurisso ainsi que Sylvia Passaquay–Rion, Irena Nikolova, Sandra Alvarez, Christophe Francey et Nils Oberhauser pour les moments partagés.

Ma profonde gratitude va naturellement à mes parents, à ma sœur Coralie et à mon amie Emilie pour leur présence, leur soutien sans faille et infiniment précieux et leur encouragements tout au long de ces années universitaires.

Et finalement, j'adresse des remerciements particuliers à mon mari Nicolas pour sa patience, sa gentillesse, son soutien et surtout pour son amour et sa présence à mes côtés.

Merci !

Table of contents

Résumé

Nous sommes de plus en plus souvent confrontés aux composés très lipophiles dans notre vie quotidienne. En effet, bien qu'une grande partie des composés très lipophiles soient des pesticides, de tels composés sont également présents dans l'industrie agroalimentaire avec les pigments, les additifs alimentaires antioxydant ou encore les compléments alimentaires. Ils sont également utilisés dans les cosmétiques comme émollients, qui forment une couche huileuse sur la peau et réduisent ainsi la perte d'eau de l'épiderme, ou en tant que filtres ultra-violet (UV). De plus, le nombre de médicaments très lipophiles augmente peu à peu.

La lipophilie étant un pré-requis pour toute nouvelle substance chimique disponible sur le marché, un grand nombre de méthodes existent afin de la mesurer telles que la méthode shake-flask (la technique de référence), la chromatographie liquide, la potentiométrie, la chromatographie de partage centrifuge ou encore l'électrophorèse capillaire. Toutefois, la limite supérieure pour ces différentes méthodes actuellement utilisées est généralement log P = 5 (coefficient de partage). Par conséquent, différentes stratégies expérimentales utilisant aussi bien la chromatographie liquide classique (HPLC) que la chromatographie liquide à ultra haute pression (UHPLC) ont été évaluées. Elles permettraient ainsi, pour la première fois, de déterminer la lipophilie de composés ayant un log P supérieur à 5. Divers co-solvants, phases stationnaires et modes d'élution ont été testés en utilisant un ensemble de composés variés. Ce groupe de composés est constitué de 38 analytes ayant des log P_{oct} (coefficient de partage obtenu dans un système eau/n-octanol) compris entre 0 à 5 ainsi que 19 composés rigides possédant un CLOGP (log P calculés) allant de 5 à 10. Tous ces composés ont été évalués sous leur forme neutre et des phases stationnaires en phase inverse ont été employées.

La dissolution de la silice à pH élevé induit une instabilité des phases stationnaires utilisées. Aussi les mesures de lipophilie pour des composés basique possédant un $pK_a > 7$ ne peuvent être effectuées en utilisant les méthodes sus-mentionnées. Dans ce but, une colonne de chromatographie liquide d'interaction hydrophile (HILIC) a été récemment évaluée sur le système HPLC. En effet, cette colonne permet la détermination de la lipophilie pour des composés basiques avec un $pK_a > 7$, par l'intermédiaire de la forme cationique plutôt que la forme neutre. Il

a ainsi été démontré que la différence entre 2 facteurs de rétention isocratique, log k_0 (extrapolé à 100% d'eau dans la phase mobile ou directement mesuré) et log k_{95} (mesuré à 95% ACN) est bien corrélée avec les valeurs de log P^N_{oct} (log P_{oct} de la forme neutre).

Bien qu'il soit admis que la cyclosporine A est un composé très lipophile, seules des faibles valeurs expérimentales de log P_{oct} (1,0 à 3,6) ont été rapportées dans la littérature. Ainsi, trois différentes méthodes développées durant cette thèse ont été utilisées pour déterminer les valeurs de log P_{oct} de la cyclosporine A et d'un analogue, la cyclosporine C. Toutefois, des valeurs différentes de log P_{oct} ont été obtenues en fonction de la phase stationnaire utilisée. Afin de permettre une explication de ces différents résultats, plusieurs composés avec des particularités spécifiques ont été évalués (des peptides linéaires ou cycliques moins lipophiles et des composés possédant un volume important avec des lipophilies variées). Suite aux résultats obtenus, une étude dynamique préliminaire a été réalisée sur la cyclosporine A pour évaluer si des changements de conformation pouvaient expliquer les différentes valeurs expérimentales de log P_{oct} obtenues.

Une évaluation de l'écotoxicité est importante pour la commercialisation d'une nouvelle molécule chimique. De plus, en raison de la directive de l'organisation de coopération et de développement économique (OCDE), une stratégie est nécessaire pour évaluer les facteurs de bioconcentration sans recours aux organismes aquatiques, en particulier les poissons. Bien que de bonnes corrélations aient été obtenues entre les facteurs de bioconcentration (log BCF) et les valeurs de log P_{oct}, de plus en plus de produits chimiques sont très lipophiles. Or aucune des méthodes expérimentales ne peut être utilisée pour l'estimation de la bioconcentration puisque ces méthodes de détermination de la lipophilie sont limitées à une valeur de 5. Puisque la colonne Hypersil™ GOLD Javelin HTS autorise une analyse rapide, cette dernière a été choisie afin d'évaluer l'utilisation de valeurs expérimentales (log k_w) pour la prédiction des facteurs de bioconcentration (log BFC).

Abstract

We are increasingly confronted to highly lipophilic compounds in everyday life. Indeed, although a large proportion of highly lipophilic compounds are pesticides, such compounds are also present in the agro-alimentary industry with pigments, antioxidant food additives or food supplements. They are also used in cosmetics as emollients, which form an oily layer on the skin and thereby reduce the epidermal loss in water, or ultra-violet (UV) filters. In addition, the number of highly lipophilic drugs is gradually increasing.

As lipophilicity is required for any new commercially available chemical, a wide number of methods exists to measure it such as the shake—flask method (the reference technique), liquid chromatography, potentiometry, centrifugal partition chromatography or capillary electrophoresis. However, the upper limitation for these different methods currently used is often around log P = 5. Therefore, different experimental strategies using both conventional liquid chromatography (HPLC) and ultra-high pressure liquid chromatography (UHPLC) were evaluated to allow, for the first time with these techniques, the lipophilicity determination of compounds with log P > 5. Various organic modifiers, stationary phases, and elution modes were tested using a well-balanced set of compounds composed of 38 analytes with log P_{oct} from 0 to 5 and 19 rigid ones with a CLogP between 5 and 10. All these compounds were evaluated under their neutral form.

Because the stationary phases used are unstable at high pH conditions due to the silica-based dissolution, the lipophilicity measurements of strongly basic compounds cannot be performed using these methods. For this purpose, a hydrophilic interaction liquid chromatography (HILIC) column was recently evaluated on HPLC system. Indeed, this column permits the determination of strongly basic compounds since the cationic form is evaluated instead of the neutral one. In fact, it was demonstrated that $\Delta\log k_{0\text{-}95}$, which corresponds to the difference between 2 isocratic log k values (log k_0, extrapolated to 100% water in mobile phase or directly measured, and log k_{95} measured at 95% ACN) were well correlated with log P^N_{oct} values (log P_{oct} of the neutral form).

Although it is known that cyclosporin A is a very lipophilic compound, only low experimental log P_{oct} values (1.0 to 3.6) were reported in literature. Thus, three

different methods developed here were applied to determine the log P_{oct} of the cyclosporins A and C. However, different log P_{oct} values were obtained depending on the stationary phase utilized. Hence, to allow an explanation for these different results, more compounds with specific particularity were evaluated (ie, linear and cyclic peptides less lipophilic and compounds with important volume and different lipophilicity). Regarding the results obtained, a preliminary dynamic study was performed on cyclosporin A to evaluate if conformational changes this compound could explained the different experimental log P values obtained.

Finally, an evaluation of the ecotoxicity is important for the commercialization of a new chemical entity and due to the Organization for Economic Co-operation and Development (OECD) Directive, a strategy to evaluate the bioconcentration factor without the employment of aquatic organisms and especially fishes is required. Although good correlations have been found between the bioconcentration factors and log P_{oct} values, more and more chemicals are very lipophilic and no experimental methods can be used for the estimation of the bioconcentration since lipophilicity determination's methods are limited to a lipophilicity value of 6. Thus, as the Hypersil™ GOLD Javelin HTS stationary phase allows the fastest analysis, this latter was chosen for the feasibility evaluation of the employment of experimental log k_w values for the prediction of bioconcentration factors (log BCF).

Abbreviations and symbols

3D	Three dimensional
Å	Angström
ACN	Acetonitrile
Acquity	Acquity UPLC™ BEH Shield RP18 column
ADMET	Absorption, distribution, metabolism, excretion and toxicity
atm	Atmosphere
b	Gradient steepness
BCF	Bioconcentration factor
BMC	Biopartitioning micellar chromatography
C_{aq}	Chemical concentration in aqueous phase
CE	Capillary electrophoresis
C_{org}	Chemical concentration in organic phase
CLogP	Calculated log P value determined using the CLOGP software
CsA	Cyclosporin A
CsC	Cyclosporin C
Discovery	Discovery® RP Amide C16 column
DME	1,2-dimethoxyethan
$\Delta\varphi$	Change in composition during the gradient run
ETA	Extended topochemical atom
EU	European union
F	Flow rate
H_2O	Water
H-bond	Hydrogen bond
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High pressure liquid chromatography
Hypersil	Hypersil™ GOLD Javelin HTS column
ID	Internal diameter
$IC_{95\%}$	95% confidence intervals
k	Retention factor
k_0	Retention factor at the initial gradient composition
k_1	Uptake

k_2	Elimination
λ_{max}	Lambda max (wavelength with the maximum of absorption)
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LEKC	Liposome electrokinetic chromatography
log BCF	Logarithm of the bioconcentration factor
log BCF_{exp}	Logarithm of the bioconcentration factor measured experimentally using fishes
log BCF_{logP}	Logarithm of the bioconcentration factor predicted using calculated log P values (Eq. 4.3)
log BCF_{logkw}	Logarithm of the bioconcentration factor predicted using experimental log k_w values (Eq. 4.4)
log k	Logarithm of the retention factor
log k_w =log k_0	Logarithm of the retention factor extrapolated to 100% water
log $k_{w/o}$	Logarithm of the retention factor measured using n-octanol in the mobile phase
log $k_{wDiscovery}$	Logarithm of the retention factor extrapolated to 100% water determined using Discovery® RP Amide C16 column
log $k_{wAcquity}$	Logarithm of the retention factor extrapolated to 100% water determined using Acquity UPLC™ BEH Shield RP18 column
log $k_{wHypersil}$	Logarithm of the retention factor extrapolated to 100% water determined using Hypersil™ GOLD Javelin HTS column
log k_{35}	Logarithm of the retention factor measured in presence of 35% tetrahydrofuran in the mobile phase
log k_{95}	Logarithm of the retention factor measured in presence of 95% acetonitrile in the mobile phase
Δlog $k_{0\text{-}95}$	Difference between retention factors measured in presence of 0 and 95% of acetonitrile in the mobile phase
log P	Logarithm of the partition coefficient
log P_{ACN}	Logarithm of the n-octanol/water partition coefficient determined using acetonitrile as organic modifier
log P_{aN}	Logarithm of the apparent permeability of neutral specie

$\log P_{grad}$	Logarithm of the n-octanol/water partition coefficient determined using methanol as organic modifier in gradient mode
$\log P_{MeOH}$	Logarithm of the n-octanol/water partition coefficient determined using methanol as organic modifier
$\log P_{MLP}$	Logarithm of the partition coefficient determined using molecular lipophilicity potential
$\Delta\log P_{MLP}$	Variations of $\log P_{MLP}$
$\log P_{oct}$	Logarithm of the n-octanol/water partition coefficient
$\log P^{N}_{oct}$	Logarithm of the partition coefficient of the neutral form in n-octanol/water system
$\log P_{THF}$	Logarithm of the n-octanol/water partition coefficient determined using tetrahydrofran as organic modifier
$\log P_{THF35}$	Logarithm of the n-octanol/water partition coefficient determined using 35% tetrahydrofran as organic modifier
LSS	Linear solvent strength
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MD	Molecular dynamics
MLP	Molecular lipophilicity potential
NCEs	New chemical entities
ns	Nano second
OECD	Organization for Economic Co-operation and Development
oct-PAMPA	n-octanol parallel artificial membrane permeability assay
p	Test de t
PAH	Polycyclic aromatic hydrocarbon
PAMPA	Parallel artificial membrane permeability assay
PCB	Polychlorinated biphenyls
PCN	Polychlorinated naphthalene
PD	Pharmacodynamic
PDA	Photo diode array
PDMS	Poly-(dimethylsoiloxane)

pK_a	Ionization constant in water
PK	Pharmacokinetic
P_M	Membrane partition
φ	Percentage of the organic modifier in the mobile phase
ps	Pico second
(Q)SAR	(Quantitative) structure activity relationship
r^2	Determination coefficient
REACH	Registration, Evaluation, Authorization of CHemicals
RESP	Restrained electrostatic potential
RMSD	Root mean square deviation
RP-like	Reversed-phase type retention
RPLC	Reversed-phase liquid chromatography
SPME	Solid-phase microextraction
THF	Tetrahydrofuran
t_0	Retention time of an unretained compound (column dead time)
t_D	System dwell time
t_{delay}	Incompressible delay time of the chromatographic system
t_G	Gradient time
THF	Tetrahydrofuran
t_r	Retention time of the solute
UHPLC	Ultra High Pressure Liquid Chromatography
UPLC	Ultra Pressure Liquid Chromatography
UV/Vis	Ultra-violet detection
UV/Vis	Ultra-violet/ visible detection
V_0	Column dead volume
v-BCF	Very bioaccumulative
V_D	System dwell volume
VEKC	Vesicle electrokinetic chromatography
V_{ext}	Extra column volume

Chapter 1: Introduction.

1.1. Highly lipophilic compounds in research process

Highly lipophilic compounds are everywhere in the current life. Indeed, they are present in the agro-alimentary industry, as for examples pigment (lutein, lycopene), antioxidant food additive (halofantrine), or food supplement (astaxanthin or halofantrine). Furthermore, more and more drugs are very lipophilic [1], such as eritoran used in treatment for severe sepsis, paclitaxel, a mitotic inhibitor used in cancer chemotherapy or cyclosporin A which is used for loads of different pharmaceuticals reasons. These compounds are also used in cosmetics with emollients, which form an oily layer on the skin and reduce the epidermal loss in water (such as isostearyl isostearate, pentaerythrityl tetraisostearate or trimethylpropane triisostearate), or ultra-violet (UV) filters such as phytofluene. However, the larger part of the highly lipophilic compounds are pesticides such as polycyclic aromatic hydrocarbon (PAH), polychlorinated biphenyls (PCB) or polychlorinated naphthalene (PCN).

Whatever the industry concerned, the research process of new interesting compounds is similar and can be divided into two main phases: (i) chemical discovery, which includes target sites identification, high-throughput screening, candidate identification and optimization; and (ii) development which contains chemical toxicity studies on the organisms targeted as well as sides effect and the submission for launch.

However, targeted compounds properties can be strongly different depending on the wanted use (pesticides, cosmetics, drugs etc..).

For pharmaceutical use, highly lipophilic compounds are usually rejected due to their poor solubility into water inducing a poor oral bioavailability. Moreover, they are retained into the different membranes due to their high affinity for the lipids present into these membranes. However, loads of techniques exist to improve the solubility and hence the bioavailability of highly lipophilic compounds. Indeed, methods can improve the cutaneous delivery such as the use of chemical penetration enhancers, novel vehicle systems (e.g. microemulsions, liposomal-based delivery systems, and supersaturated formulations), more complex physical

enhancement strategies (e.g. iontophoresis [2], sonophoresis [3], and electroporation) [4] or simply co-administration with food which increases the uptake [5]. Many examples of very lipophilic drugs on the market proved that this kind of compounds are not systematically rejected only according to their lipophilicity. Thus, the determination of lipophilicity higher than log $P > 5$ could be interesting in order to choose the adequate improvement methods.

Although highly lipophilic compounds are problematic for pharmaceutical industry they are interesting for cosmetics, agroalimentary and pesticides industries.

Indeed, personal care and cosmetic compositions can comprise lipophilic skin health benefit agent [6]. This agent is defined as any substance that possesses a high lipophilicity and provides a skin health benefit by direct interaction with the skin, such as an enhancement of skin barrier function or moisturization and a nourishment of the skin. Moreover, lipophilic compounds can also be used as UV filters. Indeed, these compounds don't have to penetrate the stratum corneum or be in the viable tissue [7]. In fact, Potard et al. [8] showed that nearly 90% of these lipophilic compounds were maintained under the skin surface. That is why lipophilic compounds are good candidates since possessing a low percutaneous penetration.

In agroalimentary industry, lipophilic compounds are used to inhibit the lipid oxidation in food products [9]. For this purpose, natural lipophilic antioxidant compositions are used in a variety of products. They can also be used as lipophilic emulsifier which worked as a viscosity control agent [10].

For pesticides use, since the epicuticular wax and the cuticle possess a lipophilic nature, foliar uptake tends to increase when the lipophilicity of the chemical compounds is increased [11, 12]. Moreover, in insecticide industry, low water solubility, usually connected to a high lipophilicity, and high adsorption to soil particles are required in order to avoid leaching [13]. Furthermore, organochlorines which are insecticide possess a high liposolubility and act into the neural central system. This liposolubility allows an easy penetration into insect organisms.

Thus, highly lipophilic compounds are interesting for these industries and hence a log P_{oct} determination technique has to be developed.

Moreover, the effect of chemicals released into the environment, on aquatic organisms and humans, is also an essential parameter which has to be determined before launching new chemical substances on the market. Furthermore, ecotoxicity evaluation is necessary if a compound's production is higher than 1 tonne per year [14]. The bioconcentration factor (BCF) is an important ecotoxicological parameter describing the accumulation in fish and other aquatic animals of chemical substances present in water [15] through non-dietary routes [16]. In fact, these chemicals can be accumulated in the aquatic media up to significant concentrations [17]. This estimation is therefore mandatory for human and environmental risk assessments whatever the targeted use concerned.

Therefore, chemical compounds fail for many reasons during the research process but some of them could be avoided. Hence development of *in vitro* methods to evaluate as early as possible chemical candidates with suitable properties [18] could permit a decrease in the attrition rate especially in toxicity studies. For this purpose, high throughput methods have been developed [19-21] such as for lipophilicity, permeability, solubility or bioconcentration determination since they are low time consuming, permit to decrease the cost of the discovery process and avoid the employment of animals.

1.2 Lipophilicity

Lipophilicity is an important physicochemical property which influence and thus can be used to predict [22, 23] many important behaviors of chemical compounds since it contributes to solubility [24], membrane permeation [25, 26], metabolism [24] or bioconcentration [27] for examples.

Moreover, log P values are required for the launch of new chemical substances.

Lipophilicity expresses the partitioning of a compound between two immiscible solvents, usually water and an organic solvent. It is commonly define by the logarithm of the partition coefficient (log P).

$$\log P = \log \frac{C_{org}}{C_{aq}} \qquad \text{Eq.1.1}$$

where C_{org} and C_{aq} are the chemical concentrations in organic and aqueous phases, respectively.

n-octanol is the most used organic solvent [28] due to structural analogy with the phospholipids found in membranes (a polar group and a long alkyl chain). Hence, the n-octanol/water system is the widely accepted reference system for the determination of lipophilicity.

1.2.1. Lipophilicity determination

The gold standard procedure to measure lipophilicity is the shake-flask method, which allows log P values measurement from -2 to 4 [29]. However, this lipophilicity range could be enlarged with other detector system such as MS (mass spectrometry). The chemical compound is mixed into two immiscible solvents, usually water and n-octanol, until reached the equilibrium. Afterwards, the two phases are settled and the concentration of the solute is measured into both water and organic phases. However, this method is time-consuming, tedious, and it required a large quantity of pure compound [30, 31]. Hence different strategies have been developed to speed up, automate and miniaturize this process, such as the work of Hitzel et al. [32] or the one of Dohta et al. [33]. However, despite all these improvements, this method still requires some time-consuming steps such as the mutual saturation and the decantation of the both phases.

As higher throughput methods were needed to evaluate potential drugs candidate as early as possible, alternatives methods were developed.

Potentiometry could be used to measure the lipophilicity of lipophilic compounds. However, even if a study of Avdeef [34] reported a large range of log P_{oct} values (-2.3 to 7.4), lipophilicity determination for log P_{oct} values higher than 5 is in practice more difficult and thus is only possible in very specific cases. Moreover, this method is only applicable for ionizable compounds, and is sensible to impurities. Furthermore, this method provides only a relatively medium throughput.

The capillary electrophoresis (CE) is an interesting technique for log P_{oct} determination. Different CE modes have been developed, namely MEKC, MEEKC and VEKC/LEKC (micellar, microemulsion and vesicle/liposome electrokinetic chromatography, respectively). The microemulsion electrokinetic chromatography (MEEKC) has been largely used [35-37] as it is the most appropriate CE mode for the lipophilicity determination. This lipophilicity determination is based on the

partitioning of compound between an aqueous and lipophilic pseudo-stationary phases. Numerous linear correlations between retention factor (log k) and log P_{oct} values were reported in the literature [38-44] and hence allow to determine with accuracy the lipophilicity of new compounds, over a large log P_{oct} range (from -1 to 6.6) [43, 45-48]. This method possesses different advantages such as a small amount of sample is required, the process is automated, rapid and insensible to impurities or degradation products [31, 49]. However, measurement at low pH require some adaptations and lipophilicity is not usable for log P_{oct} values higher than 7.

Another promising high-throughput method, oct-PAMPA (n-octanol parallel artificial membrane permeability assay), described by Faller et al. [50], is based on the 96-well format. This method is derived from the PAMPA technology [51] developed for permeability measurements where two compartments are separated by a liquid artificial membrane. Using n-octanol as artificial liquid membrane the apparent permeability of the neutral species (log P^N_a), derived from the concentration in the acceptor compartment after 4h incubation time, is used to determine log P values of new compounds. Indeed, a bilinear correlation was obtained between these two parameters. A large range of log P values can be determined (from -2 to 8) especially using the liquid chromatography mass spectrometry (LC-MS) detection (log P > 5). However, inaccurate values were obtained for compounds with log P within 0 to 2 due to a flattening of the bilinear regression. Moreover, the range of lipophilicity cannot be enlarged partly due to the limitation of the detection method induced by the small amount of product in the acceptor compartment.

Reversed-phase liquid chromatography (RP-LC) was largely used for the measurement of log P_{oct} values, as illustrated by the number of reviews published on this subject [31, 37, 49, 52-56]. This indirect approach is considered as the best alternative to the shake-flask method. Indeed, only a small amount of sample is required, the process is automated, rapid and insensible to impurities or degradation products [31, 49]. This method is principally based on the partitioning of a solute between a polar mobile phase (composed of a buffer and an organic modifier), and an apolar or less polar stationary phase. The factor used to determine the lipophilicity is the retention factor (log k) define by the following equation:

$$\log k = \log\left(\frac{t_r - t_0}{t_0}\right) \qquad \text{Eq.1.2}$$

where t_r and t_0 are the retention times of the solute and the unretained compound (uracil), respectively.

Depending on the stationary and mobile phases used, log k can then be correlated to log P_{oct} value:

$$\log P_{oct} = a \bullet \log k + b \qquad \text{Eq.1.3}$$

log k is the retention factor, and a and b are constants. Using a series of compounds with well-known log P_{oct} values, coefficients a and b can be determined for a couple of stationary and mobile phases. Then this calibration equation can be used to determine log P_{oct} value of novel compound.

Isocratic retention factors (log k) allow fast analysis and were largely used. However, because of their dependency to the percentage of organic modifier used in the mobile phase and thus potential inversions of retention [49], extrapolation to 100% water retention factors (log k_w) were preferred and numerous correlations were obtained between log k_w and log P_{oct} values either in isocratic [57-60] or gradient mode [61-63].

Isocratic or gradient mode can be used for retention factor measurements. Under isocratic conditions, log k_w is extrapolated by plotting a series of isocratic log k values against the percentage of the organic modifier used in the mobile phase. The relationship between the log k values and the percentages of organic modifier depend on the co-solvent employed (methanol (MeOH), acetonitrile (ACN) or even tetrahydrofuran (THF)). When using MeOH, it was shown that a linear extrapolation can be used [64]:

$$\log k = \log k_w - S \bullet \varphi \qquad \text{Eq. 1.4}$$

where log k_w is the intercept of the regression curve, φ is the volume fraction of organic modifier in organic-solvent mixture, and S (the slope of the regression curve) is a constant for a given solute and a given liquid chromatographic (LC) system.

With ACN or THF [65, 66], a quadratic extrapolation given by the following equation can be used:

$$\log k = \log k_w + B \bullet \varphi + A \bullet \varphi^2 \qquad \text{Eq. 1.5}$$

where A and B are constants for a given solute and a given LC system.

The gradient mode could also be used to determine the lipophilicity of new compounds. Moreover it allows a generic method without time or percentages adaptation according to the lipophilicity. Indeed, it corresponds to a gradual change of the mobile phase composition (the ratio water (buffer) / organic modifier) during the chromatographic run. The Linear Solvent Strength (LSS theory) was described by Snyder and Dolan [67] and is one of the most useful approach to determine log k_w values from gradient experiments. Hence, retention time (t_r) can be expressed with a linear-gradient separation [67] as follows:

$$t_r = \frac{t_0}{b} \bullet \log\left(2.3 \bullet k_0 \bullet b + 1\right) + t_0 + t_D \qquad \text{Eq. 1.6}$$

where t_0 is the retention time of an unretained compound (uracil), k_0 is the k value at the beginning of the gradient run (for $\varphi = \varphi_0$), t_D is the system dwell time for gradient elution (min) which can be experimentally determined [68] and b is the gradient steepness parameter described by the following relationship:

$$b = \frac{t_0 \bullet \Delta\varphi \bullet S}{t_G} \qquad \text{Eq. 1.7}$$

where t_G is the gradient time from the beginning to the end of the gradient run (min), and $\Delta\varphi$ the change in composition (φ) during the gradient run (ranging from 0 to 1).

The two unknown parameters S and k_0 can be determined from two gradient runs differing only in gradient times (t_G) and allow to calculate a log k_w value from Eq. 1.4 when MeOH is used as organic modifier. Because there is no empirical solution, values of k_0 and S have to be determined by iteration using an optimization software.

Recent development of Ultra High Pressure Liquid Chromatography (UHPLC), and especially of columns packed with small porous particles (sub-2 µm), permits to speed up the log P_{oct} determination. This system was firstly employed by Henchoz et al. [69] to determine log P_{oct} values of compounds from -1 to 5. This method is hence interesting for the lipophilicity determination of new chemical entities in the early

stage of discovery process since the analysis times are shorter than using RP-LC system.

However, the greatest number of columns used with HPLC system in these conditions have usually a pH range limited from 2 to 8 due to the instability of the silica at high pH [70-72]. Hence, log P_{oct} values of basic compounds with $pK_a > 6$ cannot be determined under their neutral form. A new approach was recently developed by Bard et al. [73] based on hydrophilic interaction liquid chromatography (HILIC) to determine log P_{oct} values for basic compounds with $pK_a > 8$. In fact, the retention factor of the cationic form can be linked to the lipophilicity of the neutral form. This log P determination is possible since three different mechanisms govern retention in this column. There are a "normal phase-like" retention, mainly hydrophilic at high percentages of organic modifier, "reversed phase-like" retention at high proportions of water, and ion exchange presents whatever the proportion of organic modifier. The correlation with log P was obtained with $\Delta\log k_{0\text{-}95}$ values which corresponds to the difference between the isocratic log k values obtained at 100% of water ($\log k_0$) and the one obtained at 95% of ACN ($\log k_{95}$). However, the log P range evaluated by Bard et al. [73] using a ZIC p-HILIC is from -1.3 to 4.6.

A summarization of the most commonly used techniques available for the lipophilicity determination and their limitations reported in the literature are represented in Fig. 1.1.

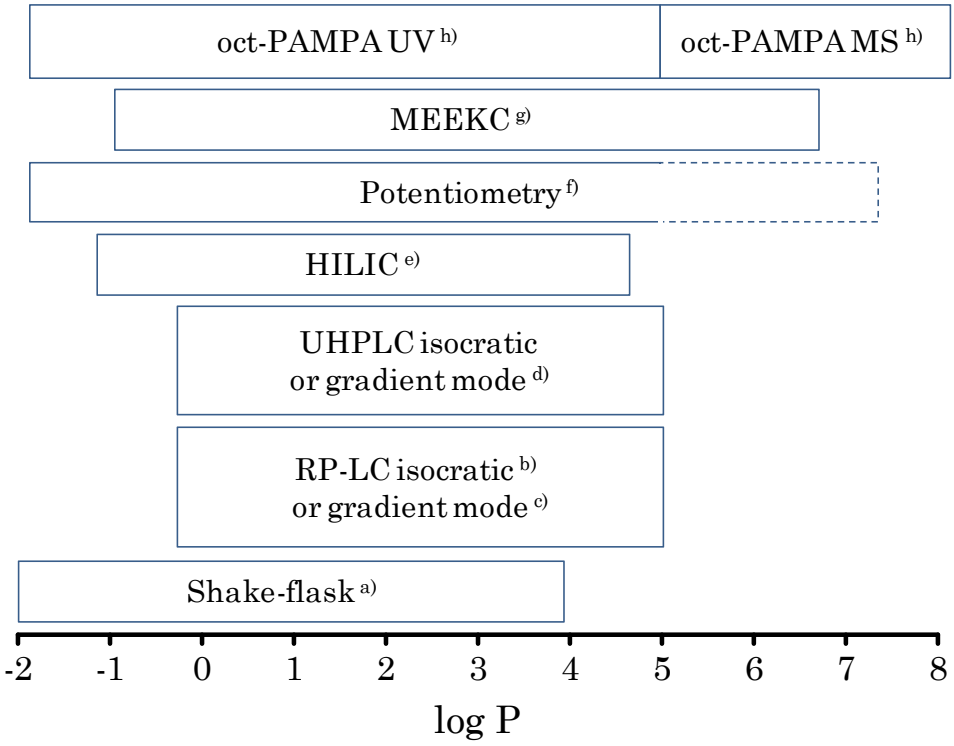


Figure1.1: log P range of in vitro techniques used to measure the lipophilicity. a)[29]; b)[57]; c)[62]; d)[69]; e)[73]; f)[34]; g)[43]; h)[74]

Lipophilicity range of RP-LC, UHPLC and HILIC can still be enlarged using more lipophilic compounds or by improving the conditions used (reduction of dimension or particle size).

This in order to work on highly lipophilic compounds which can be useful in different industries and whom number is increasing. Unfortunately, the methods discussed above are often available for log P_{oct} values of low to moderate lipophilicity. In particular the chromatographic methods which present great practical advantages remain generally limited to log P determination up to 5. A representative example is the case of cyclosporin A. In fact, although different experimental measurements of the lipophilicity of this compound were reported between 1 [75] and 3.6 [76], previous study realized in our laboratory revealed longer retention times into the column than expected with such lipophilicity values.

1.2.2. Lipophilicity and bioconcentration assessment

As already mentioned, bioconcentration is of prime importance for environmental and human risk assessment whatever the targeted use of the new chemical entities.

Because bioaccumulation and bioconcentration are often confused, these two processes are define. Thus bioaccumulation is a process in which a chemical substance is absorbed in an organism by all routes of exposure as occurs in the natural environment (dietary and ambient environment sources). However, bioconcentration only takes into account the ambient environment through the respiratory and dermal surfaces. Hence bioaccumulation is distinct from bioconcentration because chemical exposure in the diet is not included in the latter.

The bioconcentration assessment could be realized, as described by Hund-Rinke et al. [77] on aquatic organisms (European eel [78], fish [79], frog [80], salamander [81], crustaceans [82], water flea [83], insects [84], worms [85], snails [86], starfishes [87], algae [88], bacteria [89], protozoa [90], shrimp [91], crab [92], midge [93], mussel [94]), soil organisms (worms [85], fungi [95]), microorganisms (E. coli [96], S. cerevisiae [97]), plants (vegetables [98]), birds (flying sea-birds [99], penguins [100]) or mammals (seal [100], whale [100], cow [101]).

Fishes are good animals models for *in vivo* bioconcentration studies [16], and is thus the most frequently used species. Moreover, an experimental method is proposed by the European Union (EU) for bioconcentration determination in fishes [102]. Some fish species are even recommended by the Organization for Economic Co-operation and Development (OECD) guidelines 305 [103] such as fathead minnows [104], guppy [105], bluegill [106], rainbow trout [107], zebrafish [108] and carp [109]).

Nevertheless, whatever the species used, these experimental methods are time-consuming, difficult, expensive and impossible to apply to all the potential interesting chemical substances [110, 111]. Moreover, due to the Registration, Evaluation, Authorization of CHemicals (REACH) directive, *in vitro* methods are required. That is why *in vitro* methods have been developed to supply the missing data also due to several reasons, such as ethical, economic, logistical and so forth [17].

Indeed, many indices were proposed as an alternative to predict bioconcentration in fishes. For example the aqueous solubility [112, 113], the molecular

connectivity indices [15, 114], the extended topochemical atom (ETA) indices [115], the Padmakar-Ivan indices [116], the characteristic root index [117], the fragment constants [114, 118, 119] and the Fujita's inorganic and organic characters [120] were used to evaluate the bioconcentration.

However, the most common methods for predicting bioconcentration factors were based on the partitioning properties of chemical compounds. Several papers reported models based on the log P_{oct} values [116], the membrane partition indices [121], the membrane accumulation index [105], the liposome-water partition indices [122], and biopartitioning chromatography indices [17]. Indeed, bioconcentration represents the thermodynamically driven partitioning between water and the lipid phase of the fish and hydrophobicity is the principal driving force [123].

Loads of QSAR (quantitative structure-activity relationships) were reported between log BCF and n-octanol/water partition coefficient (log P_{oct}) [112, 124-127]. Most of them were linear relationships, however, if highly lipophilic compounds are taken into consideration, parabolic [128, 129] or bilinear [130, 131] correlations were thus reported. However, these methods are limited due to the lack of experimental methods for highly lipophilic compounds [132]. As an alternative, calculations methods were also used to enlarge the correlation with log BCF values introducing errors linked to *in silico* models especially for highly lipophilic compounds.

Liposome-water partition coefficient measured by solid-phase microextraction (SPME) were also utilized to predict bioconcentration [122]. In fact liposomes have been already used by several researchers to study membrane-water partitioning of chemical substances [133-137]. Indeed membranes are considered as the major target sites for partitioning of these chemical substances in organisms. Liposomes used in this study seemed to adequately mimic the partitioning of lipophilic compounds in complex lipid matrix and were hence correlated with the bioconcentration factors. The main advantages of this method are its speed, simplicity, reproducibility, and low costs. However, the log BCF prediction was limited for compounds with log $P < 7.2$.

Parallel artificial membrane permeability assay (PAMPA) was also used to evaluate the bioconcentration and two different methods were reported. The simplest one was developed by Fujikawa et al. [105, 138-141]. Since the PAMPA

permeability of lipophilic compounds was limited by their high artificial membrane accumulation, Fujikawa et al. [141] decided to use the membrane partition (P_M) as a membrane accumulation index. P_M is defined as the ratio of the amount of a compound in membrane to the amount of this compound in buffer solutions at the steady state. Hence, an excellent linear correlation was obtained between log P_M and log BCF values obtained in male guppy. However, this method was evaluated on a limited range of compounds' lipophilicity (from 2.7 to 4.8) and the detection mode is a drastic limitation of this method. Kwon et al. [121] modified the PAMPA system and used thick poly-(dimethylsiloxane) (PDMS) disks to serve as a passive dosing/sampling phase (PDMS-PAMPA). This method allows to determine how much and how fast a chemical substance accumulates in the body [142] and hence predict passive uptake/elimination rate in fish. The permeability measured with this method is proportional to the passive elimination rate constant. This method was evaluated for compounds with log P < 6.4 and even if the PAMPA system was modified, the detection mode still remains a limitation of this method.

Chromatographic separations have found a wide application in the environmental field. The retention of the chemical substances in reversed-phase liquid chromatography (RP-LC) was proposed as an alternative to *in vivo* tests. Bermudez-Saldaña et al. [17] directly used the chromatographic retention of the compounds for an evaluation of the bioconcentration. Different advantages of using log k rather than log P have been reported [143] and in particular it allows to overcome the interpolation that leads to less accurate results.

The biopartitioning micellar chromatography (BMC) was used as an *in vitro* approach to evaluate the bioconcentration factor of pesticides in fish [17]. BMC is a mode of reversed phase micellar liquid chromatography, which uses surfactant solutions above the critical micellar concentration as mobile phase. This method emulates the partitioning of chemical substances in biomembranes. The retention of compounds in the chromatographic system depends on its interactions with modified reversed stationary phase and micelles present in the mobile phase. These interactions are governed by hydrophobic, electronic and steric properties of compounds. Micelles have proven to be adequate chemical models for biomembranes mainly due to their amphiphilic and anisotropic properties [144-146]. The advantages of

this method are its speed, a good reproducibility, a low consumption of chemical substance and an insensibility to impurity. Whereas this method allows to separate the bioaccumulative compounds from the non-bioaccumulative ones, some compounds could not be sorted and others were misclassified.

1.3 Aim of the thesis

Hence, fast methods allowing an accurate measurement of highly lipophilic compounds with log P values higher than 8 is still missing. For this purpose, liquid chromatography especially used in combination with short columns and/or ultra-high pressure liquid chromatography (UHPLC) seems to be a promising method to reach such lipophilicity values.

This work was therefore devoted to the development of fast and accurate experimental tools for the measurement of highly lipophilic compounds' lipophilicity.

Thus, in the first part of this work, different stationary phases (Discovery® RP Amide C16, Acquity UPLC™ BEH Shield RP18 and Hypersil™ GOLD Javelin HTS), mobile phases (methanol, acetonitrile or tetrahydrofuran as organic modifier) and modes (isocratic or gradient) were first evaluated to determine lipophilicity higher than 5 for neutral, acidic and weakly basic compounds. Moreover, hydrophilic interaction chromatography (HILIC) was used to evaluate the lipophilicity of basic compounds with $pK_a > 8$. The lipophilicity range of this technique was enlarged to permit the log P_{oct} determination higher than 5.

Three of these methods were used to determine the lipophilicity of the cyclosporin A (CsA). However, different retention behaviors of CsA were observed on the different columns used. We hypothesized that conformational changes in lateral chains of the peptide could occurred depending on the considered stationary phase. Therefore a preliminary molecular modeling work was performed to evaluate if variations in the conformations of CsA could occurred in different environments and if these variations could explained the difference obtained in log P values and thus in chromatographic retentions.

Finally highly lipophilic compounds seem to be not toxic for human health or environment due to the low solubility or their trapping in the membrane. However,

no acceptable method is available for the bioconcentration measurement of these compounds. Hence the techniques developed in this work could be of great utility for an estimation of the bioconcentration factor, avoiding the usage of fish. Therefore the best method, developed with the Hypersil™ GOLD Javelin HTS stationary phase using methanol as organic modifier, was applied to a large number of compounds including highly lipophilic ones. The log k_w values thus obtained were evaluated as bioconcentration indices by comparing these parameters with experimental log BCF values found in the literature.

The different chapters are either submitted publications or in progress.

References

1. Neslihan Gursoy, R.; Benita, S. Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. *Biomed. Pharmacother.* **2004,** *58*, 173-182.

2. Hadgraft, J. Recent developments in topical and transdermal delivery. *Eur. J. Drug Metabol. Pharmacokin.* **1996,** *21*, 165-173.

3. Meidan, V. M.; Docker, M. F.; Walmsley, A. D.; Irwin, W. J. Phonophoresis of hydrocortisone with enhancers: an acoustically defined model. *Int. J. Pharm.* **1998,** *170*, 157-168.

4. Moser, K.; Kriwet, K.; Froehlich, C.; Naik, A.; Kalia, Y. N.; Guy, R. H. Permeation enhancement of a highly lipophilic drug using supersaturated systems. *J. Pharm. Sci.* **2001,** *90*, 607-616.

5. Nielsen, P. B.; Mullertz, A.; Norling, T.; Kristensen, H. G. The effect of alpha-tocopherol on the in vitro solubilisation of lipophilic drugs. *Int. J. Pharm.* **2001,** *222*, 217-224.

6. Maesen, T.; Kuperman, A. E.; Uckung, I. J. *Cosmetic and Personal Care Products Containing Synthetic Magnesium Alumino-Silicate Clays*; 2010.

7. Duracher, L.; Blasco, L.; Jaoued, A. A.; Vian, L.; Marti-Mestres, G. Irradiation of skin and contrasting effects on absorption of hydrophilic and lipophilic compounds. *Photochem. Photobiol.* **2009,** *85*, 1459-1467.

8. Potard, G.; Laugel, C.; Baillet, A.; Schaefer, H.; Marty, J.-P. Quantitative HPLC analysis of sunscreens and caffeine during in vitro percutaneous penetration studies. *Int. J. Pharm.* **1999,** *189*, 249-260.

9. Richards, M. P.; Lee, C. H.; Reed, J. *Methods and Lipophilic Antioxidant Compositions to Inhibit Lipid Oxidation in Food Products*; Unites States Patent: 2005.

10. Konishi, G. A.; Davy, P. F. *Aerosol Food Flavoring Compositions Containing Emulsifier Blends*; United States Patent: 1988.

11. Baker, E. A.; Hayes, A. L.; Butler, R. C. Physicochemical properties of agrochemicals - Their effects on foliar penetration. *Pestic. Sci.* **1992,** *34*, 167-182.

12. Stevens, P. J. G.; Baker, E. A.; Anderson, N. H. Factors affecting the foliar absorption and redistribution of pesticides .2. Physicochemical properties of the active ingredient and the role of surfactant. *Pestic. Sci.* **1988,** *24*, 31-53.

13. Chaton, P. F.; Lempérière, G.; Tissut, M.; Ravanel, P. Biological traits and feeding capacity of Agriotes larvae (Coleoptera: Elateridae): A trial of seed coating to control larval populations with the insecticide fipronil. *Pestic. Biochem. Physiol.* **2008,** *90*, 97-105.

14. Schreiber, R.; Altenburger, R.; Paschke, A.; Schüürmann, G.; Küster, E. A novel in vitro system for the determination of bioconcentration factors and the internal dose in zebrafish (Danio rerio) eggs. *Chemosphere* **2009,** *77*, 928-933.

15. Lu, X. X.; Tao, S.; Hu, H. Y.; Dawson, R. W. Estimation of bioconcentration factors of nonionic organic compounds in fish by molecular connectivity indices and polarity correction factors. *Chemosphere* **2000,** *41*, 1675-1688.

16. Barron, M. G. Bioconcentration. *Environ. Sci. Technol.* **1990,** *24*, 1612-1618.

17. Bermudez-Saldana, J. M.; Escuder-Gilabert, L.; Medina-Hernandez, M. J.; Villanueva-Camanas, R. M.; Sagrado, S. Modelling bioconcentration of pesticides in fish using biopartitioning micellar chromatography. *J. Chromatogr. A* **2005,** *1063*, 153-160.

18. Testa, B.; van de Waterbeemd, H.; Folkers, G.; Guy, R. H. *Pharmacokinetic Optimization in Drug Research: Biological, Physicochemical and Computational Strategies*; Wiley-VHCA: Zurich, 2001.

19. Liu, B.; Li, S.; Hu, J. Technological advances in high-throughput screening. *Am. J. Pharmacogenom.* **2004,** *4*.

20. Raveglia, L. F.; Giardina, G. A. M. Accelerating the drug-discovery process: new tools and technologies available to medicinal chemists. *Future Med. Chem.* **2009,** *1*, 1019-1023.

21. Mayr, L. M.; Bojanic, D. Novel trends in high-throughput screening. *Curr. Opin. Pharmacol.* **2009,** *9*, 580-588.

22. Smith, D. A.; Jones, B. C.; Walker, D. K. Design of drugs involving the concepts and theories of drug metabolism and pharmacokinetics. *Med. Res. Rev.* **1996,** *16*, 243-266.

23. Kerns, E. H.; Di, L. Physicochemical profiling: overview of the screens. *Drug Discovery Today: Technologies* **2004,** *4*, 343-348.

24. Wang, J. L.; Skolnik, S. Recent advances in physicochemical and ADMET profiling in drug discovery. *Chem. Biodivers.* **2009,** *6*, 1887-1899.

25. Camenisch, G.; Folkers, G.; van de Waterbeemd, H. Review of theoretical passive absorption models: historical background, recent developments and limitations. *Pharm. Acta Helv.* **1996,** *71*, 309-327.

26. Conradi, R. A.; Burton, P. S.; Borchardt, R. T. Physico-chemical and biological factors that influence a drug's cellular permeability by passive diffusion. In *Lipophilicity in Drug Action and Toxicology*; Pliska, V.; Testa, B.; van de Waterbeemd, H. Eds.; VCH Publishers: Weinheim, 1996; pp 233-252.

27. Fu, W. J.; Franco, A.; Trapp, S. Methods for estimating the bioconcentration factor of ionizable organic chemicals. *Environ. Toxicol. Chem.* **2009,** *28*, 1372-1379.

28. Giaginis, C.; Tsantili-Kakoulidou, A. Alternative measures of lipophilicity: From octanol-water partitioning to IAM retention. *J. Pharm. Sci.* **2008,** *97*, 2984-3004.

29. Pollien, P.; Roberts, D. Use of solid-phase microextraction for measuring oil-water partition coefficients and correlation with high-performance liquid chromatographic methods for lipophilicity. *J. Chromatogr. A* **1999,** *864*, 183-189.

30. Dimitrova, B.; Doytchinova, I.; Zlatkova, R. Reversed-phase high-performance liquid chromatography for evaluating the distribution of pharmaceutical substances in suppository base-phosphate buffer system. *J. Pharm. Biomed. Anal.* **2000,** *23*, 955-964.

31. Gocan, S.; Cimpan, G.; Comer, J. Lipophilicity measurements by liquid chromatography. In *Advances in Chromatography*; Grushka, E.; Grinberg, N. Eds.; Taylor & Francis Group: Boca Raton, 2006; pp 79-176.

32. Hitzel, L.; Watt, A. P.; Locker, K. L. An increased throughput method for the determination of partition coefficients. *Pharm. Res.* **2000,** *17*, 1389-1395.

33. Dohta, Y.; Yamashita, T.; Horiike, S.; Nakamura, T.; Kukami, T. A system for logD screening of 96-well plates using a water-plug aspiration/injection method combined with high-performance liquid chromatography-mass spectrometry. *Anal. Chem.* **2007,** *79*, 8312-8315.

34. Avdeef, A. Assessment of distribution-pH profiles. In *Lipophilicity in Drug Action and Toxicology*; Pliska, V.; Testa, B.; van de Waterbeemd, H. Eds.; VCH Publishers: Weinheim, 1996; pp 109-139.

35. Huie, C. W. Recent applications of microemulsion electrokinetic chromatography. *Electrophoresis* **2006,** *27*, 60-75.

36. Hanse, S. H. Recent applications of microemulsion electrokinetic chromatography. *Electrophoresis* **2003,** *24*, 3900-3907.

37. Kaliszan, R.; Nasal, A.; Markuszewski, M. J. New approaches to chromatographic determination of lipophilicity of xenobiotics. *Anal. Bioanal. Chem.* **2003,** *377*, 803-811.

38. Gluck, S. J.; Benkö, M. H.; Hallberg, R. K.; Steele, K. P. Indirect determination of octanol-water partition coefficients by microemulsion electrokinetic chromatography. *J. Chromatogr. A* **1996,** *744*, 141-146.

39. Ostergaard, J.; Hansen, S. H.; Larsen, C.; Schou, C.; Heegaard, N. H. Determination of octanol-water partition coefficients for carbonate esters and other small organic molecules by microemulsion electrokinetic chromatography. *Electrophoresis* **2003,** *24*, 1038-1046.

40. Ishihama, Y.; Oda, Y.; Uchikawa, K.; Asakawa, N. Evaluation of solute hydrophobicity by microemulsion electrokinetic chromatography. *Anal. Chem.* **1995,** *67*, 1588-1595.

41. Ishihama, Y.; Oda, Y.; Asakawa, N. A hydrophobicity scale based on the migration index from emulsion electrokinetic chromatography of anionic solutes. *Anal. Chem.* **1996,** *68*, 1032.

42. Poole, S. K.; Durham, D.; Kibbey, C. Rapid method for estimating the octanol--water partition coefficient (log P ow) by microemulsion electrokinetic chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **2000,** *745*, 117-126.

43. Klotz, W. L.; Schure, M. R.; Foley, J. P. Determination of octanol-water partition coefficients of pesticides by microemulsion electrokinetic chromatography. *J. Chromatogr. A* **2001,** *930*, 145-154.

44. Poole, S. K.; Patel, S.; Dehring, K.; Workman, H.; Dong, J. Estimation of octanol-water partition coefficients for neutral and weakly acidic compounds by microemulsion electrokinetic chromatography using dynamically coated capillary columns. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2003,** *793*, 265-274.

45. Lucangioli, S. E.; Kenndler, E.; Carlucci, A.; Tripodi, V. P.; Scioscia, S. L.; Carducci, C. N. Relation between retention factors of immunosuppressive drugs in microemulsion electrokinetic chromatography with biosurfactants and octanol-water partition coefficients. *J. Pharm. Biomed. Anal.* **2003,** *33*, 871-878.

46. Wehmeyer, K. R.; Tu, J.; Jin, Y.; King, S.; Stella, M.; Stanton, D. T.; Kenseth, J.; Wong, K. S. The application of multiplexed microemulsion electrokinetic chromatography for the rapid determination of log P_{ow} values for neutral and basic compounds. *LC. GC North America* **2003,** *21*, 1078-1088.

47. Rappel, C.; Galanski, M.; Yasemi, A.; Habala, L.; Keppler, B. K. Analysis of anticancer platinum(II)-complexes by microemulsion electrokinetic chromatography: separation of diastereomers and estimation of octanol-water partition coefficients. *Electrophoresis* **2005,** *26*, 878-884.

48. Wan, H.; Ahman, M.; Holmen, A. G. Relationship between brain tissue partitioning and microemulsion retention factors of CNS drugs. *J. Med. Chem.* **2009,** *52*, 1693.

49. Valko, K. Application of high-performance liquid chromatography based measurements of lipophilicity to model biological distribution. *J. Chromatogr. A* **2004,** *1037*, 299-310.

50. Faller, B.; Grimm, H. P.; Loeuillet-Ritzler, F.; Arnold, S.; Briand, X. High-throughput lipophilicity measurement with immobilized artificial membranes. *J. Med. Chem.* **2005,** *48*, 2571-2576.

51. Kansy, M.; Senner, F.; Gubernator, K. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. *J. Med. Chem.* **1998,** *41*, 1007-1010.

52. Nasal, A.; Siluk, D.; Kaliszan, A. Chromatographic retention parameters in medicinal chemistry and molecular pharmacology. *Curr. Med. Chem.* **2003,** *10*, 381-426.

53. Poole, S. K.; Poole, C. F. Separation methods for estimating octanol-water coefficients. *J. Chromatogr. B* **2003,** *797*, 3-19.

54. Martel, S.; Guillarme, D.; Henchoz, Y.; Galland, A.; Veuthey, J. L.; Rudaz, S.; Carrupt, P. A. Chromatographic approaches for measuring log P. In *Drug Properties: Measurement and Computation*; Mannhold, R. Ed.; Wiley-VCH: Weinheim, 2008; pp 331-356.

55. van de Waterbeemd, H.; Kansy, M.; Wagner, B.; Fischer, H. Lipophilicity measurement by reversed-phase high performance liquid chromatography. In *Lipophilicity in Drug Action and Toxicology*; Pliska, V.; Testa, B.; van de Waterbeemd, H. Eds.; VCH Publishers: Weinheim, 1996; pp 73-87.

56. Jandera, P. Correlation of retention and selectivity of separation in reversed-phase high-performance liquid chromatography with interaction indices and with lipophilic and polar structural indices. *J. Chromatogr.* **1993,** *656*, 437-468.

57. Lombardo, F.; Shalaeva, M. Y.; Tupper, K. A.; Gao, F.; Abraham, M. H. $ElogP_{oct}$: a tool for lipophilicity determination in drug discovery. *J. Med. Chem.* **2000,** *43*, 2922-2928.

58. Stella, C.; Galland, A.; Liu, X.; Testa, B.; Rudaz, S.; Veuthey, J. L.; Carrupt, P. A. Novel RPLC stationary phases for lipophilicity measurement: solvatochromic analysis of retention mechanisms for neutral and basic compounds. *J. Sep. Sci.* **2005,** *28*, 2350-2362.

59. Benhaim, D.; Grushka, E. Effect of n-octanol in the mobile phase on lipophilicity determination by reversed-phase high-performance liquid chromatography on a modified silica column. *J. Chromatogr. A* **2008,** *1209*, 111-119.

60. Barbato, F.; Cirocco, V.; Grumetto, L.; La Rotonda, M. I. Comparison between immobilized artificial membrane (IAM) HPLC data and lipophilicity in n-octanol for quinolone antibacterial agents. *Eur. J. Pharm. Sci.* **2007,** *31*, 288-297.

61. Wiczling, P.; Kawczak, P.; Nasal, A.; Kaliszan, R. Simultaneous determination of pK_a and lipophilicity by gradient RP HPLC. *Anal. Chem.* **2006,** *78*, 239-249.

62. Kaliszan, R.; Haber, P.; Baczek, T.; Siluk, D. Gradient HPLC in the determination of drug lipophilicity and acidity. *Pure Appl. Chem.* **2001,** *73*, 1465-1475.

63. Markuszewski, M. J.; Wiczling, P.; Kaliszan, R. High-throughput evaluation of lipophilicity and acidity by new gradient HPLC methods. *Comb. Chem. Highthroughput Screen.* **2004,** *7*, 281-289.

64. Horvath, C.; Melander, W. Liquid chromatography with hydrocarbonaceous bonded phases; theory and practice of reversed phase chromatography. *J. Chromato. Sci.* **1977,** *15*, 393-404.

65. Reymond, D.; Chung, G. N.; Mayer, J. M.; Testa, B. Lipophilicity measurement of nicotinates by RP-HPLC. Differences in retention

behaviour, but similarities of log k_w values, in methanol-water and acetonitrile-water eluents. *J. Chromatogr.* **1987,** *391*, 97-109.

66. Valko, K.; Snyder, L. R.; Glajch, J. L. Retention in reversed-phase liquid chromatography as a function of mobile-phase composition. *J. Chromatogr.* **1993,** *656*, 501-520.

67. Snyder, L. R.; Dolan, J. W. Initial experiments in high-performance liquid chromatographic method development. I. Use of a starting gradient run. *J. Chromatogr. A* **1996,** *721*, 3-14.

68. Dolan, J. W. Dwell volume revisited. *LC. GC North America* **2006,** *24*, 458-466.

69. Henchoz, Y.; Guillarme, D.; Rudaz, S.; Veuthey, J. L.; Carrupt, P. A. High-throughput log *P* determination by ultraperformance liquid chromatography: a convenient tool for medicinal chemists. *J. Med. Chem.* **2008,** *51*, 396-399.

70. Nawrocki, J. The silanol group and its role in liquid chromatography. *J. Chromatogr. A* **1997,** *779*, 29-71.

71. Stella, C.; Rudaz, S.; Veuthey, J. L.; Tchapla, A. Silica and other materials as supports in liquid chromatography. Chromatographic tests and their importance for evaluating these supports. Part I. *Chromatographia* **2001,** *53*, S-113-S-131.

72. Stella, C.; Rudaz, S.; Mottaz, M.; Carrupt, P. A.; Veuthey, J. L. Analysis of basic compounds at high ph values by reversed-phase liquid chromatography. *J. Sep. Sci.* **2004,** *27*, 284-292.

73. Bard, B.; Carrupt, P. A.; Martel, S. Lipophilicity of basic drugs measured by hydrophilic interaction chromatography. *J. Med. Chem.* **2009,** *52*, 3416-3419.

74. Carey, F. A.; Sundberg, R. J. Reactions of cabonyl compounds. In *Advanced Organic Chemistry. Part A: Structure and Mechanisms. Third Edition.*; Carey, F. A.; Sundberg, R. J. Eds.; Plenum Press: New York, 1991; pp 465-470.

75. Sanderson, H.; Thomsen, M. Comparative analysis of pharmaceuticals versus industrial chemicals acute aquatic toxicity classification according to the United Nations classification system for chemicals. Assessment of the (Q)SAR predictability of pharmaceuticals acute aquatic toxicity and their predominant acute toxic mode-of-action. *Toxicol. Letters* **2009,** *187*, 84-93.

76. Wolf, A.; Schramm, U.; Fahr, A.; Aicher, L.; Cordier, A.; Trommer, W. E.; Fricker, G. Hepatocellular effects of cyclosporine A and its derivative SDZ IMM 125 in vitro. *J. Pharmacol. Exp. Ther.* **1998,** *284*, 817-825.

77. Hund-Rinke, K.; Kordel, W. Underlying issues in bioaccessibility and bioavailability: experimental methods. *Ecotoxicol. Environ. Saf.* **2003,** *56*, 52-62.

78. Arleny, I.; Tabouret, H.; Rodriguez-Gonzalez, P.; Bareille, G.; Donard, O. F. X.; Amouroux, D. Methylmercury bioconcentration in muscle tissue of

the European eel (Anguilla anguilla) from the Adour estuary (Bay of Biscay, France). *Mar. Pollut. Bull.* **2007,** *54*, 1031-1036.

79. Wang, W. X.; Rainbow, P. S. Comparative approaches to understand metal bioaccumulation in aquatic animals. *Comp. Biochem. Physiol. Part C* **2008,** *148*, 315-323.

80. Bank, M. S.; Crocker, J.; Connery, B.; Amirbahman, A. Mercury bioaccumulation in green frog (Rana clamitans) and bullfrog (Rana catesbeiana) tadpoles from Acadia National Park, Maine, USA. *Environ. Toxicol. Chem.* **2007,** *26*, 118-125.

81. Bernhardt, H. *NTA: Study of the Aquatic Environmental Compatibility o Nitrilotriacetate (NTA). (NTA: Studie ueber die Aquatiche Umweltvertraeglichkeit von Nitrilotriacetat (NTA)*; Verlag Hans Richarz: Sank Angustin, 1984; pp 1-422.

82. Rainbow, P. S. Trace metal concentrations in aquatic invertebrates: why and so what? *Environ. Poll.* **2002,** *120*, 497-507.

83. Haitzer, M.; Hoss, S.; Traunspurger, W.; Steinberg, C. Effects of dissolved organic matter (DOM) on the bioconcentration of organic chemicals in aquatic organisms - A review. *Chemosphere* **1998,** *37*, 1335-1362.

84. Xie, L.; Flippin, J. L.; Deighton, N.; Funk, D. H.; Dickey, D. A.; Buchwalter, D. B. Mercury(II) Bioaccumulation and Antioxidant Physiology in Four Aquatic Insects. *Environ. Sci. Technol.* **2009,** *43*, 934-940.

85. Best, E. P. H.; Tatem, H. E.; Geter, K. N.; Wells, M. L.; Lane, B. K. Effects, uptake, and gate of 2,4,6-Trinitrotoluene sged in doil in plants and worms. *Environ. Toxicol. Chem.* **2008,** *27*, 2539-2547.

86. Legierse, K. C. H. M.; Sijm, D. T. H. M.; van Leeuwen, C. J.; Seinen, W.; Hermens, J. L. M. Bioconcentration kinetics of chlorobenzenes and the organophosphorus pesticide chlorthion in the pond snail Lymnaea stagnalis - a comparison with the guppy Poecilia reticulata. *Aquatic Toxicol.* **1998,** *41*, 301-323.

87. Eisler, R.; Gardner, G. R.; Hennekey, R. J.; Walsh, D. F.; Yevich, P. P.; Laroche, G. Acute toxicology of sodium nitrilotriacetic acid (Nta) and Nta-containing detergents to marine organisms. *Water Res.* **1972,** *6*, 1009-&.

88. Staples, C. A.; Davis, J. W. An examination of the physical properties, fate, ecotoxicity and potential environmental risks for a series of propylene glycol ethers. *Chemosphere* **2002,** *49*, 61-73.

89. Bender, J.; Phillips, P. Microbial mats for multiple applications in aquaculture and bioremediation. *Bioresource Technol.* **2004,** *94*, 229-238.

90. Karjalainen, M.; Reinikainen, M.; Lindvall, F.; Spoof, L.; Meriluoto, J. A. O. Uptake and accumulation of dissolved, radiolabeled nodularin in Baltic Sea Zooplankton. *Environ. Toxicol.* **2003,** *18*, 52-60.

91. Olusegun, A. O.; Olalekan, O. M.; Oladipo, O. J. Sediment load and bioconcentration of heavy metals by shrimp (Peaneus notalis) from Epe Lagoon, Nigeria. *Environ. Monit. Assess.* **2010,** *163*, 295-301.

92. Reichmuth, J. M.; Weis, P.; Weis, J. S. Bioaccumulation and depuration of metals in blue crabs (Callinectes sapidus Rathbun) from a contaminated and clean estuary. *Environ. Poll.* **2010,** *158*, 361-368.

93. Leslie, H. A.; Ter Laak, T. L.; Busser, F. J. M.; Kraak, M. H. S.; Hermens, J. L. M. Bioconcentration of organic chemicals: Is a solid-phase microextraction fiber a good surrogate for biota? *Environ. Sci. Technol.* **2002,** *36*, 5399-5404.

94. Gregory, M. A.; Marshall, D. J.; George, R. C.; Anandraj, A.; McClurg, T. P. Correlations between metal uptake in the soft tissue of Perna perna and gill filament pathology after exposure to mercury. *Mar. Pollut. Bull.* **2002,** *45*, 114-125.

95. Franzle, S.; Markert, B.; Wunschmann, S. Dynamics of trace metals in organisms and ecosystems: Prediction of metal bioconcentration in different organisms and estimation of exposure risks. *Environ. Poll.* **2007,** *150*, 23-33.

96. Lewis, C.; Beggah, S.; Pook, C.; Guitart, C.; Redshaw, C.; Van der Meer, J. R.; Readman, J. W.; Galloway, T. Novel use of a whole cell e-coli bioreporter as a urinary exposure biomarker. *Environ. Sci. Technol.* **2009,** *43*, 423-428.

97. Kumar, S.; Dhanaraj, P. S.; Bhatnagar, P. Bioconcentration and effects of Dieldrin, Dimethoate, and Permethrin on Saccharomyces-Cerevisiae. *Bull. Environ. Contam. Toxicol.* **1989,** *43*, 246-253.

98. McKone, T. E.; Maddalena, R. L. Plant uptake of organic pollutants from soil: Bioconcentration estimates based on models and experiments. *Environ. Toxicol. Chem.* **2007,** *26*, 2494-2504.

99. Gray, J. S. Biomagnification in marine systems: the perspective of an ecologist. *Mar. Pollut. Bull.* **2002,** *45*, 46-52.

100. Corsolini, S. Industrial contaminants in Antarctic biota. *J. Chromatogr. A* **2009,** *1216*, 598-612.

101. Wang, R. Y.; Needham, L. L. Environmental chemicals: from the environment to food, to breast milk, to the infant. *J. Tox. Environ. Health B* **2007,** *10*, 597-609.

102. *Commission Directive 98/73/EC of 18 September 1998 adapting to technical progress for the 24th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (Text with EEA relevance)*; 1998.

103. *OECD Guidelines for Testing of Chemicals. Proposal for Updating Guideline 305 - Bioconcentration: Flow-through Fish Test*; 1996.

104. Bearr, J. S.; Stapleton, H. M.; Mitchelmore, C. L. Accumulation and Dna damage in fathead minnows (Pimephales Promelas) exposed to 2

brominated flame-retardant mixtures, firemaster (R) 550 and firemaster (R) Bz-54. *Environ. Toxicol. Chem.* **2010,** *29*, 722-729.

105. Fujikawa, M.; Nakao, K.; Shimizu, R.; Akamatsu, M. The usefulness of an artificial membrane accumulation index for estimation of the bioconcentration factor of organophosphorus pesticides. *Chemosphere* **2009,** *74*, 751-757.

106. VandenHeuvel, W. J. A.; Wislocki, P. G.; Hirsch, M. P.; Porter, N. K.; Ambrose, R. T.; Robillard, K. A. Bioconcentration and metabolism of thiabendazole [2-(4-thiazolyl)-1H-benzimidazole] in Bluegill sunfish, Lepomis macrochirus. *J. Agric. Food Chem.* **1997,** *45*, 985-989.

107. Parkerton, T. F.; Stone, M. A.; Letinski, D. J. Assessing the aquatic toxicity of complex hydrocarbon mixtures using solid phase microextraction. *Toxicol. Letters* **2000,** *112*, 273-282.

108. Osterauer, R.; Haus, N.; Sures, B.; Kohler, H. R. Uptake of platinum by zebrafish (Danio rerio) and ramshorn snail (Marisa cornuarietis) and resulting effects on early embryogenesis. *Chemosphere* **2009,** *77*, 975-982.

109. De Boeck, G.; Meeus, W.; De Coen, W.; Blust, R. Tissue-specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (Oncorhynchus mykiss), common carp (Cyprinus carpio), and gibel carp (Carassius auratus gibelio). *Aquatic Toxicol.* **2004,** *70*, 179-188.

110. Mackay, D.; Fraser, A. Bioaccumulation of persistent organic chemicals: mechanisms and models. *Environ. Poll.* **2000,** *110*, 375-391.

111. Fatemi, M. H.; Jalali-Heravi, M.; Konuze, E. Prediction of bioconcentration factor using genetic algorithm and artificial neural network. *Anal. Chim. Acta* **2003,** *486*, 101-108.

112. Isnard, P.; Lambert, S. Estimating bioconcentration factors from octanol-water partition coefficient and aqueous solubility. *Chemosphere* **1988,** *17*, 21-34.

113. Banerjee, S.; Baughman, G. L. Bioconcentration factors and lipid solubility. *Environ. Sci. Technol.* **1991,** *25*, 536-539.

114. Hu, H.; Xu, F.; Li, B.; C, J.; Dawon, R.; Tao, S. Prediction of the bioconcentration factor of PCBs in fish using the molecular connectivity index and fragment constant models. *Water Environ. Res.* **2005,** *77*, 87-97.

115. Roy, K.; Sanyal, I.; Roy, P. P. QSPR of the bioconcentration factors of non-ionic organic compounds in fish using extended topochemical atom (ETA) indices. *SAR QSAR in Environ. Res.* **2006,** *17*, 563-582.

116. Khadikar, P. V.; Singh, S.; Mandloi, D.; Joshi, S.; Bajaj, A. V. QSAR study on bioconcentration factor (BCF) of polyhalogented biphenyls using the PI index. *Bioorg. Med. Chem.* **2003,** *11*, 5045-5050.

117. Saçan, M. T.; Erdem, S. S.; Özpinar, G. A.; Balcioglu, I. A. QSPR study on the bioconcentration factors of nonionic organic compounds in fish by

characteristic root index and semiempirical molecular descriptors. *J. Chem. Inf. Comput. Sci.* **2004,** *44*, 985-992.

118. Tao, S.; Hu, H.; Lu, X.; Dawson, R. W.; Xu, F. Fragment constant method for prediction of fish bioconcentration factors of non-polar chemicals. *Chemosphere* **2000,** *41*, 1563-1568.

119. Tao, S.; Hu, H.; Xu, F.; Dawson, R.; Li, B.; Cao, J. QSAR modeling of bioconcentration factors in fish based on fragment constants and structural correction factors. *J. Environ. Sci. Health* **2001,** *B36*, 631-649.

120. Nakai, S.; Saito, S.; Takeuchi, M.; Takimoto, Y.; Matsuo, M. The inorganic and organic characters for predicting bioconcentration on wide variety of chemicals in fish. *SAR QSAR in Environ. Res.* **2002,** *13*, 667-673.

121. Kwon, J. H.; Escher, B. I. A modified parallel artificial membrane permeability assay for evaluating the bioconcentration of highly hydrophobic chemicals in fish. *Environ. Sci. Technol.* **2008,** *42*, 1787-1793.

122. van der Heijden, S. A.; Jonker, M. T. O. Evaluation of liposome-water partitioning for predicting bioaccumulation potential of hydrophobic organic chemicals. *Environ. Sci. Technol.* **2009,** *43*, 8854-8859.

123. Mackay, D. Correlation of bioconcentration factors. *Environ. Sci. Technol.* **1982,** *16*, 274-278.

124. Davies, R. P.; Dobbs, A. J. The Prediction of bioconcentration in fish. *Water Res.* **1984,** *18*, 1253-1262.

125. Oliver, B. G.; Niimi, A. J. Bioconcentration factors of some halogenated organics for rainbow-trout - limitations in their use for prediction of rnvironmental tesidues. *Environ. Sci. Technol.* **1985,** *19*, 842-849.

126. Opperhulzen, A.; Vandervelde, E. W.; Gobas, F. A. P. C.; Liem, D. A. K.; Vandersteen, J. M. D.; Hutzinger, O. Relationship between bioconcentration in fish and steric factors of hydrophobic chemicals. *Chemosphere* **1985,** *14*, 1871-1896.

127. Connell, D. W.; Hawker, D. W. Use of polynomial expressions to describe the bioconcentration of hydrophobic chemicals by fish. *Ecotoxicol. Environ. Saf.* **1988,** *16*, 242-257.

128. Konemann, H.; Vanleeuwen, K. Toxico-kinetics in fish - accumulation and elimination of 6 chlorobenzenes by guppies. *Chemosphere* **1980,** *9*, 3-19.

129. Zhao, R. B.; Sun, D. Y.; Fu, S.; Wang, X. F.; Zhao, R. S. Bioconcentration kinetics of PCBs in various parts of the lifecycle of the tadpoles Xenopus laevis. *J. Environ. Sci.* **2007,** *19*, 374-384.

130. Hope, B.; Scatolini, S.; Titus, E. Bioconcentration of chlorinated biphenyls in biota from the North Pacific Ocean. *Chemosphere* **1998,** *36*, 1247-1261.

131. Pavan, M.; Netzeva, T. I.; Worth, A. P. Review of literature-based quantitative structure-activity relationship models for bioconcentration. *QSAR Comb. Sci.* **2008,** *27*, 21-31.

132. Konemann, H. Quantitative structure-activity-relationships in fish toxicity studies .1. Relationship for 50 industrial pollutants. *Toxicology* **1981,** *19*, 209-221.

133. Gobas, F. A. P. C.; Lahittete, J. M.; Garofalo, G.; Shili, W. Y. A novel method for measuring membrane-water partition comparison with 1-octanol/water partitioning. *J. Pharm. Sci.* **1988,** *77*, 265-272.

134. Vaes, W. H. J.; Ramos, E. U.; Hamwijk, C.; van Holsteijn, I.; Blaauboer, B. J.; Seinen, W.; Verhaar, H. J. M.; Hermens, J. L. M. Solid phase microextraction as a tool to determine membrane/water partition coefficients and bioavalaible concentrations in in vitro systems. *Chem. Res. Toxicol.* **1997,** *10*, 1067-1072.

135. Jabusch, T. W.; Swackhamer, D. L. Partitioning of polychlorinated biphenyls in octanol/water, triolein/water, and membrane/water systems. *Chemosphere* **2005,** *60*, 1270-1278.

136. Kwon, J. H.; Liljestrand, H. M.; Katz, L. E. Partitioning of moderately hydrophobic endocrine disruptors between water and synthetic membrane vesicles. *Environ. Toxicol. Chem.* **2006,** *25*, 1984-1992.

137. Jonker, M. T. O.; van der Heijden, S. A. Bioconcentration factor hydrophobicity cutoff: an artificial phenomenon reconstructed. *Environ. Sci. Technol.* **2007,** *41*, 7363-7369.

138. Ano, R.; Kimura, Y.; Urakami, M.; Shima, M.; Matsuno, R.; Ueno, T.; Akamatsu, M. Relationship between structure and permeability of dipeptide derivatives containing tryptophan and related compounds across human intestinal epithelial (Caco-2) cells. *Bioorg. Med. Chem.* **2004,** *12*, 249-255.

139. Ano, R.; Kimura, Y.; Shima, M.; Matsuno, R.; Ueno, T.; Akamatsu, M. Relationships between structure and high-throughput screening permeability of peptide derivatives and related compounds with artificial membranes: application to prediction of Caco-2 cell permeability. *Bioorg. Med. Chem.* **2004,** *12*, 257-264.

140. Fujikawa, M.; Ano, R.; Nakao, K.; Shimizu, R.; Akamatsu, M. Relationships between structure and high-throughput screening permeability of diverse drugs with artificial membranes: application to prediction of Caco-2 cell permeability. *Bioorg. Med. Chem.* **2005,** *13*, 4721-4732.

141. Fujikawa, M.; Nakao, K.; Shimizu, R.; Akamatsu, M. QSAR study on permeability of hydrophobic compounds with artificial membranes. *Bioorg. Med. Chem.* **2007,** *15*, 3756-3767.

142. Randall, D. J.; Connell, D. W.; Yang, R.; Wu, S. S. Concentrations of persistent lipophilic compounds in fish are determined by exchange across the gills, not through the food chain. *Chemosphere* **1998,** *37*, 1263-1270.

143. Escuder-Gilabert, L.; Martin-Biosca, Y.; Sagrado, S.; Villanueva-Camanas, R. M.; Medina-Hernandez, M. J. Biopartitioning micellar chromatography to predict ecotoxicity. *Anal. Chim. Acta* **2001,** *448*, 173-185.

144. Dorsey, J. G.; Khaledi, M. G. Hydrophobicity estimations by reversed-phase liquid chromatography. Implications for biological partitioning processes. *J. Chromatogr.* **1993,** *656*, 485-500.

145. Khaledi, M. G.; Breyer, E. D. Quantitation of hydrophobicity with micellar liquid chromatography. *Anal. Chem.* **1989,** *61*, 1040-1047.

146. Taillardat-Bertschinger, A.; Carrupt, P. A.; Barbato, F.; Testa, B. Immobilized artificial membrane (IAM)-HPLC in drug research. *J. Med. Chem.* **2003,** *46*, 655-665.

Chapter 2: High-throughput log P_{oct} determination of highly lipophilic compounds by liquid chromatography.

2.1 Neutral, acidic and weakly basic compounds

2.1.1 Introduction

Lipophilicity is a key physicochemical parameter in pharmaceutical research, since it can be used to predict both pharmacokinetic and pharmacodynamic drug behaviours. Indeed lipophilicity is involved in ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties [1], as it contributes extensively to solubility [2], membrane passive permeation [3], interactions with efflux proteins [4], plasma-protein binding [5] and metabolism [2]. It is also largely used in (quantitative) structure-activity relationship ((Q)SAR) studies [6]. Therefore, it has to be evaluated during the early stage of drug discovery. As lipophilicity is required for any new commercially available chemical, a wide number of methods exist such as shake-flask method [7] (the gold standard), liquid chromatography [8], potentiometry [9], centrifugal partition chromatography [10], and capillary electrophoresis [11]. Due to the high number of investigated compounds, high-throughput strategies with a good accuracy have been developed to replace the tedious and time-consuming shake-flask method. For this purpose, chromatographic approaches, based on correlation between partition coefficients (log P) and retention factors (log k), gained acceptance in the pharmaceutical research due to their rapidity, easy automation, low sample consumption and insensitivity to impurities [7, 8, 12-16]. Generally, the mobile phase used is a mixture of water and an organic modifier (methanol (MeOH), acetonitrile (ACN) or even tetrahydrofuran (THF)). Isocratic log k, at a fixed percentage, or extrapolated log k_w values (obtained by extrapolation to 100% water from five isocratic log k values) are usually used in lipophilicity determination. An equation correlating log P_{oct} (partition coefficient in n-octanol/water system) and log k (isocratic or extrapolated) values is established for a series of well-known compounds using specific stationary and mobile phases. This equation (Eq. 2.5) can then be used to determine log P_{oct} value of an unknown compound. Even if isocratic

log k values, at a fixed percentage, present the advantage to drastically improve the throughput, it was demonstrated that log k_w give better correlations between chromatographic retention and log P_{oct} values [13, 17]. Gradient mode, using MeOH as organic modifier, was also evaluated to further reduce highly lipophilic compounds analysis time. ACN or THF were not used as organic modifier since, as log k_w value is obtained by quadratic extrapolation, three gradient runs are required. Hence, mathematical treatment for log k_w determination from retention time is much more complicated since iteration is necessary to resolve the unknown parameters. HPLC methods performed with conventional column geometries are less suitable for lipophilicity determination of highly lipophilic compounds (log P > 5). This is mainly due to an exponential analysis time increase, and a loss of accuracy induced by peak broadening. Although these issues can be decreased using large percentages of organic modifier, they lead to extrapolation far from targeted log k_w and consequently less accurate results. Therefore, the use of very short columns with an HPLC system was proposed [18-20] and tested for a series of compounds with log P_{oct} values up to 6. More recently, the employment of short columns, packed with small particles (< 2 μm) in ultra-high pressure conditions (UHPLC), was considered for fast log P_{oct} determination of 38 diverse compounds ($0 < \log P_{oct} < 5$) without compromising the chromatographic performance [21]. These two approaches look promising for high-throughput lipophilicity determination of highly lipophilic compounds.

Hence, log P_{oct} values of 14 rigid highly lipophilic compounds and 38 reference analytes [22] were determined using short columns in conventional HPLC as well as in UHPLC. Different experimental conditions were evaluated to determine the best compromise between good log P_{oct} accuracy and throughput.

2.1.2 Materials and methods

2.1.2.1 Chemicals

Thirty-eight analytes with log P_{oct} values ranging from 0 to 5 were selected by cluster analysis to obtain a well-balanced set of compounds in terms of molecular chemical properties [22]. Fourteen highly lipophilic rigid compounds (mainly

containing aromatic ring) were added to the previous set. No experimental values were available for these compounds so they were carefully chosen according to their calculated log P (CLogP values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005) values and their chemical structure to be sure that 3D-structure effects did not occur and that any fragment was unknown in log P calculation [8].

All these compounds were obtained from commercial sources such as Acros (New Jersey, USA), Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Laubscher Labs (Miecourt, Switzerland), Merck (Darmstadt, Germany), Riedel-de-Haen (Seelze, Germany), Sigma (St-Louis, MO, USA) and Supelco (Bellefonte, USA) in the highest available purity. HPLC grade acetonitrile, tetrahydrofuran and methanol were purchased from VWR (Dietikon, Switzerland). Water was obtained with the Milli-Q Water Purification System from Millipore (Milford, MA, USA).

2.1.2.2 Buffer solutions and organic modifier

To analyse all the compounds under their neutral form, three buffers with different pH values (trifluoroacetic acid / sodium hydroxide pH 2.5, acetic acid / sodium hydroxide pH 5 and phosphoric acid / sodium hydroxide pH 7.5) were prepared. An ionic strength of 20 mM was chosen according to Phoebus software v1.0 (Analis, Namur, Belgium). Buffer solutions were filtered through a 0.22 and 0.45 µm HA Millipore filter (Millipore, Bedford, MA, USA) for UHPLC and HPLC system respectively.

The percentage range on the three stationary phases was from 10 to 80% using the MeOH and the ACN, and 5 to 75% using the THF. However, an exception was applied using the THF and the Discovery® RP Amide C16 column, since the percentage range was limited from 20 to 75%. Indeed, the quadratic extrapolation was not continued and the percentages inferior to 20% decrease the log k_w value (Fig 2.5.A). Higher percentages of THF were not tested since 75% corresponds to 104% ACN and 103% MeOH and the retention times were very close to the t_0 (retention time of the unretained compound corresponding to the dead time of the system).

2.1.2.3 Liquid chromatography instrumentations

HPLC measurements were performed on a liquid chromatography system Alliance (Waters, Milford, USA) equipped with an HPLC pump model 2690 and a dual wavelength absorbance UV/Vis detector model 2487. An Acquity UPLC (Ultra High Pressure Liquid Chromatography) system (Waters, Milford, USA) including a binary solvent manager, a sample manager with an injection loop volume of 2 µL, a photo diode array (PDA) programmable detector, and a column manager with oven were used for UHPLC measurements. Both systems were controlled by Empower Software v2.0 (Waters, Milford, MA) and the detection was performed at appropriate wavelengths (compounds λ_{max}).

Retention times measurements with the HPLC system were realized on the Discovery® RP Amide C16 column (20 x 4 mm ID, 5 µm) (Supelco, Bellefonte, PA, USA) at a flow rate of 1 mL/min at room temperature (20°C).

Discovery® RP Amide C16 column (15 cm x 4.6 mm ID, 5 µm, 100 Å) (Supelco Bellefonte, PA, USA) was also used with the HPLC system at a flow rate of 1 mL/min at room temperature (20°C). In this case, each aqueous buffer was saturated in n-octanol (stirred for 4 hours followed by a decantation during at least 3 hours) and 0.25% was added into the organic modifier. Different percentages (v/v) of methanol were used and each eluent was done and degassed off-line since no degasser was linked to the system. Moreover this procedure allows to have a constant percentages of n-octanol in the mobile phase.

The stationary phases tested with the UHPLC system were the Hypersil™ GOLD Javelin HTS (10 x 2.1 mm ID, 1.9 µm) (Thermo Scientific Runcorn, UK) and the Acquity UPLC™ BEH Shield RP18 (30 x 2.1 mm ID, 1.7 µm) (Waters, Milford, MA) at a flow rate of 1 and 0.5 mL/min, respectively, and at 30 ± 0.1°C. The flow rate of the Acquity UPLC™ BEH Shield RP18 column was lower than the one of the Hypersil™ GOLD Javelin HTS stationary phase due to the backpressure induces, especially when methanol was used as organic modifier.

The concentrations of stock solutions were 10^{-2} M in MeOH and the injected solutions vary from 10^{-2} M to 5.10^{-4} M, depending on the UV absorbance.

2.1.2.4 Columns

Based on the work of Ayouni and co-workers [20], a 20-mm Discovery® RP Amide C16 stationary phase was first investigated to widen the lipophilicity range, up to a value of 8. This column was tested as it mimics the n-octanol/water partitioning process (according to LSERs analysis [23, 24]) and good results were previously reported in terms of lipophilicity determination [23, 24]. Experiments were performed on a conventional HPLC system, at a flow rate of 1 mL/min. A 30 x 2.1 mm Acquity UPLC™ BEH Shield RP18 stationary phase was selected for log P_{oct} determination of 52 compounds according to a previous work [21]. Moreover, this column is stable for a large range of pH from 2 to 11 (2-8 for the two other columns) and hence allows the study of basic compounds with $pK_a < 8$. The chromatographic system used withstands pressures up to 1000 bar and was fully optimized to limit the external volume contributions as much as possible. To further reduce the log P_{oct} determination time for highly lipophilic compounds, the column length was also decreased. Therefore, an ultra-short commercially stationary phase available, the Hypersil™ GOLD Javelin HTS column of 10 mm length (10 x 2.1 mm) was also evaluated.

2.1.2.5 Methods for lipophilicity determination.

2.1.2.5.1 Isocratic mode

The retention time (t_r) of each compound was determined in triplicate on five different organic modifier-buffer mobile phase ratios as exemplified in Fig. 2.1. with the 1,1,1-tichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane (also known as o,p'-DDT). For each mobile phase composition, the retention factor was calculated according to the formula:

$$\log k = \log\left(\frac{t_r - t_0}{t_0}\right) = \log\left(\frac{t_r}{t_0} - 1\right) \qquad \text{Eq. 2.1}$$

where t_r and t_0 are the retention time of the solute and the unretained compound (uracil), respectively. t_0 is evaluated by an injection of uracil at 40% ACN.

It is noteworthy that, especially when using very short columns, the experimental retention factors have to be corrected by the volume of the chromatographic instrument (extra-column volume (V_{ext}) and injection delay (V_{delay})), which can be experimentally determined [25]. Indeed, the system volume could represent up to 46% of the column dead volume (V_0) using the Hypersil™ GOLD Javelin HTS column with UHPLC system. Therefore, the contribution of the former is not negligible and has to be subtracted to obtain reliable log k values. For this purpose, the following equation was systematically used to obtain the suitable retention factor of each compound:

$$\log k = \log\left(\frac{t_r - t_{delay} - (V_{ext}/F)}{t_0 - t_{delay} - (V_{ext}/F)} - 1\right) \qquad \text{Eq. 2.2}$$

where t_{delay} is the injection delay, V_{ext} the extra-column volume and F the flow rate of the mobile phase.

However, the use of isocratic retention factors is not discriminatory as these values depend on the percentage of organic modifier in the mobile phase.

Hence extrapolated retention factor ($\log k_w$), corresponding to pure water as mobile phase, is considered more relevant as lipophilicity indice. Different retention factors ($\log k$) were measured at five percentages of organic modifier, optimized according to the lipophilicity of the investigated compound. $\log k_w$ value is obtained by extrapolation to 100% water, from five isocratic $\log k$ values plotting as a function of the mobile phase composition (φ). However, it is important to notify that there is no link between the extrapolated retention factor ($\log k_w$) and the $\log k_w$ directly measured into 100% water.

With MeOH, the extrapolation to 100% water is linear [26] (as shown by Fig. 2.1.B) and given by:

$$\log k = \log k_w - S \bullet \varphi \qquad \text{Eq. 2.3}$$

where $\log k$ and $\log k_w$ are the isocratic and extrapolated retention factors respectively, φ is the composition in organic modifier and S is a constant for a given solute and fixed experimental conditions.

Using ACN and THF, the correlation between $\log k$ and organic modifier percentages in the mobile phase is quadratic [27, 28], according to:

$$\log k = \log k_w + B \bullet \varphi + A \bullet \varphi^2 \qquad \text{Eq. 2.4}$$

where A and B are constants for a given solute and a given liquid chromatographic (LC) system.

Finally, log k_w can be correlated to log P_{oct} values by the following equation:

$$\log P_{oct} = a \bullet \log k_w + b \qquad \text{Eq. 2.5}$$

where log P_{oct} is the partition coefficient of the solute in n-octanol/water system, and a and b, the linear regression coefficients. These 2 coefficients have to be determined for each pair of stationary and mobile phases. The log P_{oct} value of an unknown compound can therefore be determined (Fig. 2.1C).

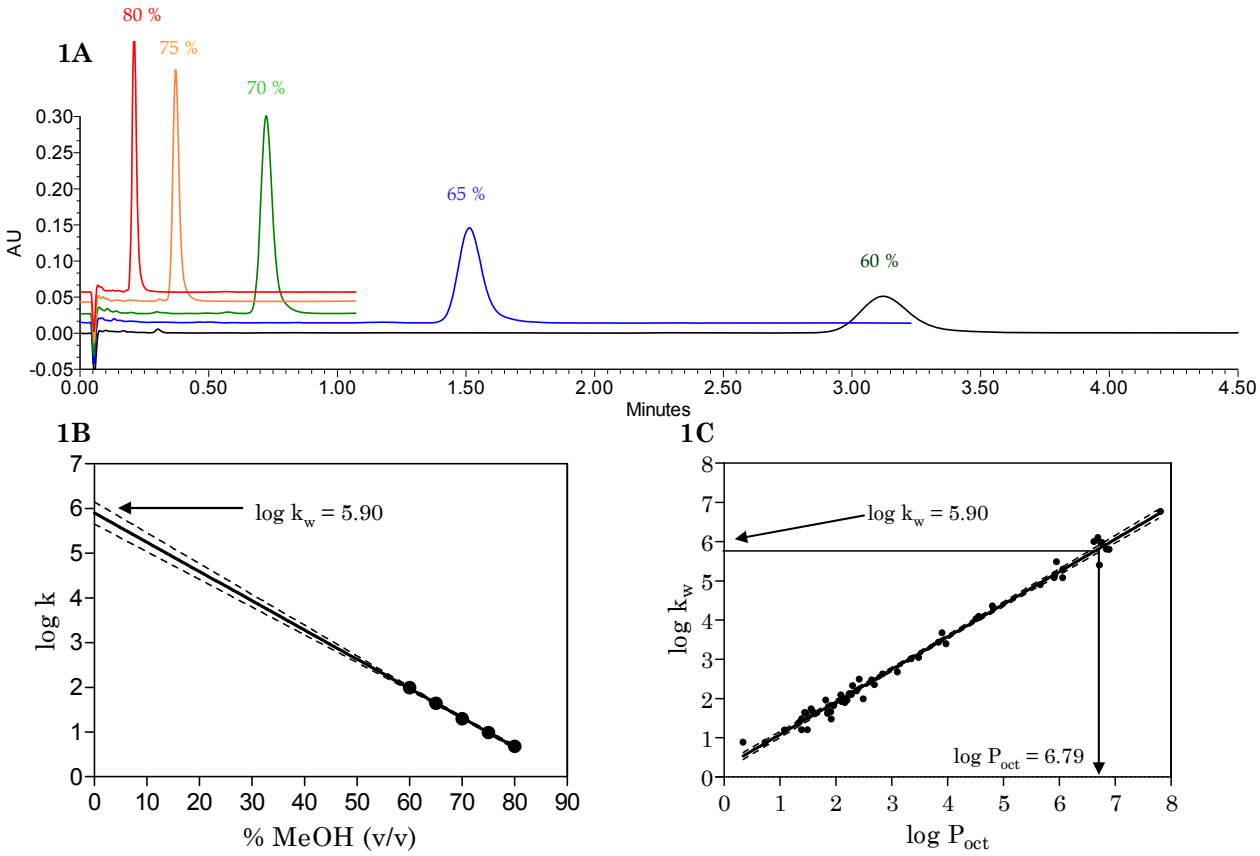


Figure 2.1: Chromatograms of o,p'-DDT's retention times (t_r) at different percentages of methanol (1A). Plot of the log k values obtained for o,p'-DDT as a function of the percentages of MeOH and extrapolation of the log k_w value to 100% water by linear regression (1B). Determination of the log P_{oct} value of o,p'-DDT using the calibration line (Eq. 2.5) and the log k_w value (1C). The straight lines correspond to linear regressions and the dot lines to the 95% confidence intervals.

2.1.2.5.2 Gradient mode

Gradient approach was also used to speed up the process of lipophilicity determination. Hence, retention time (t_r) can be expressed with a linear-gradient separation [29] as follows:

$$t_r = \frac{t_0}{b} \bullet \log\left(2.3 \bullet k_0 \bullet b + 1\right) + t_0 + t_D \qquad \text{Eq. 2.6}$$

where t_0 is the retention time of an unretained compound (uracil), k_0 is the k value at the beginning of the gradient (for $\varphi = \varphi_0$), t_D is the system dwell time for gradient elution (min) which can be experimentally determined [30] and b is the gradient steepness parameter described by the following relationship:

$$b = \frac{t_0 \bullet \Delta\varphi \bullet S}{t_G} \qquad \text{Eq. 2.7}$$

where t_G is the gradient time from the beginning to the end of the gradient run (min), S is a constant for a given solute and fixed experimental conditions and Δφ the change in composition (φ) during the gradient run (ranging from 0 to 1).

Two generic runs differing only in gradient times (t_G) were performed from 2 to 98% of MeOH. Hence, two different t_r were obtained from these two gradient runs, and were then computed in an HPLC modelling software (Osiris v.4.1.1.2, Datalys, Grenoble, France), enabling the modelling of the compounds behaviour in the whole organic modifier composition range. The two unknown parameters, S and k_0, are resolved using Eq. 2.6 and 2.7 and finally log k_w value can be determined according to Eq. 2.3 when MeOH is used as organic modifier.

For additional information about the calculation procedures used in gradient mode, readers can refer to recent reviews and papers [15, 31, 32].

2.1.3 Results and discussion

The log k_w values of the fifty-two compounds (14 highly lipophilic compounds and 38 neutral, acidic and weakly basic compounds) were measured under their neutral form with three different stationary phases (Discovery® RP Amide C16 with HPLC system, Acquity UPLC™ BEH Shield RP18 and Hypersil™ GOLD Javelin HTS with UHPLC system), and different experimental conditions were

evaluated with each column: (i) isocratic mode with methanol as organic modifier, (ii) gradient mode with methanol, (iii) isocratic mode with acetonitrile, and (iv) isocratic mode with tetrahydrofuran.

2.1.3.1 Isocratic mode with MeOH as organic modifier.

First of all, methanol was evaluated as organic modifier in isocratic mode since it appears to be the co-solvent of choice for lipophilicity determination [33]. In fact, log k_w values are obtained by a simple linear extrapolation to 100% water of different experimental log k values. Moreover the solvation of residual silanol groups with methanol reduces secondary interactions [26], leading to a better accuracy.

2.1.3.1.1 Discover® RP Amide C16

Good correlation between log P_{oct} and log k_w values (see equation parameters in Table 2.5) was obtained with the Discover® RP Amide C16 stationary phase and the log k_w values were reported as a function of log P_{oct} values for the 52 compounds in Table 2.2 and Fig. 2.2.

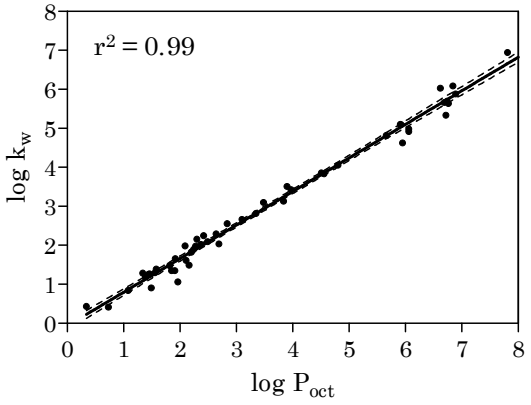


Figure 2.2: log k_w plotted as function of log P_{oct} values obtained for the 52 compounds using MeOH as organic modifier in isocratic mode with the 20-mm Discovery® RP Amide C16 column. The straight line corresponds to linear regression and the dot line to the 95% confidence intervals.

The lipophilicity determination time using the Discovery® RP Amide C16 column was lower than 30 min for compounds with log $P_{oct} < 5$ but as high as

66 min for the most lipophilic ones. Nevertheless, a determination of the lipophilicity up to 8 is experimentally attainable, with an acceptable accuracy on log P_{oct} values (95% confidence intervals ($IC_{95\%}$) ≤ 0.4), using a conventional HPLC apparatus.

Addition of n-octanol in the mobile phase

It has been already shown that addition of n-octanol in the mobile phase could improve the correlation between log P_{oct} and retention factors measured on RP-stationary phases [34, 35] such as Supelcosil LC-ABZ+Plus, 5 μm, 4.6 x 50 mm [1]. Therefore preliminary studies were performed on Discovery® RP Amide C16 15 cm with buffer saturated in n-octanol and 0.25% of n-octanol in MeOH. The correlation between log P_{oct} values and the retention factors extrapolated to 100% water with n-octanol in the mobile phase (log $k_{w/o}$) obtained with the 38 compounds were compared to the results acquired by Liu [36] using the same column in absence of the additive (log k_w).

The extrapolated log k values obtained using Discovery® RP Amide C16 stationary phase with (log $k_{w/o}$) and without (log k_w) n-octanol in the mobile phase are reported in Table 2.1.

Table 2.1: Experimental log P_{oct} values measured by shake-flask method and extrapolated retention factors measured with Discovery® RP Amide C16 column with (log $k_{w/o}$) and without (log k_w) n-octanol in the mobile phase

Solute	log P_{oct}[a]	log $k_{w/o}$	log k_w
N,N-diethylacetamide	0.34	-0.21	0.39
Ethyl acetate	0.73	0.03	0.23
Benzyl alcohol	1.08	0.59	0.72
Benzidine	1.34	0.92	1.22
4-nitroaniline	1.39	1.10	1.09
Valeric acid	1.39	0.37	0.98
Phenyl-2-propanone	1.44	1.00	1.14
Phenylacetic acid	1.46	1.28	1.12

Table 2.1: Continued

Solute	log P_{oct}[a]	log $k_{w/o}$	log k_w
Phenol	1.49	1.09	0.88
Acetophenone	1.58	1.16	1.20
Butyl acetate	1.82	1.34	1.42
Nitrobenzene	1.85	1.40	1.29
2-chloroaniline	1.91	1.48	1.25
p-nitrophenol	1.92	1.65	1.47
4-chlorobenzyl alcohol	1.96	1.63	1.51
3-chlorophenylacetic acid	2.09	2.12	1.84
Anisole	2.11	1.68	1.43
N-ethylaniline	2.16	1.61	1.38
Propiophenone	2.20	1.64	1.66
1-chloro-2-nitrobenzene	2.24	2.01	1.80
2-aminonaphtalene	2.28	2.02	1.71
2-phenylethyl acetate	2.30	1.89	1.96
m-toluic acid	2.37	2.10	1.85
4-phenylbutyric acid	2.42	2.36	2.07
3-chlorophenol	2.49	2.21	1.87
Ethyl benzoate	2.64	2.17	2.16
Toluene	2.69	2.28	1.84
2-aminobiphenyl	2.84	2.58	2.31
1-naphtoic acid	3.10	2.76	2.36
Naphtalene	3.35	2.83	2.60
Acridine	3.40	2.71	2.56
m-dichlorobenzene	3.48	2.94	2.75
Mesitylene	3.84	3.17	3.00
Biphenyl	3.90	3.43	3.25
Benzyl benzoate	3.97	3.29	3.26
1,2,4,5-tetrachlorobenzene	4.51	4.04	3.73
Pentamethylbenzene	4.56	3.95	3.66

Table 2.1: Continued

Solute	log P_{oct}[a]	log $k_{w/o}$	log k_w
Bibenzyl	4.80	4.15	3.94

a) Taken from [22]

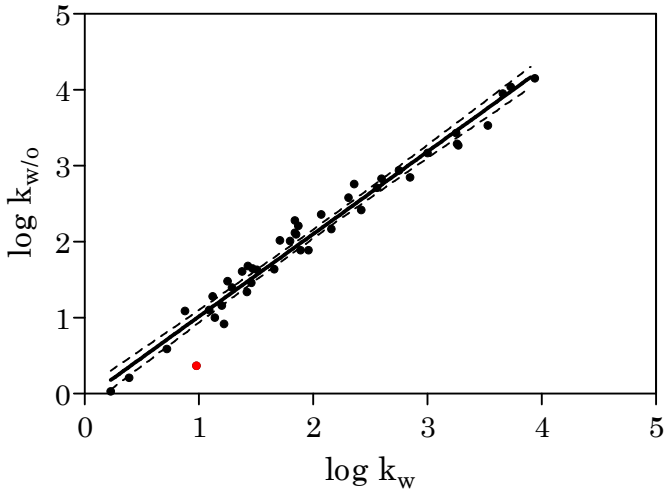


Figure 2.3: Correlation between retention factors extrapolated to 100% water obtained on Discovery® RP Amide C16 column with (log $k_{w/o}$) or without (log k_w) n-octanol in the mobile phase. The straight line corresponds to linear regression and the dot line to the 95% confidence intervals

This correlation (Fig. 2.3) shows that n-octanol had the same influence all over the lipophilicity range (lipophilicity-independent) excepted for the amide compound (N,N-diethylacetamide) (red dot). Although n-octanol decreases the retention factors of this compound and thus limits the interaction between this compound and the stationary phase, N,N-diethylacetamide is better estimated without n-octanol rather than in presence of the additive.

As shown by Fig. 2.4 and Eq. 2.8, a good correlation ($r^2 = 0.98$) was previously obtained between experimental partition coefficients measured in the n-octanol/water system (log P_{oct}) and log k_w values (without n-octanol in the mobile phase) (blue line) using the Discovery® RP Amide C16 stationary phase of 150 mm for the 38 neutral, weakly acidic and basic compounds:

$$\log k_w = 0.84\ (\pm 0.04) \bullet \log P_{oct} - 0.16\ (\pm 0.11) \qquad \text{Eq. 2.8}$$
$$n = 38,\ \ r^2 = 0.98,\ \ s = 0.14,\ \ F = 1509.$$

A good correlation was also obtained with n-octanol in the mobile phase ($\log k_{w/o}$) as given by the following equation and also shown in Fig. 2.4 (black line).

$$\log k_{w/o} = 0.95\ (\pm 0.06) \bullet \log P_{oct} - 0.30\ (\pm 0.16) \qquad \text{Eq. 2.9}$$
$$n = 38,\ \ r^2 = 0.97,\ \ s = 0.19,\ \ F = 1019$$

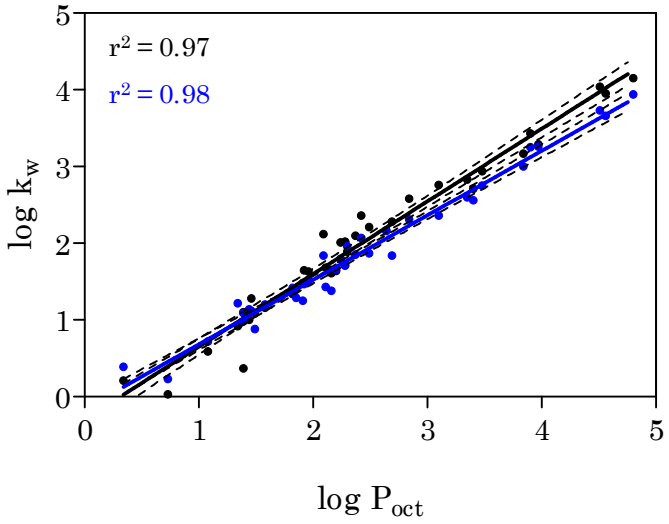


Figure 2.4: Correlation between log P_{oct} and log k_w values obtained on Discovery® RP Amide C16 column with n-octanol (●) and without n-octanol (●) in the mobile phase. The straight lines correspond to linear regressions and the dot lines to the 95% confidence intervals

Comparing Eq. 2.8 and 2.9 and Fig. 2.4, it is clear that no real improvement is obtained in presence of n-octanol. In fact, n-octanol does not modify the correlation between log P_{oct} and log k_w values obtained using the Discovery® RP Amide C16 column and it was not tested further. Indeed by taking into account the 95% confidence intervals the slope and the Y—intercept are identical.

2.1.3.1.2 UHPLC methods

Excellent correlations between log P_{oct} and log k_w values (see equation parameters in Table 2.5) were obtained with the two stationary phases. The log k_w values were reported as a function of log P_{oct} values for the 52 compounds in

Table 2.3 and Fig. 2.5A and in Table 2.4 and Fig. 2.5B for Acquity UPLC™ BEH Shield RP18 and Hypersil™ GOLD Javelin HTS stationary phases, respectively.

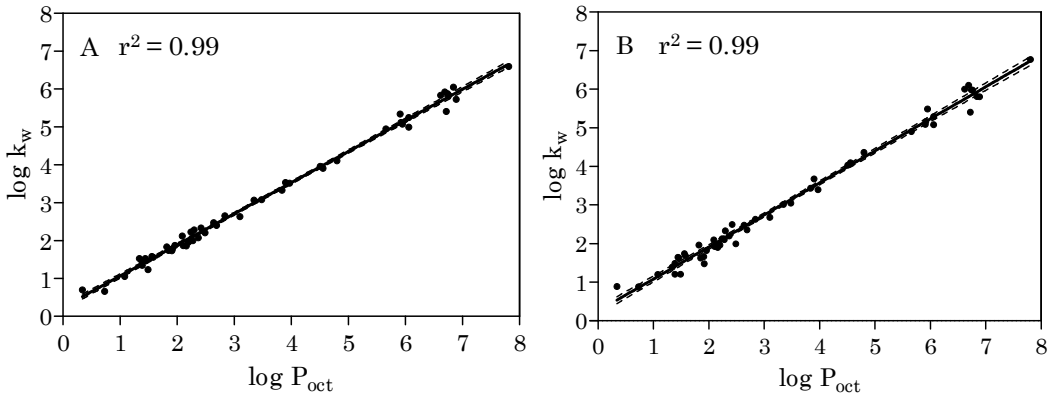


Figure 2.5: log k_w plotted as function of log P_{oct} values obtained for the 52 compounds using MeOH as organic modifier in isocratic mode with the 30-mm Acquity UPLC™ BEH Shield RP 18 stationary phase (A), and the 10-mm Hypersil™ GOLD Javelin HTS column (B). The straight lines correspond to linear regressions and the dot lines to the 95% confidence intervals.

Concerning the Acquity UPLC™ BEH Shield RP18 stationary phase, the lipophilicity determination time was lower than 22 min for compounds with log $P_{oct} < 5$ but as high as 50 min for log $P_{oct} = 8$.

Finally, using the Hypersil™ GOLD Javelin HTS column, the lipophilicity determination time was drastically decreased compared to the Discovery® RP Amide C16 or the Acquity UPLC™ BEH Shield RP18 stationary phases. Indeed, 6 min were necessary for compounds with log $P_{oct} < 5$ and only 9 min for the most lipophilic compounds, without any loss in accuracy ($IC_{95\%} \leq 0.3$). These differences were especially due to the flow rate which is 1mL/min for the Hypersil™ GOLD Javelin HTS column vs. 0.5 mL/min with the Acquity UPLC™ BEH Shield RP18 stationary phase, and the volume, which is twice lower than the one of the Acquity UPLC™ BEH Shield RP18 stationary phase (30 µL for the Hypersil™ GOLD Javelin HTS column vs. 60 µL for the Acquity UPLC™ BEH Shield RP18 stationary phase).

Therefore, the three methods developed using the three columns allow to determine the lipophilicity of highly lipophilic compounds (up to 8) with high

accuracy. While the Discovery® RP Amide C16 column presents the advantage of being usable with a conventional HPLC system, the Acquity UPLC™ BEH Shield RP18 stationary phase allows experimental determination on a large range of pH, suitable for basic compounds with a $pK_a < 8$. Moreover, the Hypersil™ GOLD Javelin HTS stationary phase permits the fastest determination of log P_{oct} values.

2.1.3.2 Gradient mode with MeOH as organic modifier.

The gradient mode was also evaluated using methanol as organic modifier to further reduce analysis time. The mathematical treatment is much more complicated than in isocratic mode and it is mandatory to take into account the system dwell volume, also called gradient delay volume (V_D). This parameter can be experimentally determined [30] and corresponds to the volume comprised between the mixing point of solvents and the head of the column. The V_D value is of prime importance since it allows the determination of the real elution percentage required for modelling purpose. Indeed, this volume induces an initial isocratic migration before the gradient elution.

Two generic runs differing only in gradient times were performed from 2 to 98% of methanol. The two times runs applied for the Discovery® RP Amide C16 column were 10.43 and 3.26 min, respectively. The two different t_r (retention time) obtained from these two gradient runs were computed using an HPLC modelling software (Osiris v.4.1.1.2, Datalys, Grenoble, France) enabling the modelling of the compounds behaviour in the whole organic modifier composition range, as explained in part 2.1.2.5.2. The two unknown parameters, S and k_0, were resolved using Eq. 2.6 and 2.7. Finally log k_w value can be determined according to Eq. 2.3 as MeOH is used as organic modifier and log k_w values are reported in Table 2.2. The correlation obtained using these conditions was less satisfactory than with the isocratic mode, even if it remained acceptable (Table 2.5 and Fig 2.6A). This could be explained by the important contribution of the dwell volume and its measurement accuracy. Indeed, the error made on its evaluation might be in the same order of magnitude as the column dead volume (around 160 µL) and thus induced inaccurate log k_w values (due to incorrect elution percentage determination). Therefore, it is important to work with an appropriate system with

reduced V_D, particularly when short columns are used. Furthermore, a limitation appears in the modelling of the behaviour of the most hydrophilic compound. Indeed, although the N,N-diethylacetamide (log P_{oct} = 0.34) was eluted just after the initial isocratic step and could be modelled, the ethyl acetate (log P_{oct} = 0.7) was eluted during this initial isocratic step (due to its low retention and the important system dwell volume) and cannot be modelled. Hence, these conditions using the Discovery® RP Amide C16 column could be used only for compounds having log P > 1. Despite these drawbacks, gradient conditions are more generic than isocratic ones and do not necessitate any adaptation of percentages or analysis times when investigating numerous different compounds [37]. In fact, the log P_{oct} determination time for each compound in gradient mode was equal to 22.5 min, whatever the analyte lipophilicity. Hence, the gradient mode is more adapted to highly lipophilic compounds in terms of analysis time (22.5 min compared to 66 min in isocratic mode). In conclusion, these conditions could be useful for a first rapid and simple evaluation of lipophilicity providing that the expected log P value is greater than 1.

The two generic gradient runs from 2 to 98% of methanol were realised in 12.8 and 4.27 min for Acquity UPLC™ BEH Shield RP18 column and in 1.92 and 0.64 min for the Hypersil™ GOLD Javelin HTS stationary phase. The log k_w values were directly obtained from an HPLC modelling software and the corresponding results are reported in Table 2.3 and 2.4, respectively, and the correlations between log k_w and log P_{oct} in Fig. 2.6B and 2.6C, respectively.

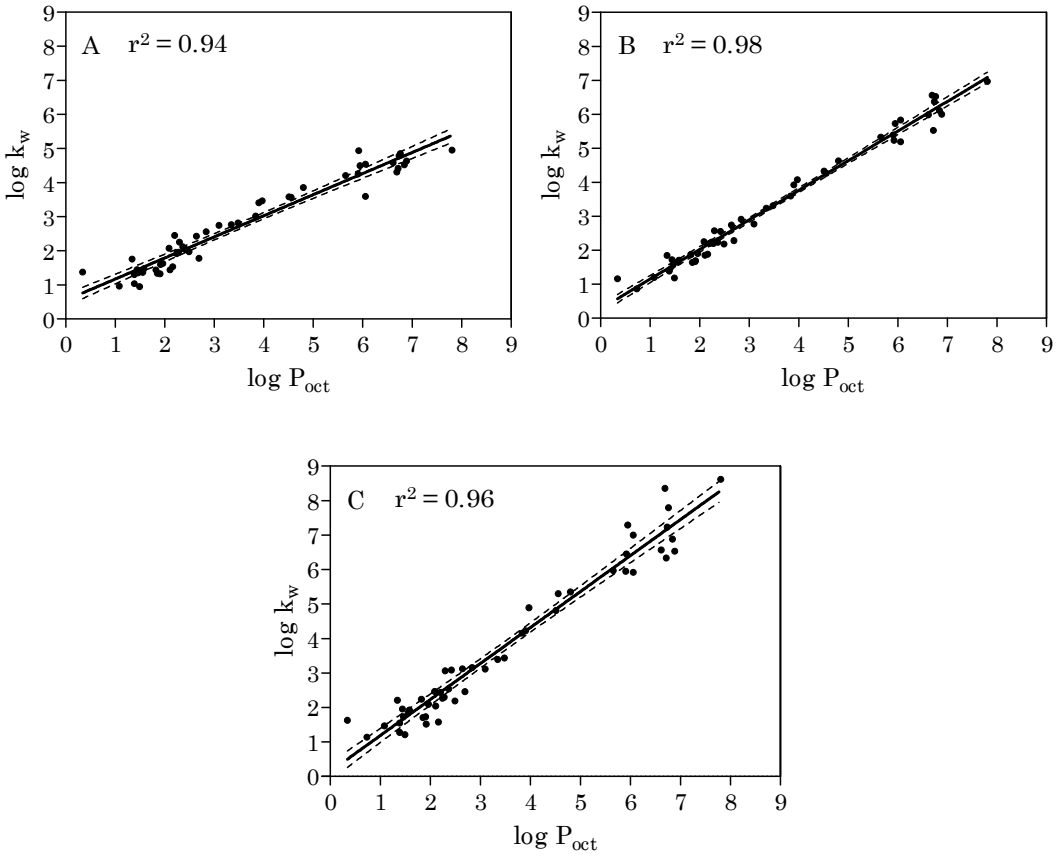


Figure 2.6: log k_w as function of log P_{oct} values obtained for the 52 compounds using MeOH as organic modifier in gradient mode with the 20-mm Discovery® RP Amide C16 column (A), the 30-mm Acquity UPLC™ BEH Shield RP 18 stationary phase (B) and the 10-mm Hypersil™ GOLD Javelin HTS column (C). The straight lines correspond to linear regressions and the dot lines to the 95% confidence intervals.

The log P_{oct} determination time for each compound represented 19.9 min using the Acquity UPLC™ BEH Shield RP18 column and 3.3 min with the Hypersil™ GOLD Javelin HTS one, even for highly lipophilic compounds as they were all eluted during these gradient runs. Therefore, the Hypersil™ GOLD Javelin HTS column allows a faster log P_{oct} determination than the Acquity UPLC™ BEH Shield RP18 stationary phase but log k_w and log P_{oct} values were less correlated (see equations parameters in Table 2.5). As already mentioned, the accurate measurement of the dwell volume is certainly the major problem, as V_D represents 3-fold the Hypersil™ GOLD Javelin HTS dead volume (100 *vs* 30 µL) but less than 2-fold the Acquity UPLC™ BEH Shield RP18 one (V_0 = 60 µL).

It is worth mentioning that results obtained with the Discovery® RP Amide C16 and the Hypersil™ GOLD Javelin HTS stationary phases were sufficient for a rapid preliminary evaluation of the lipophilicity of compounds since less than 22 min or 3.5 min were required, respectively. However, the method developed using the Discovery® RP Amide C16 column is less discriminatory than using the two others stationary phase due to a slope of 0.62. A better correlation ($r^2 = 0.98$) was obtained with the Acquity UPLC™ BEH Shield RP18 column and the lipophilicity determination is faster compared to the isocratic mode especially for highly lipophilic compounds (20 min in gradient mode for all the lipophilicity range vs. 22 for $\log P_{oct} < 5$ and 50 min for $\log P_{oct} > 5$ in isocratic mode). However, although the gradient mode is generic and thus necessitate any adaptation of percentages in organic modifier or analysis times, it is globally less accurate than the isocratic mode.

2.1.3.3 Isocratic mode with ACN as organic modifier.

Due to its better eluent strength, acetonitrile was tested as organic modifier in isocratic mode in order to reduce the analysis time with highly lipophilic compounds.

$\log k_w$ values obtained using the Discovery® RP Amide C16 column are reported in Table 2.2 and the parameters of the correlation between $\log P_{oct}$ and $\log k_w$ values in Table 2.5. An excellent correlation ($r^2=0.98$) was obtained albeit the highly lipophilic compounds were scattered around the straight line as shown in Fig. 2.7A. This result was induced by the quadratic extrapolation used with acetonitrile which is less accurate when high acetonitrile percentages are investigated, compared to the linear extrapolation used with methanol. Hence, the extrapolation error might be higher with highly lipophilic compounds. Although a slight gain in analysis time was effectively obtained using acetonitrile as organic modifier instead of methanol for the highly lipophilic compounds (56 vs. 66 min), the analysis times remain quite long at low acetonitrile percentages (34 vs. 30 min with methanol) due to the quadratic extrapolation. Moreover, the accuracy is lower than when methanol was used as organic modifier, especially for highly lipophilic

compounds. Therefore, methanol remains the co-solvent of choice for log P_{oct} determination in isocratic mode with the Discovery® RP Amide C16 column.

log k_w values obtained with the Acquity UPLC™ BEH Shield RP18 and the Hypersil™ GOLD Javelin HTS stationary phases are reported in Table 2.3 and 2.4, respectively. High acetonitrile percentages were required to elute the most lipophilic compounds using the Acquity UPLC™ BEH Shield RP18 column, leading to more critical extrapolated log k_w values. Therefore, the accuracy of the log P_{oct} determination was lower when increasing the compounds lipophilicity (Fig. 2.7B and Table 2.5). Indeed, the correlation between log P_{oct} and log k_w values was lower than the one obtained using methanol as organic modifier ($r^2 = 0.96$ vs. 0.99). Although a slight gain in analysis time was observed for the most lipophilic compounds (42 min vs. 50 min using MeOH), no interest was found to use acetonitrile instead of methanol as organic modifier. Conversely, the correlation between log k_w and log P_{oct} values obtained with the Hypersil™ GOLD Javelin HTS column (Fig. 2.7C and Table 2.5) was very good. In fact the column length (10 mm) induces shorter analysis times which allow to work at lower acetonitrile percentages and thus limit quadratic extrapolation error. Even if this method could be used for log P_{oct} determination of highly lipophilic compounds, the one obtained in isocratic mode with methanol as organic modifier is more attractive, since faster (up to 9 min vs. up to 20 min using methanol and acetonitrile, respectively) and a little bit more accurate ($r^2 = 0.99$ vs. 0.98, using MeOH or ACN, respectively).

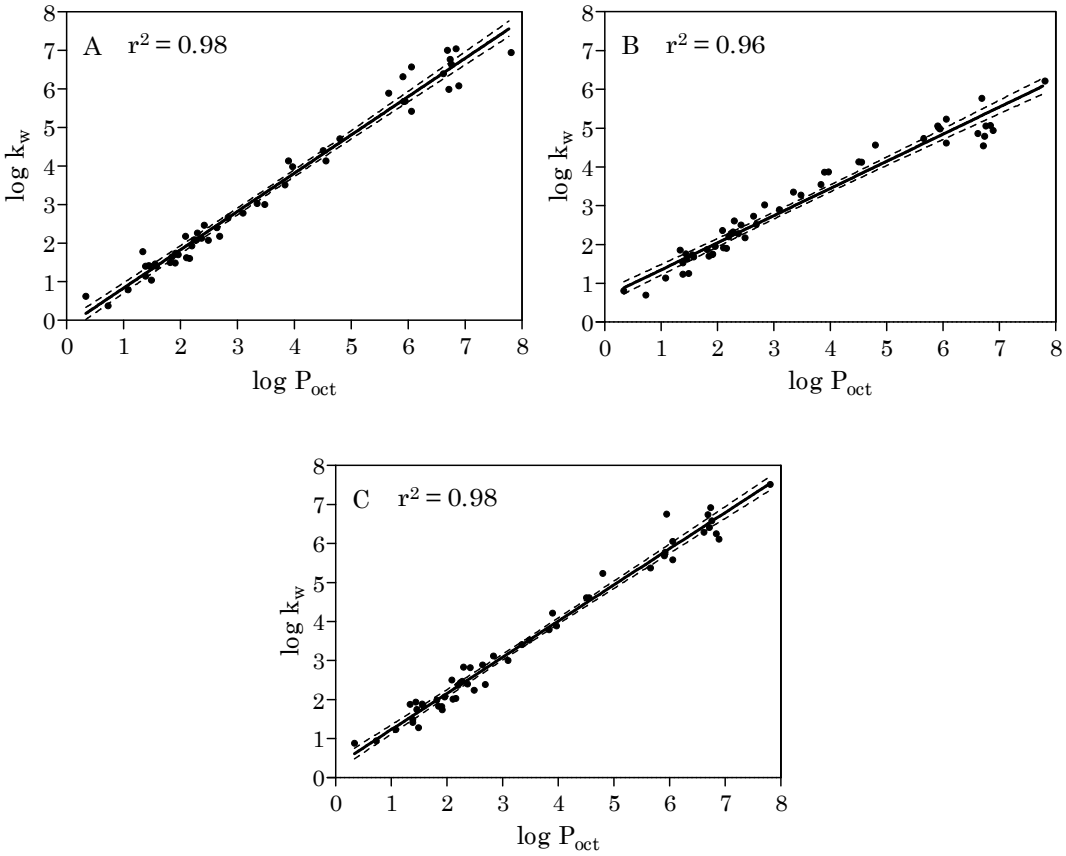


Figure 2.7: log k_w as function of log P_{oct} values obtained for the 52 compounds using ACN as organic modifier in isocratic mode with the 20-mm Discovery® RP Amide C16 column (A), the 30-mm Acquity UPLC™ BEH Shield RP 18 stationary phase (B) and the 10-mm Hypersil™ GOLD Javelin HTS column (C). The straight lines correspond to linear regressions and the dot lines to the 95% confidence intervals.

2.1.3.4 Isocratic mode with THF as organic modifier.

Tetrahydrofuran is known to have an eluent strength higher than acetonitrile one. Indeed, 52% THF corresponds to 70% ACN and 78% MeOH. Moreover, as the viscosity of THF is lower than MeOH, this organic modifier could be interesting since the flow could be increased, which will decrease the analysis time. Therefore it was tested as organic modifier for rapid lipophilicity measurements.

Retention behaviour of benzyl alcohol, one of the most hydrophilic compound with a good UV/Vis detection, was first studied at different percentages of THF (from 5 to 50%) in the mobile phase on the three stationary phases (Fig. 2.8).

Different relationships, quadratic [38] or linear [28], between isocratic log k values and percentages of THF were reported. In our case, a quadratic relation was observed on the three columns between different isocratic log k values and percentages of THF.

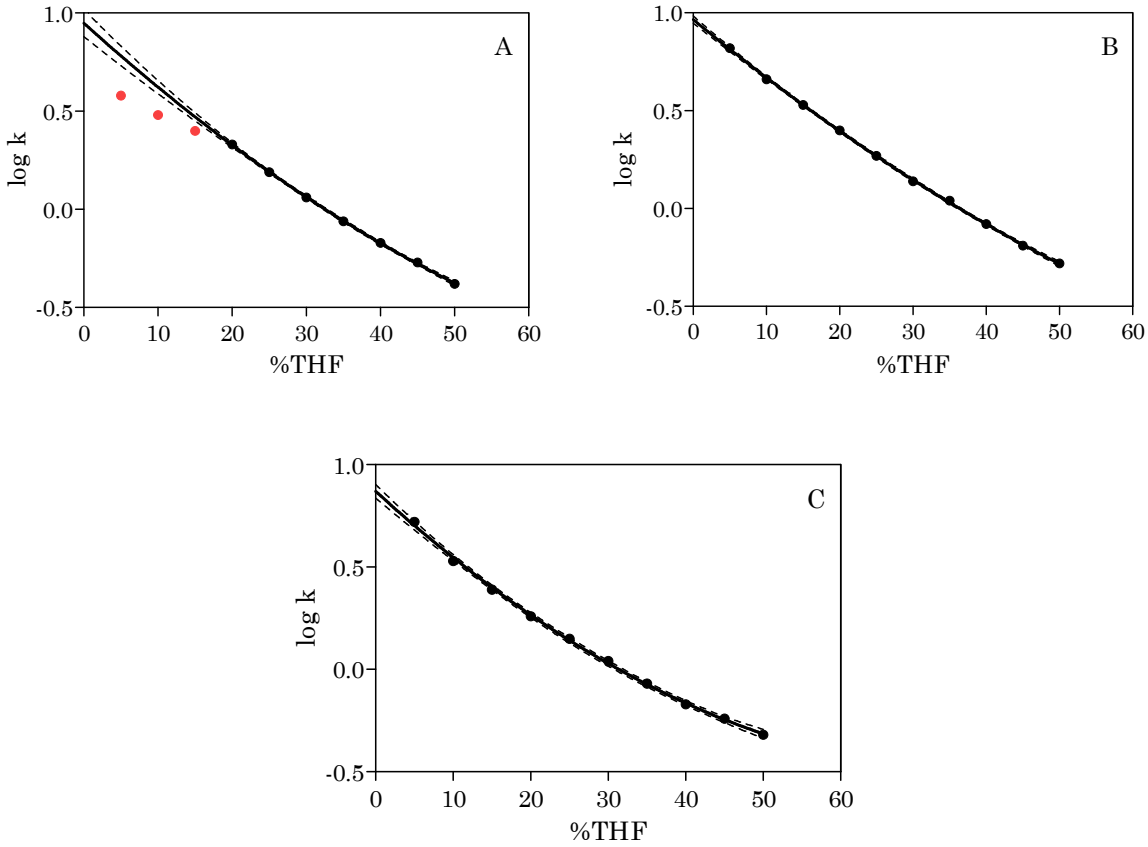


Figure 2.8: Plot of the log k values vs. THF percentages in mobile phase for benzyl alcohol (log P_{oct} = 1.08) with the 20-mm Discovery® RP Amide C16 (A), the 30-mm Acquity UPLC™ BEH Shield (B) and the 10-mm Hypersil™ GOLD Javelin HTS (C) columns. Red points on (A) correspond to the percentages unfitted with the quadratic extrapolation since less retained than expected. The solid lines correspond to the quadratic extrapolations and the dot lines to the 95% confidence intervals.

With the Discovery® RP Amide C16 stationary phase, the retention factor of benzyl alcohol at low percentages of THF (from 5 to 15%, red points in Fig. 2.8A) were lower than expected and were thus unfitted with the quadratic extrapolation. This issue is a common phenomenon in liquid chromatography [39] and could be induced by some pore exclusions due to the high aqueous environment. Moreover,

abnormal retention behaviour observed at low organic modifier composition is caused by sorption of this co-solvent [40, 41]. Hence, these different percentages could not be taken into account for the extrapolation. Therefore the THF composition used with the Discovery® RP Amide C16 column was from 20 to 75%. No similar behaviour was observed either with the Acquity UPLC™ BEH Shield RP 18 (Fig. 2.8B) or Hypersil™ GOLD Javelin HTS (Fig. 2.8C) columns since perfect quadratic relationships were obtained. Hence, although no percentage higher than 50% was analysed for the benzyl alcohol (one of the more hydrophilic compounds), the range from 5 to 75% of THF can be used with these two stationary phases for the whole range of lipophilicity.

High percentages of tetrahydrofuran were necessary with the Discovery® RP Amide C16 column to elute the most lipophilic compounds. Hence, more critical extrapolated $\log k_w$ values (Table 2.2) were obtained due to the quadratic extrapolation required with this co-solvent. Moreover, because high percentages of THF have to be used to allow the quadratic extrapolation for compounds with $\log P_{oct}$ values higher than 5, no real discrimination was done between these different compounds evaluated. For compounds having $\log P_{oct}$ from 0 to 4, the $\log k_w$ value is ranging from -0.5 to 5, whereas for compounds having $\log P_{oct}$ from 4 to 8, $\log k_w$ values is ranging from 4 to 5.5. As a consequence, a low correlation was obtained ($r^2 = 0.87$) (Fig. 2.9A and Table 2.5).

To overcome extrapolation problems, isocratic log k at a fixed percentage of THF were evaluated as lipophilicity index. Different isocratic log k were compared to $\log P_{oct}$ values, and the better correlation (Fig. 2.9B and Table 2.5) was obtained with $\log k_{35\%}$ values (reported in Table 2.2).

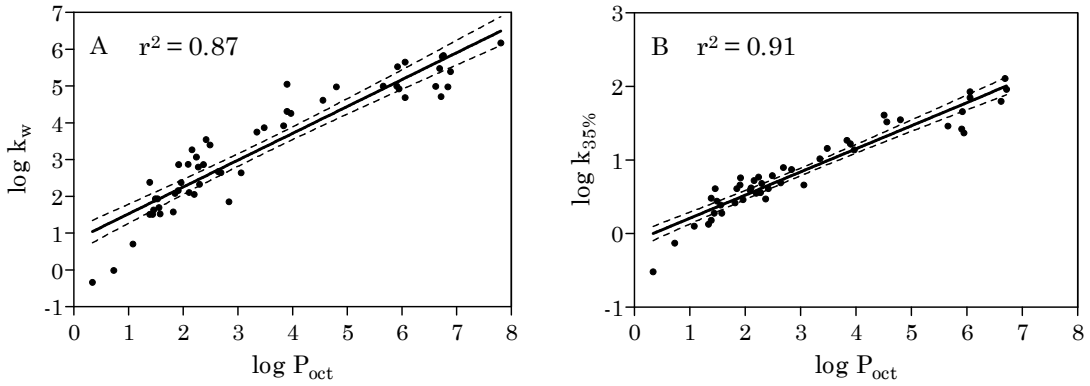


Figure 2.9: log k_w as function of log P_{oct} values obtained for the 52 compounds using THF as organic modifier in isocratic mode with the 20-mm Discovery® RP Amide C16 column (A), and log $k_{35\%}$ as function of log P_{oct} values for 47 compounds (B). The straight lines correspond to linear regressions and the dot lines to the 95% confidence intervals.

Although the analysis times were on average drastically reduced (only one log k used) in comparison with the other conditions, the correlation between log P_{oct} and these log k values obtained at a fixed THF percentage was lower ($r^2 = 0.93$) than the correlations obtained with the MeOH ($r^2 = 0.99$) or even with the ACN ($r^2 = 0.96$) as organic modifier. Moreover, this method induces a poor discrimination since 7 units of log P_{oct} values correspond to only 2.6 units of log k_w values. Furthermore, elution of highly lipophilic compounds with only 35% of THF involves long or even too long retention times, impossible to measure. Indeed log P_{oct} values of only 47 analytes, instead of 52, could be determined, and this method is only available for log $P < 6.7$. Therefore, although the correlation was lower than the one obtained with MeOH, and the method only available for a limited lipophilicity range (ie. not allowing lipophilicity measurement for highly lipophilic compounds), 35%THF with the Discovery® RP Amide C16 column could be used for a rapid preliminary lipophilicity evaluation since only one measurement is necessary.

This co-solvent was also tested on Acquity UPLC™ BEH Shield RP 18 and Hypersil™ GOLD Javelin HTS stationary phases, on a limited number of compounds from original set. In fact, because of the results obtained with the Discovery® RP Amide C16 column, only 12 selected compounds were evaluated on these two columns including 4 highly lipophilic compounds.

Similar results to the Discovery® RP Amide C16 stationary phase were obtained with the Acquity UPLC™ BEH Shield RP 18 column (Fig. 2.10A and Eq. in Table 2.5). Indeed, high THF percentages were required to elute the most lipophilic compounds, leading to a more critical extrapolated log k_w values. To further evaluate this method for log P_{oct} > 5 nine highly lipophilic compounds were evaluated. Their experimental log k_w values were reported in Table 2.3 and were correlated with the calculated log P_{oct} values determined by the CLogP software since no experimental value was available (red circles in Fig. 2.10A). As with Discovery® RP Amide C16 stationary phase method, log P_{oct} values of highly lipophilic compounds were under-estimated with this method. These results explained the low correlation obtained between log k_w and log P_{oct} values (r^2 = 0.94) compared to the use of MeOH as organic modifier. A difference of up to 0.8 units between the CLogP and log P_{THF} (determined using equation developed with the 12 compounds and reported in Table 2.5) values could be obtained, as exemplified with the 2,3,4,5,6-pentabromoethylbenzene. Hence tetrahydrofuran used as organic modifier could not be employed for the lipophilicity determination for log P_{oct} > 5, which is the aim of this work. Moreover, although a slight gain in time was effectively obtained for the highly lipophilic compounds (36 min vs. 50 min using MeOH) (Table 2.5), the analysis times remain quite long at low tetrahydrofuran percentages (31 min vs. 22 min using MeOH).

Concerning the Hypersil™ GOLD Javelin HTS column, a good correlation (r^2 = 0.96) was obtained between log k_w and log P_{oct} values with the 12 compounds of the initial limited set (Fig. 2.10B and Eq. in Table 2.5). Hence, 17 more compounds, containing 9 highly lipophilic ones, were added to confirm that this method could be also suitable for log P_{oct} determination. The experimental log k_w values (Table 2.4) (red circles in Fig. 2.10B) were correlated with the experimental log P_{oct} values measured by the shake-flask method [22] or calculated using the CLogP software for the most lipophilic compounds. Differences up to 0.8 unit were observed between log P_{oct} and log P_{THF} values, determined using equation developed with the 12 compounds and reported in Table 2.5, as for either the m-dichlorobenzene or the dibenz[a,h]anthracene. Furthermore, better correlations were obtained using either methanol or acetonitrile as organic modifier (r^2 = 0.99 and 0.98, respectively).

Moreover, the entire log P_{oct} determination was longer with tetrahydrofuran than with methanol, whatever the lipophilicity. Indeed, 17 min were required for log P_{oct} < 5 against 6 min with methanol, and up to 23 min for log P_{oct} > 5 versus only 9 min.

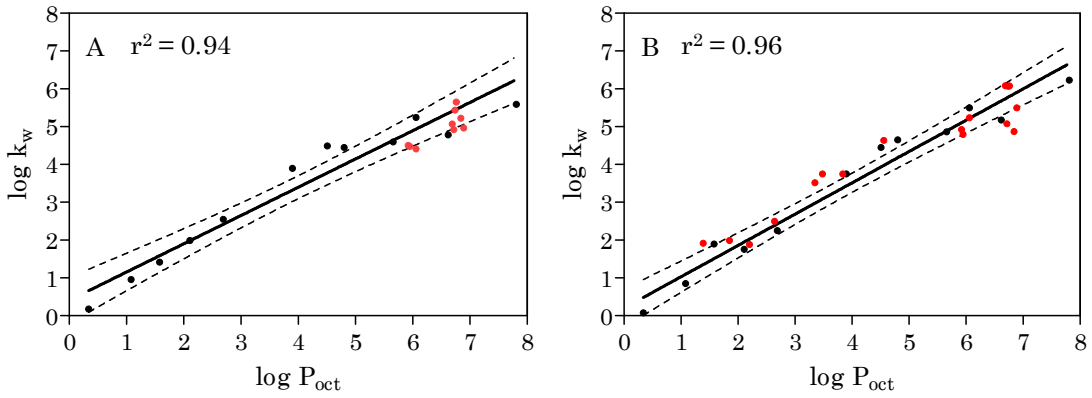


Figure 2.10: log k_w as function of log P_{oct} values obtained for the 12 compounds measured using THF as organic modifier with the 30-mm Acquity UPLC™ BEH Shield RP 18 column (A) and with the 10-mm Hypersil™ GOLD Javelin HTS one (B). Red circles are the experimental log k_w correlated with the log P_{oct} values of the added compounds used to evaluate the methods. The straight lines correspond to linear regressions and the dot lines to the 95% confidence intervals.

Therefore, there is no interest, in time or accuracy, in using tetrahydrofuran instead of methanol as organic modifier for log P_{oct} determination in isocratic mode with either the Acquity UPLC™ BEH Shield RP 18 or the Hypersil™ GOLD Javelin HTS columns. Thus, due to these results, an increase of the flow rate inducing a decrease of the analysis times, was not evaluated.

To conclude, four different conditions (cf. Part 2.1.3.1, 2.1.3.2, 2.1.3.3 and 2.1.3.4) were evaluated on the three different stationary phases and hence thirteen methods were developed. However, the best developed ones were when MeOH was used as organic modifier in isocratic mode (Part 2.1.3.1) since the more accurate.

Table 2.2: Different log P_{oct} and log k_w values obtained for the 52 compounds using the Discovery® RP Amide C16 column

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF			
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}	log k_{35}	log P_{THF35}
N,N-diethylacetamide	0.34	0.43	0.58	1.38	1.33	0.62	0.79	-0.34	-1.55	-0.52	-1.32
Ethyl acetate	0.73	0.41	0.56	c	c	0.38	0.54	-0.01	-1.11	-0.13	-0.08
Benzyl alcohol	1.08	0.84	1.06	0.96	0.67	0.79	0.96	0.70	-0.12	0.10	0.65
Benzidine	1.34	1.29	1.57	1.75	1.95	1.78	1.96	1.94	0.98	0.13	0.75
4-nitroaniline	1.39	1.20	1.47	1.03	0.78	1.14	1.31	1.51	2.18	0.18	0.91
Valeric acid	1.39	1.18	1.45	1.30	1.21	1.40	1.57	2.39	0.98	0.48	1.86
Phenyl-2-propanone	1.44	1.25	1.53	1.44	1.44	1.42	1.59	1.51	1.15	0.28	1.23
Phenylacetic acid	1.46	1.27	1.55	1.35	1.30	1.41	1.58	1.63	1.56	0.61	2.28
Phenol	1.49	0.91	1.13	0.95	0.64	1.04	1.21	1.93	1.56	0.44	1.73
Benzyl cyanide	1.56	1.30	1.58	1.37	1.32	1.46	1.63	1.70	1.23	0.39	1.58
Acetophenone	1.58	1.39	1.69	1.48	1.50	1.40	1.57	1.53	1.00	0.28	1.23
Butyl acetate	1.82	1.48	1.80	1.44	1.44	1.50	1.67	1.58	1.08	0.42	1.67
Nitrobenzene	1.85	1.35	1.64	1.34	1.27	1.64	1.82	2.08	1.76	0.61	2.28
2-chloroaniline	1.91	1.35	1.64	1.32	1.25	1.49	1.66	2.16	1.87	0.66	2.43
p-nitrophenol	1.92	1.65	2.00	1.60	1.70	1.74	1.91	2.87	2.84	0.76	2.75

Table 2.2: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF			
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}	log k_{35}	log P_{THF35}
4-chlorobenzyl alcohol	1.96	1.06	1.31	1.63	1.74	1.70	1.88	2.37	2.16	0.46	1.80
3-chlorophenylacetic acid	2.09	1.99	2.38	2.07	2.46	2.18	2.35	2.87	2.84	0.59	2.21
Anisole	2.11	1.61	1.95	1.44	1.43	1.62	1.79	2.11	1.80	0.62	2.31
N-ethylaniline	2.16	1.49	1.81	1.53	1.59	1.61	1.78	3.27	3.38	0.72	2.63
Propiophenone	2.20	1.83	2.20	2.45	3.06	1.93	2.11	2.05	1.73	0.55	2.08
1-chloro-2-nitrobenzene	2.24	1.89	2.28	1.92	2.21	2.09	2.26	3.07	3.12	0.77	2.78
2-aminonaphtalene	2.28	1.98	2.37	1.96	2.28	2.08	2.26	2.80	2.75	0.56	2.12
2-phenylethyl acetate	2.30	2.16	2.58	2.25	2.75	2.26	2.44	2.33	2.11	0.68	2.50
m-toluic acid	2.37	2.02	2.42	2.11	2.53	2.13	2.30	2.87	2.84	0.47	1.83
4-phenylbutyric acid	2.42	2.25	2.69	2.03	2.40	2.47	2.65	3.55	3.77	0.61	2.28
3-chlorophenol	2.49	2.09	2.50	1.97	2.30	2.08	2.25	3.40	3.57	0.79	2.85
Ethyl benzoate	2.64	2.29	2.74	2.42	3.03	2.41	2.58	2.67	2.57	0.69	2.53
Toluene	2.69	2.04	2.44	1.78	1.99	2.18	2.35	2.65	2.54	0.90	3.20
2-aminobiphenyl	2.84	2.55	3.04	2.56	3.24	2.67	2.85	1.86	1.45	0.87	3.10
1-naphtoic acid	3.10	2.66	3.17	2.75	3.55	2.78	2.96	2.64	2.53	0.66	2.43
Naphtalene	3.35	2.82	3.35	2.77	3.58	3.03	3.21	3.75	4.05	1.02	3.58

Table 2.2: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF			
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}	log k_{35}	log P_{THF35}
m-dichlorobenzene	3.48	3.10	3.68	2.82	3.66	3.01	3.19	3.87	4.21	1.16	4.03
Mesitylene	3.84	3.13	3.72	3.02	3.99	3.52	3.70	3.92	4.28	1.27	4.38
Biphenyl	3.90	3.51	4.15	3.40	4.61	4.14	4.32	4.31	4.82	1.22	4.22
Benzyl benzoate	3.97	3.43	4.06	3.47	4.71	3.99	4.17	4.26	5.83	1.14	3.96
1,2,4,5-tetrachlorobenzene	4.51	3.85	4.55	3.58	4.90	4.41	4.60	5.05	4.75	1.61	5.46
Pentamethylbenzene	4.56	3.84	4.53	3.57	4.87	4.13	4.32	4.61	5.23	1.52	5.17
Bibenzyl	4.80	4.05	4.78	3.85	5.34	4.71	4.90	4.98	5.73	1.55	5.27
Benz[a]anthracene	5.66 b)	4.82	5.67	4.21	5.91	5.89	6.09	5.00	5.76	1.46	4.98
Perylene	5.91 b)	5.10	6.00	4.25	5.98	6.31	6.52	5.00	5.76	1.42	4.85
p-terphenyl	5.92 b)	5.09	5.99	4.93	7.08	5.68	5.87	5.52	6.48	1.66	5.62
Tri-o-tolyl phosphate	5.95 b)	4.62	5.45	4.50	6.38	5.68	5.88	4.92	5.65	1.37	4.69
2,4'-DDD	6.06 b)	4.99	5.87	4.54	6.45	6.57	6.78	5.65	6.65	1.85	6.22
Hexachlorobenzene	6.06 b)	4.92	5.79	3.59	4.92	5.42	5.62	4.69	5.33	1.93	6.48
3-methylcholanthrene	6.62 b)	6.03	7.08	4.58	6.52	6.39	6.60	4.99	5.75	1.80	6.06
Perthan	6.69 b)	5.67	6.66	4.31	6.07	7.00	7.21	5.48	6.42	2.11	7.05
Hexabromobenzene	6.72 b)	5.34	6.27	4.42	6.25	5.99	6.19	4.71	5.37	1.96	6.57
4,4'-DDE	6.74 b)	5.70	6.69	4.78	6.84	6.77	6.98	5.80	6.86	d	d

Table 2.2: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF			
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}	log k_{35}	log P_{THF35}
4,4'-DDT	6.76 b)	5.64	6.62	4.85	6.95	6.63	6.84	5.83	6.89	d	d
Dibenz[a,h]anthracene	6.84 b)	6.08	7.14	4.52	6.41	7.04	7.25	4.98	5.73	d	d
2,3,4,5,6-pentabromoethylbenzene	6.89 b)	5.88	6.91	4.64	6.60	6.08	6.29	5.39	6.30	d	d
p-quaterphenyl	7.81 b)	6.95	8.14	4.95	7.11	6.94	7.15	6.17	7.36	d	d

a) Taken from [22]. b) CLogP values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005). c) no experimental value for this compound as explained in Part 2.3.2 Gradient mode with MeOH as organic modifier. d) no experimental value for these compounds as explained in Part 2.3.4 Isocratic mode at 35% THF in the mobile phase.

Table 2.3: Different log P_{oct} and log k_w values obtained for the 52 compounds using the Acquity UPLC™ BEH Shield RP18 column

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
N,N-diethylacetamide	0.34	0.69	0.55	1.16	1.01	0.81	0.23	0.18	-0.31
Ethyl acetate	0.73	0.66	0.51	0.86	0.67	0.70	0.07	-	-
Benzyl alcohol	1.08	1.05	0.99	1.20	1.05	1.14	0.70	0.95	0.73
Benzidine	1.34	1.52	1.56	1.85	1.80	1.86	1.73	-	-
4-nitroaniline	1.39	1.34	1.34	1.39	1.28	1.23	0.84	-	-
Valeric acid	1.39	1.42	1.44	1.42	1.32	1.53	1.26	-	-
Phenyl-2-propanone	1.44	1.53	1.56	1.72	1.66	1.76	1.59	-	-
Phenylacetic acid	1.46	1.47	1.50	1.56	1.47	1.62	1.39	-	-
Phenol	1.49	1.23	1.21	1.18	1.04	1.25	0.87	-	-
Benzyl cyanide	1.56	1.58	1.63	1.65	1.58	1.73	1.56	-	-
Acetophenone	1.58	1.56	1.61	1.71	1.64	1.68	1.48	1.42	1.35
Butyl acetate	1.82	1.83	1.94	1.88	1.83	1.87	1.75	-	-
Nitrobenzene	1.85	1.74	1.82	1.64	1.56	1.70	1.51	-	-
2-chloroaniline	1.91	1.73	1.82	1.66	1.59	1.74	1.57	-	-
p-nitrophenol	1.92	1.78	1.87	1.70	1.63	1.75	1.59	-	-
4-chlorobenzyl alcohol	1.96	1.87	1.99	1.91	1.87	1.95	1.87	-	-

Table 2.3: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
3-chlorophenylacetic acid	2.09	2.12	2.29	2.25	2.27	2.36	2.46	-	-
Anisole	2.11	1.86	1.97	1.85	1.80	1.92	1.82	1.99	2.12
N-ethylaniline	2.16	1.86	1.97	1.89	1.84	1.90	1.79	-	-
Propiophenone	2.20	1.96	2.09	2.20	2.21	2.20	2.22	-	-
1-chloro-2-nitrobenzene	2.24	2.23	2.42	2.24	2.25	2.29	2.34	-	-
2-aminonaphtalene	2.28	2.00	2.14	2.20	2.20	2.32	2.40	-	-
2-phenylethyl acetate	2.30	2.28	2.48	2.57	2.63	2.61	2.81	-	-
m-toluic acid	2.37	2.07	2.23	2.23	2.24	2.28	2.34	-	-
4-phenylbutyric acid	2.42	2.33	2.54	2.56	2.61	2.50	2.65	-	-
3-chlorophenol	2.49	2.21	2.40	2.18	2.19	2.17	2.18	-	-
Ethyl benzoate	2.64	2.47	2.72	2.75	2.83	2.73	2.98	-	-
Toluene	2.69	2.40	2.63	2.29	2.30	2.52	2.67	2.55	2.87
2-aminobiphenyl	2.84	2.65	2.93	2.91	3.02	3.02	3.40	-	-
1-naphtoic acid	3.10	2.64	2.92	2.78	2.86	2.90	3.22	-	-
Naphtalene	3.35	3.07	3.44	3.24	3.40	3.35	3.87	-	-
m-dichlorobenzene	3.48	3.08	3.46	3.31	3.48	3.28	3.76	-	-

Table 2.3: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
Mesitylene	3.84	3.32	3.75	3.60	3.81	3.55	4.15	-	-
Biphenyl	3.90	3.54	4.01	3.92	4.18	3.87	4.60	3.90	4.67
Benzyl benzoate	3.97	3.52	3.99	4.08	4.36	3.87	4.61	-	-
1,2,4,5-tetrachlorobenzene	4.51	3.96	4.53	4.33	4.65	4.13	4.98	4.49	5.46
Pentamethylbenzene	4.56	3.91	4.46	4.27	4.58	4.12	4.97	-	-
Bibenzyl	4.80	4.11	4.71	4.63	5.00	4.57	5.60	4.45	5.41
Benz[a]anthracene	5.66 b)	4.95	5.73	5.33	5.80	4.73	5.84	4.59	5.60
Perylene	5.91 b)	5.34	6.21	5.40	5.88	5.06	6.30	-	-
p-terphenyl	5.92 b)	5.12	5.93	5.23	5.69	5.01	6.24	4.50	5.48
Tri-o-tolyl phosphate	5.95 b)	5.07	5.88	5.73	6.26	4.98	6.19	4.48	5.45
2,4'-DDD	6.06 b)	5.25	6.10	5.83	6.38	5.23	6.55	5.24	6.47
Hexachlorobenzene	6.06 b)	5.00	5.79	5.20	5.64	4.61	5.67	4.41	5.35
3-methylcholanthrene	6.62 b)	5.83	6.81	5.99	6.55	4.86	6.03	4.78	5.85
Perthan	6.69 b)	5.92	6.92	6.56	7.21	5.77	7.32	5.07	6.24
Hexabromobenzene	6.72 b)	5.41	6.29	5.53	6.02	4.54	5.57	4.92	6.04
4,4'-DDE	6.74 b)	5.87	6.85	6.37	6.99	4.79	5.93	5.44	6.73

Table 2.3: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
4,4'-DDT	6.76 b)	5.84	6.81	6.53	7.17	5.06	6.31	5.65	7.02
Dibenz[a,h]anthracene	6.84 b)	6.05	7.07	6.11	6.69	5.07	6.32	5.22	6.44
2,3,4,5,6-pentabromoethylbenzene	6.89 b)	5.73	6.68	6.00	6.56	4.94	6.13	4.96	6.10
p-quaterphenyl	7.81 b)	6.60	7.74	6.97	7.67	6.22	7.96	5.59	6.94

a) Taken from [22]. b) CLogP values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005).

Table 2.4: Different log P_{oct} and log k_w values obtained for the 52 compounds using the Hypersil™ GOLD Javelin HTS column

	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
name		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
N,N-diethylacetamide	0.34	0.89	0.77	1.63	1.42	0.88	0.62	0.08	-0.15
Ethyl acetate	0.73	0.88	0.76	1.14	0.95	0.94	0.69	-	-
Benzyl alcohol	1.08	1.20	1.14	1.47	1.27	1.23	1.00	0.85	0.78
Benzidine	1.34	1.39	1.37	2.21	1.98	1.88	1.70	-	-
4-nitroaniline	1.39	1.49	1.49	1.55	1.35	1.49	1.28	-	-
Valeric acid	1.39	1.21	1.15	1.28	1.09	1.42	1.21	2.04	2.22
Phenyl-2-propanone	1.44	1.64	1.67	1.96	1.74	1.94	1.77	-	-
Phenylacetic acid	1.46	1.63	1.67	1.73	1.52	1.75	1.56	-	-
Phenol	1.49	1.20	1.15	1.21	1.02	1.28	1.06	-	-
Benzyl cyanide	1.56	1.74	1.79	1.84	1.63	1.88	1.70	-	-
Acetophenone	1.58	1.68	1.72	1.93	1.71	1.83	1.65	1.90	2.05
Butyl acetate	1.82	1.96	2.06	2.24	2.01	1.99	1.82	-	-
Nitrobenzene	1.85	1.62	1.65	1.71	1.50	1.83	1.65	2.11	2.30
2-chloroaniline	1.91	1.66	1.70	1.73	1.52	1.82	1.64	-	-
p-nitrophenol	1.92	1.48	1.48	1.52	1.32	1.74	1.55	-	-
4-chlorobenzyl alcohol	1.96	1.82	1.89	2.09	1.87	2.07	1.91	-	-

Table 2.4: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
3-chlorophenylacetic acid	2.09	2.09	2.22	2.47	2.23	2.50	2.37	-	-
Anisole	2.11	1.92	2.01	2.04	1.82	2.01	1.84	1.76	1.88
N-ethylaniline	2.16	1.90	1.98	1.58	1.37	2.03	1.86	-	-
Propiophenone	2.20	1.96	2.06	2.44	2.20	2.37	2.23	2.00	2.18
1-chloro-2-nitrobenzene	2.24	2.12	2.26	2.27	2.04	2.43	2.30	-	-
2-aminonaphtalene	2.28	2.10	2.23	2.30	2.07	2.47	2.34	-	-
2-phenylethyl acetate	2.30	2.33	2.50	3.06	2.80	2.83	2.73	-	-
m-toluic acid	2.37	2.20	2.34	2.54	2.30	2.40	2.26	-	-
4-phenylbutyric acid	2.42	2.49	2.70	3.08	2.82	2.82	2.73	-	-
3-chlorophenol	2.49	1.99	2.10	2.19	1.96	2.24	2.09	-	-
Ethyl benzoate	2.64	2.47	2.68	3.12	2.85	2.89	2.79	2.59	2.88
Toluene	2.69	2.36	2.54	2.46	2.22	2.39	2.25	2.25	2.48
2-aminobiphenyl	2.84	2.63	2.87	3.16	2.89	3.12	3.04	-	-
1-naphtoic acid	3.10	2.68	2.93	3.12	2.85	3.00	2.91	-	-
Naphtalene	3.35	3.01	3.33	3.39	3.11	3.41	3.35	3.55	4.04
m-dichlorobenzene	3.48	3.05	3.37	3.44	3.16	3.52	3.47	3.77	4.31

Table 2.4: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
Mesitylene	3.84	3.43	3.83	4.16	3.84	3.79	3.76	3.77	4.31
Biphenyl	3.90	3.67	4.12	4.23	3.91	4.21	4.22	3.75	4.29
Benzyl benzoate	3.97	3.39	3.79	4.90	4.56	3.89	3.87	-	-
1,2,4,5-tetrachlorobenzene	4.51	4.03	4.56	4.81	4.47	4.61	4.65	4.45	5.13
Pentamethylbenzene	4.56	4.10	4.63	5.30	4.94	4.61	4.65	4.61	5.33
Bibenzyl	4.80	4.36	4.95	5.35	4.99	5.23	5.32	4.65	5.37
Benz[a]anthracene	5.66 b)	4.91	5.61	5.97	5.58	5.37	5.47	4.87	5.63
Perylene	5.91 b)	5.09	5.82	5.95	5.56	5.68	5.80	-	-
p-terphenyl	5.92 b)	5.16	5.92	6.45	6.04	5.78	5.91	4.92	5.70
Tri-o-tolyl phosphate	5.95 b)	5.49	6.31	7.29	6.85	6.75	6.96	4.80	5.55
2,4'-DDD	6.06 b)	5.29	6.07	7.00	6.57	6.05	6.20	5.50	6.40
Hexachlorobenzene	6.06 b)	5.08	5.82	5.92	5.54	5.58	5.69	5.24	6.08
3-methylcholanthrene	6.62 b)	6.00	6.93	6.57	6.16	6.29	6.46	5.18	6.01
Perthan	6.69 b)	6.11	7.05	8.36	7.87	6.74	6.95	6.09	7.11
Hexabromobenzene	6.72 b)	5.41	6.21	6.34	5.94	6.41	6.59	5.08	5.89
4,4'-DDE	6.74 b)	5.98	6.90	7.23	6.80	6.91	7.13	6.07	7.09

Table 2.4: Continued

name	log P_{oct} [a]	Isocratic MeOH		Gradient MeOH		Isocratic ACN		Isocratic THF	
		log k_w	log P_{MeOH}	log k_w	log P_{grad}	log k_w	log P_{ACN}	log k_w	log P_{THF}
4,4'-DDT	6.76 b)	5.98	6.90	7.79	7.33	6.58	6.77	6.07	7.09
Dibenz[a,h]anthracene	6.84 b)	5.81	6.70	6.88	6.46	6.25	6.42	4.87	5.64
2,3,4,5,6-pentabromoethylbenzene	6.89 b)	5.81	6.69	6.53	6.12	6.11	6.27	5.50	6.40
p-quaterphenyl	7.81 b)	6.77	7.86	8.62	8.12	7.51	7.78	6.23	7.28

a) Taken from [22]. b) CLogP values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005).

Table 2.5: Equations of the different correlations obtained on the three stationary phases ($\log k_w = a \times \log P_{oct} + b$).

Methods	a	b	n	r^2	S	F	$\log P_{oct}$ determination time (min)	
Best conditions							**$\log P_{oct}<5$**	**$\log P_{oct}<8$**
Hypersil 10-mm isocratic MeOH	0.83 (± 0.02)	0.25 (± 0.09)	52	0.99	0.18	4968	6	9
Acquity 30-mm gradient MeOH	0.87 (± 0.03)	0.28 (± 0.13)	52	0.98	0.24	2842	20 a)	20 a)
Acquity 30-mm isocratic MeOH	0.82 (± 0.02)	0.24 (± 0.07)	52	0.99	0.13	9335	22	50
Discovery 20-mm isocratic MeOH	0.86 (± 0.03)	-0.07 (± 0.11)	52	0.99	0.19	4370	30	66
Less rapid conditions								
Hypersil 10-mm isocratic ACN	0.93 (± 0.04)	0.30 (± 0.15)	52	0.98	0.27	2563	11	20
Discovery 20-mm isocratic ACN	0.99 (± 0.04)	-0.16 (± 0.17)	52	0.98	0.31	2312	34	56
Less accurate conditions								
Hypersil 10-mm gradient MeOH	1.04 (± 0.06)	0.14 (± 0.26)	52	0.96	0.47	1080	3.5 a)	3.5 a)
Acquity 30-mm isocratic ACN	0.70 (± 0.04)	0.64 (± 0.17)	52	0.96	0.31	1098	22	42
Discovery 20-mm gradient MeOH	0.62 (± 0.04)	0.55 (± 0.18)	51	0.94	0.32	806	22 a)	22 a)
Discovery 20-mm 35% THF	0.31 (± 0.03)	-0.11 (± 0.10)	47	0.91	0.18	465	10	22
Discovery 20-mm isocratic THF	0.73 (± 0.08)	0.79 (± 0.33)	52	0.87	0.60	323	46	56
Hypersil 10-mm gradient THF	0.83 (± 0.10)	0.20 (± 0.46)	12	0.96	0.40	273	17	23
Acquity 30-mm isocratic THF	0.75 (± 0.12)	0.41 (± 0.54)	12	0.94	0.47	155	31	36

n is the number of compounds, r^2 the determination coefficient, s the standard deviation and F the Fisher's test value. 95% confidence intervals are given in parentheses and a) Same conditions (gradient times and mobile phase) on the whole range of $\log P_{oct}$.

2.1.3.5 Enhancement of the lipophilicity range.

Five new rigid compounds were evaluated on the three stationary phases in isocratic mode using MeOH as organic modifier to evaluate if these three methods were effectively suitable for $\log P_{oct}$ up to 10. These compounds (octachloronaphtalene, decachlorobiphenyl, 2,2',3,3',4,4',5,5'-octachlorobiphenyl, 1,2,3,4,6,7,8 heptachlorodibenzo-p-dioxin, and 1,2,3,4,6,7,8,9-octachlorodibenzofuran) were chosen according to their chemical structure and their ClogP values, as explained in the Part 2.1.2.1.

The retention factors of the five rigid compounds (pink circles) and the correlations between $\log k_w$ and $\log P_{oct}$ values were reported in Table 2.6 and Fig. 2.11A for the Discovery® RP Amide C16, Fig. 2.11B for the Acquity UPLC™ BEH Shield RP18, and finally Fig. 2.11C for the Hypersil™ GOLD Javelin HTS stationary phases. The corresponding equations are given there:

$$\log k_{w\,Discovery} = 0.87(\pm 0.02)\log P_{oct} - 0.10(\pm 0.11) \qquad \text{Eq. 2.10}$$
$$n = 57, r^2 = 0.99, S = 0.22, F = 5645$$

$$\log k_{w\,Acquity} = 0.81(\pm 0.02)\log P_{oct} + 0.28(\pm 0.07) \qquad \text{Eq. 2.11}$$
$$n = 57, r^2 = 0.99, S = 0.15, F = 10270$$

$$\log k_{w\,Hypersil} = 0.83(\pm 0.02)\log P_{oct} + 0.26(\pm 0.09) \qquad \text{Eq. 2.12}$$
$$n = 57, r^2 = 0.99, S = 0.18, F = 7310$$

In these equations, n is the number of compounds, r^2 the determination coefficient, s the standard deviation and F the Fisher's test value. 95% confidence intervals are given in parentheses.

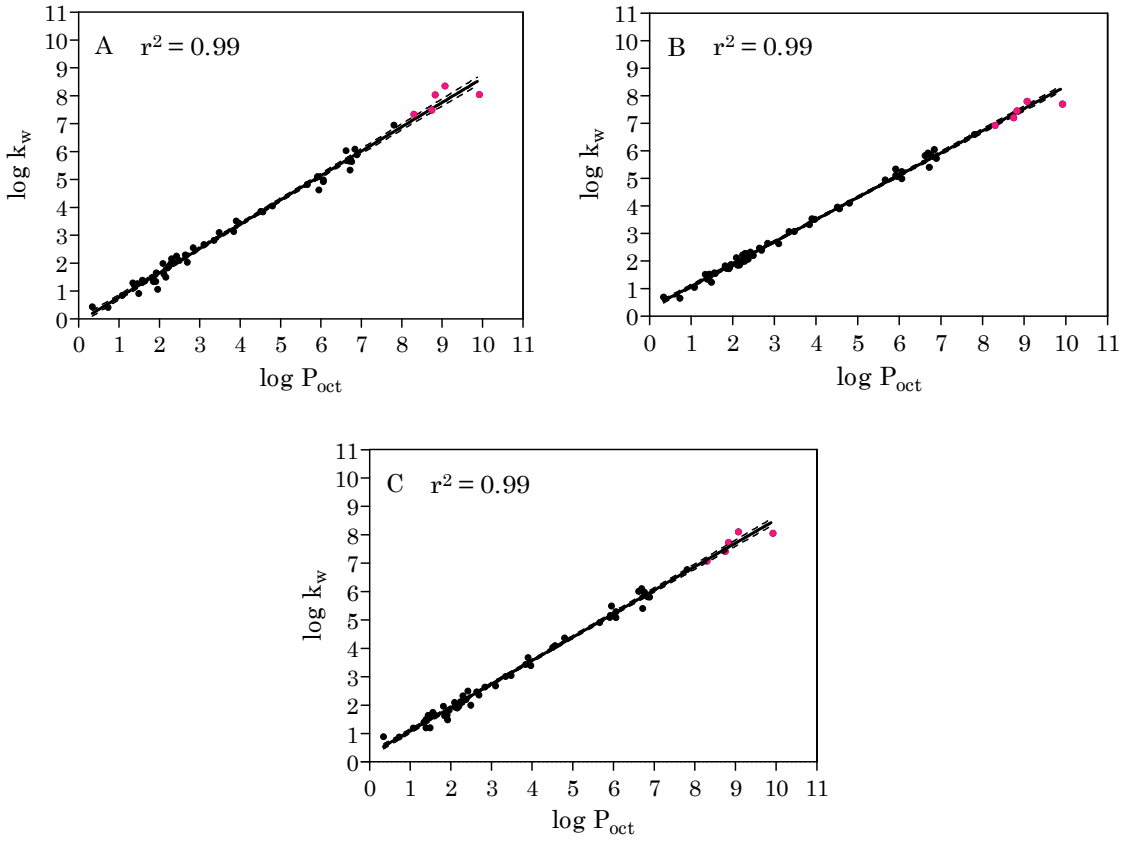


Figure 2.11: log k_w as function of log P_{oct} values obtained for the 52 compounds (black circles) using MeOH as organic modifier in isocratic mode with the 20-mm Discovery® RP Amide C16 column (A), the 30-mm Acquity UPLC™ BEH Shield RP 18 stationary phase (B) and the 10-mm Hypersil™ GOLD Javelin HTS column (C). Pink circles correspond to the five rigid compounds added for the enhancement. The straight lines corresponds to linear regressions and the dot lines to the 95% confidence intervals.

The addition of these five compounds only slightly modified the slope and/or the Y-intercept as reported in Table 2.5 (52 compounds) and in Eq. 2.10, 2.11 and 2.12 (57 analytes) for the three stationary phases.

The correlations and the equations (slope and Y—intercept) were still the same (including the IC 95%) when the five rigid compounds were added. Even if the analysis times were drastically raised for these five compounds since an increase of 2 units of log P_{oct} values induces an augmentation of 54 minutes (120 min vs. 66) for the Discovery® RP Amide C16 column, 59 minutes (109 min vs. 50) for the Acquity

UPLC™ BEH Shield RP18 stationary phase and 32 minutes (41 min vs. 9) for the Hypersil™ GOLD Javelin HTS column, such high lipophilicities are reachable with the three methods. However, the analysis time becomes important when using Discovery® RP Amide C16 and Acquity UPLC™ BEH Shield RP18 stationary phases whereas it stays reasonable with Hypersil™ GOLD Javelin HTS column. Therefore, this latter method is the method of choice for high-throughput screening with very high lipophilicity.

Table 2.6: Different log k_w and log P_{oct} values obtained for the five new compounds using the three stationary phases in isocratic mode with methanol as organic modifier

		Discovery® RP Amide		Acquity UPLC™ BEH		Hypersil™ GOLD	
name	CLogP [a]	log k_w	log P_{oct} [b]	log k_w	log P_{oct} [c]	log k_w	log P_{oct} [d]
Octachloronaphtalene	8.30	7.34	8.52	6.93	8.24	7.07	8.23
2,2',3,3',4,4',5,5'-octachlorobiphenyl	8.75	7.49	8.69	7.21	8.59	7.41	8.64
1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin	8.83	8.03	9.31	7.45	8.89	7.73	9.03
1,2,3,4,6,7,8,9-octachlorodibenzofuran	9.07	8.35	9.68	7.80	9.32	8.10	9.48
Decachlorobiphenyl	9.92	8.04	9.32	7.70	9.20	8.05	9.42

a) CLogP values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005). b) log P_{oct} values determined using Eq 2.8. c) log P_{oct} values determined using Eq. 2.9. d) log P_{oct} values determined using Eq. 2.10.

2.1.4 Conclusion

Different analytical methods (3 stationary phases evaluated with 4 different conditions) were compared to evaluate the best way to determine the lipophilicity of moderately to highly lipophilic compounds.

Acetonitrile is known to have a strong eluent strength, which could have been beneficial for highly lipophilic compounds. In fact, a gain in time was obtained for highly lipophilic compounds compared to methanol as organic modifier with Discovery® RP Amide C16 and Acquity UPLC™ BEH Shield RP18 columns. Despite this positive statement, log k_w and log P_{oct} values were often less correlated due to the inaccurate quadratic extrapolation with high acetonitrile percentages (since a low decrease of % ACN induces an important increase of t_r, sometimes not measurable) and potential secondary interactions with residual silanol groups.

Tetrahydrofuran was also evaluated because it possesses a superior eluent strength than acetonitrile, a poor viscosity inducing the possibility of increasing the flow rate and an ability to mask the residual silanol groups of the stationary phase, which decreases secondary interactions. However, an important retention time is required to restrain the inaccuracy on the log k_w determination due to the quadratic extrapolation, as for ACN. Moreover, due to the poor correlation obtained, this organic modifier is hence not interesting for the lipophilicity determination of highly lipophilic compounds, and the flow rate was not increased.

n-octanol was also evaluated on the Discovery® RP Amide C16 column as it is known to improve the correlation between log P_{oct} values and the retention factors compared to the correlation obtained in absence of this additive. However, as the correlation was already good without n-octanol, no improvement were obtained and this additive was not further study on the other columns.

The gradient mode, evaluated with methanol, presents the advantage to be more generic than isocratic mode. However, the observed correlations between log P_{oct} and log k_w values were slightly lower in gradient mode, except for the Acquity UPLC™ BEH Shield RP18 column. These results were attributed to the high dwell volume compared to the dead volumes of both Discovery® RP Amide C16 column with conventional HPLC instrument and Hypersil™ GOLD Javelin HTS stationary phase with UHPLC system. Nevertheless gradient conditions can be

useful to evaluate (with a correct accuracy) the lipophilicity of a large series of diverse compounds since it is a generic method, which does not require any time and mobile phase adaptation. To improve the results obtained using the Hypersil™ GOLD Javelin HTS stationary phase with the gradient mode, a column of 20 mm could be evaluated to limit the dwell time problem and allow a lipophilicity determination.

Finally, the isocratic mode with methanol as organic modifier is the condition of choice for the determination of log P_{oct} values up to 10. Indeed, even if MeOH possesses the lowest eluent strength, the linear extrapolation enables working at high percentages of methanol. The analysis time was thus reduced (especially for log $P_{oct} < 5$) and a good accuracy was maintained. The lowest analysis time and best correlation between log k_w and log P_{oct} values ($r^2 = 0.99$) were obtained using the Hypersil™ GOLD Javelin HTS stationary phase since 6 min were required for log $P_{oct} < 5$ and 41 min for the most lipophilic compounds (log P_{oct} up to 10). Hence, this high-throughput method allows a fast determination of lipophilicity on a wide range of log P_{oct} values (from 0 to 10) with a good accuracy. To still decrease these analysis times, MS detector could be used instead of PDA detector, since several compounds could be evaluated at the same time.

2.2 Basic compounds

2.2.1 Introduction

Although different methods were developed in the first part of this chapter, the set used was only composed of neutral, acidic and weakly basic compounds. Because the stationary phases used are unstable at high pH conditions due to the silica-based dissolution [42-44], lipophilicity measurements of basic compounds with $pK_a > 5$, using the Discovery® RP Amide C16 and the Hypersil™ GOLD Javelin HTS stationary phases, or $pK_a > 8$, with the Acquity UPLC™ BEH Shield RP18 column, cannot be performed using these methods.

Hydrophilic interaction chromatography (HILIC) was recently evaluated on HPLC system [45] for lipophilicity measurements of basic compounds with $pK_a > 7$. Indeed, this column permits the determination of basic compounds with $pK_a > 7$ [46] since the retention time of the cationic form is measured instead of the neutral one.

In fact, it was demonstrated that $\Delta \log k_{0\text{-}95}$, which corresponds to the difference between 2 isocratic log k values measured for the cationic form ($\log k_0$, directly measured or extrapolated to 100% water in mobile phase, and $\log k_{95}$ measured at 95% ACN) were well correlated with $\log P^N_{oct}$ values ($\log P_{oct}$ of the neutral form).

HILIC retention mechanism of cationic form of compounds was reported to be a complex combination of different interactions [47], depending on the co-solvent proportion:

- an hydrophilic type retention [45, 48-51] when high proportions of organic modifier are used in the mobile phase
- a reversed-phase type retention ("RP-like") [52, 53] (i.e. a decrease of retention by increasing organic modifier proportion) when the mobile phase contains high proportions of water
- and an additional ion exchange mechanism [54] which is present all over the percentage range of co-solvent.

Since lipophilicity expresses the balance of polarity and hydrophobicity, the particularity of ionized compounds on HILIC retention was exploited to obtain n-octanol/water partition coefficients of the neutral form of basic compounds. Indeed, two diametrically opposite mobile phase compositions were measured for the cationic form (i.e. 0% and 95% (v/v) of ACN in the mobile phase) [46].

This previous study was realized using 39 moderate to strong basic compounds ($7.1 < pK_a < 11$) with $\log P^N_{oct}$ values ranging from -1.3 to 4.6. A new set containing five highly lipophilic basic compounds ($4.9 < \log P_{oct} < 7.7$ and $pK_a > 7.0$) was evaluated to enlarge the lipophilicity range determined by Bard et al. [46] on ZIC-*p*HILIC stationary phase.

2.2.2 Materials and methods

2.2.2.1 Chemicals

All compounds were obtained from Sigma (St-Louis, MO, USA) in the highest available purity. HPLC grade acetonitrile was purchased from VWR (Dietikon, Switzerland). Water was obtained with the Milli-Q Water Purification System from Millipore (Milford, MA, USA).

2.2.2.2 Retention time measurements

To study basic compounds under their cationic form with the HILIC column, a trifluoroacetic acid / ammonia pH 2 buffer was realised. Indeed, as organic modifier increases the pH of the mobile phase and decreases the pK_a of basic compounds [55], the lowest pH supported by the stationary phase was used. In fact, at 95% ACN the pH measured for the mixture buffer/co-solvent was 2.97 instead of 2.00 obtained with the buffer. Using Rosés corrections [56], the real pH value for the hydro-organic solution was 3.68. Hence, only basic compounds with $pK_a > 7$ were evaluated to be sure that each compound was in cationic form. An ionic strength of 100 mM was fixed since basic compounds retention decreases, in the RP—like part, when ionic strength increases [47]. Buffer solutions were filtered through a 0.45 µm HA Millipore filter (Millipore, Bedford, MA, USA).

HPLC measurements were performed on a liquid chromatography system Alliance (Waters, Milford, USA) equipped with an HPLC pump model 2690 and a dual wavelength absorbance UV/Vis detector model 2487. The system was controlled by Empower Software v2.0 (Waters, Milford, MA) and the detection was performed at appropriate wavelengths (compounds λ_{max}).

Retention times measurements were performed on the ZIC®-pHILIC column (Sulfoalkylbetaine phase grafted on a polymeric support, 100 x 4.6 mm, 5 µm) from SeQuant (Umeå, Sweden) and flow rate was set to 1 ml/min at room temperature (20°C) using the HPLC system.

The concentrations of stock solutions were 10^{-2} M in ACN and the injected solutions vary from 10^{-2} M to 5.10^{-4} M, depending on the UV absorbance.

The retention time (t_r) of each compound was determined in triplicate. For each mobile phase composition, the retention factor was calculated according to the formula:

$$\log k = \log\left(\frac{t_r - t_0}{t_0}\right) = \log\left(\frac{t_r}{t_0} - 1\right) \qquad \text{Eq. 2.13}$$

where t_r and t_0 are the retention times of the solute and the unretained compound (1,2-dimethoxyethan (DME)), respectively. t_0 is evaluated by an injection of DME at 60% ACN.

Due to the high lipophilicity of compounds evaluated, extrapolation was required to obtain $\log k_0$. Hence, at least five different organic modifier-buffer mobile phase ratios, chosen according to the lipophilicity (between 15 to 50% ACN), were measured and quadratic extrapolation was realised according to the following equation:

$$\log k = \log k_0 + B \bullet \varphi + A \bullet \varphi^2 \qquad \text{Eq. 2.14}$$

where $\log k$ and $\log k_0$ are the isocratic and extrapolated retention factors respectively, A and B are constants for a given solute and a given liquid chromatographic (LC) system and φ is the composition in organic modifier.

$\Delta \log k_{0\text{-}95}$ ($\log k_{0\%} - \log k_{95\%}$) were compared to $\log P^{N}_{oct}$ values obtained by traditional methods or calculated.

2.2.3 Results and discussion

Three different retention mechanisms (hydrophilic, "RP-like", and ion-exchange mechanisms) are responsible of the retention of cationic compounds on HILIC stationary phase [47]. This HILIC column particularities are illustrated in Fig. 2.12, with two opposite relationships between log k values and percentages of acetonitrile. The two behaviours ("RP-like" retention in black and hydrophilic retention in green) are present for all compounds, independently of their lipophilicity. However, it is noteworthy that the "RP-like" part is more marked for lipophilic compounds while hydrophilic retention could be higher for hydrophilic solutes.

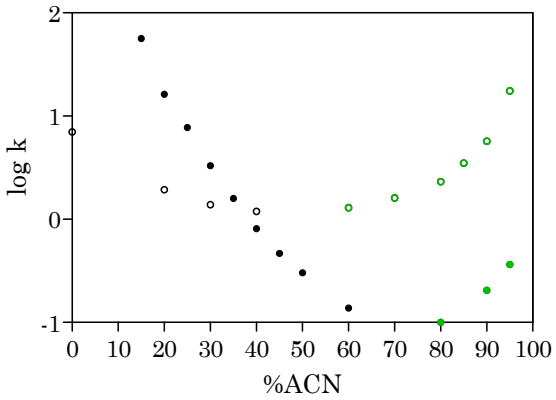


Figure 2.12: log k values plotted as a function of ACN percentages for atenolol (empty circles) (log P_{oct} = 0.22) and for amitriptyline hydrochloride (full circles) (log P_{oct} = 4.92). The black symbols correspond to the "RP-like" retention, and the green ones to the hydrophilic retention.

Five highly lipophilic and basic ($4.9 < \log P^{N}_{oct} < 7.7$ and $7.0 < pK_a < 9.6$) compounds were evaluated to enlarge the lipophilicity range of the method previously developed on 39 basic compounds with lipophilicity ranging from 1.3 to 4.6 [46]. The log k_0 (at 100% of water) extrapolated from 5 isocratic log k values and the log k_{95} (measured at 95% ACN) values of the 5 drugs were measured under their cationic form with ZIC®-pHILIC stationary phase. The difference $\Delta \log k_{0\text{-}95}$ was compared to log P^{N}_{oct} values measured by traditional methods and represented by pink circle in Fig. 2.13. All data are reported in Table 2.7.

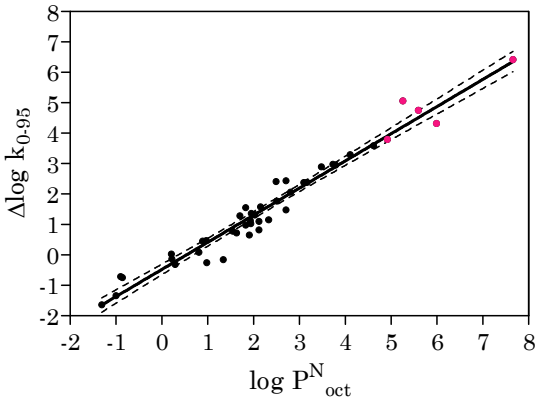


Figure 2.13: $\Delta \log k_{0\text{-}95}$ as function of log P^{N}_{oct} values obtained for the 39 compounds (black circles) evaluated by Bard et al. [46] and the 5 highly lipophilic bases (pink circles) using ACN as organic modifier in isocratic mode with a 100 x 4.6 mm ZIC®-pHILIC column. The straight line corresponds to linear regression and the dot line to the 95% confidence interval.

As shown in Fig. 2.13, the correlation obtained with 44 compounds including highly lipophilic basic compounds (Eq. 2.16) is close to the one obtained previously [46] with 39 compounds (Eq. 2.15).

$$\Delta \log k_{0\text{-}95} = 0.87(\pm 0.08) \log P^{N}_{oct} - 0.45(\pm 0.18) \qquad \text{Eq. 2.15}$$
$$n = 39, r^2 = 0.93, S = 0.34, F = 480$$

$$\Delta \log k_{0\text{-}95} = 0.89(\pm 0.06) \log P^{N}_{oct} - 0.48(\pm 0.17) \qquad \text{Eq. 2.16}$$
$$n = 44, r^2 = 0.96, S = 0.36, F = 935$$

However, it is noteworthy that even if cationic form is more polar than the neutral one, the lipophilicity range could not be enlarged indefinitely. In fact, it is difficult to evaluate the higher lipophilicity potentially measurable using this method. It was first thought the more lipophilic the analyte, the shorter and less marked the hydrophilic retention part and the closer to the t_0 the retention time at 95% ACN. However, although highly lipophilic compounds are globally less retained with 95% ACN compared to moderate and low lipophilic compounds, the more lipophilic compounds did not induce the lower log k_{95} values. Furthermore, in the "RP-like" retention part, the analysis times drastically increase with lipophilicity.

Hence, organic modifier percentages used to extrapolate log k_0 can be relatively high, increasing thus experimental error due to the quadratic regression.

Hence, HILIC can be used for lipophilicity measurements of basic compounds with $pK_a > 7$ and log P values now ranging from -1.3 to 7.7.

Table 2.7: Physicochemical properties and data obtained for the 44 tested basic compounds.

Compounds	pK_a	log P^N_{oct}	log k_{95}	log k_0	$\Delta\log k_{0\text{-}95}$
Ethanolamine [a)]	8.92 b)	-1.31 m)	1.097	-0.542	-1.639
Triethanolamine [a)]	7.76 b)	-1.00 m)	0.763	-0.574	-1.337
Octopamine [a)]	8.81 c)	-0.90 b)	1.148	0.437	-0.711
Morpholine [a)]	8.49 b)	-0.86 m)	0.475	-0.267	-0.742
Serotonine [a)]	–	0.21 m)	1.154	1.186	0.032
Atenolol [a)]	9.54 d)	0.22 d)	0.556	0.424	-0.132
Tyrosine methyl ester [a)]	–	0.29 n)	0.571	0.258	-0.313
Nadolol [a)]	9.67 d)	0.81 d)	0.503	0.587	0.084
Pseudoephedrine [a)]	10.25 b)	0.89 o)	0.130	0.583	0.453
Butylamine [a)]	10.77 b)	0.97 m)	0.286	0.755	0.469
Scopolamine [a)]	7.75 c)	0.98 c)	-0.542	-0.800	-0.258
Nicotine [a)]	3.4; 8.2 e)	1.34 e)	0.137	-0.020	-0.157
Methylephedrine [a)]	9.00 f)	1.55 f)	-0.140	0.637	0.777
Homatropine [a)]	9.81 g)	1.63 g)	-0.073	0.645	0.718
Rilmenidine [a)]	9.22 g)	1.70 g)	-0.412	0.877	1.289
Pindolol [a)]	9.54 d)	1.83 d)	0.080	1.634	1.554
Atropine [a)]	9.43 c)	1.83 m)	-0.111	0.872	0.983

Table 2.7: Continued.

Compounds	pK_a	log P^N_{oct}	log k_{95}	log k_0	$\Delta logk_{0-95}$
N,N-dimethylbenzylamine [a)]	9.02 g)	1.91 g)	-0.226	0.429	0.655
Strychnine [a)]		1.93 m)	0.056	1.182	1.126
Mepivacaine [a)]	7.6 h)	1.94 g)	-0.177	0.854	1.031
Metoprolol [a)]	9.63 d)	1.95 d)	-0.268	1.097	1.365
Acebutolol [a)]	9.92 d)	2.02 d)	-0.071	1.260	1.331
Procaine [a)]	9.0 h)	2.03 g)	-0.259	1.053	1.312
Timolol [a)]	9.53 d)	2.12 d)	0.077	0.895	0.818
Prilocaine [a)]	7.8 h)	2.12 g)	-0.171	0.930	1.101
Bisoprolol [a)]	9.57 d)	2.15 d)	-0.413	1.168	1.581
Lidocaine [a)]	7.9 h)	2.33 g)	-0.395	0.760	1.155
Apomorphine [a)]	–	2.49 m)	0.332	2.742 r)	2.41
Oxprenolol [a)]	9.57 d)	2.51 d)	-0.343	1.43	1.773
MPTP [a)]	–	2.71 m)	-0.181	1.296	1.477
Tacrine [a)]	9.95 b)	2.71 m)	0.164	2.601 r)	2.437
Metipranolol [a)]	9.54 d)	2.81 d)	-0.498	1.565	2.063
Alprenolol [a)]	9.59 d)	3.10 d)	-0.359	2.017 r)	2.376
Moxisylyte [a)]	8.72 c)	3.17 p)	-0.857	1.529	2.386
Propranolol [a)]	9.53 d)	3.48 d)	-0.190	2.709 r)	2.899

Table 2.7: Continued.

Compounds	pK_a	log P^N_{oct}	log k_{95}	log k_0	$\Delta logk_{0-95}$
Carazolol [a)]	9.52 d)	3.73 d)	-0.009	2.972 r)	2.981
Verapamil [a)]	8.92 c)	3.79 m)	-1.265	1.689 r)	2.954
Carvedilol [a)]	7.97 d)	4.11 d)	-0.200	3.099 r)	3.299
Penbutolol [a)]	9.92 d)	4.62 d)	-0.511	3.078 r)	3.589
Amitriptyline hydrochloride	9.01 i)	4.92 m)	-0.440	3.353 r)	3.793
Ritanserin	7.75 j)	5.26 j)	-0.411	4.652 r)	5.063
Lidoflazine	6.98 j)	5.60 q)	-0.519	4.235 r)	4.754
Thioridazine hydrochloride	9.59 k)	5.99 k)	-0.142	4.179 r)	4.321
Clofazimine	8.51 l)	7.66 l)	-0.630	5.790 r)	6.420

a) Compounds already published in [57]. b) Taken from [58]. c) Taken from [59]. d) Taken from [60]. e) Taken from [61]. f) Taken from [62]. g) Taken from [63]. h) Taken from [64]. i) Taken from [65]. j) Calculated using Advanced Chemistry Development (ACD/Labs) Software V8.0. k) Taken from [66]. l) Taken from [67]. m) Taken from [68]. n) Taken from [69]. o) Taken from [70]. p) Taken from [71]. q) Taken from [72]. r) obtained by quadratic extrapolation.

2.2.4. Conclusion and perspective

Thanks to the HILIC stationary phase, it was demonstrated that the lipophilicity range could successfully be enlarged up to $\log P_{oct} = 7.7$, for the lipophilicity determination of basic compounds with $pK_a > 7$. Hence, a correlation between $\log P^N_{oct}$ and $\Delta\log k_{0\text{-}95}$ values allows the determination of n-octanol/water partition coefficient, with $\Delta\log k_{0\text{-}95}$ value corresponding to the difference between $\log k_{95}$ and $\log k_0$ (obtained by extrapolation due to the high lipophilicity) of the cationic form of the compounds. This is an especially relevant way to proceed for basic compounds with $pK_a > 7$ which are sorely measurable under unionized form using RPLC methods because of chemical instability of many stationary phases at high pH conditions.

Thus, more compounds would be evaluated to confirm this result for this lipophilicity enlargement up to $\log P^N_{oct} = 7.7$ and maybe evaluate the upper-limit of this method.

2.3 General conclusion

To summarize all the different methods evaluated in this chapter, a synthetic scheme was realised to help the readers choosing the more adapted method for his needs. Indeed, depending on the type of compounds, basic with $pK_a > 7$ or not, there are two possibilities. For the former type, only the HILIC method is available. For neutral, acidic and weakly basic compounds, depending on the precision (evaluation or determination), the pH, the system available (RPLC or UHPLC) or the rapidity of the method, four methods are available as shown in Fig. 2.14. Indeed, a first approximation of the lipophilicity can be obtained using Acquity UPLC™ BEH Shield RP18 column in gradient mode. This method can be very useful for a first screening since it is a generic and rapid method. This stationary phase should also be chosen for more accurate lipophilicity measurements in isocratic mode if buffer solution of relatively high pH are needed since this stationary phase can withstand pH up to 10.5. But in other cases, Hypersil™ GOLD Javelin HTS of 10 mm is the stationary phase of choice since the analysis time remains reasonable even for very highly lipophilic compounds.

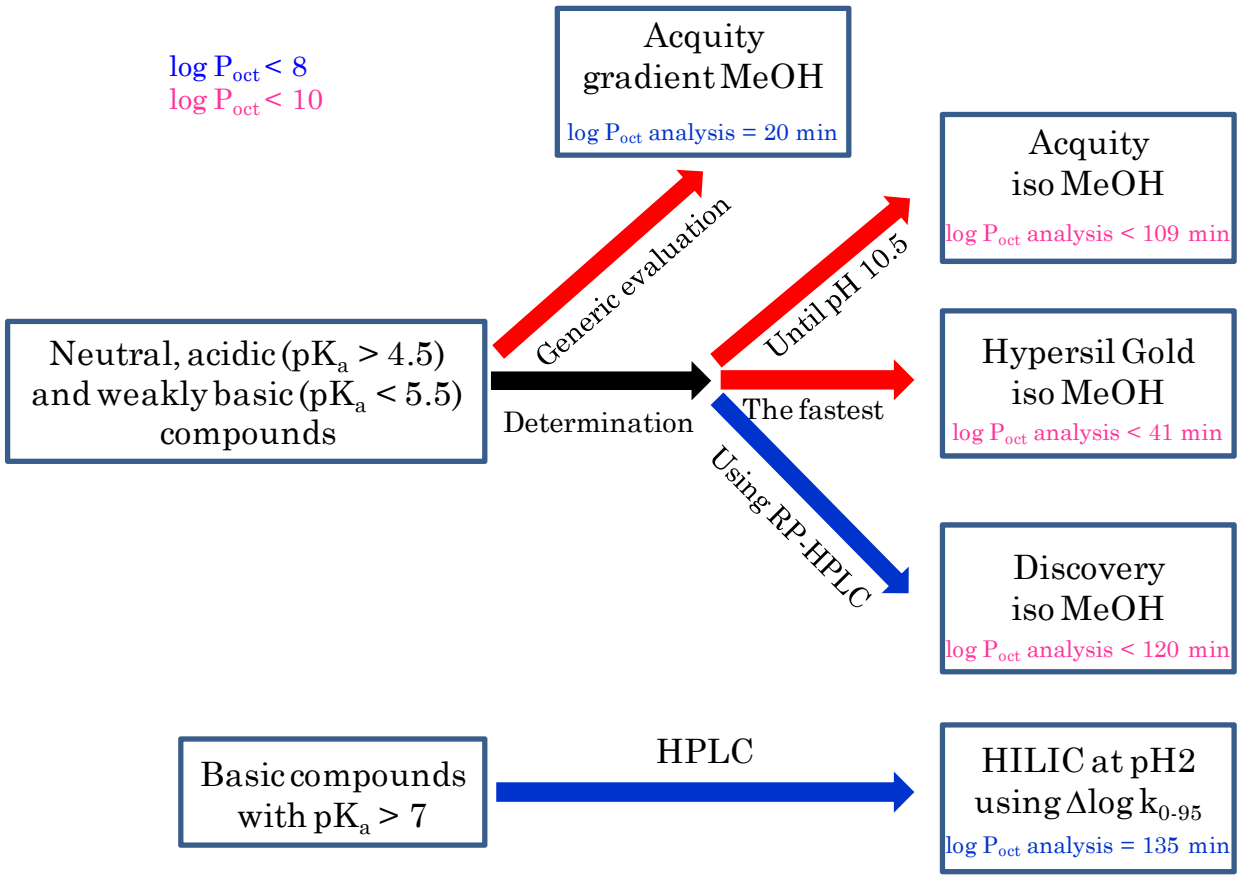


Fig. 2.14: Decisional tree depending on the needs and the compounds evaluated. Red and blue arrows are for UHPLC and HPLC systems, respectively.

References

1. Lombardo, F.; Shalaeva, M. Y.; Tupper, K. A.; Gao, F.; Abraham, M. H. $ElogP_{oct}$: a tool for lipophilicity determination in drug discovery. *J. Med. Chem.* **2000,** *43*, 2922-2928.

2. Wang, J. L.; Skolnik, S. Recent advances in physicochemical and ADMET profiling in drug discovery. *Chem. Biodivers.* **2009,** *6*, 1887-1899.

3. Camenisch, G.; Folkers, G.; van de Waterbeemd, H. Review of theoretical passive absorption models: historical background, recent developments and limitations. *Pharm. Acta Helv.* **1996,** *71*, 309-327.

4. Pedersen, J. M.; Matsson, P.; Bergström, C. A. S.; Norinder, U.; Hoogstraate, J.; Artursson, P. Prediction and identification of drug interactions with the human ATP-binding cassette transporter multidrug-resistance associated protein 2 (MRP2;ABCC2). *J. Med. Chem.* **2008,** *51*, 3275-3287.

5. Lobell, M.; Sivarajah, V. In silico prediction of aqueous solubility, human plasma protein binding and volume of distribution of compounds from calculated pK(a) and AlogP98 values. *Mol. Diversity* **2003,** *7*, 69-87.

6. Bajda, M.; Boryczka, S.; Malawska, B. Investigation of lipophilicity of anticancer-active thioguinoline derivatives. *Biomed. Chromatogr.* **2007,** *21*, 123-131.

7. Gocan, S.; Cimpan, G.; Comer, J. Lipophilicity measurements by liquid chromatography. In *Advances in Chromatography*; Grushka, E.; Grinberg, N. Eds.; Taylor & Francis Group: Boca Raton, 2006; pp 79-176.

8. Martel, S.; Guillarme, D.; Henchoz, Y.; Galland, A.; Veuthey, J. L.; Rudaz, S.; Carrupt, P. A. Chromatographic approaches for measuring log P. In *Drug Properties: Measurement and Computation*; Mannhold, R. Ed.; Wiley-VCH: Weinheim, 2008; pp 331-356.

9. Avdeef, A.; Testa, B. Physicochemical profiling in drug research: a brief survey of the state-of-the-art of experimental techniques. *Cell. Mol. Life Sci.* **2002,** *59*, 1681-1689.

10. Tsai, R. S.; Lisa, G.; Carrupt, P. A.; Testa, B. Centrifugal partition chromatography for lipophilicity measurements. In *Lipophilicity in Drug Action and Toxicology*; Pliska, V.; Testa, B.; van de Waterbeemd, H. Eds.; VCH Publishers: Weinheim, 1996; pp 89-107.

11. Hanse, S. H. Recent applications of microemulsion electrokinetic chromatography. *Electrophoresis* **2003,** *24*, 3900-3907.

12. Nasal, A.; Kaliszan, R. Progess in the use of HPLC for evaluation of lipophilicity. *Curr. Computer-Aided Drug Des.* **2006,** *2*, 327-340.

13. van de Waterbeemd, H.; Kansy, M.; Wagner, B.; Fischer, H. Lipophilicity measurement by reversed-phase high performance liquid

chromatography. In *Lipophilicity in Drug Action and Toxicology*; Pliska, V.; Testa, B.; van de Waterbeemd, H. Eds.; VCH Publishers: Weinheim, 1996; pp 73-87.

14. Poole, S. K.; Poole, C. F. Separation methods for estimating octanol-water coefficients. *J. Chromatogr. B* **2003,** *797*, 3-19.

15. Kaliszan, R.; Haber, P.; Baczek, T.; Siluk, D.; Valko, K. Lipophilicity and pK_a estimates from gradient high-performance liquid chromatography. *J. Chromatogr. A* **2002,** *965*, 117-227.

16. Nasal, A.; Siluk, D.; Kaliszan, A. Chromatographic retention parameters in medicinal chemistry and molecular pharmacology. *Curr. Med. Chem.* **2003,** *10*, 381-426.

17. Kaliszan, R.; Kaliszan, A.; Wainer, I. W. Deactivated hydrocarbonaceous silica and immobilized artificial membrane stationary phases in high-performance liquid chromatographic determination of hydrophobicities of organic bases: relationship to log P and CLOGP. *J. Pharm. Biomed. Anal.* **1993,** *11*, 505-511.

18. Lambert, W. J. Modeling oil-water partitioning and membrane permeation using reversed-phase chromatography. *J. Chromatogr.* **1993,** *656*, 469-484.

19. Donovan, S. F.; Pescatore, M. C. Method for measuring the logarithm of the octanol-water partition coefficient by using short octadecyl-poly(vinyl alcohol) high-performance liquid chromatography columns. *J. Chromatogr. A* **2002,** *952*, 47-61.

20. Ayouni, L.; Cazorla, G.; Chaillou, D.; Herbreteau, B.; Rudaz, S.; Lanteri, P.; Carrupt, P. A. Fast determination of lipophilicity by HPLC. *Chromatographia* **2005,** *62*, 251-255.

21. Henchoz, Y.; Guillarme, D.; Rudaz, S.; Veuthey, J. L.; Carrupt, P. A. High-throughput log *P* determination by ultraperformance liquid chromatography: a convenient tool for medicinal chemists. *J. Med. Chem.* **2008,** *51*, 396-399.

22. Pagliara, A.; Khamis, E.; Trinh, A.; Carrupt, P. A.; Tsai, R. S.; Testa, B. Structural properties governing retention mechanisms on RP-HPLC stationary phase used for lipophilicity measurements. *J. Liquid Chromatogr.* **1995,** *18*, 1721-1745.

23. Stella, C.; Galland, A.; Liu, X.; Testa, B.; Rudaz, S.; Veuthey, J. L.; Carrupt, P. A. Novel RPLC stationary phases for lipophilicity measurement: solvatochromic analysis of retention mechanisms for neutral and basic compounds. *J. Sep. Sci.* **2005,** *28*, 2350-2362.

24. Coym, J. W. Comparison of retention on traditional alkyl, polar endcapped, and polar embedded group stationary phases. *J. Sep. Sci.* **2008,** *31*, 1-7.

25. Henchoz, Y.; Guillarme, D.; Martel, S.; Rudaz, S.; Veuthey, J. L.; Carrupt, P. A. Fast log determination by ultra high pressure liquid chromatography coupled with UV and mass spectrometry detections. *Anal. Bioanal. Chem.* **2009,** *394*, 1919-1930.

26. Horvath, C.; Melander, W. Liquid chromatography with hydrocarbonaceous bonded phases; theory and practice of reversed phase chromatography. *J. Chromato. Sci.* **1977,** *15*, 393-404.

27. Reymond, D.; Chung, G. N.; Mayer, J. M.; Testa, B. Lipophilicity measurement of nicotinates by RP-HPLC. Differences in retention behaviour, but similarities of log k_w values, in methanol-water and acetonitrile-water eluents. *J. Chromatogr.* **1987,** *391*, 97-109.

28. Valko, K.; Snyder, L. R.; Glajch, J. L. Retention in reversed-phase liquid chromatography as a function of mobile-phase composition. *J. Chromatogr.* **1993,** *656*, 501-520.

29. Snyder, L. R.; Dolan, J. W. Initial experiments in high-performance liquid chromatographic method development. I. Use of a starting gradient run. *J. Chromatogr. A* **1996,** *721*, 3-14.

30. Dolan, J. W. Dwell volume revisited. *LC. GC North America* **2006,** *24*, 458-466.

31. Guillarme, D.; Nguyen, D. T. T.; Rudaz, S.; Veuthey, J. L. Method transfer for fast liquid chromatography in pharmaceutical analysis: Application to short columns packed with small particle. Part II: Gradient experiments. *Eur. J. Pharm. Biopharm.* **2008,** *68*, 430-440.

32. Jandera, P. Can the theory of gradient liquid chromatography be useful in solving practical problems? *J. Chromatogr. A* **2006,** *1126*, 195-218.

33. Snyder, L. R.; Kirkland, J. J. *Introduction to Modern Liquid Chromatography*; Wiley-Interscience: West Sussex, 1979.

34. Giaginis, C.; Theocharis, S.; Tsantili-Kakoulidou, A. Octanol/water partitioning simulation by reversed-phase high performance liquid chromatography for structurally diverse acidic drugs: Effect of n-octanol as mobile phase additive. *J. Chromatogr. A* **2007,** *1166*, 116-125.

35. Liu, X.; Tanaka, H.; Yamauchi, A.; Testa, B.; Chuman, H. Determination of lipophilicity by reversed-phase high-performance liquid chromatography. Influence of 1-octanol in the mobile phase. *J. Chromatogr. A* **2005,** *1091*, 51-59.

36. Liu, X. *New approaches to measure lipophilicity: applications and interpretation*; Thesis: University of Lausanne, 2003.

37. Valko, K.; Du, C. M.; Bevan, C.; Reynolds, D. P.; Abraham, M. H. Rapid method for the estimation of octanol/water partition coefficient (log P_{oct}) from gradient RP-HPLC retention and a hydrogen bond acidity term ($\Sigma\alpha_2^H$). *Curr. Med. Chem.* **2001,** *8*, 1137-1146.

38. Klopman, G.; Stefan, L. R.; Saiakhov, R. D. ADME evaluation. A computer model for the prediction of intestinal absorption in humans. *Eur. J. Pharm. Sci.* **2002,** *17*, 253-263.

39. Tate, P. A.; Dorsey, J. G. Column selection for liquid chromatographic estimation of the k'_w hydrophobicity parameter. *J. Chromatogr. A* **2004,** *1042*, 37-48.

40. Hsieh, M. M.; Dorsey, J. G. Accurate determination of log k'_w in reversed-phase liquid chromatography. Implications for quantitative structure-retention relationships. *J. Chromatogr.* **1993,** *631*, 63-78.

41. Schoenmakers, P. J.; Billiet, H. A. H.; de Galan, L. Description of solute retention over the full range of mobile phase compositions in reversed-phase liquid chromatography. *J. Chromatogr. A* **1983,** *282*, 107-121.

42. Kirkland, J. J.; van Straten, M. A.; Claessens, H. A. High pH mobile phase effects on silica-based reversed-phase high performance liquid chromatographic columns. *J. Chromatogr. A* **1995,** *691*, 3-19.

43. Nawrocki, J. The silanol group and its role in liquid chromatography. *J. Chromatogr. A* **1997,** *779*, 29-71.

44. Giaginis, C.; Tsantili-Kakoulidou, A. Current state of the art in HPLC methodology for lipophilicity assessment of basic drugs. A review. *J. Liquid Chromatogr. Rel. Technol.* **2008,** *31*, 79-96.

45. Alpert, A. J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **1990,** *499*, 177-196.

46. Bard, B.; Carrupt, P. A.; Martel, S. Lipophilicity of basic drugs measured by hydrophilic interaction chromatography. *J. Med. Chem.* **2009,** *52*, 3416-3419.

47. McCalley, D. V. Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds? *J. Chromatogr. A* **2007,** *1171*, 46-55.

48. Hemström, P.; Irgum, K. Hydrophilic interaction chromatography. *J. Sep. Sci.* **2006,** *29*, 1784-1821.

49. Orth, P.; Engelhardt, H. Separation of sugars on chemically modified silica gel. *Chromatographia* **1982,** *15*, 91-96.

50. Verhaar, L. A. Th.; Kuster, B. F. M. Contribution to the elucidation of the mechanism of sugar retention on amine-modified silica in liquid chromatography. *J. Chromatogr. A* **1982,** *234*, 57-64.

51. Nikolov, Z. L.; Reilly, P. J. Retention of carbohydrates on silica and amine-bonded silica stationary phases: application of the hydration model. *J. Chromatogr.* **1985,** *325*, 287-293.

52. Bidlingmeyer, B. A.; Del Rios, J. K.; Korpl, J. Separation of organic amine compounds on silica gel with reversed-phase eluents. *Anal. Chem.* **1982,** *54*, 442-447.

53. Cox, G. B.; Stout, R. W. Study of the retention mechanisms for basic compounds on silica under "pseudo-reversed-phase" conditions. *J. Chromatogr.* **1987,** *384*, 315-336.

54. McKeown, A. P.; Euerby, M. R.; Lomax, H.; Johnson, C. M.; Ritchie, H. J.; Woodruff, M. The use of silica for liquid chromatographic/mass

spectrometric analysis of basic analytes. *J. Sep. Sci.* **2001,** *24*, 835-842.

55. Avdeef, A.; Comer, J. E. A.; Thomson, S. J. pH-metric log P.3. glass electrode calibration in methanol-water, applied to pKa determination of water-insoluble substances. *Anal. Chem.* **1993,** *65*, 42-49.

56. Roses, M.; Bosch, E. Influence of mobile phase acid-base equilibria on the chromatographic behaviour of protolytic compounds. *J. Chromatogr.* **2002,** *982*, 1-30.

57. Bard, B. *Development of new experimental tools for the fast determination of solubility and lipophilicity*; PhD Thesis: University of Geneva, 2008.

58. Perrin, D. D.; Dempsey, B.; Serjeant, E. P. *pK$_a$ Prediction for Organic Acids and Bases*; Chapman and Hall: New York, 1981.

59. Sangster, J. *Octanol-Water Partition Coefficients: Fundamentals and Physical Chemistry*; Wiley & Sons: Chichester, 1997.

60. Caron, G.; Steyaert, G.; Pagliara, A.; Crivori, P.; Gaillard, P.; Carrupt, P. A.; Avdeef, A.; Box, K. J.; Girault, H. H.; Testa, B. Structure-lipophilicity relationships of the neutral and cationic forms of β-blockers. Part I. Partitioning in isotropic systems. *Helv. Chim. Acta* **1999,** *82*, 1211-1222.

61. Reymond, F.; Chopineaux-Courtois, V.; Steyaert, G.; Bouchard, G.; Carrupt, P. A.; Testa, B.; Girault, H. H. Ionic partition of ionisable drugs: pH-lipophilicity profiles, transfer mechanisms and charge effects on solvation. *J. Electroanal. Chem.* **1999,** *462*, 235-250.

62. Reymond, F.; Carrupt, P. A.; Testa, B.; Girault, H. H. Charge and delocalisation effects on the lipophilicity of protonable drugs. *Chem. Eur. J.* **1999,** *5*, 39-47.

63. Galland, A. *Towards the validation of in silico models and physicochemical filters to identify and characterize new chemical entities*; PhD Thesis: University of Lausanne, 2004.

64. Barbato, F.; La Rotonda, M. I.; Quaglia, F. Chromatographic indexes on immobilized artificial membranes for local anesthetics: relationships with activity data on closed sodium channels. *Pharm. Res.* **1997,** *14*, 1699-1705.

65. Delmar Cantu, M.; Hillebrand, S.; Carrilho, E. Determination of the dissociation constants (pK_a) of secondary and tertiary amines in organic media by capillary electrophoresis and their role in the electrophoretic mobility order inversion. *J. Chromatogr.* **2005,** *1068*, 99-105.

66. Dross, K. P.; Mannhold, R.; Rekker, R. F. Drug lipophilicity in QSAR practice. II. Aspects of RM-determinations; critics of RM-corrections; interrelations with partition coefficients. *Quant. Struct. -Act. Relat.* **1992,** *11*, 36-44.

67. Quigley, J. M.; Fahelelbom, K. M. S.; Timoney, R. F.; Corrigan, O. I. Temperature dependence and thermodynamics of partitioning of clofazimine analogues in the n-octanol/water system. *Int. J. Pharm.* **1990,** *58*, 107-113.

68. Hansch, C.; Leo, A. *Exploring QSAR. Fundamentals and Applications in Chemistry and Biology*; ACS Professional Reference Book, American Chemical Society: Washington, 1995; pp 1-557.

69. Chong, H. H.; Ryohei, K.; Rima, B.-N.; Anwar, H. Mechanism of nasal absorption of drugs II: absorption of L-tyrosine and the effect of structural modification on its absorption. *J. Pharm. Sci.* **2006,** *74*, 1298-1301.

70. Kril, M. B.; Fung, H. L. Influence of hydrophobicity on the ion exchange selectivity coefficients for aromatic amines. *J. Pharm. Sci.* **1990,** *79*, 440-443.

71. Dallet, P.; Dubost, J. P.; Colleter, J. C.; Audry, E.; Creuzet, M. H. QSAR in a series of α-adrenolytic molecules: optimization of physicochemical parameters. *J. Med. Chem.* **1985,** *20*, 551-557.

72. Zerara, M.; Brickmann, J.; Kretschmer, R.; Exner, T. Parameterization of an empirical model for the prediction of n -octanol, alkane and cyclohexane/water as well as brain/blood partition coefficients. *J. Comput. Aided Mol. Des.* **2009,** *23*, 105-111.

Chapter 3: Experimental log P_{oct} value of cyclosporin A and C by LC.

3.1 Introduction

Cyclosporin A (CsA) is a cyclic undecapeptide isolated from the fungal species Tolypocladium inflatum gams. This drug is an immunosuppressant widely used in post-allogeneic organ transplant to reduce the activity of the patient's immune system and, hence the risk of organ rejection. It has been largely studied in transplants of heart [1, 2], kidney [3, 4], liver [5, 6], lung [7, 8], pancreas [9, 10], and bone marrow [4, 11]. Cyclosporin A is also an effective drug for the treatment of rheumatoid arthritis [12, 13], inflammatory bowel disease [14, 15] (systemic immune-related disorders [16, 17]), and in dermatological [18, 19] and ophthalmological [20, 21] disorders.

Large number of studies and reviews was devoted to cyclosporin A and lipophilicity experimental values reported were ranging from 1.0 to 3.6 [22-24]. Furthermore, a large range of calculated values of the lipophilicity of the cyclosporin A was reported from 1.0 (EPI Suite software) to 14.4 (CLOGP software) and the very low aqueous solubility [25-28] seems to involve a high lipophilicity. Hence, because of these different values and properties not in accordance for the lipophilicity, the log P_{oct} value of this compound and the cyclosporin C (CsC) (very similar) were evaluated using the different methods developed in chapter 2 to determine high lipophilicity.

Indeed, isocratic mode with methanol as organic modifier was used in this chapter since it is the method of choice for the log P_{oct} determination using the three stationary phases, tested in chapter 2. Hence, the 20-mm Discovery® RP Amide C16 column was used on the HPLC (high pressure liquid chromatography) system. The UHPLC (ultra high pressure liquid chromatography) approach was also employed as it reduces analysis time. Consequently, the 30-mm Acquity BEH UPLC™ RP18 and the 10-mm Hypersil™ GOLD Javelin HTS (inducing the most rapid and accurate

results) stationary phases were used. Different log P_{oct} values were obtained for CsA and CsC depending on the stationary phase utilized. Therefore, more compounds with specific particularity (ie. linear and cyclic peptides less lipophilic and compounds with important volume and different lipophilicity) were also evaluated. Regarding the results obtained, preliminary dynamic study was performed on cyclosporin A to evaluate the conformational implication on expected experimental log P values.

3.2 Material and methods

3.2.1 Chemicals

Cyclosporin A was purchased from Fluka (Buchs, Switzerland). Cyclosporin C was obtained from Toronto Research Chemicals (Ontario, Canada). Beauvericin and isovaleryl-Phe-Nle-Sta-Ala-Sta-OH were purchased from Bachem (Bubendorf, Switzerland). Bromophenol blue and digitoxin were obtained from Sigma-Aldrich (Buchs, Switzerland). 5,5'-[1,4-phenylenebis(oxy)]bis[2-(3-hydroxypropyl)-1H-isoindole-1,3(2H)-dione] was purchased from ChemBridge (Sna Diego, CA, USA). 3-benzo[1,3]dioxol-5-yl-N-{3-[(3-benzo[1,3]dioxol-5-yl-2-cyano-acryloylamino)-methyl]-benzyl}-2-cyano-acrylamide was obtained from Enamine (Kiev, Ukraine). HPLC grade methanol was purchased from VWR (Dietikon, Switzerland). Water was obtained with the Milli-Q Water Purification System from Millipore (Milford, MA, USA).

3.2.2 Buffer solutions and organic modifier

To analyse all the compounds under their neutral form, two buffers with different pH values (trifluoroacetic acid / ammonia pH 2 and acetic acid / ammonia pH 5) were prepared. An ionic strength of 20 mM was chosen according to Phoebus software v1.0 (Analis, Namur, Belgium). Buffer solutions were filtered through a 0.22 μm HA Millipore filter (Millipore, Bedford, MA, USA).

Methanol (MeOH) was used as organic modifier since it was the co-solvent of choice in chapter 2. Indeed, better correlations with shorter analysis times were obtained with this organic modifier rather than acetonitrile or tetrahydrofuran.

3.2.3 Liquid chromatography instrumentations

HPLC measurements were performed on a liquid chromatography system Alliance (Waters, Milford, USA) equipped with an HPLC pump model 2690 and a dual wavelength absorbance UV/Vis detector model 2487. An Acquity UPLC system (Waters, Milford, USA) including a binary solvent manager, a sample manager with an injection loop volume of 2 µL, a photo diode array (PDA) programmable detector, and a column manager with oven, was used for UHPLC measurements. Both systems were controlled by Empower Software v2.0 (Waters, Milford, MA) and the detection was performed at appropriate wavelengths (compounds λ_{max}).

Retention times measurements with the HPLC system were realised using a Discovery® RP Amide C16 column (20 x 4 mm ID, 5 µm) (Supelco, Bellefonte, PA, USA) at a flow rate of 1 mL/min and at room temperature (20°C).

The stationary phases tested on the UHPLC system were the Hypersil™ GOLD Javelin HTS (10 x 2.1 mm ID, 1.9 µm) (Thermo Scientific Runcorn, UK) and the Acquity UPLC™ BEH Shield RP18 (30 x 2.1 mm ID, 1.7 µm) (Waters, Milford, MA) at a flow rate of 1 and 0.5 mL/min (due to the backpressure), respectively, and at 30 ± 0.1°C.

The concentrations of stock solutions were 10^{-2} M in MeOH and the injected solutions vary from 10^{-2} M to 5.10^{-4} M, depending on the UV absorbance.

3.2.4 Method for lipophilicity determination

The retention time (t_r) of each compound was determined in triplicate on five different organic modifier-water mobile phase ratios. For each

mobile phase composition, the retention factor was calculated according to the formula (as explained in part 2.1.2.5.1 of chapter 2):

$$\log k = \log\left(\frac{t_r - t_{delay} - (V_{ext}/F)}{t_0 - t_{delay} - (V_{ext}/F)} - 1\right) \qquad \text{Eq 3.1}$$

where t_r and t_0 are the retention times of the solute and the unretained compound (uracil), respectively. t_{delay} is the injection delay, V_{ext} the extra-column volume and F the flow rate of the mobile phase. t_0 is evaluated by an injection of uracil at 40% ACN.

As explained in chapter 2 §2.1.2.5.1, extrapolated retention factors corresponding to pure water as mobile phase ($\log k_w$) were used for the determination of $\log P_{oct}$ values. Thus, five retention factors ($\log k$) were measured at different percentages of methanol (optimized according to the lipophilicity of the investigated compound) and $\log k_w$ value is obtained by extrapolation to 100% water (from these five isocratic log k values plotting as a function of the mobile phase composition (φ)). With MeOH, the extrapolation to 100% water is linear [29] and given by:

$$\log k = \log k_w - S \bullet \varphi \qquad \text{Eq 3.2}$$

where $\log k$ and $\log k_w$ are the isocratic and extrapolated retention factors respectively, φ the mobile phase composition in organic modifier and S (the slope) a constant for a given solute and fixed experimental conditions.

$\log k_w$ can finally be correlated to $\log P_{oct}$ values (the partition coefficient of the solute in n-octanol/water system) by the following equations determined using the three stationary phases:

$$\log k_{w\,Discovery} = 0.87(\pm 0.02)\log P_{oct} - 0.10(\pm 0.11)$$
$$n = 57, r^2 = 0.99, S = 0.22, F = 5645 \qquad \text{Eq. 3.3}$$

$$\log k_{w\,Acquity} = 0.81(\pm 0.02)\log P_{oct} + 0.28(\pm 0.07)$$
$$n = 57, r^2 = 0.99, S = 0.15, F = 10270 \qquad \text{Eq. 3.4}$$

$$\log k_{w\,Hypersil} = 0.83(\pm 0.02)\log P_{oct} + 0.26(\pm 0.09)$$
$$n = 57, r^2 = 0.99, S = 0.18, F = 7310 \qquad \text{Eq. 3.5}$$

In these equations, n is the number of compounds, r^2 the determination coefficient, s the standard deviation and F the Fisher's test value. 95% confidence intervals are given in parentheses.

These equations were analysed and discussed in chapter 2 § 2.1.3.5.

3.2.5 Molecular modelling

3.2.5.1 Molecular lipophilicity potential

Molecular lipophilicity potential (MLP) calculations were performed on a dot-filled molecular surface around all cyclosporin A snapshots from the molecular dynamics (MD) simulations (without solvent molecules). The MLP was developed as a molecular interaction field describing the distribution of lipophilicity around a conformation. It relies on a fragmental system built from experimental partition values measured in the n-octanol/water biphasic system ($\log P_{oct}$) (refer to [30-32] for details). Besides giving a qualitative three-dimentional (3D) map of hydrophobic and polar zones around cyclosporin A in the two solvents, the MLP allows the prediction of n-octanol/water partition coefficient in a conformation-dependant manner. Indeed, a mathematically integrating MLP values born by all points of the surface to get a global $\log P_{MLP}$ (partition coefficient determined using MLP) for the conformation was used.

3.2.5.2 Molecular dynamics

Geometry optimizations and molecular dynamics simulations were carried out with the Amber11 package [33]. For standard amino acids, the force field parameters and the charges were taken from the Amber ff99SB set. For the non-standard amino acids, the general amber force field (gaff) [34] with RESP (restrained electrostatic potential) charges computed after an Hartree-Fock (HF/6-31g*) optimization was used. The starting point for the simulations was a cyclosporin A structure extracted from a crystallographic complex with cyclophilin D (from the protein data bank,

PDB ID: 2Z6W) [35]. Two different octahedral solvent boxes were built around this structure, the first made of 1092 water molecules and the second of 667 methanol molecules. All two systems were first geometrically optimized. Thereafter, the cyclosporin A molecule was frozen and the solvent boxes were first heated up to 300 K at constant volume during 50 ps (picoseconds) and then equilibrated at constant pressure of 1 atmosphere (atm) during 50 ps. Following this, the two whole systems were equilibrated at constant temperature and constant pressure during 200 ps without any constraint. Finally, the molecular dynamics simulation was carried out during 9.8 nanoseconds (ns) at constant temperature and pressure, snapshots being taken each 10 ps.

3.3 Results and discussion

3.3.1 log k_w measurement of the cyclosporins A and C using the three different stationary phases

The retention factors extrapolated to 100% water (log k_w) of cyclosporins A and C were measured, on the three stationary phases (Discovery® RP Amide C16, Acquity UPLC™ BEH Shield RP18 and Hypersil™ GOLD Javelin HTS) in isocratic mode using methanol as organic modifier. log P_{oct} values of these two compounds were determined using the corresponding equations (see materials and methods §3.2.4). The different log k_w and log P_{oct} values for the two cyclosporins were reported in Table 3.1.

Table 3.1: Different log k_w and log P_{oct} values obtained for the two cyclosporins in isocratic mode with MeOH as organic modifier using Discovery® RP Amide C16 (Discovery), Acquity UPLC™ BEH Shield RP18 (Acquity) and Hypersil™ GOLD Javelin HTS (Hypersil) columns

		Discovery		Acquity		Hypersil	
Solute	ClogP[a]	log k_w	log P_{oct}[b]	log k_w	log P_{oct}[c]	log k_w	log P_{oct}[d]
Cyclosporin A	14.36	7.83	9.09	8.54	10.24	9.35	10.99
Cyclosporin C	13.19	7.46	8.66	7.98	9.54	9.02	10.59

a) CLogP values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005). b) log P_{oct} values determined using Eq. 3.3. c) log P_{oct} values determined using Eq. 3.4. d) log P_{oct} values determined using Eq. 3.5.

The log P_{oct} values obtained for the two cyclosporins were ranking as expected, since the cyclosporin C (CsC) is less lipophilic, due to the addition of a hydroxy group, than the cyclosporin A (CsA).

However, different log P_{oct} values were obtained for both compounds depending on the stationary phase used. Indeed, a difference of 1.9 units between log P_{oct} values determined using Discovery® RP Amide C16 and Hypersil™ GOLD Javelin HTS columns was obtained for the two cyclosporins.

Several parameters were thought to be implicated in these different log P_{oct} values obtained for the two cyclosporins such as the very high lipophilicity, the important volume (from 3.12 to 6.20 compared to the reference compounds volume from 0.75 to 2.79), or the flexibility induce by the structure of the molecule.

Thus, 7 additional compounds were also evaluated to explain these diverging results.

3.3.2 Evaluation of the behaviour of additional compounds on the three different stationary phases

To evaluate if the important volume of the two cyclosporins are responsible of these differences between the log P_{oct} values determined with the three stationary phases, 7 more compounds with an important volume but different lipophilicity (from 2.8 to 14.8) and varied structures were tested. Hence, the bromophenol blue, the digitoxin, the 5,5'-[1,4-phenylenebis(oxy)]bis[2-(3-hydroxypropyl)-1H-isoindole-1,3(2H)-dione], the 3-benzo[1,3]dioxol-5-yl-N-{3-[(3-benzo[1,3]dioxol-5-yl-2-cyano-acryloylamino)-methyl]-benzyl}-2-cyano-acrylamide and the octaphenylcyclotetrasiloxane were chosen. The last compound was added to exclude the involvement of the too high lipophilicity into the differences obtained for the two cyclosporins.

Moreover, two peptides were also tested, the beauvericin (dipepsipeptide) and the isovaleryl-Phe-Nle-Sta-Ala-Sta-OH. The former is a cyclopeptide, and the latter is a linear peptide. No intra molecular hydrogen-bond (H-bond) could be realised in beauvericin whereas in cyclosporins H-bond could occurred. Although the second one is linear, this compound is hence flexible as the cyclosporins, with possible intra molecular H-bond [36].

All these additional compounds were evaluated on the three stationary phases in isocratic mode using methanol as organic modifier and the different results were reported in Table 3.2.

Table 3.2: log k_w and log P_{oct} values of the 7 additional compounds obtained with Discovery® RP Amide C16 (Discovery), Acquity UPLC™ BEH Shield RP18 (Acquity) and Hypersil™ GOLD Javelin HTS (Hypersil) columns.

		Discovery		Acquity		Hypersil	
Solute	ClogP[a)]	log k_w	log P_{oct}[b)]	log k_w	log P_{oct}[c)]	log k_w	log P_{oct}[d)]
Compounds with important volume (green circles in Fig. 3.1, 3.2 and 3.3)							
Digitoxin	2.85	4.65	5.44	4.62	5.38	4.82	5.51
3-Benzo[1,3]dioxol-5-yl-N-{3-[(3-benzo[1,3]dioxol-5-yl-2-cyano-acryloylamino)-methyl]-benzyl}-2-cyano-acrylamide	3.65	4.09	4.80	4.28	4.96	4.24	4.81
5,5'-[1,4-phenylenebis(oxy)]bis[2-(3-hydroxypropyl)-1H-isoindole-1,3(2H)-dione]	4.03	4.14	4.85	4.05	4.67	4.59	5.24
Bromophenol blue	5.51	3.36	3.96	3.73	4.28	3.88	4.37
Peptides (red circles in Fig. 3.1, 3.2 and 3.3)							
Isovaleryl-Phe-Nle-Sta-Ala-Sta-OH	4.22	4.92	5.75	5.12	6.00	5.52	6.36
Beauvericin	9.57	5.92	6.89	6.25	7.40	6.77	7.87
The more lipophilic compound (blue circle in Fig. 3.1, 3.2 and 3.3)							
Octaphenylcyclotetrasiloxane	14.82	11.54	13.33	11.60	14.03	11.89	14.06

a) CLogP values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005). b) log P_{oct} values determined using Eq. 3.3. c) log P_{oct} values determined using Eq. 3.4 d) log P_{oct} values determined using Eq. 3.5.

$\log k_w$ values were used for the comparison between the different columns in order to better visualise the differences and to suppress the inaccuracy induce by the determination of $\log P_{oct}$ values. The different relationships were reported in Fig. 3.1, 3.2 and 3.3.

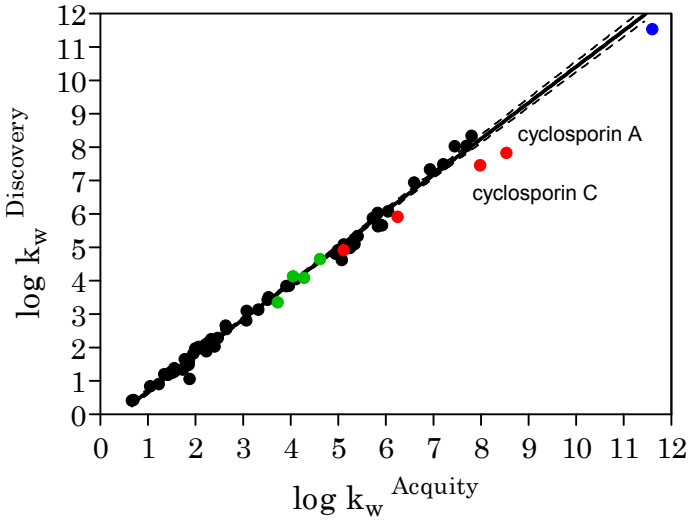


Figure 3.1: Relationship between $\log k_w$ values measured on Discovery® RP Amide C16 and Acquity UPLC™ BEH Shield RP 18 stationary phases. The 57 reference compounds are in black. Green circles are the compounds with important volume, red ones are the different peptides (including the cyclosporins) and blue circle is for the octaphenylcyclotetrasiloxane. The straight line represents the correlation obtained with the 57 reference compounds

$\log k_w^{Discovery} = 1.08\ (\pm 0.02) \bullet \log\ k_w^{Acquity} - 0.40\ (\pm 0.10)$

$n = 57,\ r^2 = 0.994,\ s = 0.18,\ F = 8374$ and the dot line the 95% confidence intervals.

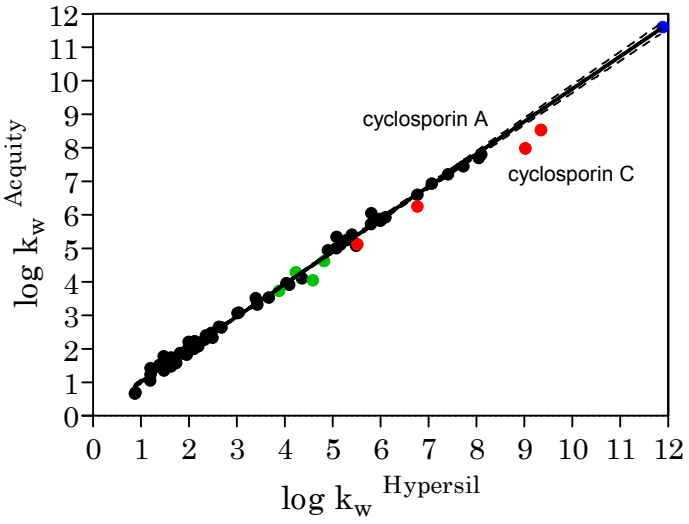


Figure 3.2: Relationship between $\log k_w$ values measured on Acquity UPLC™ BEH Shield RP18 and Hypersil™ GOLD Javelin HTS stationary phases. The legend is as explained in Fig. 3.1. The straight line represents the correlation obtained with the 57 reference compounds $\log k_w^{Acquity} = 0.97\ (\pm 0.02) \bullet \log\ k_w^{Hypersil} + 0.04\ (\pm 0.07)$ $n = 57,\ r^2 = 0.996,\ s = 0.14,\ F = 12080$ and the dot line the 95% confidence intervals.

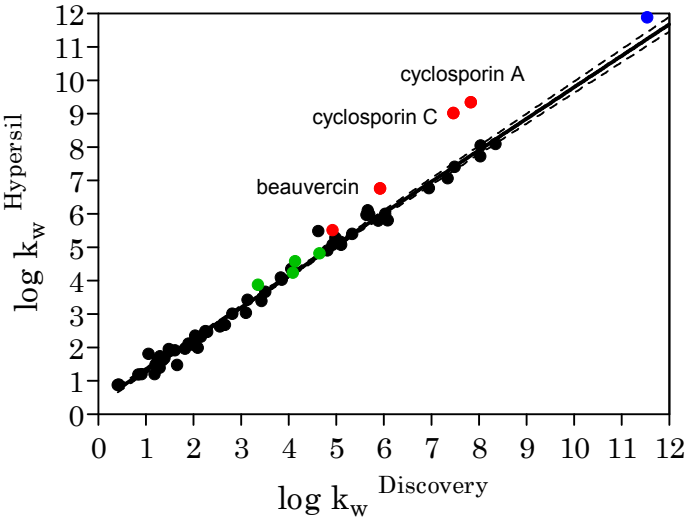


Figure 3.3: Relationship between log k_w values measured on Discovery® RP Amide C16 and Hypersil™ GOLD Javelin HTS stationary phases. The legend is as explained in Fig. 3.1. The straight line represents the correlation obtained with the 57 reference compounds

$\log k_w^{Hypersil} = 0.94\ (\pm 0.02) \bullet \log\ k_w^{Discovery} + 0.37\ (\pm 0.10)$

$n = 57,\ \ r^2 = 0.991,\ \ s = 0.20,\ \ F = 6035$ and the dot line the 95% confidence intervals.

First of all, the log k_w values obtained for the compounds with important volume (in green in Fig. 3.1, 3.2 and 3.3) have the same behaviour on the different stationary phases, independently of their lipophilicity. Indeed they correlate whatever the stationary phases employed. Thus, these results suggest that important volume is not involved in the differences observed for the log P_{oct} values of the two cyclosporins.

No difference was observed for the isovaleryl-Phe-Nle-Sta-Ala-Sta-OH whatever the stationary phases used. However, differences between determined log P_{oct} values were obtained for the beauvercin (1 unit) on the Discovery® RP Amide C16 and the Hypersil™ GOLD Javelin HTS columns. The difference between the two log P_{oct} values was therefore probably induced by the different embedded group of the two stationary phases.

A small difference between log P_{oct} values measured for the octaphenylcyclotetrasiloxane on Discovery® RP Amide C16 and Hypersil™ GOLD Javelin HTS columns was obtained. However, the log P_{oct} values obtained using the Acquity UPLC™ BEH Shield RP18 and the Hypersil™ GOLD Javelin HTS stationary phases are comparable. Thus the value measured with the Discovery® RP Amide C16 column seems to be under-estimated maybe because of experimental error induced by important analysis times.

As explained before, the two cyclosporins possess different behaviours on the three stationary phases and hence do not correlate with the 64 compounds (57 reference and 7 added compounds). However, this study excluded the involvement of important volume and too high lipophilicity albeit close to the upper-limit of the methods. Nevertheless, another explanation for the cyclosporins could be advanced. The hypothesis was a possibility of conformational changes depending on the stationary phases used. Hence, a conformational study was realised for the cyclosporin A.

3.3.3 Evaluation of the different conformations and their log P values of the cyclosporin A

A preliminary computational study was undergone to challenge the hypothesis that the different behaviors of the cyclosporin A in the various liquid chromatography columns could be linked to a conformation-dependant variation of lipophilicity of this molecule in different environments, especially a total or partial interconversion [37]. The conformational fate of cyclosporin A in two different solvents (water and methanol) was simulated by molecular dynamics and the 3D expression of lipophilicity of the conformers obtained was described and quantified by the molecular lipophilicity potential (MLP).

In fact, numerous studies [38-44] were realized about the possibility of interconversion induces by the stationary phase. This phenomenon could happen at room temperature inside the column, even if this conformational equilibrium is slow for cyclic peptides [45].

Indeed, Huisden et al. [38] compared the effect of the stationary phase (ligand) to the solvent effect. Hence, even if the mobile phase is identical (isocratic mode with the same % MeOH), the differences on the stationary phases, such as the embedded group, could induce modifications of the conformation.

Thus, a preliminary study was realised in diverse solvents (methanol and water) to evaluate the possible difference of lipophilicity value between different conformations.

Fig. 3.4 shows the repartition of lipophilicity values as returned by the back-calculation of MLP ($\log P_{MLP}$) for all conformations of cyclosporin A from both simulations in the explicit solvents (water and methanol). The first consideration is that the $\log P_{oct}$ values themselves are not physically meaningful since outside of the validity domain. This is not surprising as the linear model linking MLP values on a surface and partition coefficient in n-octanol/water system ($\log P_{oct}$) was trained on a set of drug-like compounds. Thus that narrows the validity domain of such prediction to small-to-medium size molecules from which the large and flexible cyclosporin A diverges [31]. As one compares MLP values from geometries of a unique chemical structure, the focus will be on the variations of $\log P_{MLP}$ ($\Delta\log P_{MLP}$) and relative predicted values.

The main information enclosed in cyclosporin A $\Delta\log P_{MLP}$ values between the simulations in methanol and water shows that cyclosporin A has the tendency to express different $\log P_{oct}$ values (so to say different apparent lipophilicity) depending on its environment (here, solvent). This first attempt of quantification defines an average global log P measured using the conformers determined in MeOH being significantly higher by 0.47±0.014 unit compared to water (unpaired t-test $p < 0.0001$). The trend is intuitively acceptable since far from being totally different due to the similarities between methanol and water. However, methanol is a more apolar solvent than water and so the cyclosporin A should favor more hydrophobic conformations in this solvent.

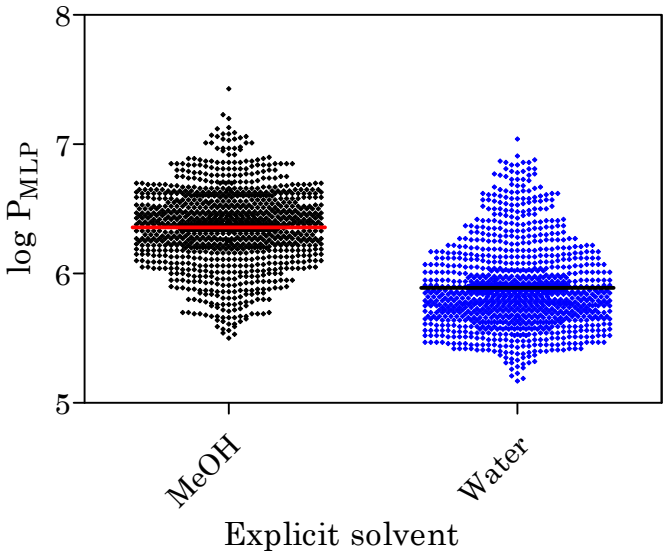


Figure 3.4: Range of n-octanol/water partition coefficients predicted by back-calculation of MLP computation on molecular surface (log P_{MLP}) around all geometries of cyclosporin A from molecular dymanics in methanol (MeOH) (black dots) and water (blue dots). X—axis refers to the number of conformers. The red and black horizontal lines represent the average of log P_{MLP} for conformers in MeOH and water, respectively.

Moreover the spread of predicted n-octanol/water partition for all possible geometries within a system is large but similar between solvents (1.93 and 1.87 log P units in methanol and water, respectively). This illustrates the intrinsic tendency of solvated cyclosporin A to be in conformations of significantly diverse apparent lipophilicities.

Overall and regardless the solvents, the Δlog P_{MLP} between the most hydrophobic to the most polar conformations is 2.26 units (respectively on the left and right hand side of Fig. 3.5). This substantial variation should encourage more in depth exploration of the conformation lipophilicity phenomena.

Furthermore, comparing the MLP maps and conformations (Fig. 3.5), such sizeable Δlog P_{MLP} is for geometries that displays only minor conformational variations (1.29 Å RMSD (root mean square deviation) on heavy atoms). Globally, the most apolar conformation of cyclosporin A

obtained (in methanol) extends slightly the hydrophobic side chains (tert-butyl, ethyl and methyl) towards the outside (the solvent) to shield the polar backbone (the amide groups). This is particularly visible for both carbonyls pointed by arrows in Fig. 3.5. They are solvent accessible and as such display polar contribution to lipophilicity for the most polar conformation (circled red zones on the right-hand side) whereas shielded by carbon chain for the most hydrophobic conformation (circled blue zones on the left-hand side).

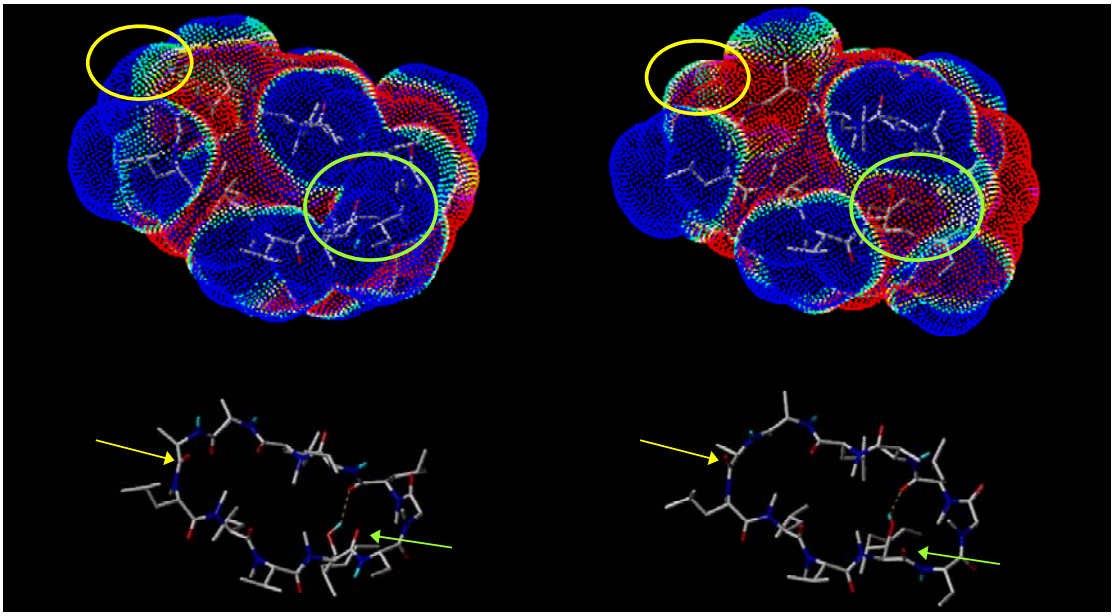


Figure 3.5: MLP maps (up) on the molecular surface around two conformations bearing extreme computed lipophilicity (RMSD = 1.29Å; $\Delta\log P_{MLP}$ = 2.26) and in sticks and atom-type colors (down) (with polar hydrogen omitted for clarity) for the most hydrophobic (measured in MeOH) (left) and the most polar (measured in water) (right) conformations. The dots color-coded are as follows for polar values: red, magenta, orange, and yellow (in decreasing order) and for hydrophobic values: blue, green-blue, green and cyan (in decreasing order) and white for near zero values. Arrows point the most conformational changes that impact mostly the MLP, translated in diversely colored circled zones.

Significant changes of geometries were not expected between the conformers obtained in MeOH and water because i) the methanol and

water are not drastically different solvents (as the tested LC columns are not radically different environments) and ii) the molecular simulations were performed from a unique structural starting point at a level where large energy barrier cannot be jumped (room temperature and constant energy). But surprising (and finally supporting the working hypothesis) is the significant variation of global lipophilicity and its 3D distribution computed from these close conformations.

Hence, these first results seem to confirm that a slight difference in solvent (water and MeOH) induce different conformations and also differences in lipophilicity. Thus a slight difference, as in the different stationary phases (embedded groups) used, could explain the different log P_{oct} values determined.

3.4 Conclusion and Perspectives

Thus, these three analytical methods confirm that the cyclosporin A is highly lipophilic with log P_{oct} values superior or equal to 9.1. These results explain the absence of this compound in the brain and especially its poor aqueous solubility.

However, the two cyclosporins had different behaviour compared to the 64 compounds tested in this study (57 reference compounds and 7 added ones). Moreover these differences were not induced by the important volume or the high lipophilicity of the cyclosporin family. Hence, different interactions between compound and ligand of stationary phases could induce these diverging results. Furthermore these different interactions could be induced by conformational changes in cyclosporins. Preliminary analysis of the impact of different conformations on calculated log P tends to be directed to this end.

Even if different log P_{oct} values were obtained for the cyclosporins, these differences could be due to conformational changes, and hence all these three results are correct determinations of the two cyclosporins lipophilicity. Additional compounds also showed that the three developed methods could be used for very highly lipophilic compounds. For a lipophilicity determination, if only a RPLC system was available, the

Discovery® RP Amide C16 column is advised up to 10. For the fastest analysis, the Hypersil™ GOLD Javelin HTS stationary phase on the UHPLC system has to be employed since it is the fastest method.

The preliminary molecular modeling study encourages exploring more thoroughly the conformational surface of cyclosporin A by performing high temperature molecular dynamics or Monte Carlo methodologies. This latter technique starts from diverse geometries of cyclosporin A. There is a strong likelihood that bigger conformational moves shall broaden the range of apparent lipophilicity within a simulation, as well as between the different solvents (environments).

Another perspective is to increase gradually the complexity of the simulated environment to approach the properties of the liquid chromatographic (LC) systems under study. A first step will be to assess the impact of different methanol-water mixtures on the computed conformations and their apparent lipophilicity. A longer term work and much more demanding tasks will be to assess the feasibility of modeling the molecular structure of LC columns and performing simulation in a hugely complex atomic system. This latter study could hence model the different ligands' stationary phases to confirm that the different embedded groups could induce these differences of log P_{oct} values.

References

1. Monchaud, C.; Marquet, P. Pharmacokinetic optimization of immunosuppressive therapy in thoracic transplantation: part II. *Clin. Pharmacokin.* **2009,** *48*, 489-516.

2. Aliabadi, A. Z.; Zuckermann, A. O.; Grimm, M. Immunosuppressive therapy in older cardiac transplant patients. *Drugs & Aging* **2007,** *24*, 913-932.

3. Marcen, R. Immunosuppressive drugs in kidney transplantation impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. *Drugs* **2009,** *69*, 2227-2243.

4. Beauchesne, P. R.; Chung, N. S. C.; Wasan, K. M. Cyclosporine A: a review of current oral and intravenous delivery systems. *Drug Dev. Ind. Pharm.* **2007,** *33*, 211-220.

5. Tredger, J. M.; Brown, N. W.; Dhawan, A. Calcineurin inhibitor sparing in paediatric solid organ transplantation - Managing the efficacy/toxicity conundrum. *Drugs* **2008,** *68*, 1385-1414.

6. Schrem, H.; Luck, R.; Becker, T.; Nashan, B.; Klempnauer, J. Update on liver transplantation using cyclosporine. *Transplant. Proc.* **2004,** *36*, 2525-2531.

7. Watts, A. B.; Williams, R.; Peters, J. I. Recent developments in drug delivery to prolong allograft survival in lung transplant patients. *Drug Dev. Ind. Pharm.* **2009,** *35*, 259-271.

8. Snell, G. I.; Westall, G. P. Immunosuppression for lung transplantation. *Drugs* **2007,** *67*, 1531-1539.

9. Egidi, F. M. Management of hyperglycaemia after pancreas transplantation - Are new immunosuppressants the answer? *Drugs* **2005,** *65*, 153-166.

10. Berney, T.; Buhler, L. H.; Majno, P.; Mentha, G.; Morel, P. Immunosuppression for pancreatic islet transplantation. *Transplantation Proceedings* **2004,** *36*, S362-S366.

11. Duncan, N.; Craddock, C. Optimizing the use of cyclosporin in allogeneic stem cell transplantation. *Bone Marrow Transplant* . **2006,** *38*, 169-174.

12. Diaz-Borjon, A. Guidelines for the use of conventional and newer disease-modifying antirheumatic drugs in elderly patients with rheumatoid arthritis. *Drugs & Aging* **2009,** *26*, 273-293.

13. Galeazzi, M.; Bellisai, F.; Giannitti, C.; Manganelli, S.; Morozzi, G.; Sebastiani, G. D. Safety of cyclosporin a in HCV-infected patients. Experience with Cyclosporin A in patients affected by rheumatological disorders and concomitant HCV infection. *Ann. N. Y. Acad. Sci.* **2007,** *1110*, 544-549.

14. Domenech, E. Inflammatory bowel disease: current therapeutic options. *Digestion* **2006,** *73*, 67-76.

15. Sands, B. E. Immunosuppressive drugs in ulcerative colitis: twisting facts to suit theories? *Gut* **2006,** *55*, 437-441.

16. Galeazzi, M.; Bellisai, F.; Manganelli, S.; Morozzi, G.; Sebastiani, G. D. Cyclosporine A for the treatment of autoimmune disorders in HCV infected patients. *Autoimmun. Rev.* **2006,** *5*, 493-498.

17. Poggi, A.; Zocchi, M. R. Cyclosporin A regulates human NK cell apoptosis induced by soluble HLA-1 or by target cells. *Autoimmun. Rev.* **2005,** *4*, 532-536.

18. Silverberg, N. B. Pediatric psoriasis: an update. *Ther. Clin. Risk Man.* **2009,** *5*, 849-586.

19. Fife, D. J.; Maibach, H. I. Gender differences in the pharmacokinetics of oral dermatologic medications. *Cutan. Ocul. Toxicol.* **2005,** *23*, 119-133.

20. Kashani, S.; Mearza, A. A. Uses and safety profile of ciclosporin in ophthalmology. *Exp. Opin. Drug Saf.* **2008,** *7*, 79-89.

21. Bertelmann, E.; Pleyer, U. Immunomodulatory therapy in ophthalmology - Is there a place for topical application? *Ophthalmologica* **2004,** *218*, 359-367.

22. Sanderson, H.; Thomsen, M. Comparative analysis of pharmaceuticals versus industrial chemicals acute aquatic toxicity classification according to the United Nations classification system for chemicals. Assessment of the (Q)SAR predictability of pharmaceuticals acute aquatic toxicity and their predominant acute toxic mode-of-action. *Toxicol. Letters* **2009,** *187*, 84-93.

23. Wolf, A.; Schramm, U.; Fahr, A.; Aicher, L.; Cordier, A.; Trommer, W. E.; Fricker, G. Hepatocellular effects of cyclosporine A and its derivative SDZ IMM 125 in vitro. *J. Pharmacol. Exp. Ther.* **1998,** *284*, 817-825.

24. El Tayar, N.; Mark, A. E.; Vallat, P.; Brunne, R. M.; Testa, B.; van Gunsteren, W. F. Solvent-dependance conformation and hydrogen-bonding capacity of cyclosporin A: evidence from partition coefficients and molecular dynamics simulations. *J. Med. Chem.* **1993,** *36*, 3757-3764.

25. Italia, J. L.; Bhardwaj, V.; Kumar, M. N. V. R. Disease, destination, dose and delivery aspects of ciclosporin: the state of the art. *Drug Discovery Today* **2006,** *11*, 846-854.

26. Czogalla, A. Oral cyclosporine A - the current picture of its liposomal and other delivery systems. *Cell. Mol. Biol. Lett.* **2009,** *14*, 139-152.

27. Lallemand, F.; Felt-Baeyens, O.; Besseghir, K.; Behar-Cohen, F.; Gurny, R. Cyclosporine A delivery to the eye: a

pharmaceutical challenge. *Eur. J. Pharm. Biopharm.* **2003,** *56*, 307-318.

28. Liu, H.; Wang, Y.; Li, S. Advanced delivery of Ciclosporin A: present state and perspective. *Exp. Opin. Drug. Del.* **2007,** *4*, 349-358.

29. Horvath, C.; Melander, W. Liquid chromatography with hydrocarbonaceous bonded phases; theory and practice of reversed phase chromatography. *J. Chromato. Sci.* **1977,** *15*, 393-404.

30. Gaillard, P.; Carrupt, P. A.; Testa, B.; Boudon, A. Molecular lipophilicity potential, a tool in 3D-QSAR. Method and applications. *J. Comput. Aided Mol. Des.* **1994,** *8*, 83-96.

31. Gaillard, P. *La modélisation moléculaire en chimie thérapeutique: Apports de la lipophilie et de la flexibilité en 3D-QSAR*; Thesis: University of Lausanne, 1994.

32. Carrupt, P. A.; Gaillard, P.; Billois, F.; Weber, P.; Testa, B.; Meyer, C.; Pérez, S. The molecular lipophilicity potential (MLP): a new tool for log P calculations and docking, and in comparative molecular field analysis (CoMFA). In *Lipophilicity in Drug Action and Toxicology*; Pliska, V.; Testa, B.; van de Waterbeemd, H. Eds.; VCH Publishers: Weinheim, 1996; pp 195-217.

33. Case, D. A.; Cheatham III, T. E.; Simmerling, C. L.; Wang, J.; Duke, R. E.; Luo, R.; Darden, T. A.; Zhang, W.; Merz, K. M.; Roberts, B.; Wang, B.; Hayik, S.; Walker, R. C.; Seabra, G.; Kolossvary, I.; Wong, K. F.; Paesani, F.; Vanicek, J.; Liu, J.; Roitberg, A.; Brozell, S. R.; Steinbrecher, T.; Gohlke, H.; Cai, Q.; Ye, X.; Wang, J.; Wu, X.; Cui, G.; Roe, D. R.; Mathews, D. H.; Seetin, M. G.; Sagui, C.; Babin, V.; Hsieh, J. J.; Gusarov, S.; Kovalenko, A.; Kollman, P. A.; Luchko, T. Amber 11. 2010. San Francisco, University of San Francisco.

34. Wang, J. M.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and testing of a general amber force field. *J. Comp. Chem.* **2004,** *25*, 1157-1174.

35. Kajitani, K.; Fujihashi, M.; Kobayashi, Y.; Shimizu, S.; Tsujimoto, Y.; Miki, K. Crystal structure of human cyclophilin D in complex with its inhibitor, cyclosporin A at 0.96-+à resolution. *Proteins: Struct. Funct. Bioinf.* **2008,** *70*, 1635-1639.

36. Vallat, P.; Gaillard, P.; Carrupt, P. A.; Tsai, R. S.; Testa, B. Structure-lipophilicity and structure-polarity relationships of amino acids and peptides. *Helv. Chim. Acta* **1995,** *78*, 471-485.

37. Purcell, A. W.; Aguilar, M. I.; Hearn, M. T. W. Conformational effects in reversed-phase high-performance liquid-chromatography of polypeptides .1. Resolution of insulin variants. *J. Chromatogr. A* **1995,** *711*, 61-70.

38. Huisden, R. E.; Kraak, J. C.; Poppe, H. Method for the characterization of stationary phases for the separation of proteins by high-performance liquid-chromatography. *J. Chromatogr.* **1991,** *537*, 1-16.

39. Takakuwa, T.; Kurosu, Y.; Sakayanagi, N.; Kaneuchi, F.; Takeuchi, N.; Wada, A.; Senda, M. Direct combination of a high-performance liquid chromatograph and a circular-dichroism spectrometer for separation and structural-analysis of proteins. *J. Liquid Chromatogr.* **1987,** *10*, 2759-2769.

40. Krull, I. S.; Stuting, H. H.; Krzysko, S. C. Conformational studies of bovine alkaline phosphatase in hydrophobic interaction and size-exclusion chromatography with linear diode array and low-angle laser light scattering detection. *J. Chromatogr. A* **1988,** *442*, 29-52.

41. Lu, X. M.; Figueroa, A.; Karger, B. L. Intrinsic fluorescence and Hplc measurement of the surface dynamics of lysozyme adsorbed on hydrophobic silica. *J. Am. Chem. Soc.* **1988,** *110*, 1978-1979.

42. Lau, S. Y. M.; Taneja, A. K.; Hodges, R. S. Synthesis of a model protein of defined secondary and quaternary structure - effect of chain-length on the stabilization and formation of 2-stranded alpha-helical coiled-coils. *J. Biol. Chem.* **1984,** *259*, 3253-3261.

43. Hearn, M. T. W. Reverse phase liquid chromatography of β-endorphins and related peptides. *Life Sci.* **1987,** *41*, 897-900.

44. Katzenstein, G. E.; Vrona, S. A.; Wechsler, R. J.; Steadman, B. L.; Lewis, R. V.; Middaugh, C. R. Role of conformational-changes in the elution of proteins from reversed-phase Hplc columns. *Proc. Natl. Acad. Sci. U. S. A.* **1986,** *83*, 4268-4272.

45. Gesquiere, J. C.; Diesis, E.; Cung, M. T.; Tartar, A. Slow isomerization of some proline-containing peptides inducing peak splitting during reversed-phase high-performance liquid-chromatography. *J. Chromatogr.* **1989,** *478*, 121-129.

Chapter 4: Estimation of bioconcentration factors by UHPLC

4.1 Introduction

An evaluation of the ecotoxicity is important for the commercialization of a new chemical entity since at the end of 2006, new legislation was adopted by the European Council and European Parliament [1, 2]. This novel system was called Registration, Evaluation, Authorization of CHemicals (REACH). Due to this directive, chemicals' toxicity has to be evaluated for compounds' production or importation higher than one tonne per year [3] in order to avoid the possibility of intolerable consequences for human health or environment. Moreover, a strategy to evaluate the bioconcentration factor without the employment of aquatic organisms and especially fishes is recommended.

For this purpose, bioconcentration factor (BCF) has been used for a while as a quantitative index for a possible accumulation [4]. The bioconcentration is a process of chemicals' accumulation by organisms, but only through the non-dietary routes [5]. Hence, BCF indicates the future effect of a substance on organisms, which can be a toxic compound (e.g. PCB) or a drug [4, 6, 7]. Thus, this factor is used as an important parameter in the risk assessment of environmental contaminants. Moreover, this factor is important for the pharmaceuticals' firms to evaluate the accumulation of a drug in the organism or for the cosmetics such as solar creams or dyeing products.

The bioconcentration factor of a product, in aquatic ecosystems, is defined as the ratio of the concentration of the chemical substance in a target organism to its concentration in water, at steady-state conditions [8, 9]. However, it can also be viewed as the ratio of the uptake (k_1) to the elimination (k_2) from the animal ($BCF = k_1/k_2$) [10, 11]. Usually, fishes are the targets organisms for the bioconcentration assessments due to their presence in humans' chain food source and the availability of standardized testing protocols [5].

The experiment is divided into two phases: (i) the uptake or exposure phase, and (ii) the depuration or post-exposure phase. In the first phase, different groups of fishes of the same species are exposed to, normally, two concentrations of the chemical substances. After this exposure, fishes are transferred to a medium free of the chemical substance for the clearance phase. Generally, the exposure phase is run for 28 days, excepted if the steady state is reached earlier [12].

A conceptual model of bioconcentration in fish was proposed by Barron [5] and was adapted (Fig. 4.1) to better understand the progression of chemical substances into fish body. So, before being accumulate in lipid storage sites, a chemical substance penetrates into the fish via the gill. Because of diffusion barriers (e.g. membranes), the chemical substance moves from the water into the blood, and is hence transported to lipoidal tissues, or excludes after the metabolisation into less lipophilic compound.

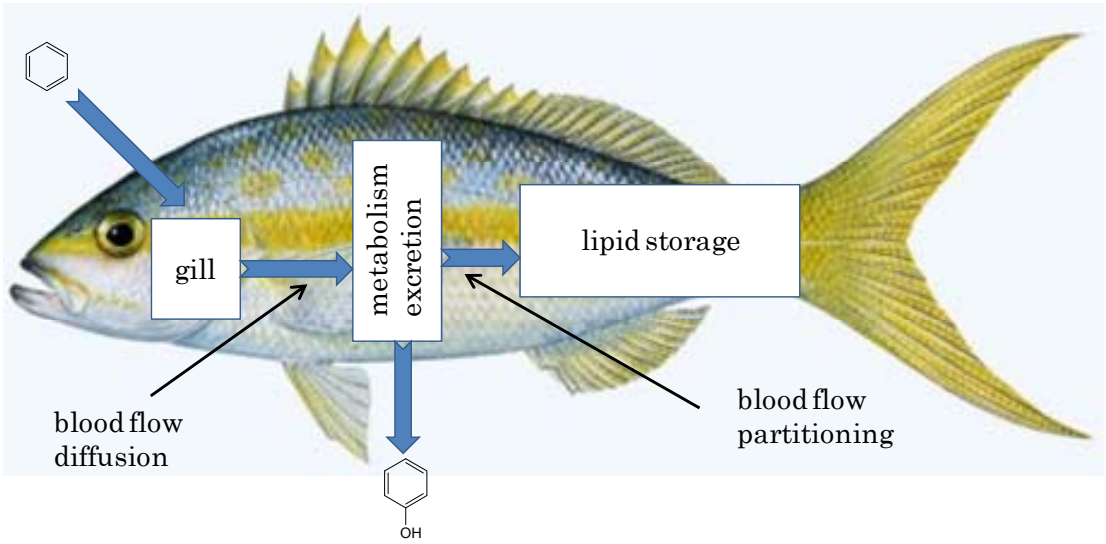


Figure4.1: Conceptual model of bioconcentration in fish adapted from [16]

However, non-animal testing is encouraged in REACH directive. A strategy to evaluate the bioconcentration factor without the employment of aquatic organisms and especially fishes is required, especially in cosmetic industry since analysis on animal are definitely forbidden. Moreover, experimental determination of BCF is time consuming, expensive and experimental measurements of bioconcentration for all the

chemical substances that have a potential interest is simply not possible [6, 13].

Therefore, BCF-models were largely developed to overcome regulatory constraints coupled to high cost. Particularly since REACH legal text encourages the use of QSAR models to predict environmental and toxicological risk [14].

The widely used model for the prediction of chemicals' bioconcentration factors is the employment of a correlation between BCF data and the partition coefficients obtained in the n-octanol/water system ($\log P_{oct}$). Indeed hydrophobicity is one of the main driving force of bioconcentration. However, other factors are also implied in bioconcentration process such as metabolism or transport through membranes [15]. Nevertheless, a large number of models based on $\log P_{oct}$ values were reported to predict log BCF measured in fish [9, 16-27]. It was largely demonstrated that for compounds with $\log P_{oct}$ up to 6, bioconcentration can be modelled using linear regression. On the other hand, for highly lipophilic compounds ($\log P_{oct} > 6$), the bioconcentration tends to decrease when lipophilicity increases. Many reasons can be advised to explain this behaviour such as the low aqueous solubility or the reduced diffusion through membranes. Therefore several non-linear models implying the $\log P_{oct}$ values were proposed to predict log BCF for such compounds. Among these models, polynomial [22] but especially quadratic [24, 25] or bilinear models proposed by Kubinyi [28], or Bintein et al. [23] were considered as reasonably good models for BCF predictions. It is known that experimental variations in BCF measurements can occur for highly lipophilic compounds that participate to pejorative effect on QSAR models as for example the steady state equilibrium not being reached or increasing adsorption. However, the lack of accuracy in experimental log P values especially for highly lipophilic compounds is also a great handicap in developing such models. Yet, more and more chemicals are very lipophilic and calculated log P methods have proved their limitations for this kind of compounds.

Therefore, methods developed to determine the lipophilicity even for highly lipophilic compounds in chapter 2 [29] could be an interesting alternative to perform BCF-models. The Hypersil™ GOLD Javelin HTS stationary phase, employed in isocratic mode with MeOH as organic modifier, allows the fastest analysis. Moreover, the lipophilicity range of this method could still be enlarged (Fig. 4.2). Hence, this column was chosen to evaluate the employment of experimental values for the correlation with log BCF, even for highly lipophilic compounds, to replace calculated log P values in bioconcentration models.

Hence, bioconcentration factors measured in different fish species found in literature were directly correlated with retention factors (log k_w) obtained on a UHPLC stationary phase. The 85 compounds retention factors (log k_w) were evaluated as an *in vitro* alternative index to model the bioconcentration in fish (log BCF).

4.2 Material and methods

4.2.1 Chemicals

All the compounds were obtained from commercial sources (Acros (New Jersey, USA), Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Laubscher Labs (Miecourt, Switzerland), Merck (Darmstadt, Germany), Riedel-de-Haen (Seelze, Germany), Sigma (St-Louis, MO, USA) and Supelco (Bellefonte, USA)) in the highest available purity.

HPLC grade methanol was purchased from VWR (Dietikon, Switzerland). Water was obtained with the Milli-Q Water Purification System from Millipore (Milford, MA, USA).

4.2.3 Mobile phase composition and UHPLC instrumentation

To analyse all the compounds under their neutral form, three buffers with different pH values (trifluoroacetic acid / sodium hydroxide pH 2.5, acetic acid / sodium hydroxide pH 5 and phosphoric acid / sodium hydroxide pH 7.5) were prepared. A ionic strength of 20 mM was chosen according to Phoebus software v1.0 (Analis, Namur, Belgium). Buffer

solutions were filtered through a 0.22 µm HA Millipore filter (Millipore, Bedford, MA, USA). The percentage range using MeOH as organic modifier was from 10 to 80%. However, as shown by the Fig.4.2, this range could still be enlarged. Indeed, for highly lipophilic compounds, no loose of accuracy or quadratic curve were obtained. Hence, the lipophilicity of the compounds could be increased since this method does not reach its upper-limit yet.

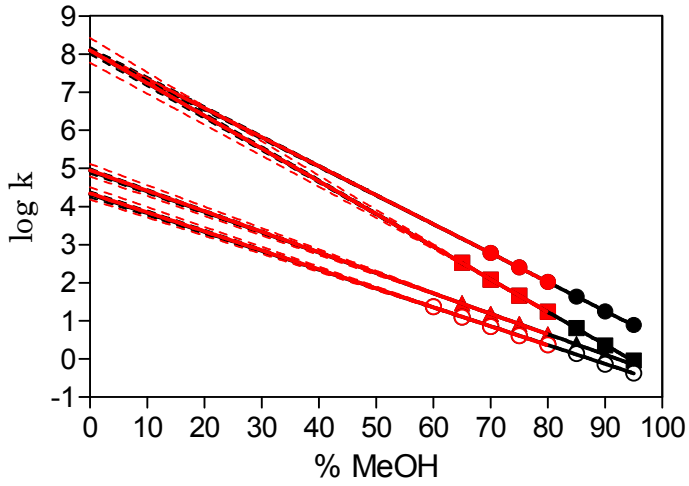


Figure 4.2: Linear extrapolation obtained with Hypersil™ GOLD Javelin HTS column. Red symbols represent the percentages < 80% MeOH and the black ones the percentages > 80% MeOH. Full circles were for octachlorodibenzofuran (log k_w = 8.1), squares for bis(2-ethylhexyl) phthalate (log k_w = 8.11), triangles for benzo[a]anthracene (log k_w = 4.93) and empty circles for bibenzyl (log k_w = 4.36). The straight lines represent linear extrapolations and the dot lines the 95% confidence intervals.

An Acquity UPLC system (Waters, Milford, USA) including a binary solvent manager, a sample manager with an injection loop volume of 2 µL, a photo diode array (PDA) programmable detector, and a column manager with oven, was used. The system was controlled by Empower Software v2.0 (Waters, Milford, MA) and the detection was performed at appropriate wavelengths (compounds λ_{max}).

Retention measurements were performed on a Hypersil™ GOLD Javelin HTS stationary phase (10 x 2.1 mm ID, 1.9 µm) (Thermo Scientific

Runcorn, UK) at a flow rate of 1 mL/min, and at 30 ± 0.1°C. This column was chosen since it allows the fastest lipophilicity's determination (chapter 2 and [29]).

The concentrations of stock solutions were 1000 ppm in MeOH and the injected solutions vary from 500 to 50 ppm, depending on the UV absorbance.

4.2.4 Measurements of retention factors

4.2.4.1 Generic gradient

First of all, a generic gradient was realised to allow a fast estimation of the compounds' lipophilicity in order to adapt methanol percentages used for isocratic measurements. Only one generic gradient run was performed in 1.4 min. One minute for the gradient run from 2 to 98% of MeOH, 0.15 min for a stage at 98% to be sure that all the compounds, even the more lipophilic ones, were eluted. And finally a conditioning at 2% of MeOH was realised during 0.2 min to equilibrate the column in the initial condition.

Hence, a t_r corresponding to the retention time of each compound was obtained and permits a comparison between the different analytes.

Due to this generic method, a realistic estimation of the lipophilicity was done and an appropriate range of percentages in organic modifier could be chosen according to Table 4.1.

Table 4.1: Isocratic run's times (in minute) and MeOH percentages recommended for new chemical entities (NCE's) depending on the t_r obtained during the generic gradient run.

t_r mini	t_r max	10%	20%	30%	40%	50%	60%	65%	70%	75%	80%
	0.47	1'	1'	1'	1'	1'	1'				
0.47	0.55		1'	1'	1'	1'	1'	1'			
0.55	0.60			1'	1'	1'	1'	1'	1'		
0.64	0.72				1'	1'	1'	1'	1'	1'	
0.72	0.82					1'	1'	1'	1'	1'	1'
0.82	0.90						1'	1'	1'	1'	1'
0.91	0.94						2'	1'	1'	1'	1'
0.94	1.00							2'	1'	1'	1'
1.01	1.06							5'	3'	2'	1'
1.06	1.11							12'	5'	2'	1'
1.11	1.18								12'	6'	3'
1.18	-								20'	10'	5'

4.2.4.2 Isocratic mode

Retention factor is given by the following equation when short stationary phases are used:

$$\log k = \log\left(\frac{t_r - t_{delay} - (V_{ext}/F)}{t_0 - t_{delay} - (V_{ext}/F)} - 1\right) \quad \text{Eq. 4.1}$$

where t_r and t_0 are the retention times of the solute and the unretained compound, respectively. t_{delay} is the injection delay, V_{ext} the extra-column volume and F the flow rate of the mobile phase. An injection of uracil at 40% ACN allows to evaluate the t_0. t_r was determined in triplicate.

Extrapolated retention factors, corresponding to a mobile phase of pure water (log k_w), were employed in this study and were directly used for the correlation with the log BCF values.

Methanol (MeOH) was used as organic modifier. Retention factors (log k) were measured at, at least, three different percentages of methanol, and up to six, chosen according to the retention time obtained during the generic gradient. These values were plotted as a function of the mobile phase composition (φ). log k_w values were obtained using the following equation:

$$\log k = \log k_w - S \bullet \varphi \quad \text{Eq. 4.2}$$

where log k and log k_w are the isocratic and extrapolated retention factors respectively, φ is the composition in organic modifier and S is a constant for a given solute and fixed experimental conditions.

4.3 Results and discussion

Considering only one species of fish for this study was not possible since the number of highly lipophilic compounds is limited in literature. Therefore, data obtained on different species were used and the repartition of the number of bioconcentration factors obtained on different species is represented in Fig. 4.3. 85% of these data were measured on

species recommended in the Organization for Economic Co-operation and Development (OECD) guidelines (carp, fathead minnows, zebrafish and guppy) [12].

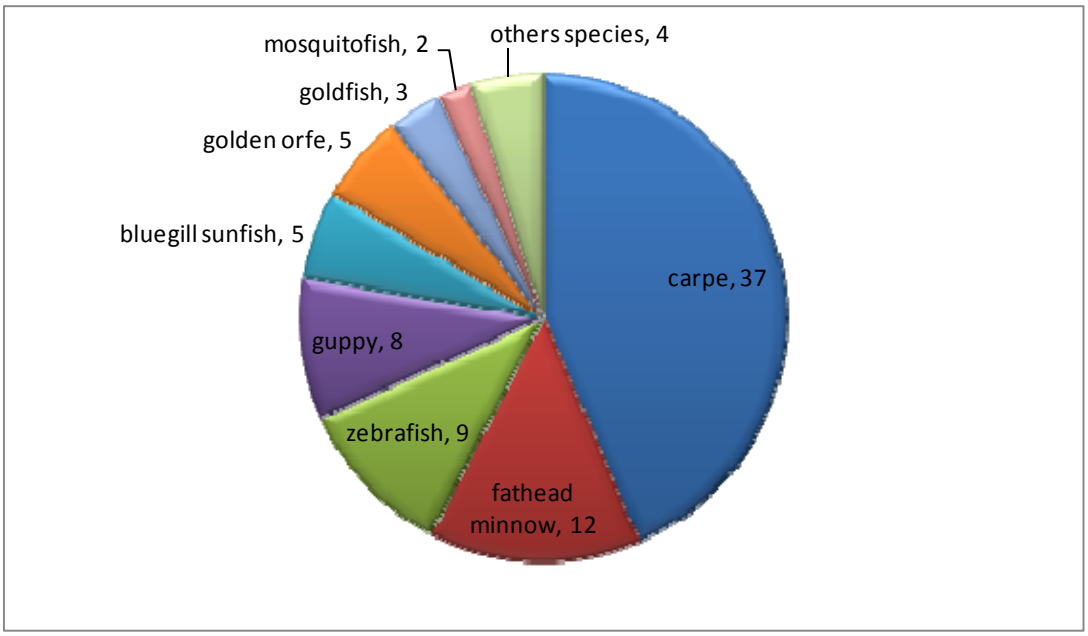


Figure 4.3: Number of bioconcentration data per fish species used in this study.

The new experimental method to determine the lipophilicity for compounds with log P ranging from 0 to 10, developed in chapter 2, was therefore applied. Experimental values obtained were compared to experimental log BCF data for compounds with a large range of lipophilicity. Indeed, since correlation has been described between partition coefficients (log P_{oct}) and retention factors (log k_w) [29], this latter was directly used for the estimation of the bioconcentration data. Thus, the loss of accuracy creates by lipophilicity's interpolation is suppressed. In parallel, calculated log P values (EPI Suite software) were also compared to bioconcentration factors. This software was used in this chapter since it allows a searching by CAS number and a calculation of the bioconcentration. Eighty-five compounds with experimental log BCF values from 0.3 to 6.0 were tested.

In Table 4.2 are reported the experimental bioconcentration factors (log BCF), calculated log P_{oct} values (log P), retention data (log k_w) and

log BCF interpolated from log P values using Eq. 4.3 (calculated log P) and from log k_w values using Eq. 4.4 (UHPLC method) for the 85 compounds. The publications sources of the log BCF values were also reported in this table.

Table 4.2: Data for the 85 compounds

name	log BCF [a)]	log P [x)]	log BCF_{logP} [y)]	log k_w [z)]	log BCF_{logkw} [aa)]
Phenol	0.28 [b)]	1.51	0.71	1.16	0.53
Chlorobromomethane	0.39 [c)]	1.43	0.65	0.94	0.35
Aniline	0.41 [d)]	1.08	0.39	0.86	0.28
4,6-dinitro-o-cresol	0.46 [c)]	2.27	1.27	1.62	0.92
4-nitroaniline	0.64 [d)]	1.47	0.68	1.16	0.53
2,4-toluenediamine	0.70 [c)]	0.16	-0.29	0.37	-0.12
Chloroform	0.78 [e)]	1.52	0.72	1.65	0.94
Furan	0.81 [c)]	1.36	0.60	0.92	0.33
2-chlorophenol	0.81 [b)]	2.16	1.19	1.81	1.07
Thiophene	0.86 [c)]	1.81	0.93	1.38	0.72
Benzophenone	0.89 [f)]	3.15	1.92	2.91	1.98
Quinoline	0.90 [g)]	2.14	1.18	2.05	1.27
2-nitroaniline	0.91 [d)]	2.02	1.08	1.61	0.91
4-chloroaniline	0.91 [d)]	2.16	1.19	1.71	1.32

Table 4.2: Continued

name	log BCF [a)]	log P [x)]	log BCF_{logP} [y)]	log k_w [z)]	log BCF_{logkw} [aa)]
Di-nitrotoluene	0.92 [c)]	2.18	1.20	2.06	1.28
N-ethylaniline	0.95 [c)]	2.40	1.37	1.91	1.44
N-methylaniline	1.00 [f)]	2.11	1.15	1.53	1.15
m-methylphenol	1.03 [h)]	2.06	1.11	1.74	1.02
3-chloroaniline	1.06 [i)]	1.72	0.86	1.68	0.97
3-chlorophenol	1.06 [f)]	2.16	1.19	2.07	1.29
p-bromophenol	1.17 [f)]	3.18	1.95	2.26	2.08
2-chloroaniline	1.18 [d)]	1.72	0.86	1.68	0.96
Nitrobenzene	1.18 [j)]	1.81	0.93	1.71	0.99
2-chloro-1-nitrobenzene	1.19 [k)]	2.46	1.41	2.18	1.38
1-naphtylamine	1.20 [c)]	2.25	1.26	2.11	1.32
3-nitrotoluene	1.20 [l)]	2.36	1.33	2.36	1.53
m-bromophenol	1.20 [c)]	2.40	1.37	2.30	1.47
2,4,6-trichlorophenol	1.30 [b)]	3.45	2.14	2.98	2.04
2-nitrophenol	1.34 [f)]	1.91	1.00	1.69	0.97
Chlorobenzene	1.36 [c)]	2.64	1.54	2.43	1.58

Table 4.2: Continued

name	log BCF [a)]	log P [x)]	log BCF_{logP} [y)]	log k_w [z)]	log BCF_{logkw} [aa)]
4-chlorophenol	1.38 [c)]	4.08	2.61	2.11	0.84
3-nitrophenol	1.40 [m)]	1.91	1.00	1.56	0.87
2-aminobiphenyl	1.42 [f)]	2.84	1.69	2.65	1.77
3,4-dichloroaniline	1.48 [d)]	2.37	1.34	2.46	1.61
Carbon tetrachloride	1.48 [e)]	2.44	1.40	2.58	1.71
1-naphtyl-N-methylcarbamate	1.53 [n)]	2.35	1.33	2.62	1.75
p-chlorotoluene	1.58 [c)]	1.72	0.86	3.03	0.99
Benzotrifluoride	1.63 [f)]	2.96	1.78	2.91	1.98
Bromobenzene	1.68 [c)]	2.88	1.72	2.57	1.70
Molinate	1.68 [o)]	2.91	1.74	2.99	2.05
o-chlorotoluene	1.68 [c)]	3.18	1.95	3.03	2.08
Dimethyl phtalate	1.76 [e)]	1.66	0.82	2.31	1.49
Naphtalene	1.90 [j)]	3.17	1.93	2.96	2.03
1,2-dichlorobenzene	1.95 [e)]	3.28	2.02	2.93	2.00
1,3-dichlorobenzene	1.99 [p)]	3.28	2.02	3.07	2.12
Carbazole	2.07 [f)]	3.23	1.98	2.95	2.01

Table 4.2: Continued

name	log BCF [a)]	log P [x)]	log BCF_{logP} [y)]	log k_w [z)]	log BCF_{logkw} [aa)]
Acridine	2.10 [j)]	3.32	2.05	2.46	1.61
Diuron	2.16 [m)]	2.67	1.57	2.69	1.80
Diazinon	2.27 [q)]	3.86	2.45	3.81	2.73
Fenitrothion	2.39 [q)]	3.30	2.03	3.34	2.34
Fenthion	2.68 [m)]	4.08	2.61	3.82	2.74
Octachlorodibenzofuran	2.77 [m)]	8.87	3.80	8.10	3.19
9H-fluorene	2.78 [f)]	4.02	2.56	3.64	2.59
Triphenylphosphate	2.79 [r)]	4.70	3.06	4.25	3.09
Butyl benzyl phtalate	2.89 [j)]	4.84	3.16	4.58	3.36
Bis(2-ethylhexyl)phtalate	2.93 [j)]	8.39	3.89	8.11	3.18
4-chlorobiphenyl	2.95 [s)]	4.40	2.84	4.15	3.01
1,3,5-trichlorobenzene	2.97 [f)]	3.93	2.49	3.70	2.64
Anthracene	2.99 [f)]	4.35	2.80	3.99	2.88
Dibenzothiphene	3.04 [c)]	4.17	2.67	3.78	2.71
Pentachloronitrobenzene	3.06 [n)]	5.03	3.30	4.49	3.29
Chlornitrofen	3.11 [f)]	4.96	3.25	4.79	3.53

Table 4.2: Continued

name	log BCF [a)]	log P [x)]	log BCF_{logP} [y)]	log k_w [z)]	log BCF_{logkw} [aa)]
Dibenzofuran	3.13 [j)]	3.71	2.34	3.47	2.45
1,3,5-tribromobenzene	3.23 [m)]	4.66	3.03	4.08	2.95
Phenanthren	3.25 [t)]	4.35	2.80	3.80	2.72
Fenvalerate	3.43 [u)]	6.76	4.08	7.09	4.14
1,2,4,5-tetrachlorobenzene	3.45 [f)]	4.57	2.97	4.06	2.94
Pentachlorobenzene	3.49 [f)]	5.22	3.42	4.60	3.38
1,2,3,4–tetrachlorodibenzo-p-dioxin	3.50 [f)]	6.92	4.09	6.14	4.37
p,p'-dibromobiphenyl	3.57 [f)]	5.54	3.63	5.09	3.76
Tricresyl phosphate	3.57 [v)]	6.34	4.01	5.49	4.05
Iodofenphos	3.62 [m)]	5.39	3.54	4.84	3.57
Methoxychlor	3.91 [w)]	5.67	3.71	4.96	3.67
Decachlorobiphenyl	3.92 [m)]	10.20	3.53	7.95	3.35
2,4',5-trichlorobiphenyl	3.95 [f)]	5.69	3.72	5.13	3.79
Heptachlor	3.98 [w)]	5.86	3.82	5.41	3.99
Hexachlorobenzene	4.16 [j)]	5.86	3.81	5.08	3.76
2,2',3,3'-tetrachlorobiphenyl	4.17 [f)]	6.34	4.01	5.10	3.77

Table 4.2: Continued

name	log BCF [a)]	log P [x)]	log BCF_{logP} [y)]	log k_w [z)]	log BCF_{logkw} [aa)]
Hexabromobiphenyl	4.26 [w)]	9.1	3.75	6.55	4.39
2,2',3,3',4,4',5,5'-octachlorobiphenyl	4.33 [m)]	8.91	3.79	7.44	3.85
p,p'-DDT	4.47 [w)]	6.79	4.08	5.98	4.32
o,p'-DDT	4.57 [w)]	6.79	4.08	6.03	4.34
2,3',4',5-tetrachlorobiphenyl	4.62 [m)]	6.34	4.01	5.65	4.15
2,2',4,4',5,5'-hexachlorobiphenyl	4.62 [s)]	7.62	4.02	6.47	4.4
p,p'-DDE	4.71 [w)]	6	3.88	5.98	4.32

a) Experimental values of bioconcentration factor measured using fish species. b) Taken from [30]. c) Taken from [31]. d) Taken from [32]. e) Taken from [33]. f) Taken from [34]. g) Taken from [35]. h) Taken from [36]. i) Taken from [37]. j) Taken from [38]. k) Taken from [39]. l) Taken from [40]. m) Taken from [41]. n) Taken from [42]. o) Taken from [43]. p) Taken from [44]. q) Taken from [18]. r) Taken from [45]. s) Taken from [46]. t) Taken from [47]. u) Taken from [48]. v) Taken from [49]. w) Taken from [9]. x) log P_{oct} values calculated using the software EPI Suite v3.12. y) log BCF predicted using Eq. 4.3. z) retention factor measured using a Hypersil™ GOLD Javelin HTS column with MeOH as organic modifier. aa) log BCF predicted using Eq. 4.4.

4.3.1 Correlation between experimental log BCF and calculated log P values

Calculated log P values obtained using EPI Suite software (v3.12), for the entire range of lipophilicity, were correlated with the log BCF data. A high-polynomial correlation was proposed by Connell and Hawker [22] when lipophilic compounds (log P > 6) were employed since this model eliminated the influence of non-equilibrium conditions [22, 50]. However, because the statistical validity of this model is questionable [23, 50], a model was recalculated and a parabolic relationship was obtained. It was emphasized that according to the experimental artifacts which occurred when measuring log BCF values for highly lipophilic compounds, a conservative non-linear approach is recommended. Indeed, parabolic [26, 27] or bilinear models [51, 52] are thus more appropriated [25] for lipophilic compounds. Moreover, since bilinear model provides a better fit than other models, and because it is recommended by the OECD guidelines [12], this latter model was chosen in this study. The OECD criteria to classify bioaccumulative compounds are as follow: "very bioaccumulative" when log BCF is higher than 3.7 and "bioaccumulative" when log BCF is between 3.3 and 3.7 [53]. These thresholds are represented by the red and the green lines, respectively, in Fig. 4.4.

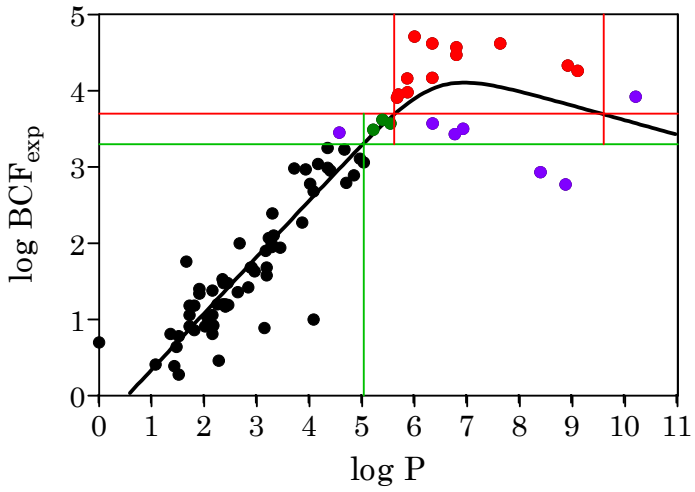


Figure 4.4: Correlation between log BCF$_{exp}$ (experimental) and calculated log P values (obtained using EPI Suite software v3.12). Red and green lines represent the regulatory limits: log BCF = 3.7 and log BCF = 3.3, respectively. Black, green, red and purple dots are for non—bioaccumulative, bioaccumulative, very—bioaccumulative and wrongly evaluated compounds respectively.

$$\log BCF_{exp} = 0.74(\pm 0.07) \bullet \log P - 0.94(\pm 0.21) \log(4.34 \bullet 10^{-7} \bullet P + 1) - 0.41(\pm 0.25) \quad \text{Eq. 4.3}$$

$n = 85,\ r^2 = 0.88,\ s = 0.44,\ F = 200.$

In this and following equations, n is the number of compounds, r^2 the squared correlation coefficient, s the standard deviation and F the Fisher's test value. 95% confidence intervals are given in parentheses.

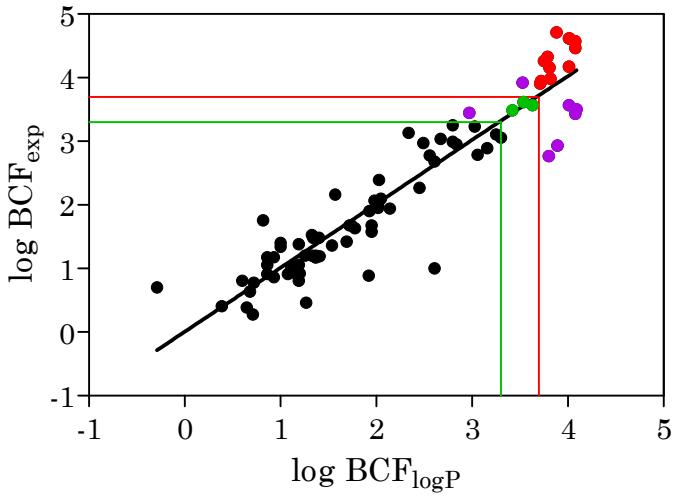


Figure 4.5: Comparison between experimental ($\log BCF_{exp}$) and predicted log BCF values (using bilinear model based on calculated log P ($\log BCF_{logP}$))
$\log BCF_{exp} = 1.01(\pm 0.08) \bullet \log BCF_{logP} - 0.01(\pm 0.20)$
$n = 85,\ r^2 = 0.88,\ s = 0.44,\ F = 611$. Red and green lines represent the regulatory limits: log BCF = 3.7 and log BCF = 3.3, respectively. Black, green, red and purple dots are for non—bioaccumulative, bioaccumulative, very—bioaccumulative and wrongly evaluated compounds respectively.

As shown in Fig. 4.4 and 4.5, some compounds were predicted more bioaccumulative regarding the calculated log P than evaluated with the log BCF values such as the fenvalerate, the tricresyl phosphate and the 1,2,3,4-tetrachlorodibenzo-p-dioxin which were estimated very bioaccumulative (v-BCF) whereas their log BCF values evaluated them as bioaccumulative. The octachlorodibenzofuran and the bis(2-ethylhexyl)phthalate were estimated very bioaccumulative (v-BCF) whereas their log BCF values evaluated them as non-bioaccumulative. These two compounds were thus estimated as false-positive. On the other hand, some compounds were estimated less or even

non-bioaccumulative with the calculated log P compared to the log BCF values such as the decachlorobiphenyl. The worse situation was obtained with the 1,2,4,5-tetrachlorobenzene since this compound was estimated as non-bioaccumualtive with the calculated log P value whereas the log BCF evaluated it as bioaccumulative and is thus a false-negative.

A good example of the poor prediction of log BCF using calculated log P values is given by the octachlorodibenzofuran which is not bioaccumulated in fishes whereas its calculated log P value of 8.87 predicts it as very-bioaccumulative. In the same way, the 2,2',3,3',4,4',5,5'-octachlorobiphenyl possesses the same calculated log P value (8.91) and is classified in very-bioaccumulative compounds. Therefore, the calculated log P values could reach erroneous predictions in particular for highly lipophilic compounds for which variation between experimental and calculated log P values are often observed.

Furthermore, the interval of bioconcentration is not clear for the high lipophilicity due to a lack of compound with log P values higher than 10 and a safety range is thus difficult to express. Even if a decrease in bioaccumulation has been reported for highly lipophilic compounds, the model proposed with calculated log P values seems not to satisfy this assumption. Hence, to avoid risks of toxicity for human health and environment, only compound with log P values inferior to 5.0 could be accepted for further development.

4.3.2 Correlation between experimental log BCF and log k_w values obtained on Hypersil™ GOLD Javelin HTS column

log k_w values were used in this study especially to avoid the inaccuracy induce by the determination of log P_{oct} values and were directly compared to the bioconcentration factors obtained using different fish species (Fig. 4.6).

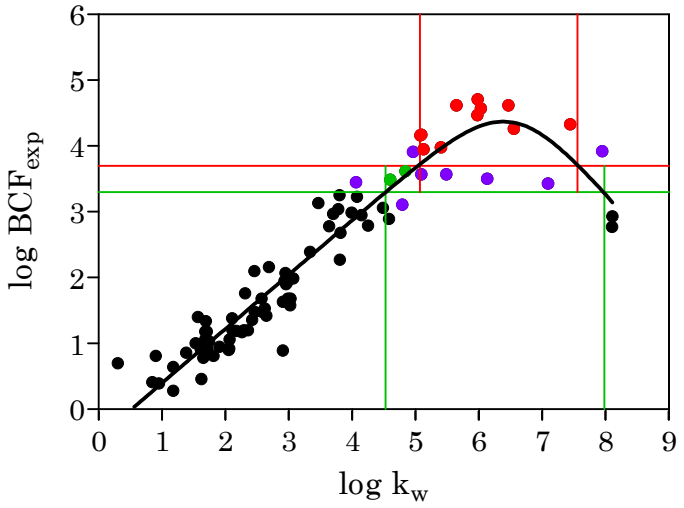


Figure 4.6: Correlation between log BCF_{exp} and log k_w values obtained on Hypersil™ GOLD Javelin HTS column. Red and green lines represent the regulatory limits: log BCF = 3.7 and log BCF = 3.3, respectively. Black, green, red and purple dots are for non—bioaccumulative, bioaccumulative, very—bioaccumulative and wrongly evaluated compounds respectively.

$$\log BCF_{exp} = 0.83(\pm 0.06) \bullet \log k_w - 1.95(\pm 0.33) \log(3.03 \bullet 10^{-7} \bullet k_w + 1) - 0.43(\pm 0.19) \quad \text{Eq. 4.4}$$

$n = 85,\ r^2 = 0.92,\ s = 0.36,\ F = 316.$

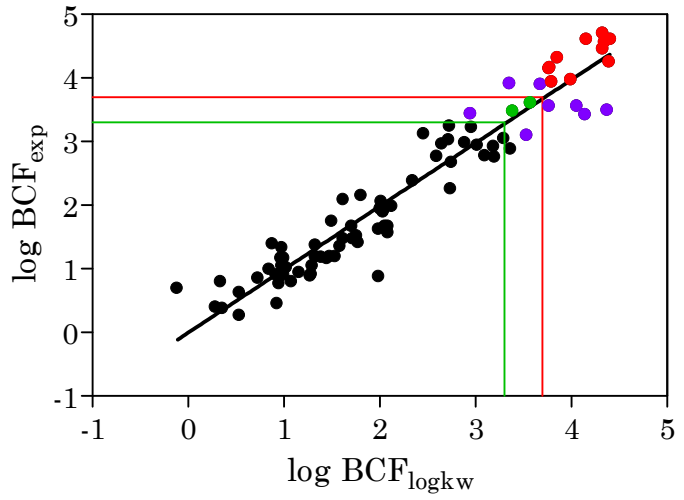


Figure 4.7: Comparison between experimental (log BCF_{exp}) and predicted log BCF values (using bilinear model based on experimental log k_w (log BCF_{logkw}))

$\log BCF_{exp} = 0.99(\pm 0.06) \bullet \log BCF_{logkw} - 0.00(\pm 0.16)$

$n = 85,\ r^2 = 0.92,\ s = 0.36,\ F = 975$. Red and green lines represent the

regulatory limits: log BCF = 3.7 and log BCF = 3.3, respectively. Black, green, red and purple dots are for non—bioaccumulative, bioaccumulative, very—bioaccumulative and wrongly evaluated compounds respectively.

It has already been demonstrated that bioconcentration rate decreases when the lipophilicity is sufficiently high. Different reasons were set out to explain these results as for example, the low solubility of highly lipophilic compounds that restricted the chemicals dissolved in water and then the bioconcentration factor. Moreover, due to the very important lipophilicity, the analytes could be trapped in the membrane [54] and thus were not measured into the lipid part of the animals. Hence, these compounds are not bioaccumulated into the organism as generally shown by log BCF measurements.

This characteristic seems to take shape when log k_w values are used as descriptor as shown in Figure 4.6.

Some compounds were problematic like the 1,2,4,5-tetrachlorobenzene since this compound was predicted as non-bioaccumualtive regarding its retention factor (log k_w) whereas the log BCF value evaluated it as bioaccumulative. Identical problem was obtained with the calculated log P value. This compound induces thus a false-negative evaluation, as in 1% of cases. On the other hand, two compounds, the chlornitrofen and the butyl benzyl phthalate were predicted bioaccumulative whereas their log BCF values evaluated them as non-bioaccumulative and were hence false-positive. However, other parameters than lipophilicity can influence the bioconcentration and an in-depth study should be made on these outliers.

However, with experimental log k_w values, more than half poor-predicted compounds were estimated very-bioaccumulative instead of bioaccumulative or bioaccumulative instead of very-bioaccumulated, but all these compounds would be *a priori* rejected from advanced development since bioaccumulative. Using calculated log P values, more than half compounds were predicted non-bioaccumulated instead of bioaccumulated (or very-bioaccumulated) or reverse which is more problematic.

Furthermore, predicted log BCF values were better estimated using the retention factors measured with a Hypersil™ GOLD Javelin HTS stationary phase

all over the log P range compared to predicted log BCF values estimated using calculated log P as shown with Figure 4.7 and Figure 4.5.

Right now, too few compounds with very high log P and known log BCF values have been tested to confirm this model. But this rapid experimental method could be used to first estimate bioaccumulation capacity in screening process. The preliminary model suggests that a chemical is not considered bioaccumulative if its retention factor is lower than 4.5 or higher than 8. In this interval the compound should not be retained for a drug development as it could induce adverse effect for human and environmental problems. Hence, a bioaccumulation classification of chemicals could be done with a good confidence using experimental log k_w values measured on Hypersil™ GOLD Javelin HTS stationary phase that is REACH compatible as, according to the OECD directive, no animal were used for this evaluation of the toxicity assessment.

4.4 Conclusion

The experimental method using the Hypersil™ GOLD Javelin HTS stationary phase seems to be promising for the measurement of the chemicals' risk assessment which is hence consistent with the legislative requirement (REACH). Indeed, as analytical measurement were used, no fish were required for the bioconcentration evaluation.

Hence, a compound can be considered as very-bioaccumulative if its $5.0 < \log k_w < 7.6$ and bioaccumulative if $4.5 < \log k_w < 5.0$ and $7.6 < \log k_w < 8.0$. Thus, for log k_w values inferior to 4.5 or higher than 8.0, all the compounds could be developed without problem of bioconcentration. The method developed in chapter 2 and used in this study allows an evaluation of bioconcentration more comparable than experimental assessment in animals. In fact, all these log k_w values will be done using the same stationary phase and hence reduced the errors. Moreover, an interval of confidence could be obtained for log k_w values which could increase the accuracy of log BCF data. Indeed, at our knowledge, only few publications [55] were realised about this subject and no interval of error could be given for log BCF values.

Finally it is relevant to say that the estimation of the bioconcentration via the measurement of the log k_w values is low time consuming as shown in Table 4.1.

Furthermore, this time is necessary for reliable results concerning bioconcentration, especially when it concerns the development of new drugs.

References

1. Regulation (EC) No 1907/2006 of the European parliament and of the council. 2006.
   Ref Type: Patent

2. *Directive 2006/121/EC of the European Parliament and of the Council*; 2006.

3. Nordberg, A.; Ruden, C. The usefulness of the bioconcentration factor as a tool for priority setting in chemicals control. *Toxicol. Letters* **2007,** *168*, 113-120.

4. Rand, G. *Fundamentals of Aquatic Toxicology. Second Edition: Effects, Environmental Fate and Risk Assessment*; Taylor & Francis: London, 1995; pp 1-128.

5. Barron, M. G. Bioconcentration. *Environ. Sci. Technol.* **1990,** *24*, 1612-1618.

6. Meylan, W. M.; Howard, P. H.; Boethling, R. S.; Aronson, D.; Printup, H.; Gouchie, S. Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. *Environ. Toxicol. Chem.* **1999,** *18*, 664-672.

7. Mackay, D.; Fraser, A. Bioaccumulation of persistent organic chemicals: mechanisms and models. *Environ. Poll.* **2000,** *110*, 375-391.

8. Hamelink, J. L.; Spacie, A. Fish and chemicals - process of accumulation. *Ann. Rev. Pharmacol. Toxicol.* **1977,** *17*, 167-177.

9. Veith, G. D.; Defoe, D. L.; Bergstedt, B. V. Measuring and estimating the bioconcentration factor of chemicals in fish. *Journal of the Fisheries Research Board of Canada* **1979,** *36*, 1040-1048.

10. Branson, D. R.; Blau, G. E.; Alexander, H. C.; Neely, W. B. Bioconcentration of 2,2',4,4'-Tetrachlorobiphenyl in rainbow-trout as measured by an accelerated test. *Trans. Am. Fish. Soc.* **1975,** *104*, 785-792.

11. Hamelink, J. L.; Waybrant, R. C.; Ball, R. C. Proposal - exchange equilibria control degree chlorinated hydrocarbons are biologically magnified in lentic environments. *Trans. Am. Fish. Soc.* **1971,** *100*, 207-&.

12. *OECD Guidelines for Testing of Chemicals. Proposal for Updating Guideline 305 - Bioconcentration: Flow-through Fish Test*; 1996.

13. Fatemi, M. H.; Jalali-Heravi, M.; Konuze, E. Prediction of bioconcentration factor using genetic algorithm and artificial neural network. *Anal. Chim. Acta* **2003,** *486*, 101-108.

14. *White Paper. Strategy for a Future Chemicals Policy*; Commission of the European Communities: 2001; pp 1-32.

15. Nenzda, M. *Structure-Activity Relationships in Environmental Sciences*; Chapman & Hall: Padstow,Cornwall, 1998; pp 1-271.

16. Neely, W. B.; Branson, D. R.; Blau, G. E. Partition-coefficient to measure bioconcentration potential of organic chemicals in fish. *Environ. Sci. Technol.* **1974,** *8*, 1113-1115.

17. Mackay, D. Correlation of bioconcentration factors. *Environ. Sci. Technol.* **1982,** *16*, 274-278.

18. Kanazawa, J. Bioconcentration potential of pesticides by aquatic organisms. *Jap. Pest. Inf.* **1981,** *39*, 12-16.

19. Schuurmann, G.; Klein, W. Advances in bioconcentration prediction. *Chemosphere* **1988,** *17*, 1551-1574.

20. Isnard, P.; Lambert, S. Estimating bioconcentration factors from octanol-water partition coefficient and aqueous solubility. *Chemosphere* **1988,** *17*, 21-34.

21. Samiullah, Y. *Prediction of the Environmental Fate of Chemicals*; British Petroleum Company: 1990; pp 1-284.

22. Connell, D. W.; Hawker, D. W. Use of polynomial expressions to describe the bioconcentration of hydrophobic chemicals by fish. *Ecotoxicol. Environ. Saf.* **1988,** *16*, 242-257.

23. Bintein, S.; Devillers, J.; Karcher, W. Nonlinear dependence of fish bioconcentration on n-octanol/water partition coefficient. *SAR QSAR in Environ. Res.* **1993,** *1*, 29-39.

24. Pavan, M.; Netzeva, T. I.; Worth, A. P. Review of literature-based quantitative structure-activity relationship models for bioconcentration. *QSAR Comb. Sci.* **2008,** *27*, 21-31.

25. Hope, B.; Scatolini, S.; Titus, E. Bioconcentration of chlorinated biphenyls in biota from the North Pacific Ocean. *Chemosphere* **1998,** *36*, 1247-1261.

26. Konemann, H.; Vanleeuwen, K. Toxico-kinetics in fish - accumulation and elimination of 6 chlorobenzenes by guppies. *Chemosphere* **1980,** *9*, 3-19.

27. Zhao, R. B.; Sun, D. Y.; Fu, S.; Wang, X. F.; Zhao, R. S. Bioconcentration kinetics of PCBs in various parts of the lifecycle of the tadpoles Xenopus laevis. *J. Environ. Sci.* **2007,** *19*, 374-384.

28. Kubinyi, H. Liphophilicity and biological activity. Drug transport and drug distribution in model systems and in biological systems. *Arzneim. - Forsch.* **1979,** *29*, 1067-1080.

29. Guillot, A.; Henchoz, Y.; Moccand, C.; Guillarme, D.; Veuthey, J. L.; Carrupt, P. A.; Martel, S. Lipophilicity determination of highly lipophilic compounds by liquid chromatography. *Chem. Biodivers.* **2009,** 1828-1836.

30. Kobayashi, K.; Akitake, H.; Hiroshi, M.; Manabe, K. Studies on the metabolism of chlorophenols in fish. X. Relation between toxicity and accumulation of various chlorophenols in goldfish. *Nippon Suisan Gakkaishi* **1979,** *45*, 173-175.

31. CITI Biodegradation and bioaccumulation data of existing chemicals based on the CSL Japan. *Chemicals Inspection and Testing Institute Japan* **2002**.

32. Kalsch, W.; Nagel, R.; Urich, K. Uptake, elimination, and bioconcentration of ten anilines in zebrafish (Brachydanio rerio). *Chemosphere* **1991,** *22*, 351-363.

33. Barrows, M. E.; Petrocelli, S. R.; Kenneth, J.; Carroll, J. Bioconcentration and elimiation of selected water pollutants by bluegill sunfish. *J. Am. Chem. Soc.* **1978,** *18*, 345-346.

34. Dimitrov, S.; Dimitrova, N.; Parkerton, T.; Comber, M.; Bonnell, M.; Mekenyan, O. Base-line model for identifying the bioaccumulation potential of chemicals. *SAR QSAR in Environ. Res.* **2005,** *16*, 531-554.

35. Southworth, G. R.; Keffer, C. C.; Beauchamp, J. J. The accumulation and disposition of benz(A)acridine in the fathead minnow, pimephales-promelas. *Arch. Environ. Contamination Toxicol.* **1981,** *10*, 561-569.

36. Shiu, W. Y.; Ma, K. C.; Varhanickova, D.; Mackay, D. Chlorophenols and alkylphenols: a review and correlation of environmentally relevant properties and fate in an evaluative environment. *Chemosphere* **1994,** *29*, 1155-1224.

37. Zok, S.; Gorge, G.; Kalsch, W.; Nagel, R. Bioconcentration, metabolism and toxicity of substituted anilines in the zebrafish (Brachydanio-Rerio). *Sci. Total Environ.* **1991,** *109*, 411-421.

38. Veith, G. D.; Kosian, P. Estimating bioconcentration potential from octanol/water partition coefficients. *Phys. Behav.* **1983,** 269-282.

39. Deneer, J. W.; Sinnige, T. L.; Seinen, W.; Hermens, J. L. M. Quantitative structure-activity-relationships for the toxicity and bioconcentration factor of nitrobenzene derivatives towards the guppy (Poecilia-Reticulata). *Aquatic Toxicol.* **1987,** *10*, 115-129.

40. Canton, J. H.; Slooff, W.; Kool, H. J.; Struys, J.; Pouw, T.; Wegman, R. C. C.; Piet, G. J. Toxicity, biodegradability, and accumulation of a number of Cl/N-containing compounds for classification and establishing water quality criteria. *Regul. Toxicol. Pharmacol.* **1985,** *5*, 123-131.

41. Devillers, J.; Bintein, S.; Domine, D. Comparison of BCF models based on log P. *Chemosphere* **1996,** *33*, 1047-1065.

42. Freitag, D.; Geyer, H.; Kraus, A.; Viswanathan, R.; Kotzias, D.; Attar, A.; Klein, W.; Korte, F. Ecotoxicological profile analysis : VII. Screening chemicals for their environmental behavior by comparative evaluation. *Ecotoxicol. Environ. Saf.* **1982,** *6*, 60-81.

43. Tsuda, T.; Aoki, S.; Kojima, M.; Harada, H. Pesticides in water and fish from rivers flowing into Lake Biwa. *Toxicol. Environ. Chem.* **1991,** *34*, 39-55.

44. Carlson, A. R.; Kosian, P. A. Toxicity of chlorinated benzenes to fathead minnows (pimephales-promelas). *Arch. Environ. Contamination Toxicol.* **1987,** *16*, 129-135.

45. Muir, D. C. G.; Yarechewski, A. L.; Grift, N. P. Environmental dynamics of phosphate esters. III. Comparison of the bioconcentration of four triaryl phosphates by fish. *Chemosphere* **1983,** *12*, 155-166.

46. Leifer, A.; Brink, R. H.; Thom, G. C.; Partymiller, K. G. Environmental transport and transformation of polychlorinated biphenyls. *Gov. Rep. Announce* **1984,** *84*, 70.

47. Freitag, D.; Ballhorn, L.; Geyer, H.; Korte, F. Environmental hazard profile of organic chemicals : An experimental method for the assessment of the behaviour of organic chemicals in the ecosphere by means of simple laboratory tests with 14C labelled chemicals. *Chemosphere* **1985,** *14*, 1589-1616.

48. Spehar, R. L.; Tanner, D. K.; gibson, J. H. Effects of Kelthane and Pydrin on early life stages of fathead minnows (Pimephales promelas) and amphipods (Hyalella azteca). *ASTM Spec. Tech. Publ.* **1982,** *766*, 234-244.

49. Boethling, R. S.; Cooper, J. C. Environmental fate and effects of triaryl and tri-alkyl aryl phosphate-esters. *Res. Rev.* **1985,** *94*, 49-99.

50. Pavan, M.; Worth, A. P.; Netz, T. I. *Review of QSAR Models for Bioconcentration*; European Commission: 2006; pp 1-142.

51. Kubinyi, H. Quantitative structure-activity-relationships .4. Nonlinear dependence of biological-activity on hydrophobic character - new model. *Arzneim. -Forsch.* **1976,** *26*, 1991-1997.

52. Kubinyi, H. Non-linear dependence of biological activity on hydrophobic character: the bilinear model. *Pharmacochem. Libr.* **1977,** *2*, 239-249.

53. Tyle, H.; Larsen, H. S.; Wedebye, E. B.; Sijm, D.; Pedersen Krog, T.; Niemelä, J. *Identification of potential PBTs and vPvBs by use of QSARs*; Danish EPA, SHC/TS 2-3/029: 2002.

54. Akamatsu, M.; Fujikawa, M.; Nakao, K.; Shimizu, R. In silico prediction of human oral absorption based on QSAR analyses of PAMPA permeability. *Chem. Biodivers.* **2009,** *6*, 1845-1866.

55. Bailer, A. J.; Walker, S. E.; Venis, K. J. Estimating and testing bioconcentration factors. *Environ. Toxicol. Chem.* **2000,** *19*, 2338-2340.

Conclusions and outlooks

The knowledge of the lipophilicity of new chemical entities (NCEs) allows the prediction of many important behaviors of chemical compounds since it contributes to solubility, membrane permeation, metabolism or bioconcentration for examples. Thus this parameter is mandatory for pharmaceutical, cosmetics, agroalimentary or pesticides industries and has to be measured in the early stages of research process using high-throughput methods. In spite of the progresses realized in lipophilicity measurements, the lipophilicity of highly lipophilic compounds was still hardly measurable, especially for log P_{oct} > 6 or for basic compounds with pK_a > 7.

In the current thesis, high throughput screening RPLC (reversed-phase liquid chromatography) and UHPLC (ultra high pressure liquid chromatography) methods were first optimized to obtain log P_{oct} values up to 10 for neutral, acidic and weakly basic compounds, using short columns and up to 7.7 for basic compounds with pK_a > 7. Indeed, different analytical methods (Discovery® RP Amide C16, Acquity UPLC™ BEH Shield RP18 and Hypersil™ GOLD Javelin HTS stationary phases in isocratic mode with methanol, acetonitrile or tetrahydrofuran as organic modifier and in gradient mode with methanol) were compared to evaluate the best way to determine the lipophilicity of moderately to highly lipophilic compounds. The lowest analysis time and best correlation between retention factor extrapolated to 100% water (log k_w) and the partition coefficient obtained in n-octanol/water system (log P_{oct}) values were obtained using the Hypersil™ GOLD Javelin HTS stationary phase in isocratic mode with methanol as organic modifier. More lipophilic compounds could be evaluated to still enlarge this lipophilicity range. Since the stationary phases used are instable at high pH, hydrophilic interaction chromatography (HILIC) showed its capacity to determine the lipophilicity of basic compounds with pK_a > 7 and log P_{oct} up to 7.7. More compounds would be evaluated to confirm this result for this lipophilicity enlargement and maybe evaluate the upper-limit of this method. Furthermore, zwitterionic compounds could be evaluated using this column and hence allow for the first time the log P_{oct} determination of these particular and problematic compounds.

To summarize, depending on the type of compounds, different conditions should be used. For basic compounds with pK_a > 7, only the HILIC method is

available. For neutral, acidic and weakly basic compounds, a first approximation of the lipophilicity can be obtained using Acquity UPLC™ BEH Shield RP18 column in gradient mode which is very useful for a first screening since this method is generic and rapid. This stationary phase should also be chosen for more accurate lipophilicity measurements in isocratic mode if buffer solution of relatively high pH are needed (pH up to 10.5). But in other cases, Hypersil™ GOLD Javelin HTS of 10 mm is the stationary phase of choice since the analysis times remain reasonable even for very high lipophilic compounds.

The optimized high throughput screening methods (Discovery® RP Amide C16, Acquity UPLC™ BEH Shield RP18 and Hypersil™ GOLD Javelin HTS stationary phases in isocratic mode with methanol as organic modifier) were then used to measure the log P_{oct} values of the cyclosporins A and C. The three analytical methods used confirm that the cyclosporin A is highly lipophilic with log P_{oct} values superior or equal to 9.1. These results explain its low membrane permeation and especially its poor aqueous solubility. Although the two cyclosporins had different behaviours on the different stationary phases used compared to the other compounds tested, these differences were not due to method's limitation (too important volume or the high lipophilicity of the cyclosporin family) but probably to different interactions between compound and component of stationary phases because of conformational changes in cyclosporins. Preliminary analysis of the impact of different conformations on calculated log P tends to be directed to this end and hence all these three experimental results could be considered as correct determination of the two cyclosporins lipophilicities. Thus the preliminary molecular modeling study encourages exploring more thoroughly the conformational surface of the cyclosporin A. High temperature molecular dynamics or Monte Carlo methodologies are currently performed and the complexity of the simulated environment to approach the properties of the liquid chromatographic (LC) columns under study will be gradually increased to:

- assess the impact of different methanol-water mixtures on the computed conformations and their apparent lipophilicity.
- assess the feasibility of modeling the molecular structure of LC columns and hence model the different ligands' stationary

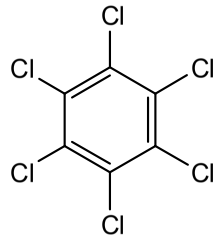
phases to confirm that the different embedded groups could induce these differences of log P_{oct} values.

Finally, the method developed with the Hypersil™ GOLD Javelin HTS stationary phase seems to be promising for the measurement of the chemicals' risk assessment. Hence, the method allows to predict the bioconcentration capacity from the retention factor measured by UHPLC and we determined that a compound can be considered as very-bioaccumulative if its $5.0 < \log k_w < 7.6$ and bioaccumulative if $4.5 < \log k_w < 5.0$ and $7.6 < \log k_w < 8.0$. Thus, for $\log k_w$ values inferior to 4.5 or higher than 8.0, compounds could be developed further. The advantages of this method is that experimental $\log k_w$ values will be done using the same stationary phase reducing the errors and is low time consuming. More compounds with high lipophilicity are under study to confirm these first results and in a second time be validated and officially replaced experiments on fishes as recommended by the Registration, Evaluation, Authorization of CHemicals (REACH) directive.

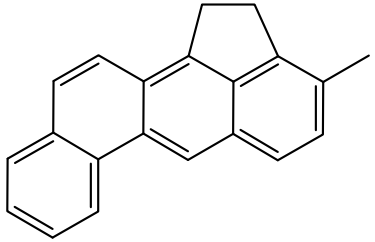
Structures of molecules used

O
N
N,N-diethylacetamide
O
O
Ethyl acetate
OH
Benzyl alcohol
H2N
NH2
Benzidine
O
OH
4-nitroaniline
NH2
NO2
Valeric acid
O
O
Phenyl-2-propanone
O
OH
Phenylacetic acid
OH
Phenol
N
Benzyl cyanide
O
Acetophenone
O
O
Butyl acetate
NO2
Nitrobenzene
NH2
Cl
2-chloroaniline
OH
O2N
p-nitrophenol
OH
Cl
4-chlorobenzyl alcohol
O
Anisole
H
N
N-ethylaniline
O
Propiophenone
Cl
NO2
1-chloro-2-nitrobenzene
O
OH
Cl
3-chlorophenylacetic acid
NH2
2-aminonaphtalene

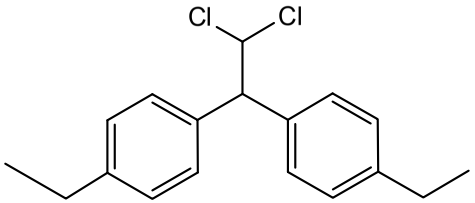
2-phenylethyl acetate
m-toluic acid
4-phenylbutyric acid
3-chlorophenol
Ethyl benzoate
Toluene
2-aminobiphenyl
1-naphtoic acid
Naphtalene
m-dichlorobenzene
Mesitylene
Biphenyl
Benzyl benzoate
1,2,4,5-tetrachlorobenzene
Pentamethylbenzene
Bibenzyl
Benz[a]anthracene
Perylene
p-terphenyl
Tri-o-tolyl phosphate
2,4'-DDD



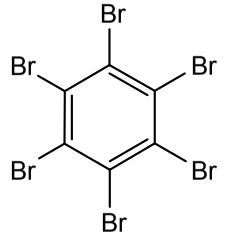
Hexachlorobenzene



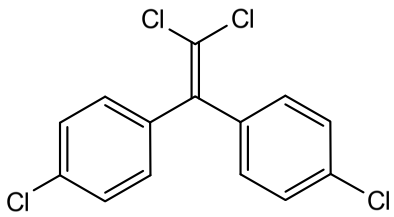
3-methylcholanthrene



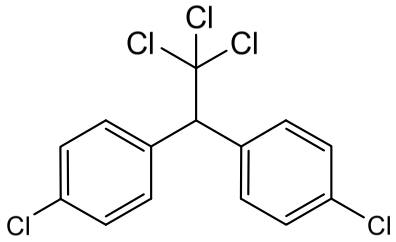
Perthan



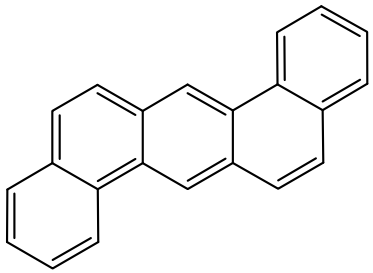
Hexabromobenzene



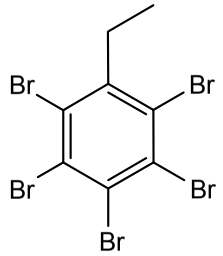
4,4'-DDE



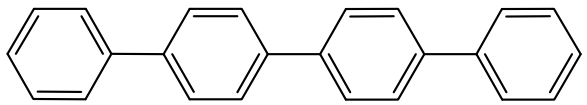
4,4'-DDT



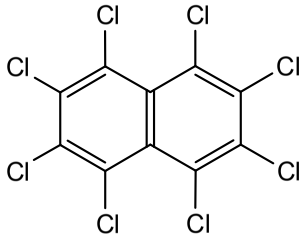
Dibenz[a,h]anthracene



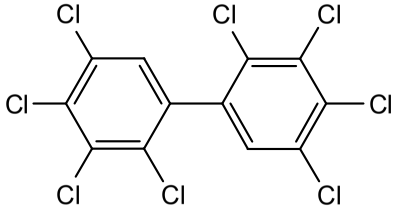
2,3,4,5,6-pentabromoethylbenzene



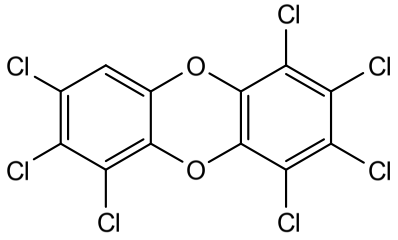
p-quaterphenyl



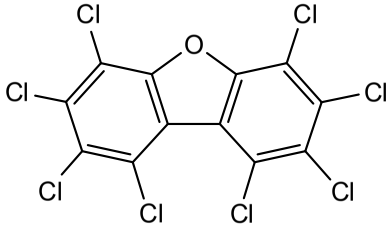
Octachloronaphtalene



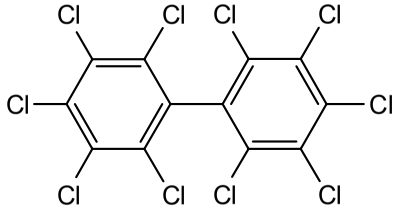
2,2',3,3',4,4',5,5'-octachlorobiphenyl



1,2,3,4,6,7,8–heptachlorodibenzo-p-dioxin



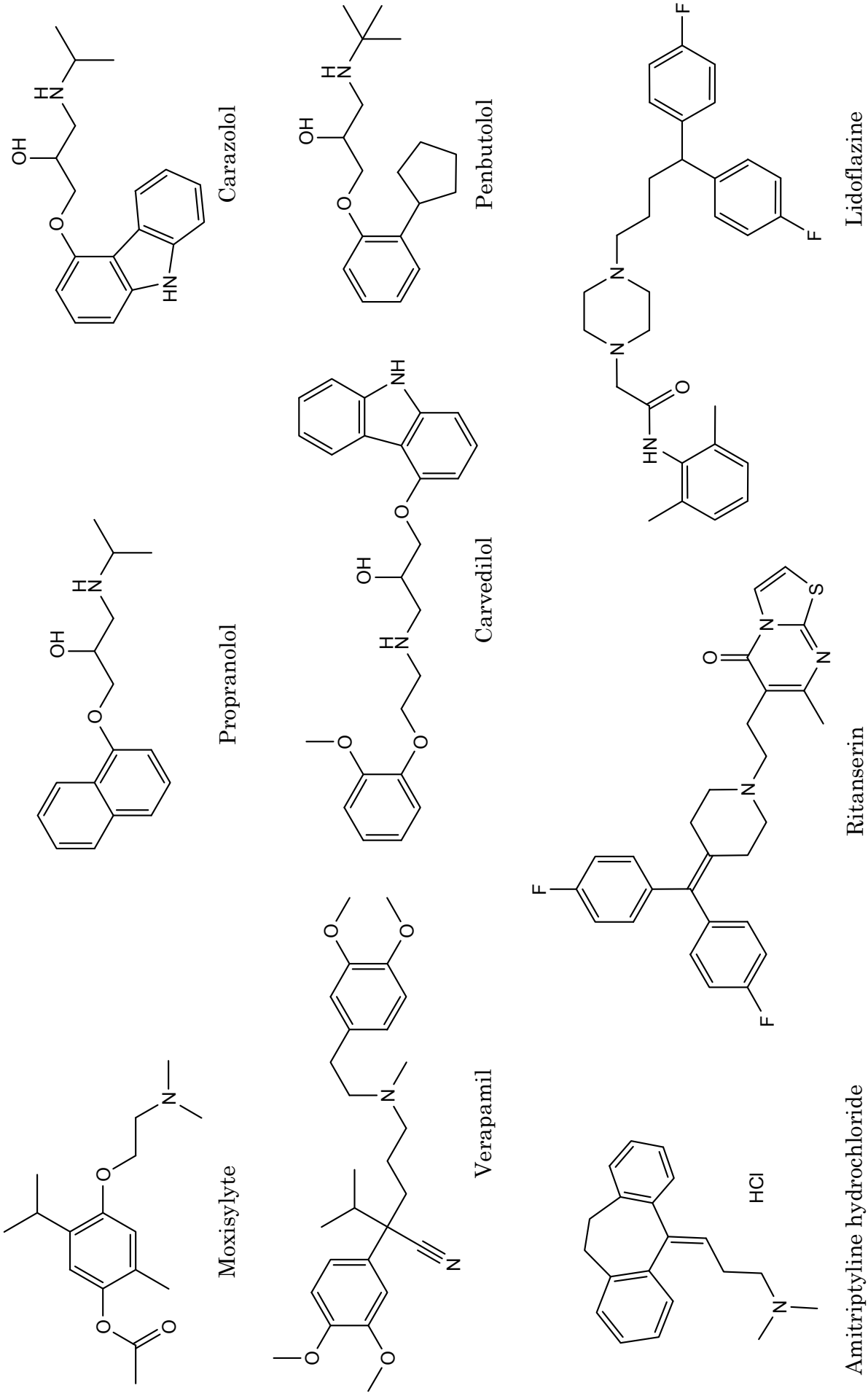
1,2,3,4,6,7,8,9–octachlorodibenzofuran

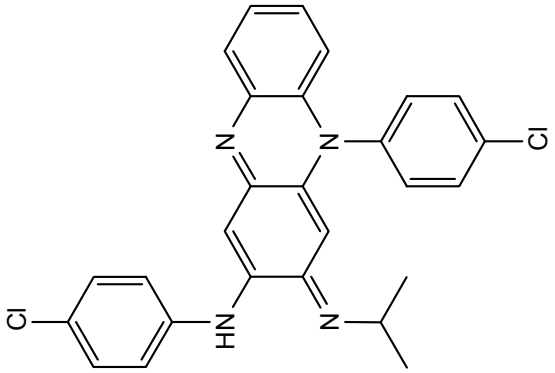


Decachlorobiphenyl

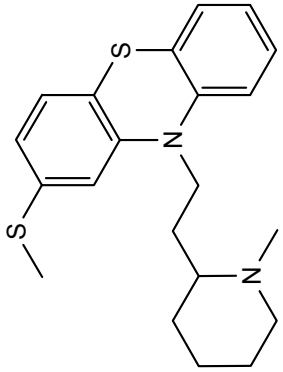
Ethanolamine
Triethanolamine
Octopamine
Morpholine
Serotonine
Pseudoephedrine
Tyrosine methyl ester
Nadolol
Atenolol
Homatropine
Methylephedrine
Nicotine
Scopolamine
Atropine
Butylamine
Pindolol
Rilmenidine
N,N-dimethylbenzylamine

Strychnine
Acebutolol
Metoprolol
Mepivacaine
Procaine
Timolol
Prilocaine
Apomorphine
Lidocaine
Oxprenolol
Metipranolol
Alprenolol
Bisoprolol
Tacrine
MPTP


Carazolol
Penbutolol
Lidoflazine
Propranolol
Carvedilol
Ritanserin
Moxisylyte
Verapamil
Amitriptyline hydrochloride

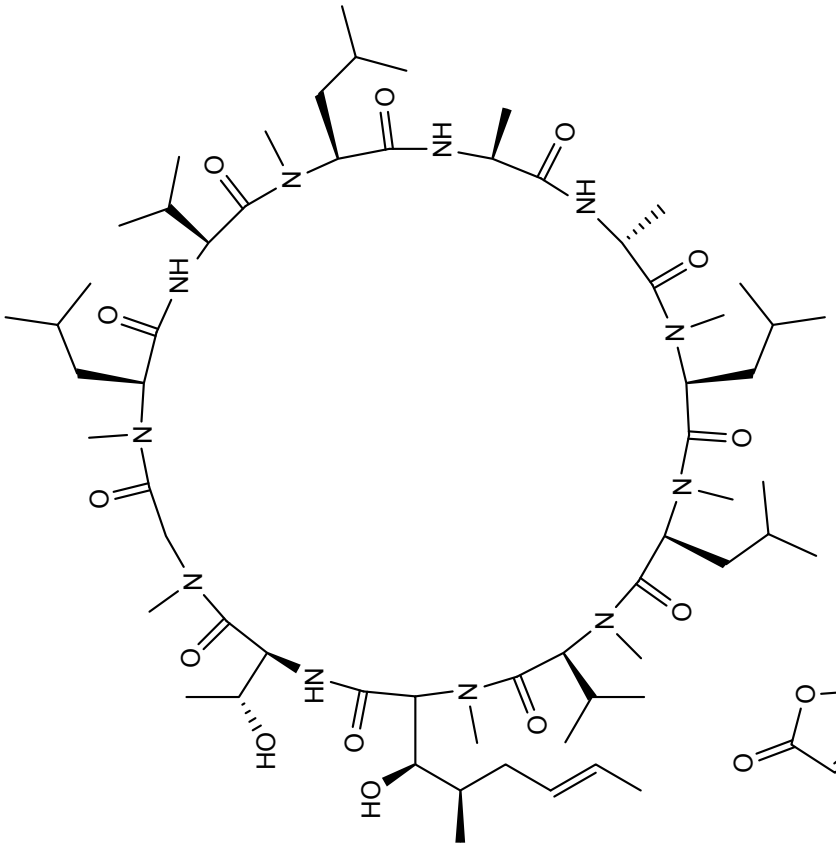


Clofazimine

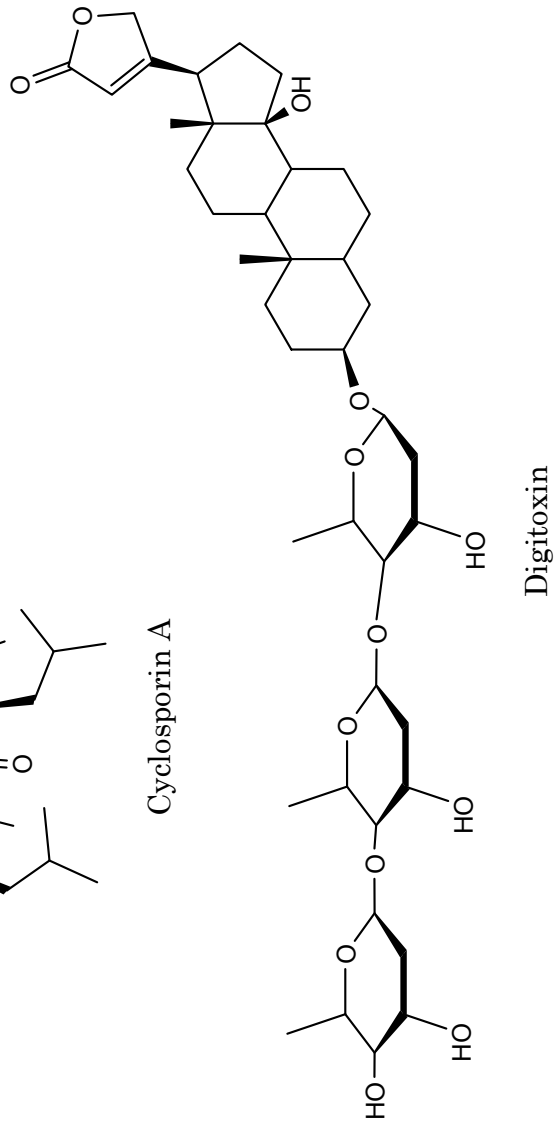


HCl

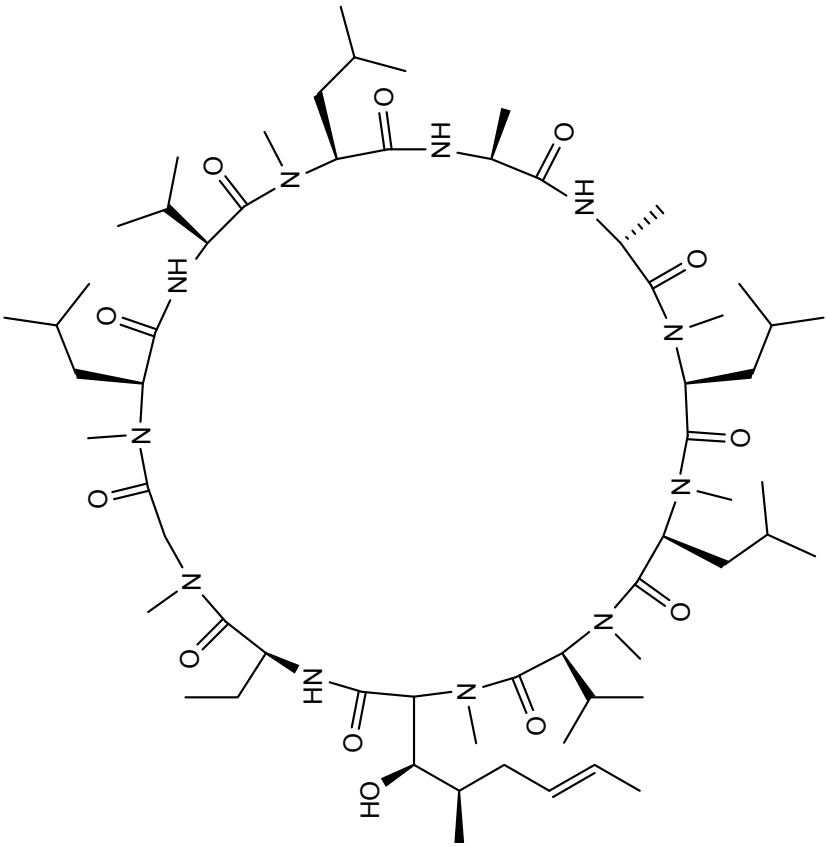
Thioridazine hydrochloride



Cyclosporin C



Digitoxin

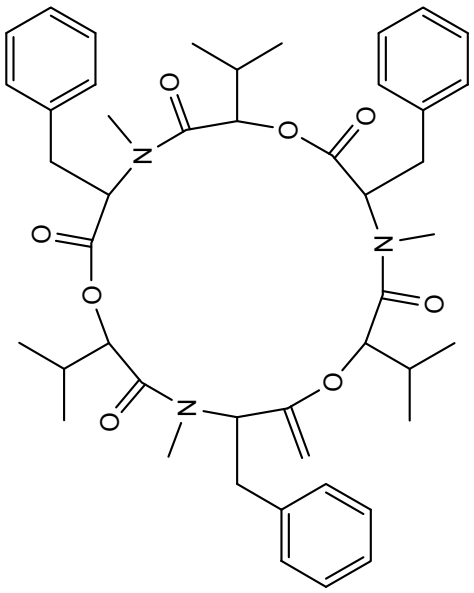


Cyclosporin A

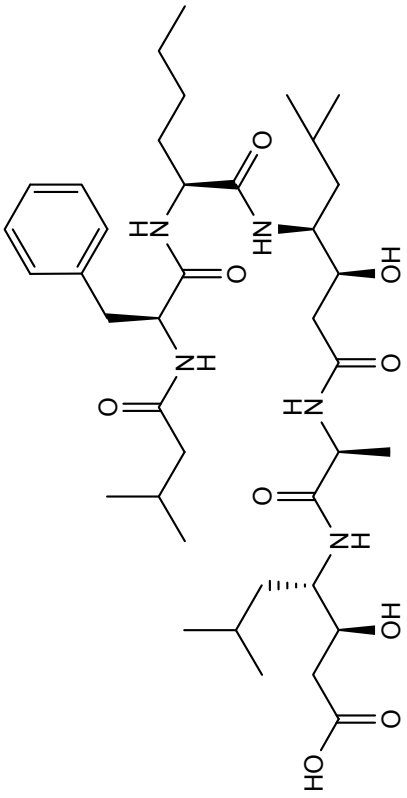
5,5'-[1,4-phenylenebis(oxy)]bis[2-(3-hydroxypropyl)
-1H-isoindole-1,3(2H)-dione]

3-Benzo[1,3]dioxol-5-yl-N-{3-[(3-benzo[1,3]dioxol-5-yl-2-cyano-
acryloylamino)-methyl]-benzyl}-2-cyano-acrylamide

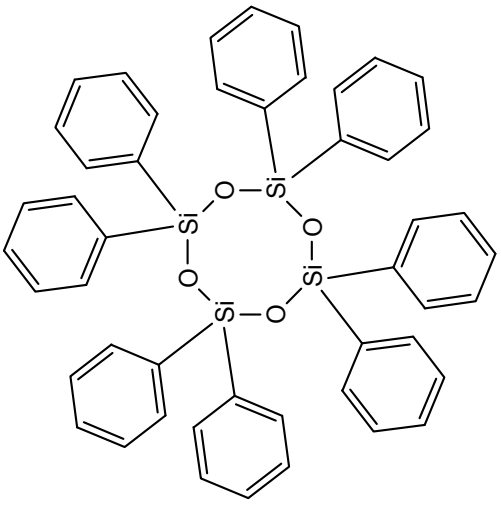
Bromophenol blue



Beauvericin



Isovaleryl-Phe-Nle-Sta-Ala-Sta-OH



Octaphenylcyclotetrasiloxane

OH
Phenol
Br
Cl
Chlorobromomethane
NH2
Aniline
OH
NO2
NO2
4,6-dinitro-o-cresol
NH2
O2N
4-nitroaniline
NH2
H2N
2,4-toluenediamine
Cl
Cl
Cl
Chloroform
O
Furan
Cl
OH
2-chlorophenol
S
Thiophene
O
Benzophenone
N
Quinoline
H2N
O2N
2-nitroaniline
NH2
Cl
4-chloroaniline
NO2
NO2
2,4-dinitrotoluene
HN
N-ethylaniline
NH
N-methylaniline
Cl
Cl
3-chloroaniline
Br
HO
p-bromophenol
Cl
NH2
2-chloroaniline
OH
m-methylphenol
NO2
Nitrobenzene
Cl
NO2
2-chloro-1-nitrobenzene

1-naphthylamine 3-nitrotoluene m-bromophenol 2,4,6-trichlorophenol 2-nitrophenol Chlorobenzene

4-chlorophenol 3-nitrophenol 2-aminobiphenyl 3,4-dichloroaniline Carbon tetrachloride

1-naphthyl-N-methylcarbamate p-chlorotoluene Benzotrifluoride Bromobenzene Molinate

o-chlorotoluene Dimethyl phtalate Naphtalene 1,2-dichlorobenzene 1,3-dichlorobenzene Carbazole

Acridine

Diuron

Diazinon

Fenitrothion

Fenthion

Octachlorodibenzofuran

9H-fluorene

Triphenyl phosphate

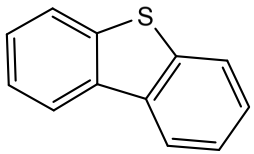
Butyl benzyl phthalate

Bis(2-ethylhexyl) phthalate

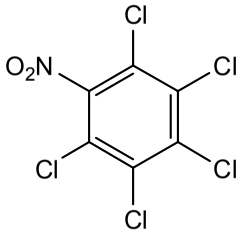
4-chlorobiphenyl

1,3,5-Trichlorobenzene

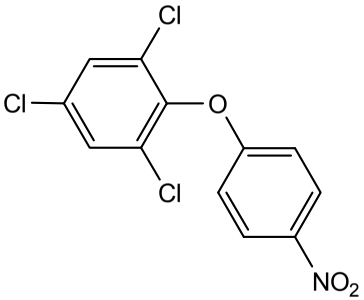
Anthracene



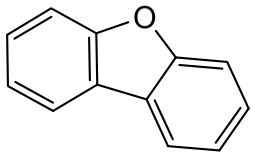
Dibenzothiophene



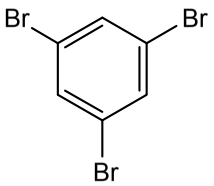
Pentachloronitrobenzene



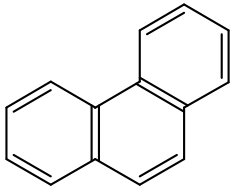
Chlornitrofen



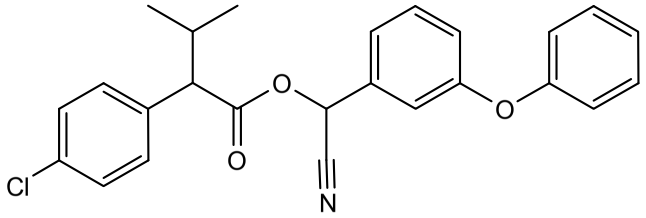
Dibenzofuran



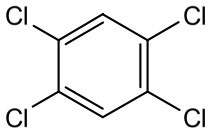
1,3,5-tribromobenzene



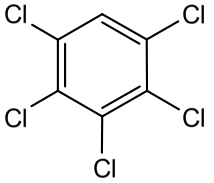
Phenanthren



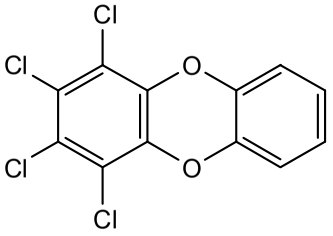
Fenvalerate



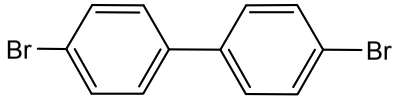
1,2,4,5-tetrachlorobenzene



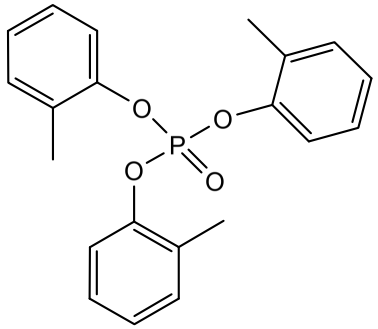
Pentachlorobenzene



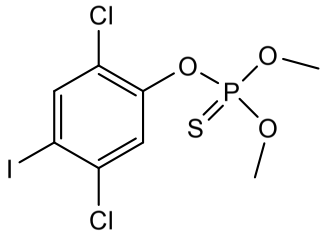
1,2,3,4-tetrachlorodibenzo-p-dioxin



p,p'-dibromobiphenyl



Tricresyl phosphate



Iodfenphos

Hexachlorobenzene

Heptachlor

2,4',5-trichlorobiphenyl

p,p'DDT

2,2',3,3',4,4',5,5'-octachlorobiphenyl

p,p'-DDE

Decachlorobiphenyl

2,2',4,4',5,5'-hexachlorobiphenyl

Methoxychlor

2,2',4,4',6,6'-hexabromobiphenyl

2,2'3,3'-tetrachlorobiphenyl

2,3',4',5-tetrachlorobiphenyl

o,p'-DDT