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Department of Analytical Chemistry

THESIS

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Use of Chiral Separations for the Determination of Enzyme Enantioselectivity

THESIS

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Obzor kdo v očích nemá, nic nepochopil
(Who does not see behind horizon, does not understand anything)

Jiří Wabi Ryvola (1935 - 1995)

I honestly declare that all results summarized in this thesis are based either on my own experimental work or on the team work where my contribution was substantial. Information gathered from the experiments conducted by the other parties is properly referenced. This dissertation is not substantially the same as any work that has been, or is being submitted to any other university for any degree, diploma or any other qualification.

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List of Attached Publications

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-V.

I. Honzatko A., Brichac J., Murphy T. C., Reberg A., Kubátová A., Smoliakova I. P., Picklo M. J. Sr.: Enantioselective metabolism of *trans*-4-hydroxy-2-nonenal by brain mitochondria. *Free Radical Biology & Medicine* **2005**, *39*, 913-924.

II. Brichac J., Ho K. K., Honzatko A., Wang R., Lu X., Weiner H., Picklo M. J. Sr.: Enantioselective oxidation of *trans*-4-hydroxy-2-nonenal is aldehyde dehydrogenase isozyme and Mg^{2+}-dependent. *Chemical Research in Toxicology* **2007**, *20*, 887-895.

III. Brichac J., Honzatko A., Picklo M. J.: Direct and indirect high-performance liquid chromatography enantioseparation of *trans*-4-hydroxy-2-nonenoic acid. *Journal of Chromatography A* **2007**, *1149*, 305-311.

IV. Honzatko A., Brichac J., Picklo M. J.: Quantification of *trans*-4-hydroxy-2-nonenal enantiomers and metabolites by LC-ESI-MS/MS. *Journal of Chromatography B* **2007**, *857*, 115-122.

V. Kotik M., Brichac J., Kyslik P.: Novel microbial epoxide hydrolases for biohydrolysis of glycidyl derivatives. *Journal of Biotechnology* **2005**, *120*, 364-375.

Key words

chiral separations
HPLC
GC
LC-MS/MS

enzyme enantioselectivity
enzyme kinetic resolution

trans-4-hydroxy-2-nonenal (HNE)
trans-4-hydroxy-2-nonenoic acid (HNEA)
aldehyde dehydrogenases (ALDHs)
oxidative stress

epoxide hydrolases

List of Abbreviations

ACN	acetonitrile
ADH	alcohol dehydrogenases
AGD	3-allyloxy-1,2-propanediol
AGE	allyl glycidyl ether
AKR	aldo-keto reductase
ALDH	aldehyde dehydrogenase
ANPAD	(1*S*,2*S*)-(+)-2-amino-1-(4-nitrophenyl)-1,3-propanediol
BGD	3-benzyloxy-1,2-propanediol
BGE	benzyl glycidyl ether
BuGE	butyl glycidyl ether
CD	circular dichroism
CS	chiral selector
CSP	chiral stationary phase
CE	capillary electrophoresis
CEC	capillary electrochromatography
CTPC	cellulose *tris*(phenylcarbamate)
DNA	deoxyribonucleic acid
E	enantiomeric ratio of the kinetic resolution
ECD	electronic circular dichroism
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
ees	enantiomeric excess of the substrate
eep	enantiomeric excess of the product
EPR	electron paramagnetic resonance
ER	enantiomeric ratio of the mixture
ESI	electrospray ionization
FDA	Food and Drug Administration
FID	flame ionization detector
FLEC	1-(9-fluorenyl)ethylchloroformate
GC	gas chromatography
GITC	2,3,4,6-tetra-*O*-acetyl-*β*-*D*-glucopyranosyl isothiocyanate
GST	glutathione S-transferase
HBT	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HNE	*trans*-4-hydroxy-2-nonenal
HNEA	*trans*-4-hydroxy-2-nonenoic acid
IL	ionic liquid

IUPAC	International Union of Pure and Applied Chemistry
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
MTPA-Cl	Mosher's acid chloride
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NMR	nuclear magnetic resonance
NP	normal-phase
ORD	optical rotatory dispersion
PO	polar organic
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	reversed-phase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFC	supercritical fluid chromatography
SPE	solid phase extraction
TBDMS	*tert*-butyldimethylsilyl
TBD	3-*tert*-butoxy-1,2-propanediol
TBE	*tert*-butyl glycidyl ether
TLC	thin-layer chromatography
UV	ultraviolet
VCD	vibrational circular dichroism
VIS	visible
*	denotes the chiral center

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1. INTRODUCTION

1.1 Preface

The key elements of the life – replication, information processing, and metabolism –occur largely by molecular recognition mechanisms. Molecular recognition is based on specific non-covalent interaction between biomolecules, including receptor-ligand, enzyme-substrate, antigen-antibody, DNA-protein and sugar-lectin interactions. The biological activity of a molecule often depends upon its stereochemistry.

Most of the molecular components of living organisms are nonsuperimposable on its mirror image, the property known as chirality. All living organisms are inheretently chiral. Superior three-dimensional structure of proteins often allows them to distinguish between two enantiomers. As a result, enzymes and receptors are able to stereospecifically recognize chiral substrates and chiral ligands. The chiral recognition or the enantioselectivity is a subtle aspect of the broader subject of above mentioned molecular recognition.

Chiral compounds, including drugs, pharmaceuticals, agrochemicals, pheromones, food additives, flavors, fragrances, new chiral materials and catalysts represent classes of chemicals with high economic and scientific impact and there is increasing demand for optically pure compounds.

Chiral separations are the most important methods for analysis of chiral compounds. Despite the fact, that chiral separations found many applications in biomedicine and biotechnology and became a classical technique during last two decades, the extensive research related to enantioseparation is still on-going. Development and application of novel separation methods using commercially available stationary phases is broadening the range of the applications. New chiral stationary phases (CSPs) are synthesized, analyte-chiral selector (CS) interactions are studied and effort is devoted to miniaturization of chiral separation on chip.

1.2 Enantioselectivity

1.2.1 Stereochemistry of chiral compounds

Isomers that possess identical constitution but which differ in the arrangement of their atoms in space are called **stereoisomers.**[1] Interactions between biological molecules are almost always stereospecific[2] and arrangement of a molecule's atom in three-dimensional space plays key role in biochemistry of living organisms.

When a carbon atom has four different substituents, the substituent groups can be arranged in two different ways generating two stereoisomers. Such asymmetric carbon represents **chiral center** of the molecule[2] and molecule exists as two **enantiomers**. In general, a molecule with *n* chiral centers has a maximum of 2^n stereoisomers.[3]

Enantiomers are two identical molecular species that represent nonsuperimposable mirror images of each other.[2] For example, glyceraldehyde exists as a pair of enantiomers (see Figure 1). Unless exposed to chiral environment, enantiomers display identical physical and chemical properties.[1] Due to enantiomers having exactly the same physical properties, they are extremely difficult to separate. Separation can only be achieved by using chiral agents in the separation process.[4]

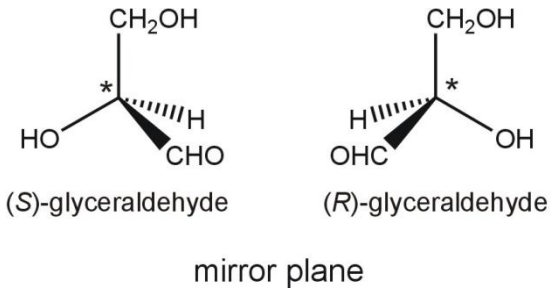


Figure 1. Glyceraldehyde is a chiral molecule and exists as (*R*)- and (*S*)-enantiomer.

The presence of a carbon atom bonded to four different groups is the most common (although not the only) cause of chirality in organic molecules.[3] In general, **chirality** is the geometric property of a rigid object of being nonsuperimposable on its mirror image[5] and is caused by the presence of one or more chirality elements (chirality axis, chirality plane,

or chirality center) in their structure.[1] A molecule may be chiral, and therefore optically active, only if it does not possess an axis of improper rotation, S_n. However, such an axis may be implied by other symmetry elements that are present. Thus, a molecule may be chiral if it does not have a centre of inversion *i* or a mirror plane σ.[6] The simplest example of chiral objects are left and right hand. When a right hand is hold hand up to a mirror, the image we see looks like a left hand. Most biologically important molecules are chiral, and usually only a single stereoisomer is found in nature.[3]

Optical activity is property of chiral compounds due to the asymmetric structure of the molecules themselves and is observed when passing plane-polarized light through an enantiomerically enriched solution. Optically active molecules rotate the plane of polarization of a plane-polarized light.[5] The amount and direction of rotation can be measured with an instrument known as a **polarimeter**. When viewed in the direction facing the oncoming light beam, an enantiomer causing rotation to the right (clockwise direction) is called dextrorotatory, whereas other rotates polarized light to the left (counterclockwise) is said to be levorotatory. By convention, rotation to the right is given a plus sign (+), and rotation to the left is given a minus sign (-).[3]

Racemate is an equimolar mixture (enantiomeric ratio of the mixture *ER* is 50:50) of a pair of enantiomers, which is optically inactive.[1] Racemate is denoted either by the symbol (±) or by the prefix *d,l* to indicate a mixture of dextrorotatory and levorotatory forms. The physical properties of racemate usually differ from those of the two enantiomers.[3]

Diastereomers are stereoisomers that are not mirror images of each other. Diastereomers have opposite configurations at one or more chiral centers, but have the same configuration at others. Enantiomers, by contrast, have opposite configurations at all stereogenic centers.[3] Diastereomers are characterized by differences in physical and chemical properties, and thus can often be resolved by conventional separation techniques.[1, 4]

1.2.2 Specification of configuration

Absolute configuration is the spatial arrangement of the atoms in the molecule. In contrast to the conformation, the molecular configuration of enantiomers can be changed only by temporarily breaking one or more covalent bonds.[1]

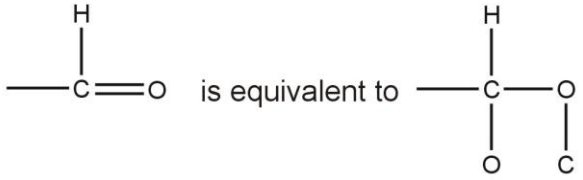
For **specification of configuration** at a chiral center, **Cahn-Ingold-Prelog sequence rules** are used for the purpose of unambiguous designation of stereoisomers:[3, 4]

1. If all the atoms directly attached to the chiral atom are different, then the priority sequence is determined by the atomic number of each element. The atom with highest atomic number is ranked first; the atom with lowest atomic number is ranked fourth.

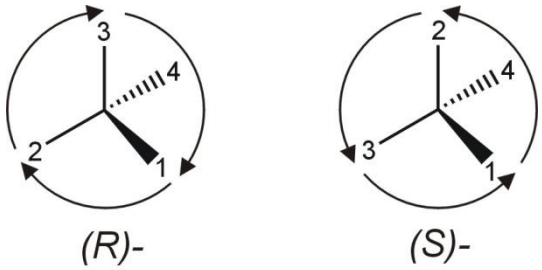
2. If a decision about priority can't be reached by applying rule l, atomic numbers of the second atoms in each substituent are compared continuing on as necessary through the third or fourth atoms until a point of difference is reached.

3. Multiple-bonded atoms are considered equivalent to the same number of single-bonded atoms. If a double bond, or triple bond, is present, the specific atom(s) attached to the double bond are considered to be doubled or tripled, respectively (see Figure 2).

When the priorities are assigned, the molecule is visualized so that the lowest priority atom or group points away from the viewer into the paper. Labeling each atom or group in order of their priority sequence, if the decrease in priority order is clockwise, the substance is defined as (*R*). If the decrease in priority is anti clockwise it is defined as (*S*) (see Figure 2). Note that the sign of optical rotation, (+) or (-), is not related to the *R*, *S* designation.



(a)



(b)

Figure 2. Cahn-Ingold-Prelog sequence rules: (a) multiple-bonded atoms are equivalent to the same number of single-bonded atoms, (b) application of the rules to the specification of the configuration.

1.2.3 Enantioselectivity, enantioselective enzymes and chiral selectors

According to International Union of Pure and Applied Chemistry (IUPAC), enantioselectivity is the preferential formation in a chemical reaction of one enantiomer over another.[5] Unfortunately, this definition is too narrow to cover various aspects of this phenomenon. Therefore, we define **enantioselectivity**[*] as an ability of chiral environment to distinguish between two enantiomers. Enantioselective enzyme, catalyst, receptor, antibody or chromatographic chiral selector (CS) preferentially interacts with or preferentially form in a chemical reaction one enantiomer over the other.

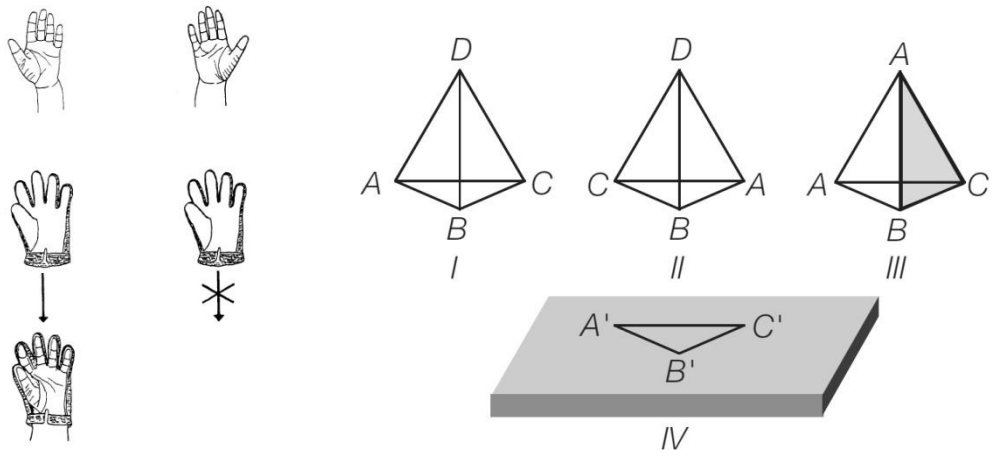
Chemical or biological activity of a substance often depends upon its stereochemistry and chiral recognition (enantioselectivity) is a subtle aspect of the broader subject of molecular recognition.[7] Chiral recognition is based on **three-point interaction**. In three-point attachment model (see Figure 3), when three groups (*A*, *B* and *C*) of the

[*] In this thesis, we mention **enantioselectivity of an enzyme** (quantitatively described by enantiomeric ratio *E*, see (1-8)) and **enantioselectivity of a chiral separation system** (quantitatively described by separation factor α, see (1-14))

tetrahedral carbon atom (enantiomer *I*) bind to a protein surface (*IV*) at specific sites *A'*, *B'* and *C'*, it is impossible to bind the equivalent groups *A*, *B* and *C* of its mirror image (enantiomer *II*) at the same three sites. If there are two *A* groups to produce a prochiral molecule (*III*), the model can distinguish between the two identical groups.[8, 9]

One of the most remarkable enantioselective species are enzymes. An **enantioselective enzyme** prefers one enantiomer over the other as a substrate and/or preferentially forms one enantiomer over the other as a product. In general, enzymes show high substrate and product stereoselectivity and regioselectivity.[10] Enantioselectivity arises because of enzymes inherent chirality. Proteins consist of only *L*-amino acids, therefore they form asymmetric active sites.[11] A chiral molecule must fit into a chiral active site, much as a hand fits into a glove. But just as a right hand can fit only into a right-hand glove, so a particular stereoisomer can fit only into an active site having the proper complementary shape (see Figure 3).[3]

Enantioselective interaction of **analyte with chiral selector (CS)** embedded in stationary phase or presented in mobile phase is also based on three-point interaction. Dalgliesh[12] postulates that three interactions have to take effect and at least one of them has to be stereoselective to obtain chiral recognition.



(a) (b)

Figure 3. (a) Illustration of enantioselectivity: the left hand fits into the left glove properly, but the right hand does not, (b) chiral recognition of enantiomers *I* and *II* and a prochiral molecule *III* by three-point interaction. The protein surface to which the molecules bind is shown in *IV*.

1.2.4 Why to study enantioselectivity: implications of chirality in biomedicine and biotechnology

In organisms, amino acids are almost exclusively *L* isomers, and sugars are almost exclusively *D* isomers. However, the origins of this preference remain a mystery. Question "what is the origin of homochirality in nature" was recently selected as one of the most 125 emerging questions of contemporary science by prestigious scientific journal Science.[13]

The chiral nature of living systems has evident implications on biologically active compounds interacting with them.[14] Examples bellow illustrate, that chirality and enantioselectivity is of interest in numerous fields of contemporary life sciences.

Pharmacology and physiology

Interaction of **enantioselective enzyme** and chiral drug might lead to different metabolic pathways of individual enantiomers. Enantioselective interaction of ligand with its **receptor**[15] or enantioselective interaction of **antigen with its specific antibody**[16-18] might lead to different physiological or immunological response. Stereoisomers may differ in **pharmacodynamics properties** (interaction of bioactive agents with enzymes and receptors, pharmacological and toxicological activity), in **pharmacokinetic properties** (absorption, distribution, metabolic conversion, biotransfotmation, and excretion of the drug)[19] and in **clinical pharmacology properties** (therapeutic index and response, bioavailability, adverse drug reactions, drug-drug interactions). As a result, enantiomers may show quantitatively or qualitatively different **pharmacologic or toxicologic effects**.[14, 20] Very often one of enantiomer represents the more active isomer for a given action, while the other one might be even active in a different way, contributing to side-effects, displaying toxicity, or acting as antagonist.[14]

One of the key event through which people recognized the importance of evaluating the difference in pharmacological behaviors of enantiomers of a drug is tragedy of thalidomide. (±)-thalidomide (see Figure 4) had been used as a hypnotic drug and sedative under the name Contergan. Unfortunately, thalidomide is also teratogen and teratogenic action may be caused only by the (*S*) isomer, whereas the (*R*) isomer may not show any teratogenic behavior.[21]

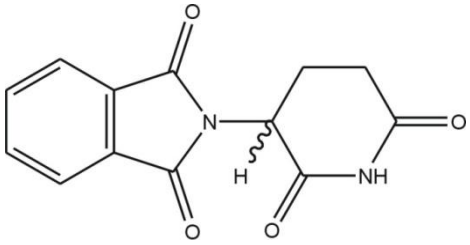


Figure 4. (±)-thalidomide

The (*S*)-enantiomers of *α*-arylpropionic acids, including (*S*)-ibuprofen, are responsible for the anti-inflammatory effect useful in treatment of rheumatoid arthritis.[3] On the other hand (*R*)-ibuprofen is inactive. However, since the (*R*)-isomers undergo metabolic inversion of configuration by racemase to form the active (S)-isomers, these drugs have been administrated as racemates.[19]

Only penicillin V (2*S*, 5*R*, 6*R*) has biologic effect. Its enantiomer is inactive and does not occure naturally.[3] *d*-sotalol has a class III antiarrhythmic activity while *l*-sotalol is a β-blocker.[20] (*R*) enantiomer of the anticancer prodrug ifosfamid is metabolised enantioselectively and exerts greater anticancer activity than (*S*)-ifosfamid.[22]

Other example comes from zoology. Chiral **pheromones** are used to chemical communication between elephants. The *ER* of released pheromon frontalin enables Asian elephants to distinguish both the maturity of male elephants and phase of the annual period of heightened sexual activity. In other words, mature male Asian elephants secrete a chiral chemical in a ratio that is especially attractive to females.[23]

The United States **Food and Drug Administration (FDA)** recommends that unless it proves particularly difficult, the pharmacologic activities of the individual enantiomers should be characterized. To evaluate the pharmacokinetics of a single enantiomer or mixture of enantiomers, manufacturers should develop quantitative assay for individual enantiomers in *in vivo* samples early in drug development. [1, 20]

Where little difference is observed in activity and disposition of the enantiomers, racemates may be developed. However, in some situations, development of a single enantiomer is particularly desirable (e.g., where one enantiomer has a toxic or undesirable pharmacologic effect and the other does not). Differences in pharmacokinetic behavior

may not pose a major therapeutic problem although it can make non-chiral blood level assays difficult to interpret.[20]

Biotechnology

Enantioselective enzymes are implied in **biotechnology and organic chemistry synthesis**. Enzyme kinetic resolution of racemate or enzymatic asymmetric synthesis might serve for cheap synthesis of optically pure compounds. This topic is discussed in chapter 1.2.6.2.

Food and fragrances

Some enantiomers are distinguished by **sensory receptors** for smell and taste in humans. For example, (*R*)-carvone has the characteristic fragrance of spearmint, on the other hand, (*S*)-carvone smells like caraway. Aspartame (*L*-Aspartyl-*L*-phenylalanine methyl ester), the artificial sweetener, is easily distinguishable by taste receptors from its bitter-tasting enantiomer (*D*-Aspartyl-*D*-phenylalanine methyl ester).[2]

Agrochemistry

A large number of examples of differences in biological activities between enantiomers of chiral agrochemicals have been observed.[24] Enantioselectivity of pollutant degradation by microorganisms, including degradation of some phenoxy acid herbicides, organophosphorus insecticides and DDT derivatives, is influenced by environmental changes.[25]

Special applications

In the archeology and forensic science, measurements of the degree of racemisation of specific amino acids are used to date the age of human remains such as bones and teeth.[14, 26]

Enantioselective chromatography might be a powerful tool for the discrimination of biotic and abiotic transformation processes of chiral environmental pollutants.[27]

1.2.5 Enzymes

Subject of this thesis is implication of enantioselectivity in enzymology, therefore enzymes properties are briefly discussed.

Enzyme is protein that acts as a catalyst.[28] Like all catalysts, enzymes act by lowering the **activation energy** $\Delta G^{\ddagger}$ for the reaction being catalyzed[11], thus dramatically accelerate the rate of the reaction. Enzymes are not consumed by the reactions they catalyze and they do not affect reaction equilibria.[2]

The distinguishing feature of an enzyme-catalyzed reaction is that it takes place within the confines of a pocket on the enzyme called the **active site**. The molecule that is bound in the active site and is a reactant in a catalyzed reaction is called the **substrate**. The surface of the active site is lined with amino acid residues with substituent groups that bind the substrate and catalyze its chemical transformation. Often, the active site encloses a substrate, sequestering it completely from solution.[2] Enzymes are classified according to reaction they catalyze (see Table 1).

Table 1. Enzyme Classification According to Reaction Type[11]

Classification	Type of Reaction Catalyzed	Examples [a]
1. Oxidoreductases	Oxidation-reduction reactions	aldehyde dehydrogenase catalase superoxide dismutase
2. Transferases	Transfer of functional groups	glutathione transferase hexokinase RNA polymerase I
3. Hydrolases	Hydrolysis reactions	epoxide hydrolase phospholipase A triacylglycerol lipase trypsin
4. Lyases	Group elimination to form double bond	pyruvate decarboxylase
5. Isomerases	Isomerization	alanine racemase aldose 1-epimerase
6. Ligases	Bond formation coupled with ATP hydrolysis	DNA ligase (ATP) tyrosine-tRNA ligase

[a] Enzyme nomenclature database 13.10.2007; http://www.expasy.org/enzyme/

Kinetics is the study of the rates at which chemical reactions occur. Enzyme kinetics plays important role in the studying of enantioselective enzymes.

The simple enzymatic reaction is composed of two elementary reactions in which the substrate forms a complex with the enzyme that subsequently decomposes to products an enzyme. *E*, *S*, *ES*, and *P* symbolize the enzyme, substrate, enzyme-substrate complex, and products, respectively:[11]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E \qquad \text{(1-1)}$$

The **Michaelis-Menten equation**, the basic equation of enzyme kinetics, is derived with simplifying assumption that [*ES*] maintains steady state during reaction. $[E]_0$ is the total concentration of an enzyme:

$$v_0 = \frac{k_2[E]_0[S]}{K_M + [S]} = \frac{V_{max}[S]}{K_M + [S]} \qquad \text{(1-2)}$$

Initial velocity of the reaction v_0 depends on the **substrate concentration** [*S*] and has the form of a rectangular hyperbola (see Figure 5).[11] The use of the initial velocity (operationally taken as the velocity measured before more than ~10% of the substrate has been converted to product) rather than just the velocity minimizes such complicating factors as the effects of reversible reactions, inhibition of the enzyme by product, and progressive inactivation of the enzyme.[11]

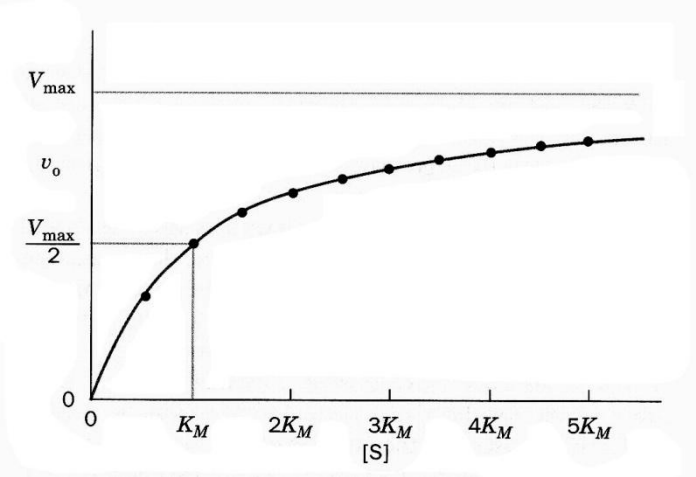


Figure 5. Plot of the initial velocity v_0 of a simple Michaelis-Menten reaction versus the substrate concentration [*S*].

K_M and V_{max} describe the catalytic properties of the enzyme. **Michaelis constant** is defined as ratio of rate constants:

$$K_M = \frac{(k_{-1} + k_2)}{k_1} = K_S + \frac{k_2}{k_1} \quad \text{(1-3)}$$

K_M is the substrate concentration at which the reaction velocity is half-maximal. If an enzyme has a small value of K_M, it achieves maximal catalytic efficiency at low substrate concentrations. K_M is also a measure of the affinity of the enzyme for its substrate providing k_2/k_1 is small compared with dissociation constant K_S.[11]

The maximal velocity of a reaction, V_{max}, occurs at high substrate concentration when the enzyme is saturated, that is, when it is entirely in the ES form.[11] Note that V_{max} is directly proportional to total amount of enzyme used.

$$V_{max} = k_2[E]_0 \quad \text{(1-4)}$$

Catalytic constant k_{cat} of an enzyme is defined as:

$$k_{cat} = \frac{V_{max}}{[E]_0} \quad \text{(1-5)}$$

k_{cat} is also called **turnover number**, because it reflects the number of substrate molecules converted per unit time by a single enzyme molecule (or by a single active centre). For the Michaelis-Menten model, k_{cat} is equal to k_2. For enzymes with more complicated mechanisms, k_{cat} may be a function of several rate constants.[11]

When concentration of substrate is very low ($[S] << K_M$), very little *ES* is formed and equation (1-2) reduces to a second-order rate equation:

$$v_0 \approx \frac{k_{cat}}{K_M}[E][S] \quad \text{(1-6)}$$

Specificity constant k_{cat}/K_M is the apparent second-order rate constant of the enzymatic reaction; the rate of the reaction varies directly with how often enzyme and substrate encounter one another in solution. The quantity k_{cat}/K_M is therefore a measure of an enzyme's catalytic efficiency.[11] k_{cat}/K_M is useful for comparing different enzymes

against each other, or the same enzyme with different substrates. This ratio is considered to be the best measure of substrate specificity.[29]

1.2.6 Preparation of optically pure compounds

Reaction between two achiral reactants always leads to optically inactive products – either racemic or meso-compounds.[3] Therefore, processes leading to a enantiopure compound have to involve some form of chiral environment.

A chiral material might be prepared from optically pure intermediates. However, this approach only moves the problem of purification to a point higher in the synthetic chain.[4] A more common solution is to employ a biosynthetic procedure[30] using organism (e.g. a yeast or bacteria) or enzyme that will selectively cleave or chemically alter one enantiomer only.[4] Thus, optically pure compound might be prepared by **asymmetric (stereoselective) synthesis** or by **kinetic resolution of racemate**. Pure enantiomers might be also prepared by achiral synthesis followed by separation on a preparative scale using either liquid chromatography (LC) or supercritical fluid chromatography (SFC). Special crystallization procedures might also lead to optically pure compounds.

1.2.6.1 Asymmetric synthesis

Asymmetric (stereoselective) synthesis is a chemical reaction (or reaction sequence) in which one or more new elements of chirality are formed in a substrate molecule and which produces the enantiomeric products in unequal amounts.[5] Prochiral molecule can be made chiral by the addition of a new atom or achiral group to the trigonal system.[5] The specific attachment of a prochiral center to an enzyme binding site permits the enzyme to differentiate between prochiral groups.[11] For example, asymmetric reduction of achiral aromatic ketones using secondary alcohol dehydrogenase from *Thermoanaerobacter ethalonicus* leads to enantioselective formation of secondary alcohols.[31]

Enantiomeric ratio *E* of asymmetric catalysis is defined as ratio of catalytic constant[32]:

$$E = \frac{k_{cat}^{R}}{k_{cat}^{S}} \quad (1\text{-}7)$$

1.2.6.2 Kinetic resolution of racemate by enantioselective enzymes

Preparation of optically pure compounds by **kinetic resolution of racemate** is based on unequal rates of reaction of the enantiomers with an enantioselective enzyme (or enantioselective catalyst).[5] Enantioselective enzyme transfers the enantiomers at a different rate into new chemical entities (see Figure 6).[1] Two resulting chemically different chiral compounds can be separated by common achiral separation techniques, for example by a column chromatography on silicagel (see **I**). When the catalyst is absolutely selective, 50% of enantiopure product will be obtained while 50% of enantiopure substrate will remain at the end of the reaction. For lower enantioselectivity, the required enantiomeric purity of a substrate may still be reached at higher conversions.[32] Example of use of enzyme kinetic resolution in chemical industry includes production of optically pure amino acids by aminopeptidases.[30]

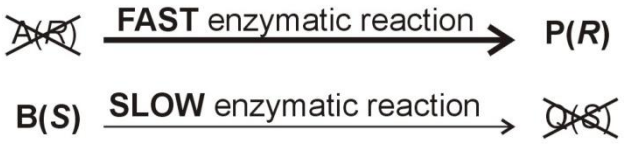


Figure 6. Kinetic resolution of a racemate (represented by enantiomers *A* and *B*) by an enantioselective enzyme

The enantioselective performance of kinetic resolution during enzymatic reaction is quantitatively described by **enantiomeric ratio *E**.** *E* is a measure of discrimination

* Unfortunatelly, term "enantiomeric ratio" is used for two different variables in scientific literature. In further text, we distinguish between **enantiomeric ratio of the kinetic resolution *E*** (ratio of kinetic konstants, see equation (1-7) and (1-8)) and **enantiomeric ratio of the mixture *ER*** (mole or weight fractions, e.g. $ER_{R\text{-}} = n_{R\text{-}}/(n_{R\text{-}} + n_{S\text{-}})$)

between two competing enantiomers, where *A* is the fast and *B* is the slow reacting enantiomer. *E* is dependent on the ratio of the specificity constants and is independent of substrate concentrations.[33] *E* is intrinsic property of the enzyme and cannot be changed unless the values of K_M or k_{cat} change.[32]

(1-8) $$E = \frac{k_{cat}^{A} / K_{M,A}}{k_{cat}^{B} / K_{M,B}} \approx \frac{V_{\max,A} / K_{M,A}}{V_{\max,B} / K_{M,B}}$$

Several methods for determination of *E* exist and can be found in review by Straathof, et al.[32]

E might be determined by two different approaches. First approach is based on monitoring of enzyme kinetic resolution of racemate using analytical chiral separations. Second approach is based on measurement of enzyme kinetics of individual enantiomers.

When a **kinetic resolution of racemate** is performed, four different quantities can be monitored: degree of conversion (ξ), enantiomeric excess of the substrate (*ees*), enantiomeric excess of product (*eep*) and time (*t*). All known methods to determine *E* rely on a combination of the measurements of two out of these four quantities.[32] Chiral chromatography plays crucial role in the determination of enantiomeric excess values.[32]

The relationship between ξ and *ees* for various values of *E* is governed by:[32, 33]

(1-9) $$E = \frac{\ln[(1-\xi)(1-ees)]}{\ln[(1-\xi)(1+ees)]}$$

Where $$\xi = 1 - \frac{[A]+[B]}{[A]_0+[B]_0}$$ and $$ees = \frac{[B]-[A]}{[A]+[B]}$$

After derivation, the relationship between *ees* versus ξ allows the determination of *E* by non-linear fitting of multiple data into equation[32] (see **V**):

(1-10) $$\xi = 1 - \left(\frac{1-ees}{(1+ees)^{E}}\right)^{\frac{1}{E-1}}$$

A graphical representation of the equation (1-10) is very helpful to estimate the amount of enantiopure remaining substrate that can be obtained (see Figure 7). When *ees*

has increased to the required value, the reaction should be terminated in order to obtain the maximum yield.

When optically pure enantiomers are available, *E* can be determined by **measuring of the enzyme kinetic of individual enantiomers.** The initial rates of reaction of the individual enantiomers are fitted to the Michaelis-Menten equation (1-2). Parameters V_{max} and K_M of the pure enantiomers are obtained and *E* is calculated according equation (1-8)[32]. In this case, achiral chromatography is used for monitoring the enzymatic reactions (see **II**).

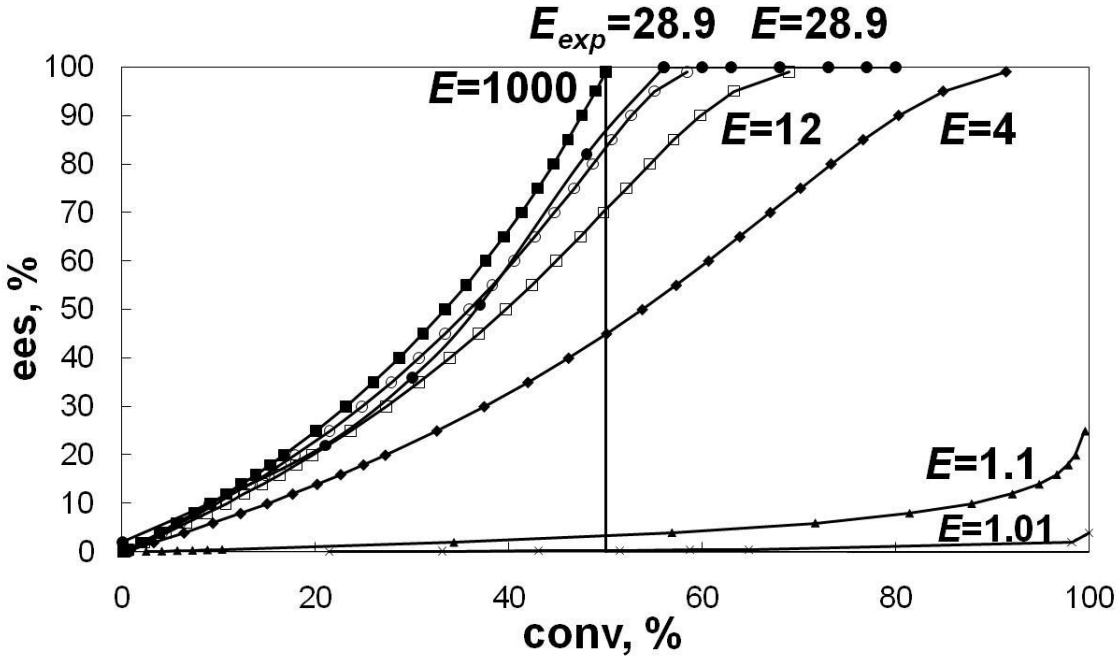


Figure 7. Dependence of enantiomeric excess of the substrate *ees* versus degree of conversion ξ is affected by enantiomeric ratio *E*. Theoretical values are calculated according equation (1-10). Experimentally determined curve (closed circles, *E* = 28.9) of enantioselective hydrolysis of *(R,S)-tert*-butyl glycidyl ether using *Aspergillus niger* M200 is depicted (for details see **V**) together with theoretical curve (open circles, *E* = 28.9).

1.2.6.3 Thermodynamic resolution (direct chiral separation)

The thermodynamic resolution using enantioselective **CSs** plays major role in analytical chiral separations and is discussed in the chapter 1.3. However, LC and SFC are also used for the isolation of chiral compounds from racemates on a preparative scale.[1, 34]

1.2.6.4 Crystallization procedures

Optically pure compounds can be also prepared indirectly using derivatization by chiral agent. For example, reaction of (*R*,*S*)-lactic acids with (*R*)-phenylethylamine results in formation of diastereomers with different chemical and physical properties. The **diastereomers can be separated by crystallization** or some other means. Once separated, acidification of the two diastereomeric salts with mineral acid allows to isolate the two pure enantiomers of lactic acid and to recover the chiral amine.[3]

In some cases, **spontaneous resolution** might occur. During spontaneous resolution **racemic conglomerate**, an equimolar mechanical mixture of crystals is formed. Each one of the crystals contains only one of the two enantiomers. Pure or nearly pure enantiomers can often be obtained from the conglomerate by sorting[5]. Such process stayed behind discovery of molecular chirality by Louis Pasteur (1822-1895).[35] Pasteur was able to separate racemic tartaric acid into its (+) and (-) enantiomers, using fractional crystallization and sorting crystals carefully with a pair of tweezers.[3] Less-well appreciated is the fact that in 1857 Pasteur made also first discovery of enantioselectivity in a biological process – he observed the enantioselective microbial metabolism of tartaric acid.[35]

A limited number of crystallization processes have been successfully established, where a concentrated racemic solution is carefully maintained at a critical temperature and then seeded with one isomer only. Under these conditions the crystals produced are almost entirely of that of a single isomer only.[4]

Formation of diastereomers is also used in indirect analytical separation (see chapter 1.3.5).

1.2.7 Analysis of chiral compounds

Determination of **the enantiomeric composition** might be performed either by chiral separations (see chapter 1.3) or by methods not involving separation (polarimetry, NMR and enzyme techniques).[36]

Most often, chiral analyses are based on comparison of optically active analyte with standard (e.g. comparison of retention times in chromatography) or on relative measurement of optical purity (e.g. expression of *ER* by mole fraction). Nevertheless, when standard of known absolute configuration is not available, structural analysis might be necessary. **Absolute configuration of enantiomers** can be determined[37] directly by X-ray diffraction analysis[36], vibrational circular dichroism (VCD), electronic circular dichroism (ECD), Raman optical activity and optical rotation measurements or indirectly by nuclear magnetic resonance (NMR)[38] and enzymatic transformations.[37] Use of optical rotatory dispersion (ORD), VCD and ECD for determination of absolute configuration require a reference compound of known absolute configuration[36] or the comparison of measured ECD and VCD spectra with simulated spectra obtained by quantum mechanical methods.[38]

All **chiroptical methods**[37] depend on the different interaction of an optically active compound with left- and right-handed circularly polarized light. If these components interact differently with a chiral medium, the medium is said to be optically active. This can be manifested in different ways. (a) Because of a difference in velocity through the medium, a circular birefringence or anisotropic refraction is obtained, i.e. $(n_L - n_R) \neq 0$, observed as a rotation of the plane of polarization. (b) Because of a difference in absorption by the medium, a circular dichroic effect or anisotropic absorption can also be registered, i.e. $(A_L - A_R) \neq 0$, hence $\Delta\varepsilon \neq 0$.

Polarimetry is based on measurement of optical activity. This method is using the ability of an optically active substance to rotate the plane of polarization of plane-polarized light.[6] Light consists of a sinusoidally changing electric field normal to, and in phase with, a sinusoidally changing magnetic field. The plane of the electric vector in unpolarized light

takes no particular orientation. However, in **plane-polarized light**, light waves oscillate in a single plane.[4] When the electrical component interacts with an asymmetric molecule, the direction of the field is rotated because of the dissymmetry of the molecule. Since enantiomers exist as mirror images, they interact with plane-polarized light to an equal but opposite extent, rotating the plane either to the right or the left.

Optical activity is measured using a polarimeter (see Figure 8). Light passes through a polarizer which produces a beam of plain polarized light. The polarized light then passes through a sample tube containing the optically active substance. The plane of polarization is turned by an angle, the magnitude of which is determined by the nature of the substance and its concentration in solution. The light then passes through a second polarizer that is adjusted by rotation. The angle through which the second polarizer is rotated is called the optical rotation of the sample.[4]

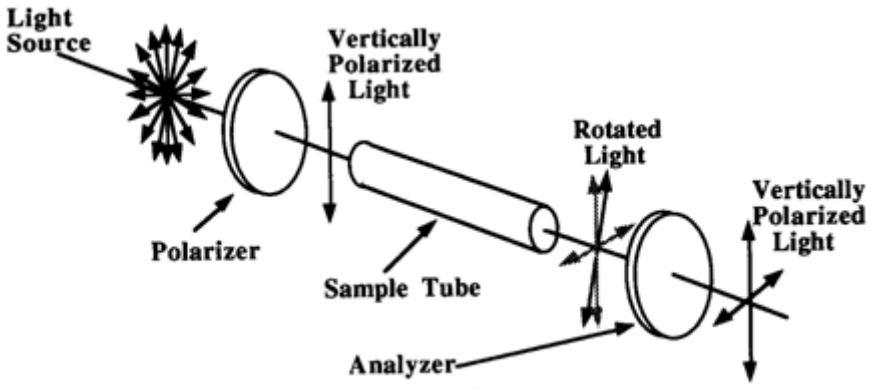


Figure 8. Polarimeter

Specific rotation of substance, $[\alpha]_{\lambda}^{t}$ at a given temperature and a given light wavelength is defined by equation (1-11), where α is the observed rotation (degrees), l is the length of the sample cell (dm), and d is the concentration of the sample (g/ml).[4]

$$[\alpha]_{\lambda}^{t} = \frac{\alpha}{ld} \qquad (1\text{-}11)$$

Enantiomeric content might be expressed by the **optical purity *P*.** If the specific rotation of an optical pure compound is denoted by $[\alpha]_{max}$; it follows that the optical purity, P (%), of a given sample of specific rotation $[\alpha]$ can be obtained from equation:

(1-12) $P = 100\ [\alpha] / [\alpha]_{max}$

Circular dichroism (CD) is caused by the difference in the adsorption characteristics of a compound to the left and right circularly polarized light. CD spectra are usually measured as the differential absorption of left and right circularly polarized light, i.e. (A_L-A_R) and is usually reported as the differential molar extinction coefficient ($\Delta\varepsilon$)

(1-13) $$\Delta\varepsilon = (\varepsilon_L - \varepsilon_R) = \frac{(A_L - A_R)}{cl}$$

where *l* is the length of the cell (cm) and *c* is the molarity of solute.[4]

Recently, CD thermal lens microscope for sensitive chiral analysis on microchip was developed.[39]

HPLC chiral detectors are based on polarimetry or CD[40]. Both positive and negative signals are produced, depending on the direction of rotation. This means that the isomers need to be completely resolved to obtain a quantitative estimation of individual enantiomers.[4]

1.3 Analytical chiral separations

1.3.1 Chiral separations

Chiral separations (also called enantioseparations or enantioselective separations) are the separations of enantiomeric species[1].

The major separation techniques employed for chiral separations[41] are high-performance liquid chromatography (HPLC), gas chromatography (GC)[42], SFC[43], capillary electrophoresis (CE)[44], capillary electrochromatography (CEC)[44], microchip electrophoresis[45-47] and thin-layer chromatography (TLC)[48].

This thesis is devoted to chiral HPLC and chiral GC.

The application of chiral separations includes enantiomer purity control in synthesis, check for racemization processes, pharmaceutical[49] quality control, pharmacokinetic, pharmacodynamic and metabolism studies and toxicological analysis.[41]

Some of the typical **applications of chiral GC** include drug analysis and analysis of essential oils, food, beverages, flavors and perfumes. Essential oils contain many chiral compounds and one enantiomer may be entirely responsible for a particular taste or odor, whereas the complementary enantiomer has an entirely different olfactory effect. Thus, chiral GC might be used for quality control and monitoring product stability.[4]

The most important **applications of chiral HPLC** come from the pharmaceutical and biochemical industries and from the clinical laboratories.[4]

1.3.2 Molecular interaction

Molecular interactions (also called intermolecular forces or non-covalent interactions) control retention and enantioselectivity during chiral separations. Therefore the list of these interactions is given in Table 2. All molecular interactions are electrical in nature.[4]

According IUPAC, the contributions to **van der Waals interaction (forces)** include dipole-dipole interaction, dipole-induced dipole interaction and dispersion interaction.[50] The term van der Waals interaction will be avoided, because that phrase incorporates several of the interactions listed in Table 2, without decomposing the interactions in a meaningful way. Moreover, the term is sometimes used loosely for the totality of nonspecific attractive or repulsive intermolecular forces.

Hydrophobic interactions (hydrophobic effect) is the tendency of hydrocarbons (or of lipophilic hydrocarbon-like groups in solutes) to form intermolecular aggregates and associate in an aqueous medium. The hydrophobic interaction is entropy driven process based on disruption of highly organized water clathrate cages formed around individual molecule of hydrocarbon. The terms hydrophobic is often used to describe the overall interactive character of a large molecule as opposed to the individual group interactions.[4]

Table 2. Characteristics of molecular interactions.[4, 6, 50, 51]

Interaction	Also called	Strength, typical energy ($kJ.mol^{-1}$)	Direction	Range	Description
ionic interaction	Coulomb interaction ionic forces charge-charge interaction	Very strong ~ 250	Attractive or repulsive	Medium ($1/d^2$)	Ions possessing a net charge interact with ions having an opposite charge.
hydrogen bond	-	Very strong ~20	Attractive	Long	A hydrogen bond is an attractive interaction between two species that arises from a link of the form A - H ... B, where A and B are highly electronegative elements and B possesses a lone pair of electrons. Hydrogen bonding is conventionally regarded as being limited to N, O, and F. The formation of a hydrogen bond can be regarded either as the approach between a partial positive charge of H and a partial negative charge of B or as a particular example of delocalized molecular orbital formation in which A, H, and B each supply one atomic orbital from which three molecular orbitals are constructed.
steric hindrance	repulsive interaction	Very strong	Repulsive	Very short	Steric hindrances are due to the intrinsic room needed per atom or group of atoms. The repulsive interactions arise from Coulombic repulsions and, indirectly, from the Pauli principle and the exclusion of electrons from regions of space where the orbitals of neighboring species overlap.
π-π interaction	charge transfer complexation π - π stacking	Strong	Attractive or repulsive	Medium	When π-electron molecular assemblies (mainly aromatic rings) interact with each other, π – π interactions might be observed. A π-accepting group of the enantiomer interacts with a π -donating group of the selector, or vice versa.

ion – dipole interaction	charge - dipole interaction	Strong ~ 15	Attractive	Short	Coulomb force between the ion and the partial charge of the dipolar molecule. It is always attractive because a permanent dipole structure combines a partial positive charge with an equal partial negative charge.
dipole - dipole interaction	dipole stacking	Intermediate ~ 2	Attractive	Short ($1/d^3$)	Intermolecular or intramolecular interaction between molecules or groups having a permanent electric dipole moment. The strength of the interaction depends on the distance and relative orientation of the dipoles.
dipole - induced dipole interaction	-	Weak	Attractive	Very short ($1/d^6$)	A polar molecule with dipole moment can induce a dipole to neighbor polarizable molecule. The induced dipole interacts with the permanent dipole of the first molecule, and the two are attracted together. The strength of the interaction depends on the dipole moment of the first molecule and the polarizability of the second. Example of polarizable molecule is benzene containing π electrons in aromatic nucleus.
dispersion interaction	dispersion force London forces instantaneous induced dipole-induced dipole interaction hydrophobic force hydrophobic interaction	Very weak ~ 2	Attractive	Very short ($1/d^6$)	The interaction between nonpolar molecules arises from the transient dipoles that all molecules possess as a result of fluctuations in the instantaneous positions of electrons. The strength of the dispersion interaction depends on the polarizabilities of the two molecules. Polar molecules also interact by a dispersion interaction. However, the time average of each fluctuating instantaneous dipole corresponds to the permanent dipole. Such molecules therefore interact both through their permanent dipoles and through instantaneous fluctuations in these dipoles. Dispersion interaction responsible for the hydrophobic effect and for entropy driven forces that cause oil to separate from water.

1.3.3 Direct chiral separations

Enantiomers might be separated either directly using CSs or indirectly after derivatization to corresponding diastereomers.

Direct chiral separations[4, 36, 41, 52-56] are based on interaction of analytes with **CSs.** CS is the chiral component of the separation system (chromatographic or electrophoretic) capable of interacting enantioselectively with the enantiomers to be separated. CS is either an appropriate chiral molecule or a chiral surface.[1]

Direct chiral separations are usually performed on chiral column containing **chiral stationary phase (CSP)**. CS can be either incorporated into the stationary phase or chemically bonded to or immobilized onto the surface of a solid support or column wall, or simply dissolved in the liquid stationary phase.[1] Less often, HPLC separation is performed in chiral mobile phase with added CS.

In CE, chiral selector is added as a **chiral additive** into electrophoretic medium.[1]

Interaction of the CS with the enantiomers of the solute results in the formation of two labile diastereomers. Chiral separation between two enantiomers will occur when the diastereomeric labile complexes formed with the chiral support differ in stability.[38] This occur if at least three active points of the CS participate in the interaction with corresponding sites of the solute molecule and at least one of them is stereoselective (the principles of three-point interaction are illustrated in chapter 1.2.3.). Interaction of CS with an analyte is a thermodynamically enantioselective process and does not modify the enantiomeric species to be separated.[1] Interaction in this context can be attractive as well as repulsive, and might also include steric hindrance. Although the interactions between CS and analyte may be dominated by one type of interaction, chiral recognition always needs a set of interactions.[57]

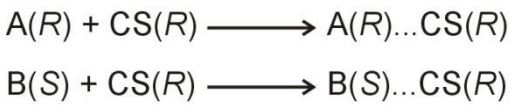


Figure 9. Direct chiral separation by interaction of analyte with CS to form two labile diastereomers which differ in their thermodynamic stability.

In the case of CSPs, the enantiomer that forms the more stable association with the chiral selector will be the more strongly retained species of the racemate. On the other hand, a chiral mobile phase reduces the retention of the solute enantiomer which forms a stronger association with the chiral selector.[1]

The enantioselectivity of the chiral separation system is expressed by **separation factor *α*** (ratio of the retention factors of the two enantiomers):

(1-14) $$\alpha = \frac{k_2}{k_1}$$

The total enantioselectivity of given separation system can depend strongly on the composition, pH and temperature of the mobile phase [1].

1.3.4 Classification of CSs

The most important chiral selectors are listed in Table 3. Molecular interaction participating on retention and separation depends on type of CS and on the type of CS derivatization. In case of HPLC, participating molecular interactions depend on chromatographic mode. Non-polar organic eluents in normal-phase (NP) mode enhance dipole-dipole and π-π interactions, whereas aqueous eluents in reversed-phase (RP) mode induce dispersion (hydrophobic) interactions.[57]

In HPLC, most common CSPs are based on polysaccharides, proteins, macrocyclic glycopeptides, cyclodextrins and π-complex CSs.[4] In GC, majority of CSPs are based on cyclodextrin CSs.[4]

CS might be categorized according their chemical structure (see Table 3). However, CSs are also classified according their source to **synthetic selectors**[51] **(**ligand exchange, π-complex, molecularly imprinted polymers, chiral crown ethers, synthetic polymers), **semisynthetic chiral selectors** (modified polysaccharides)[49] and **natural selectors**[51] **(**proteins, macrocyclic glycopeptides, cyclodextrins, cinchona alkaloids). Natural selectors are very often modified by derivatization or covalently binded to stationary phase.

Group of CSs based on **inclusion complexing**[57, 58] is also defined and includes cyclodextrins, synthetic polymers and chiral crown ethers. At this CS, formation of

inclusion complexes between chiral analytes and chiral host molecules might occure.[57] **Inclusion complex** is a complex in which one component (the host) forms a cavity in which molecular entities of a second chemical species (the guest) are located. There is no covalent bonding between a guest and a host[50], the attraction being generally due to dipole-dipole interaction, hydrogen bond and dispersion interaction.[57]

CSPs are also divided to molecular and polymeric CSPs.[38] This classification emphasizes concept of the "independent" chiral selectors in opposition to "cooperative" chiral selectors. **Molecular ("brush type") CSPs[59]** consists of simple chiral molecule or more complex one (e.g. macrocyclic antibiotics) but always presents a well-defined and delineated molecular structure. Usually linked to a silica inert support, this well-defined structure will operate on its own and independently from the next anchored chiral selector which will duplicate the same molecular recognition behavior.[38] **Polymeric CSPs** is composed of synthetic or naturally occurring chiral polymers (e.g. polysaccharides). A large number of chiral centers are in a close proximity and thus the three-dimensional structure of the polymer will play a decisive role in the multiplicity and geometry of the chiral sites. The variability of the three-dimensional structures and the resulting occurrence of several interdependent chiral sites in these polymers may be at the origin of their remarkable range of successful applications in terms of molecular diversity [38].

Table 3. Classification of CSs[4, 36, 41, 51, 57, 58, 60]

CS	Mechanism	Molecular interaction	Techniques and HPLC modes
Amino acids derivatives	Formation of labile diastereomers via hydrogen bonding	Hydrogen bond	GC, HPLC
Polysaccharides	Insertion into helical structures	**Hydrogen bond** **Dipole - dipole interaction** **Steric hindrance** π-π interaction Dispersion (hydrophobic) interaction	HPLC (NP, RP, PO), SFC, CEC, CE, TLC
Cyclodextrins	Inclusion complexation	**Hydrogen bond** Dipole – dipole interaction Dispersion (hydrophobic) interaction	HPLC (NP, RP, PO), GC, SFC, CE, MEKC, CEC, TLC
Proteins	Multiple binding sites	Dispersion (hydrophobic) interaction Dipole - dipole interaction Hydrogen bond Ionic interaction	HPLC (RP), TLC, CE, CEC
Macrocyclic antibiotics	Multiple binding sites Inclusion	Hydrogen bond Dipole - dipole interactions Dipole - induced dipole interactions Steric hindrance Ionic interactions π-π interactions Dispersion (hydrophobic) interaction	HPLC (NP, RP, PO), CE, TLC
Cinchona alkaloids	Ion pairing	**Ionic interaction** π-π interaction Hydrogen bond Steric hindrance	HPLC, CEC

π-complex	Transient three-point selector-analyte association	**π-π interaction** Hydrogen bond Dipole - dipole interaction Dipole - induced dipole interaction Dispersion (hydrophobic) interaction Steric interactions	HPLC (NP, RP), CEC
Ligand exchange	Formation of diastereoisomeric selector-metal ion-analyte complex	**Ionic interaction** **Ion - dipole interaction**	HPLC (RP), GC, CE, CEC, TLC
Molecularly imprinted polymers	Key-and-lock association	Selective shape interaction with the imprint	HPLC, TLC, CEC
Chiral crown ethers	Inclusion complexation	**Ion - dipole interaction** Hydrogen bond Dipole - dipole interaction	HPLC (RP), CZE
Synthetic polymers	Diastereoisomeric selector–analyte complex	**Hydrogen bond**	HPLC
Calixarenes	Inclusion complexation	Hydrogen bond Dipole - dipole interaction	GC, CE, CEC
Chiral micels of biological and synthetic source	Difference in electroforetic mobility due to analyte competitive interactions with miceles and CSs	Depends on type of miceles and CSs	MEKC
Ion pairs formed witch chiral counter ions	Chiral ion-pair chromatography using CS added into mobile phase as a counter ions	Ionic interactions	HPLC, CZE
Ionic liquids	Diasteriomeric interactions between analyte and chiral ILs	Dipole - dipole interactions	GC, HPLC, CE

1.3.4.1 Amino acids derivatives

GC

The first separation of enantiomers by GC on a CSP was discovered by Gil-Av in 1966.[61] His group developed chiral GC phases based on ***N*-trifluoroacetyl-*L*-isoleucine lauryl ester** and resolved *N*-trifluoroacetyl amino acids. The separation is based on the formation of multiple hydrogen bonds.[41, 42] However, these types of stationary phases had very limited temperature stability and the optimum temperature for separation was often in excess of that at which the stationary phase was stable.[4]

Later, Bayer's group prepared a GC phase based on valine diamide linked to polysiloxanes, which was commercialized under the name **Chirasil-Val** (see Figure 10).[62] Such chiral polysiloxane stationary phase combines chiral selectivity of *L*-valine-*t*-butylamide with the high thermal stability and low volatility of the polysiloxanes.[4]

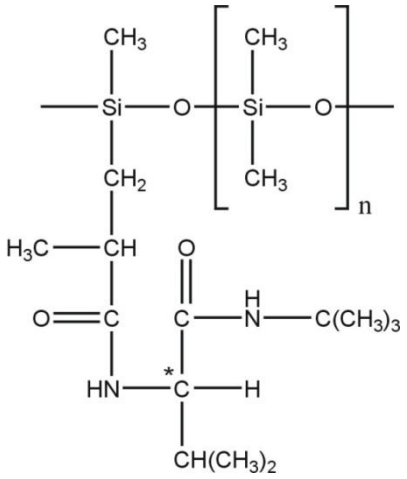


Figure 10. Chirasil-Val CSP.

HPLC

HPLC phases using amino acid amides as CSs were also prepared and derivatives of amino acids, hydroxy acids, and amino alcohols were resolved.[41, 63]

1.3.4.2 Polysaccharides

HPLC

Polysaccharide-based CSPs[64-66] appear to be among the most popular CSPs[21] and are commercially available under name Chiralpak and Chiralcel. Cellulose consists of *D*-glucose units linked by 1,4'-*β*-glycoside bonds. Several thousand glucose units are linked to form one large molecule, and different molecules form a large structure held together by hydrogen bonds. Amylose consists of *D*-glucose units linked by 1,4'-*α*-glycoside bonds.[3] Both cellulose and amylose unit contains 5 chiral centers. As a result the polymers contain a large number of chiral active sites and thus a relatively high probability of chiral site interaction with the solute.[4] Native polysaccharides showed only weak chiral recognition ability.[41] However, **cellulose and amylose derivatives** coated onto a silica support show distinct enantioselectivity.[21, 67-69]

Main **cellulose derivatives** that are used in chiral HPLC are cellulose triacetate, *tris*(3,5-dimethylphenyl carbamate) and *tris*(4-methyl benzoate).[21] Tribenzoate and phenylcarbamate structures contain polar and dispersive interactive sites but, in addition, the aromatic nuclei can provide polarizability and thus will offer strong polar interactions with any strongly polar group appropriately situated on the solute molecule.[4] (see Figure 11).

Evidence seems to point to an inclusion mechanism for enantioselectivity on cellulose triacetate CTA-I CS by shape-selective adsorption into chiral cavities in the polymer network. It is the size and shape of the molecule, rather than specific functionality, that are important for enantioseparation.[7] X-ray, NMR studies, and computer simulations brought some insight into the chiral recognition mechanism of phases based on the cellulose *tris*(phenylcarbamate) (CTPC) CS.[21] CTPC has a left-handed 3/2-helical conformation, and the glucose residues are regularly arranged along the helical axis. A chiral helical groove with polar carbamate groups exists parallel to the main chain. The polar carbamate groups are preferably located inside, and hydrophobic aromatic groups are placed outside the polymer chain so that polar enantiomers may predominantly interact with the carbamate residues in the groove through hydrogen-bond formation. In addition to these polar interactions, π-π interactions between phenyl groups of a CTPC derivative and aromatic groups of a solute may play a role.[21]

When amylose is used instead of cellulose, different enantioselectivity is observed. Main **amylose derivatives** that are used in HPLC include *tris*(3,5-dimethylphenyl carbamate) and *tris*((S)-α-methylbenzyl carbamate).[4]

Polysacharides CSPs are used under NP mode conditions. However certain derivatives render the coating less vulnerable to solvent dissolution and can be used in RP mode.[4, 70]

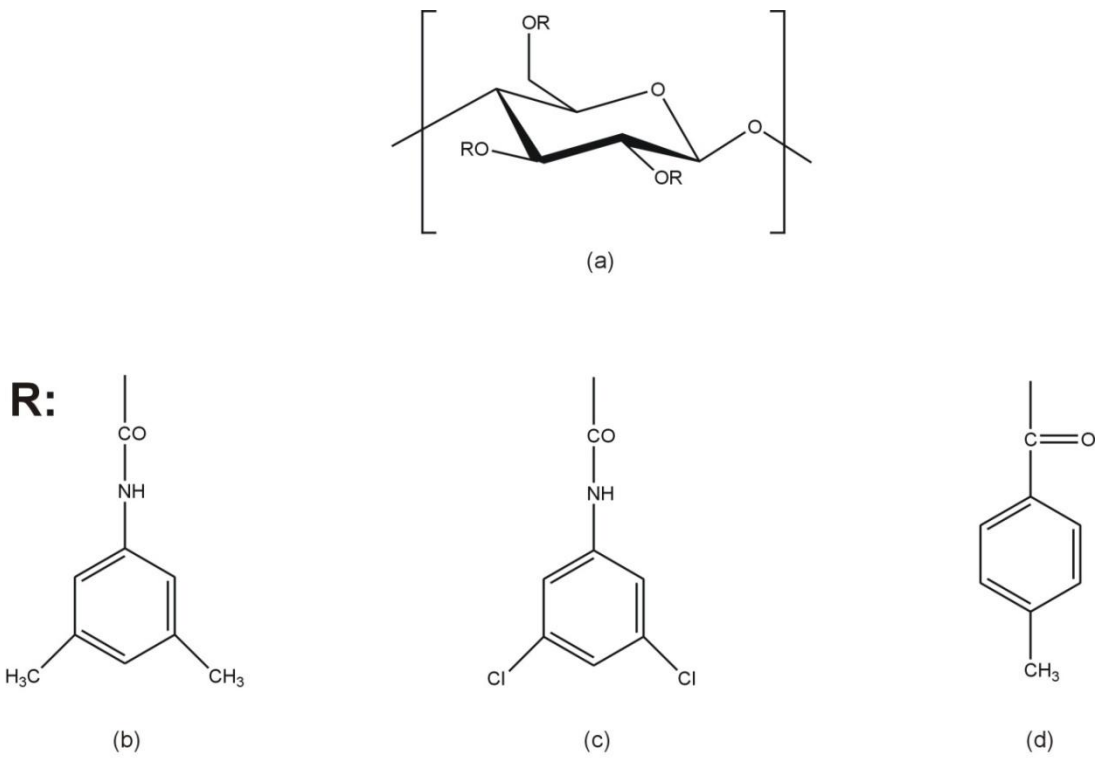


Figure 11. (a) Cellulose derivatives used as CSs: (b) *tris*(3,5-dimethylphenyl carbamate), (c) *tris*(3,5-dichlorophenyl carbamate) and (d) *tris*(4-methyl benzoate).

1.3.4.3 Cyclodextrins

Cyclodextrins are the most important CSs based on inclusion complexing[41] and their use in chiral separations is very common. The cyclodextrins are produced by the partial degradation of starch followed by the enzymatic coupling of the glucose units into crystalline, toroidal structures of different molecular size. These cyclic chiral oligosaccharides contain the *D*-glucose residues bonded through *α*-(l-4)glycosidic linkages.

α-, *β*-, *γ*- cyclodextrins are consisting of six, seven and eight glucose units, respectively.[4] Glucopyranose units form a truncated cone with a hydrophobic cavity. The outer surface is hydrophilic.[41] The mouth of the torus-shaped cyclodextrin molecule has a larger circumference than at the base and is linked to secondary hydroxyl groups of the C2 and C3 atoms of each glucose unit (see Figure 12). The primary hydroxyl groups are located at the base of the torus on the C6 atoms. As these hydroxyl groups are free to rotate, they partially block the base aperture. The size of the cavity increases with increasing number of glucose units.[4]

The secondary hydroxyl groups (positions 2 and 3) can be derivatized to introduce further interactive character to the cyclodextrin molecule.[4] Derivatizing the 6-hydroxyl position is used for anchoring the cyclodextrins to silica gel in HPLC CSPs. In GC, 6-position is usually alkylated and as the group is free to rotate, it blocks the base aperture to the cone and thus the 6-position offers no chiral selectivity. In addition, the alkylation of the 6-position can increase the solubility of the cyclodextrin in the polysiloxane matrix.

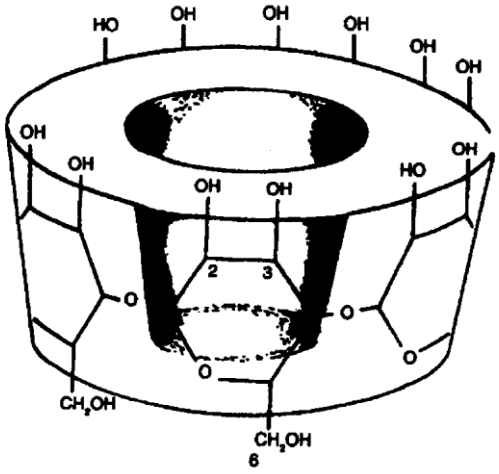


Figure 12. A molecular model of *α*-cyclodextrin

There are two different interactive processes leading to retention and resolution of enantiomers on cyclodextrins. Firstly, inclusion of a bulky hydrophobic group of the analyte, preferably aromatic groups, into the hydrophobic cavity of the cyclodextrin might occur. In this case the proper cavity size must be chosen to achieve the desired selectivity. Secondly, analyte might interact with the CS by formation of hydrogen bonds or dipole-

dipole interactions between the hydroxyl groups at the mouth of the cyclodextrin and polar groups of the analyte.[4, 41]

GC

The cyclodextrins are probably the most effective stationary phases presently available for the GC separation of stereoisomers.[4]

CSPs are based on α, β or γ- cyclodextrin and a range of derivatives have been introduced that are designed to enhance the separation of enantiomers of different types and classes of compounds.[71, 72] Derivatization of position 2 and 3 can strongly affect selectivity. Alkylation (pentylation) on the 2, 3-positions introduces strong dispersive interactions with the solutes. In contrast the introduction of polar groups (e.g. trifluoroacetylation) will increase the polar interactions. Derivatization in the 3-position removes the size selectivity of the cyclodextrin molecule. The typical cyclodextrin derivatives used as a CSs include 2,6-di-*O*-pentyl cyclodextrin, permethyl-2-hydroxypropyl cyclodextrin and 2,6-di-*O*-pentyl-3-*O*-trifluoroacetyl cyclodextrin.[4]

The derivatized cyclodextrins are incorporated into appropriate polysiloxanes which are then coated on the column walls or onto an inert support. The level of the cyclodextrin component in the stationary phase varies between about 8% and 15% depending on the solubility of the cyclodextrin in the particular polysiloxane. Some derivatized cyclodextrins can be extremely vulnerable to degradation. Therefore, it is imperative that all carrier gases and samples are completely dry and oxygen free gasses must be used.[4]

The selection of the best size of the cyclodextrin and best cyclodextrin derivative for a given separation rely on experiment and experience. If the solute is largely hydrophobic then a cyclodextrin derivative that is also dispersive might be appropriate. Conversely, a polar solute might interact more strongly with a polar or polarizable group associated with the cyclodextrin.[4]

HPLC

Use of the cyclodextrins in HPLC[73] become very popular due to their stability, reproducibility and high chiral selectivity.[4] Cyclodextrins can be used either as CSPs or as chiral mobile phase additives.[41] Cyclodextrin type CSPs may be operated in RP mode

(retention and selectivity result from dispersive interaction), in NP mode (retention and selectivity result from dipole - dipole and ionic interactions) and in polar organic (PO) mode.[4]

1.3.4.4 Proteins

HPLC

Protein based CSPs[74, 75] consist of natural proteins bonded to a silica matrix. Proteins contain a large number of chiral centers of one configuration and are known to bind small analytes and drugs stereoselectively.[4, 41]

Chiral recognition is due to interactions with receptor-type sites of proteins.[57] Therefore, proteins show enantioselectivity for a broad spectrum of compounds. However, prediction of chromatographic behavior of particular analyte is hardly possible.[41] Dipole-dipole interactions, hydrogen bonds, and hydrophobic interactions are assumed to be the main interactions. Dependent on pH proteins might be negatively or positively charged and ionic interaction may play the role in enantioseparation.[41]

Typical mobile phase is phosphate buffer containing small amount (1 % - 10 %) of 2-propanol, ethanol or acetonitrile.[4] Ionic strength and pH, type, and concentration of organic modifiers were found to affect strongly retention and resolution.[41]

There are a number of commercially available protein based CSPs which have been used to separate a wide range of chiral substances, including α_1-acid glycoprotein, ovalbumin, cellobiohydrolase I and human serum albumin.[4] Penicillin G-acylase[76], antibodies, fatty acid binding protein and streptavidin were introduced recently as CSs.[75]

1.3.4.5 Macrocyclic antibiotics

HPLC

Ansamycins and macrocyclic glycopeptides are two main groups of **macrocyclic antibiotics**[41, 77, 78] frequently used as CSs.

Macrocyclic glycopeptides based CSs consist of aglycon and carbohydrate part. An aglycon part form a hydrophobic basket shape and consists of three or four fused macrocyclic rings composed of linked amino acids and substituted phenols. Macrocyclic glycopeptides contain a large number of chiral centers, together with molecular cavities in

which hydrophobic parts of an analyte may enter.[4, 78] Pendant polar arms might form hydrogen bonds and dipole-dipole interactions with polar groups of the analyte. Furthermore, ionic interactions and π-π-interactions might support the separation.[41]

The most common macrocyclic glycopeptides CSs include vancomycin, teicoplanin, avoparcin and ristocetin A. Avoparcin, ristocetin A and teicoplanin are not pure compounds but exist as mixtures of known composition. Teicoplanin is unique among the glycopeptides in that it has a hydrophobic acyl side chain.[78]

Vancomycin (see Figure 13) contains 18 chiral centers surrounding three cavities which are bridged by five aromatic rings. The cavities are shallower than those in the cyclodextrins and thus interactions are weaker. However, this allows more rapid solute exchange between the phases, and thus higher column efficiencies. Strong polar groups are proximate to the ring structures to offer strong polar interactions with the solutes. CS vancomycin is covalently bond to the surface of silica gel particles. Vancomycin has a number of ionizing groups and thus can be used over a range of different pH values (pH 4.0 to 7.0) and exhibit a wide range of retention characteristics and chiral selectivities.[4]

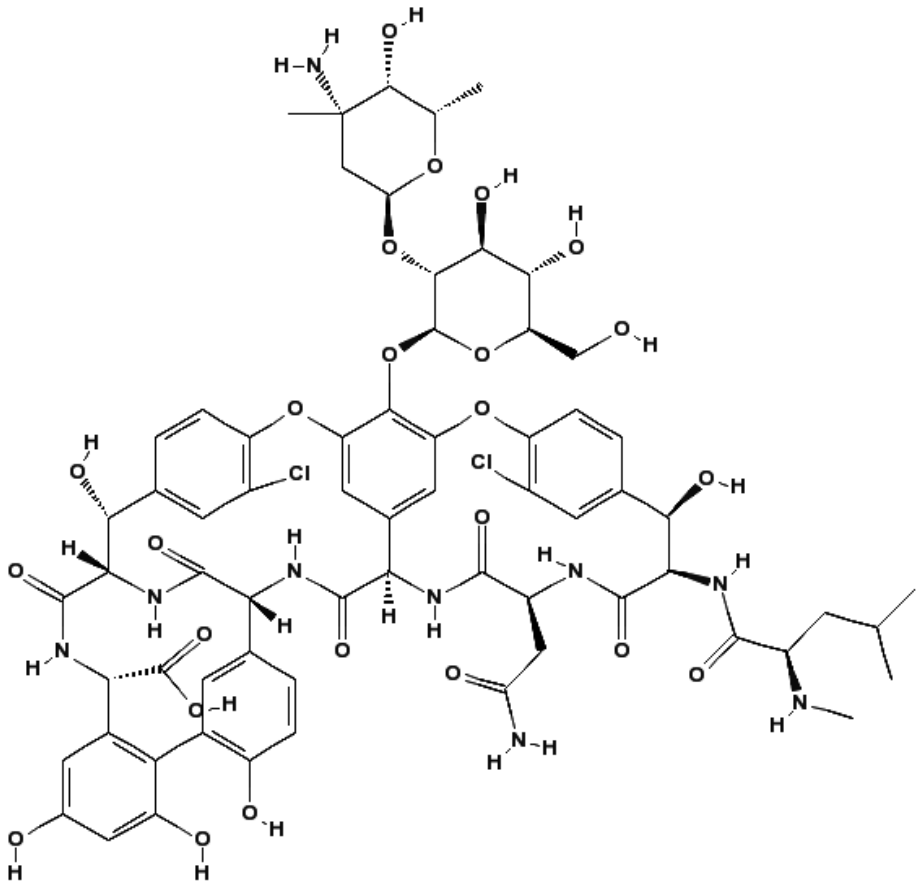


Figure 13. Structure of vancomycin.

The most common **ansamycins** used as CSs are rifamycin B and rifamycin SV.[41] They have a characteristic structure consisting of a ring structure or chromophore spanned by an aliphatic bridge. The aliphatic chain can be highly substituted, and the ansamycins differ by the type and position of substituents on their naphthohydroquinone ring.[78]

Macrocyclic antibiotics based CSPs might be used in various chromatographic modes. In **RP mode** (e.g. tetrahydrofuran-water mixtures), retention and selectivity depend on dispersive (hydrophobic) interactions between the solute and the stationary phase. In **NP mode** (e.g. hexane-ethanol mixtures), the presence of the strongly dispersive hydrocarbon completely swamps any possible competing dispersive interactions with the stationary phase and thus the solutes are retained almost solely by polar and ionic interactions. In **PO mode** (e.g. ethanol or methanol with addition of acetic acid and

triethylamine) all interactions mentioned above might participate on retention and enantioselectivity. The acid/base concentration is quite critical.[4]

1.3.4.6 Cinchona alkaloids

HPLC

Cation-exchange-based CSPs using **cinchona alkaloids**[79, 80] as CS were used in HPLC. Quinine is a natural alkaloid extracted from the bark of the South American cinchona tree that is commonly used as an antimalarial drug and can easily be derivatized to prepare CPS.[51] Beside ionic interactions, π-π-interactions and hydrogen bonds are additional interactions.[41]

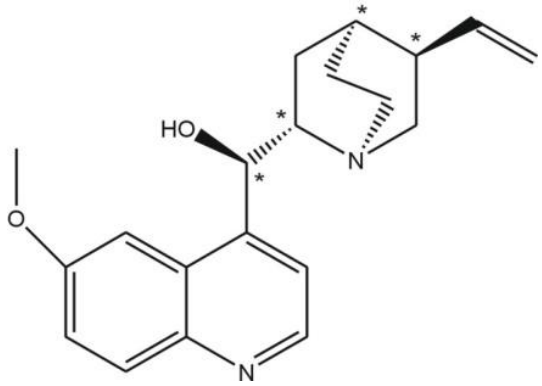


Figure 14. Quinine

1.3.4.7 π-complex

HPLC

π-complex[59, 81, 82] **CSPs** (also called Pirkle-type or brush-type CSPs) consist of small molecular weight chiral molecules containing polar and polarizable groups bonded to silica. Each bonded group has a limited number of chiral centers available. However, due to their small size, there can be a large number of groups bonded to the silica and thus a relatively high probability of the solute interacting with a chiral center is maintained. π-complex CSPs are quite stable, and exhibit good chiral selectivity to a wide range of solute types.[4] π-complex CSPs are best suited for use under normal phase conditions.[58]

The mechanism for enantioseparation includes attractive π-π interactions between activated (electron-rich) and deactivated (electron-poor) aromatic rings[57]. A **π-donor** will

have a tendency to lose an electron because the resulting positive charge will be accommodated by the π-system. Conversely, a **π-acceptor** can readily stabilize a negative charge and has therefore a tendency to accept an additional electron in its π-system. In this way a π-donor/acceptor pair will form a complex when a charge can be transferred from the donor to the acceptor molecule.[36]

Example of a π-acceptor CSP is an (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine bonded to silica (see Figure 15).[41] Analytes separable on this CSP almost always contain π-donor functionality or dipolar π-groups conformationally influenced by the stereogenic centers.[7] Example of a π-donor CSP is an *N*-butanoyl-(*R*)-*p*-hydroxyphenylglycine propyl amide immobilized on silica gel via its phenolic oxygen.[83] The most popular π-complex CSP today is Whelk-*O*-1 [4] containing a naphthyl π donating group and a dinitrobenzoyl π accepting group.[38]

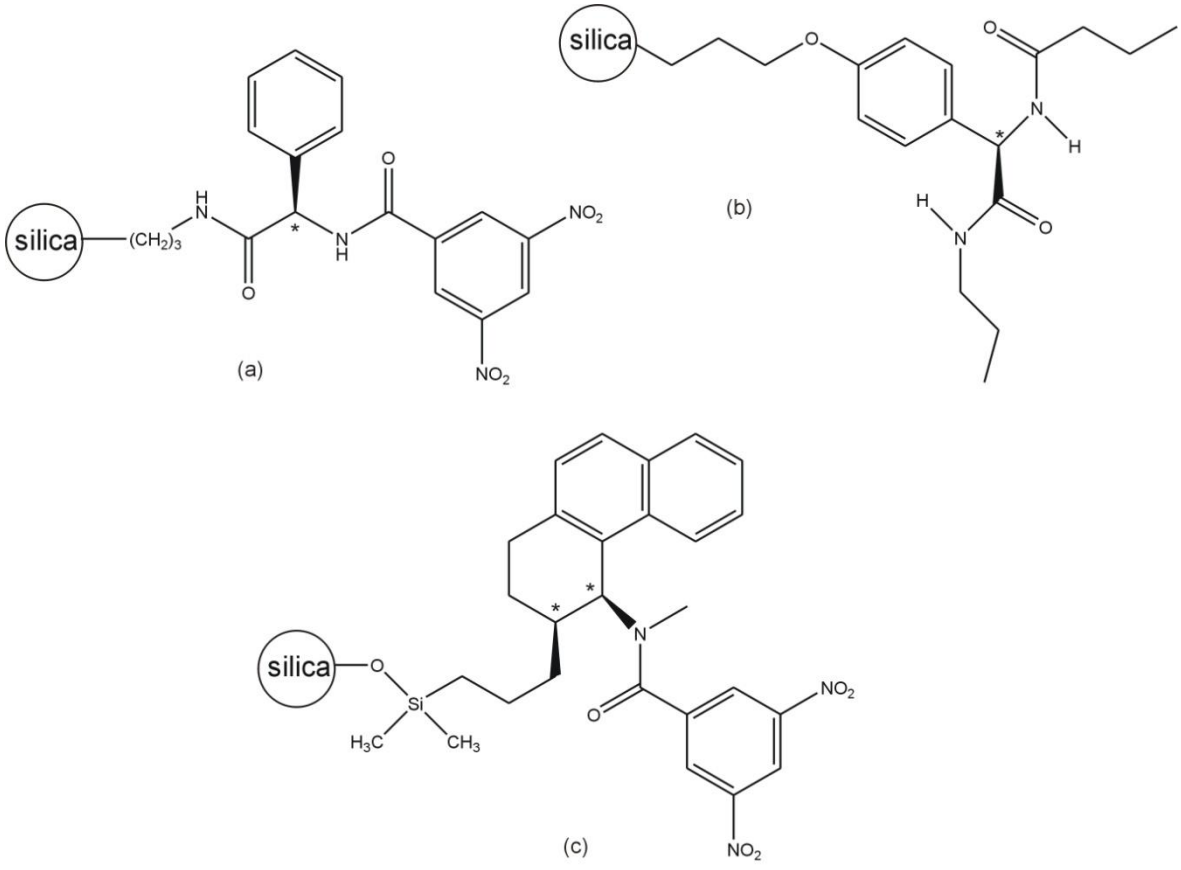


Figure 15. (a) (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine based CSP, (b) *N*-butanoyl-(*R*)-*p*-hydroxyphenylglycine propyl amide based CSP, (c) Whelk-*O*-1 CS

CSPs containing an **ergot alkaloid** (see Figure 16) were prepared, including 1-(3-aminopropyl) derivative of (+)-(5*R*, 8*S*, 10*R*)-terguride[84] and 1-allyl derivative of (+)-(5*R*, 8*S*, 10*R*)-terguride bonded to mercaptopropyl silanized silica gel[85]. The terguride belongs to the group of low molecular mass CSs and resulting CSPs belongs to brush type phases. The molecule of terguride contains several groups, which can interact with analyte in a different mode of action, including π-π interaction, hydrogen bond and steric hindrance.[86] Ergot alkaloid based CSPs shown to be effective in the resolution of acidic compounds in buffered aqueous media. Good levels of enantioselectivity were observed for 2-aryloxypropionic acids, chrysanthemic acid, and profens.[87, 88]

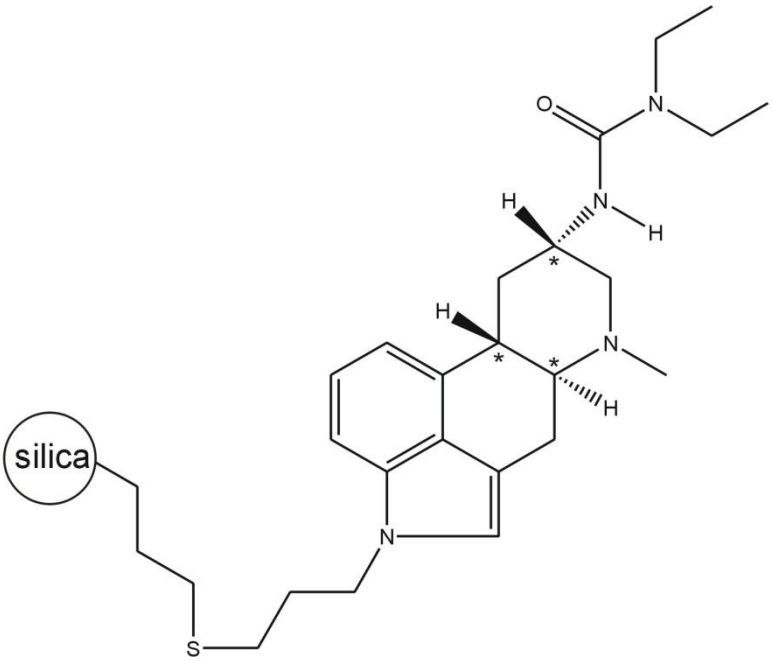
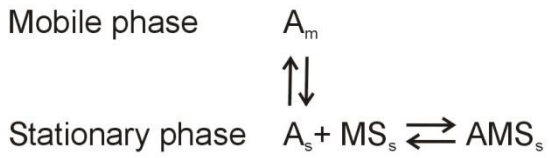


Figure 16. Ergot alkaloid based CSP.

1.3.4.8 Ligand exchange

Chiral recognition in ligand exchange chromatography[89, 90] is based on the formation of ternary mixed metal complexes between a CS ligand and the analyte ligand (see equation (1-15)). The different complex stability constants of the mixed complexes with *D*- and *L*-enantiomers are responsible for separation[41, 42].

(1-15)



A represents the analyte; M represents the metal; and S represents the selector.

HPLC

The first chiral ligand exchange chromatography phases were based on polystyrene-divinylbenzene polymers containing amino acid residues complexed with metal ions. Chemically bonded phases on silica gel basis were prepared latter.[41]

CSPs *N*,*S*-dioctyl-(*D*)-penicillamine and *L*-proline-copper complex bonded to silica[91] (see Figure 17) are suitable for chiral analysis of amino acids and peptides. Elution on the former CSP is carried out with a copper sulfate solution.[38]

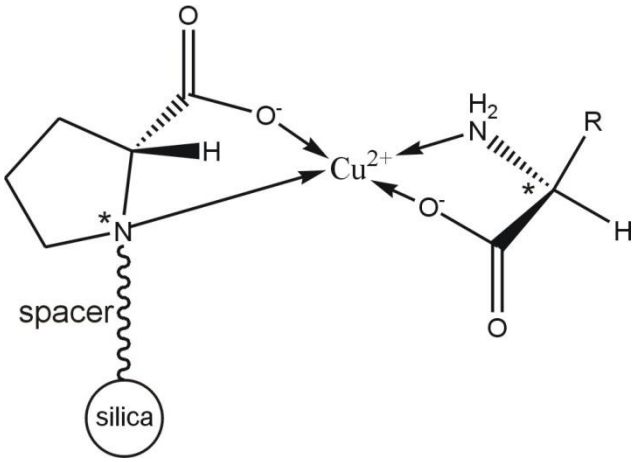


Figure 17. Enantioseparation of amino acids during ligand exchange chromatography is achieved due to formation of ternary complexes between CS (*L*-proline), Cu^{2+} ion and analyte (α-amino acid).

GC

Metal complexes, such as rhodium and nickel camphorates and 1,3-diketonate-bis-chelates of manganese(II), cobalt(II), and nickel(II) derived from perfluoroacetylated terpene-ketones were used for chiral GC separation of pheromones, flavors and oxiranes.[41, 42, 92]

1.3.4.9 Molecularly imprinted polymers

HPLC

Molecularly imprinted polymers[93, 94] are synthetic polymers containing "grafted" chiral cavities.[36] A monomer is polymerized with a crosslinker in the presence of a chiral template molecule. After removing the template molecule, a chiral imprinted cavity remains, which shows stereoselectivity to the template or closely related molecules.[41]

1.3.4.10 Chiral crown ethers

HPLC

Crown ethers[95] (see Figure 18) are macrocyclic polyethers that form host-guest complexes with alkali metal, earth alkali metal, and ammonium cations. Crown ethers might also include enantioselectively primary amines.[41] Therefore, several crown ethers based CSPs were prepared.[96-98]

As a chiral recognition mechanism, the formation of hydrogen bonds between the three hydrogens attached to the amine nitrogen and the dipoles of the oxygens of the macrocyclic ether is postulated. Furthermore, the substituents of the crown ether are arranged perpendicular to the plane of the macrocyclic ring, forming a kind of chiral barrier. [41]

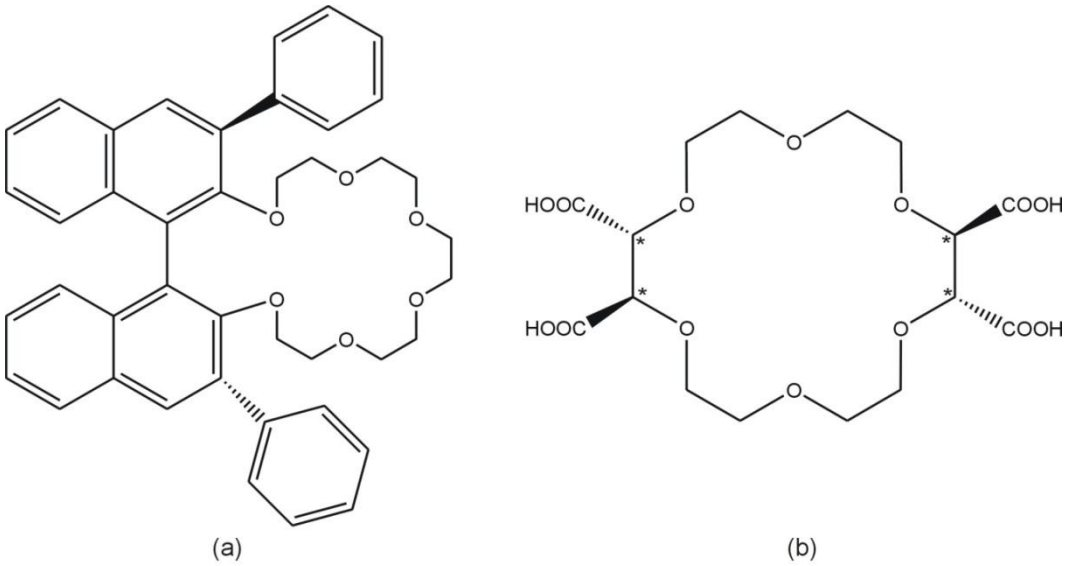


Figure 18. Crown ethers CSs. (a) (3,30-diphenyl-1,10-binaphthyl)-22-crown-6, (b) (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid

1.3.4.11 Synthetic polymers

HPLC

Synthetic, optically active polymers might be used as CSPs.[99] **Polyacrylamide based CPSs** contains poly(*N*-acryloyl-*S*-phenylalanine ethyl ester) coated on silica gel particles (see Figure 19).[100] Polymer prepared by asymmetric polymerization of **triphenylmethyl methacrylate** exhibits a helical chirality and is capable separating the enantiomers of compounds that are themselves helically chiral (i.g. helicenes) or that have an aromatic substituent at the stereogenic center.[101]

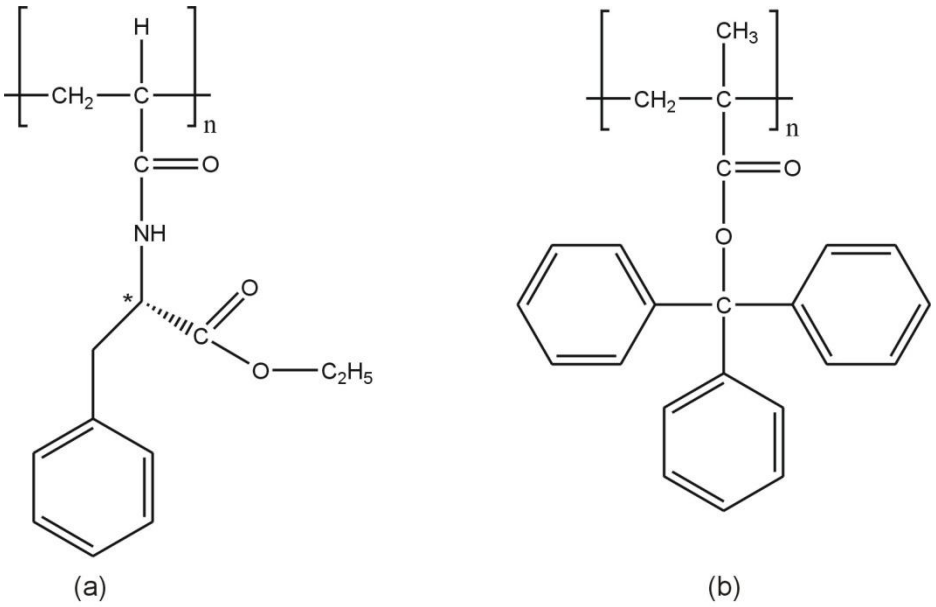


Figure 19. (a) polyacrylamide based CPSs, (b) helical polytriphenylmethyl methacrylate CSP

1.3.4.12 Calixarenes

GC

Calixarenes are GC synthetic selectors showing high thermal stability and unique cavity-type supramolecular shape. Chiral GC phases based on resorc[4]arene (see Figure 20) were used for the chiral separation of amino acids derivatives.[102] An inclusion mechanism supported by dipole-dipole interactions and hydrogen bonds might be assumed as the chiral recognition basis.[41]

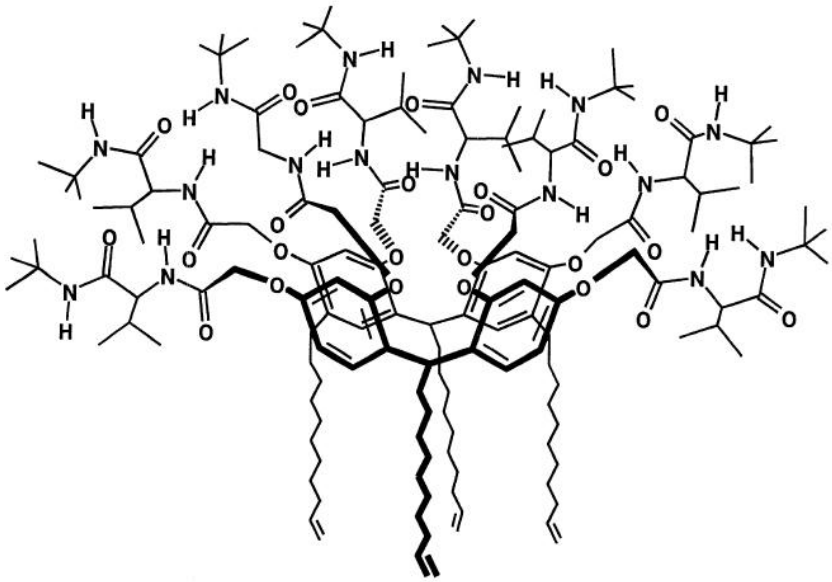


Figure 20. Calixarene. A highly ordered supramolecular structure has been prepared by linking chiral *L*-valine *tert*-butylamide moieties to the eight hydroxy groups of a resorc[4]arene basked-type structure.

1.3.4.13 Ionic Liquids

Ionic liquids (ILs) are class of compounds that are semiorganic salts, consisting entirely of ionic components with melting points at or close to room temperature (below 100 °C). Room temperature ILs usually consist of bulky nitrogen or phosphorous containing organic cations such as alkyl ammonium, alkyl imidazolium, alkyl pyridinium, alkyl pyrrollidinium, alkyl phosphonium with an associated inorganic (e.g., Cl^-, PF_6^-, BF_4^-) or organic (e.g., $[(CF_3SO_2)_2\ N]^-$, $[(CF_3CO_2)]^-$) counteranion. ILs have an extended range of solubility in both polar and nonpolar solvents.[103] ILs have gained popularity in recent years as chiral separation media for GC, HPLC, and CE.[56]

GC

The room temperature ILs have negligible vapor pressure with densities generally greater than water and viscosities often 2–3 orders of magnitude greater than conventional solvents. These properties indicate compatibility of ILs as GC stationary phases. ILs show "dual nature" properties. They separate nonpolar compounds as if they were nonpolar stationary phases and separate polar compounds as if they were polar stationary phases.[104]

Using ILs for chiral GC might be done in two ways: either CS can be dissolved in an achiral IL, or the IL liquid itself can be chiral.[103] Chiral ephedrinium-based IL (see Figure 21) was used as the GC CSP for separation of alcohols, diols, sulfoxides, epoxides, and acetylamines.[104]

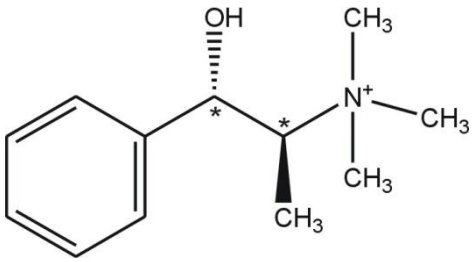


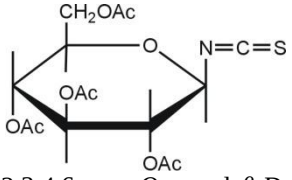
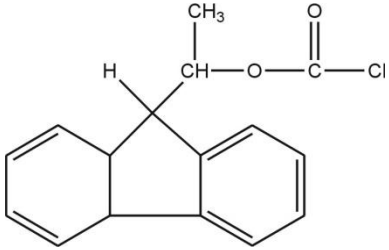
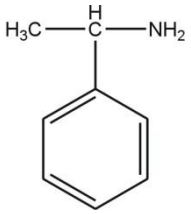
Figure 21. Structure of (*1S,2R*)-(+)-*N,N*-dimethylephedrinium ion

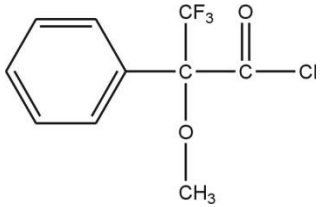
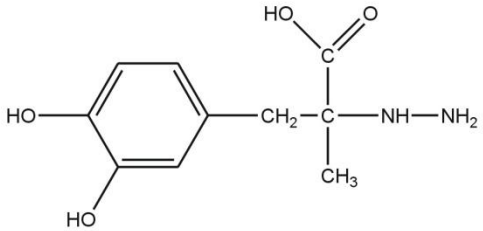
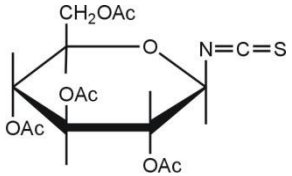
1.3.5 Indirect chiral separations

Indirect chiral separations[19, 105, 106] involve the coupling of the enantiomers with an auxiliary chiral reagent to convert them into diastereomers. Because diastereomers differ in physical and chemical properties[4], they can be separated by any achiral separation technique.[1] A broad spectrum of chiral derivatization reagents have been developed for GC, HPLC[107], and CE. Examples of derivatization agents for HPLC are given in Table 4.

To demonstrate how formation of diastereomers allows separation in achiral environment, we can again use example with gloves. Stereochemically, the situation is analogous to what happens when a hand (analyte) puts on a right-handed glove (a chiral derivatization agent). Left and right hands do not put on the glove in the same way. The products - right hand in right glove versus left hand in right glove - are not mirror images, they're altogether different.[3]

Table 4. Examples of chiral derivatization agents for indirect chiral HPLC.[19, 49, 105, 106]

Functional group of analyte	**Chiral derivatization agent**	**Diasteromeric product**
primary and secondary amines	 2,3,4,6-tetra-*O*-acetyl-*β*-*D*-glucopyranosyl isothiocyanate (GITC)	thioureates
primary and secondary amines	 1-(9-fluorenyl)ethylchloroformate (FLEC)	carbamates
carboxylic acids	 1-phenylethylamine	amides

Hydroxyls	 α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's acid chloride, MTPA-Cl)	esters
Carbonyls	 α-hydrazino-3,4-dihydroxy-2-methylbenzenepropanoic acid (carbidopa)	hydrazones
Thiols	 2,3,4,6-tetra-*O*-acetyl-*β*-*D*-glucopyranosyl isothiocyanate (GITC)	dithiocarbamates

There are several **requirements for reliable indirect separation method**[19, 41, 49, 108]:

- **Racemization does not occur during derivatization** procedure. Moreover, good **chemical stability of the resulting diastereomers** (at least 1 day) is required for many analyses, because automated analysis are usually carried out overnight.
- **High optical purity of a chiral derivatization agent** is necessary. Since the opposite enantiomer contaminating the reagent also produces a corresponding diastereomer, it is obvious that erroneous results will be obtained with the use of impure reagents (see Figure 22).
- There is **absence of kinetic resolution** (rate of the reaction should be the same for both enantiomers) or chiral derivatization reaction proceeds completely for both enantiomers.
- There should be **equal detector response** to both diastereomers.
- **Distance between the two asymmetric centers** in the substrate and the reagent **should be minimized** for the best separation. Derivatives with distances exceeding 3 bonds are rarely used in indirect separation methods. The conformational rigidity around the chiral centers is another important factor for the separation. A resolving reagent in which free rotation near the asymmetric center of the substrate is hindered by the formation of the diastereomer is recommended. Resolution is usually improved when bulky groups are attached to the chiral centers.
- The derivatization reagent posess specificity for the target functional group and **quantitatively labels the analyte under mild conditions.**
- Derivatization agent should be **soluble** in water or miscible with aqueous solvents as methanol (MeOH) and acetonitrile (ACN), because most of the bioactive chiral molecules are analyzed in aqueous solutions.

- Both enantiomers of the reagent are commercially available or easily obtained by simple synthesis, because the **elution order can be controlled by the selection of the reagent enantiomer**. This is necessary when the determination a trace of enantiomers is required in the presence of a large amount of the oposit enantiomer.

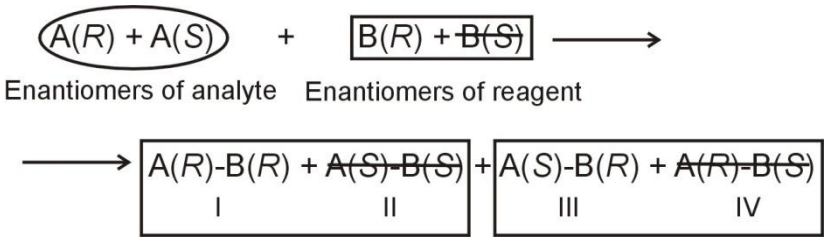


Figure 22. Derivatization of analyte enantiomers *A* using chiral derivatization agent *B*(*R*) contaminated with opposite enantiomer *B*(*S*). The enantiomers of *A* are to be separated and determined as their diastereomeric derivatives (I and III), which can be resolved on achiral stationary phases. However, the peaks II and IV produced by *B*(*S*) impurity interfere with the analysis. In this case, peaks I and II, and peaks III and IV are enantiomeric pairs and are not separable on achiral stationary phases.

1.3.6 Selection of separation system and developing of chiral separation methods

Chiral method development is a challenge due to the number of available CSPs[38] and complexities in the retention mechanism. Evaluating multiple columns and mobile phases is expensive in terms of time, materials, and effort.

HPLC versus GC

Chiral HPLC is used for the separation of involatile substances that cannot be made volatile by derivatization. Such substances include those that are highly polar or ionic, or those substances that have relatively high molecular weights.[4] HPLC, carried out at around room temperature, is suitable for chiral resolution, because the possible racemization during separation on the column is negligible.[19]

Chiral GC should be selected as the separation technique if the materials are volatile and stable at elevated temperatures. GC offers much higher efficiencies than HPLC.[4]

The fundamental difference between separations that are achieved by HPLC as opposed to GC is that in HPLC, there can be strong interactions between the solute and the mobile phase, in addition to those between the solute and the stationary phase. In GC, interactions between the solute molecules and those of the gas phase are weak, and relatively infrequent. This means that the retention and selectivity can be modified by solely changing the nature and/or composition of the mobile phase. In HPLC, the solutes no longer need to be volatile, thus ionic interactions can be used to control retention, in addition to dispersive and polar interactions.[4]

Direct versus indirect separation method[19, 106]

Direct separation methods are suitable for pharmaceutical analysis of traces of one enantiomer in majority of the opposite enantiomer, e.g. for the determination of an optical purity of a drug substance. Direct separations are also suitable for preparative purposes. In the biomedical analysis, direct separations are suitable for analysis of the endogenous compounds, drugs and its metabolites in complex matrices of biological samples (plasma, blood, urine, serum, saliva). However, tedious and time-consuming sample pretreatment is necessary in this case.[49]

Advantages of direct separation are:

- Simple preparation of analytes due to absence of a derivatization step.
- Absence of racemization and kinetic resolution.
- The purity of CS is not critical.

Disadvantages of direct separation are:

- No universal column exists. The choice of the best column for the separation of each racemate is difficult, because the separation highly depends on the interaction between CSP and enantiomer. Moreover, CSPs are very sensitive to chromatographic conditions, so method development is rather difficult.
- The theoretical plate number of the CSP is small.
- High cost of CSPs.

- The elution order of enantiomers depends upon the CSP column used, and cannot be changed easily.

Indirect separation methods are suitable for trace analysis of enantiomers in biological samples, because of the possibility of the introduction of a highly sensitive UV-VIS or fluorescence tag. The indirect methods are not suitable for the analysis of a enantiomer in a standard sample or during pharmaceutical preparations, where a low amount of enantiomer (at a level of 0.1 or 0.05%) is to be determined.

Advantages of indirect separation are:

- Good chromatographic, chromophoric or fluorophoric properties of derivatives.
- Low cost of achiral column. However, the economics of chiral separation should be calculated carefully considering all the costs involved. It is very easy to ignore facts, that price of CSPs may be insignificant compared with the time taken to prepare samples. Moreover, achiral columns exposed to samples containing derivatization mixtures are often deteriorated more quickly, because of excessive amount of reactive derivatization agent is presented.
- Possibility of selection of the elution sequence.

Disadvantages of indirect separation are:

- The possibility of racemization or kinetic resolution.
- The excess of reagent and side products may interfere with the separation.
- Additional derivatization step might be time-consuming.

Selection of suitable CS

The selection of suitable CS is fundamental and mostly empirical process.

All enantioselective chiral mechanisms involve a combination of interactions. The strongest one may be as important as the weakest one for enantiomer discrimination. However, the choice of CS is mainly controlled by the strongest interaction between the CS and the analyte.[51] Selection of particular CSP might include automated **screening strategies**, during which several CSPs with different properties are screened for enantioselectivity. The conditions for separation are further optimized using the most promising CSP.

Although chiral selectivity may be anticipated based on the nature of the interaction between a chiral solute and a chiral stationary phase, the **elution order** of the enantiomeric pair will remain unknown unless experiment is performed. (*R*)-isomer in the stationary phase will not determine that the *(R*) or (*S*) isomer of the solute will be eluted first.[4] In some applications, it is important to be able to detect traces of the inactive enantiomer, beside a high excess of the active enantiomer. To avoid overlapping with the tailing of the large peak of the active enantiomer, the inactive enantiomer should appear always as first peak. However, in direct HPLC, the possibilities for changing the **enantiomeric elution order** are restricted, since only few chiral phases exist in both enantiomeric forms.[41]

ChirBase HPLC and **ChirBase GC** and **ChirBase CE** are chiral applications databases which allow locate separation methods for similar compounds that have been studied before. The databases can be searched by structure or structure similarity and are updated regularly using both published and unpublished data. ChirBase HPLC contains method information for 30,000 unique structures linked to over 1400 unique CSs. ChirBase GC and CE contain information for more than 8000 and 2000 unique structures, respectively (Advanced Chemistry Development, http://www.acdlabs.com; 3.3.2009).

Biological samples

The chiral analysis of compounds in **biological samples** usually requires intensive sample pretreatment and preconcentration steps (liquid–liquid extraction, dialysis, column switching techniques, achiral separation steps in off-line mode, solid phase extraction (SPE))[49] and use of sensitive detection systems such as laser-induced fluorescence detection[41] or liquid chromatography-mass spectrometry (LC-MS).[109]

GC

There are two approaches to **derivatization** in chiral GC. The first approach is to derivatize analyte with a **chiral reagent to produce diastereomers** which can be separated on conventional GC columns. The second approach is to derivatize with an **achiral reagent to increase volatility** and separate the derivatives on a column with a CSP.[4]

Major factors that control chiral selectivity in GC are the chemical structure and the stereoisomerism of a CS, the CS loading on the column and the operating temperature.[4]

After the CSP has been selected, the only important variable left to the analyst, is the operating temperature, or the **temperature program**. In practice, the sample should be injected onto the column and the temperature programmed from about 50°C to 200°C (depending on the upper thermal limit of the CSP). From the temperature program, the best isothermal temperature can be estimated and the optimum operating temperature can be assessed by iteration.[4]

HPLC

In HPLC, after choosing the CSP from a wide range of possibilities, the optimum mobile phase composition must be selected. Manufacturers of CSPs usually provide an **experimental step-by-step protocol for method development** using their particular products.[4]

Temperature should be controlled during chiral HPLC separations. The isoelution temperature is determined by a balance of enthalpic and entropic contributions to the chromatographic enantioselectivity. Consequently, when temperature increases, selectivity of chiral separations decreases until enantiomers coelute at an isoelution temperature. Above this temperature, elution order should reverse and selectivity will increase with temperature.[110]

Strategies for method development in NP and RP HPLC using polysaccharide-based stationary phases were suggested by Matthijs et al., including a screening and an optimization stage. The screening stage allows a fast evaluation of separation possibilities and enantioselectivity for many compounds in a short period of time, while the optimization stage gives the opportunity to enhance, if needed, the initially obtained separation. For example, compounds are screened using a 3 × 2 experimental design. Three CSPs are tested using two type of organic modifier resulting in six experiments. When required, after the initial screening a further optimization can be performed starting with the best conditions (e.g. giving the highest resolution). Screening is intended for systems having both eluent and column switching valves allowing complete automatization. However, performing the full screening procedure is unpractical and time consuming when no automatic switching device is available. For such systems, a step-by-step approach is proposed, which is derived from the full screening strategy.[111]

1.4 Oxidative stress

Most of this thesis is devoted to studying of enantioselectivity of *trans*-4-hydroxy-2-nonenal (HNE) detoxification. HNE is product of lipid peroxidation[112] and is implicated in diseases involving oxidative stress[113]. Therefore, these terms are briefly discussed. Moreover, the most important analytical method that are used for measurement of oxidative stress are described.

1.4.1 Oxidative stress and lipid peroxidation

Oxidative stress occurs when cells are subjected to excess levels of reactive oxygen species (ROS)[‡]. An increase in intracellular oxidants results in damage to various cell components and in activation of specific signaling pathways[114].

The major ROS are **superoxide radical**, $O_2^{\bullet -}$, **hydrogen peroxide**, H_2O_2 and **hydroxyl radical**, $OH^{\bullet}$. Hydroxyl radical is extremely reactive oxidizing radical[115], responsible for most of the damage to biological macromolecules. Damage is restricted to a small radius limited by the diffusion of the radical. In cell, the hydroxyl radical is produced by the Fenton reaction[116]:

(1-16) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$

ROS are produced in all aerobic organisms[114] as a by-product of respiration[116] and mitochondrial electron transport chain is the major ROS source[117]. Outside mitochondria, ROS are generated during biotransformation of various xenobiotics and drugs, inflammation, and by UV and ionic irradiation[115].

Under normal circumstances, cells are able to balance the production of oxidants and antioxidants, resulting in redox equilibrium[114]. Antioxidant defense is mediated by defense enzymes, low-molecular weight antioxidants and deoxyribonucleic acid (DNA) and protein repair. Major protective enzymes against damage by ROS are **superoxide dismutase** (catalyzes reaction $2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$), **catalase** (converts the hydrogen peroxide to water and oxygen $2H_2O_2 \rightarrow O_2 + 2H_2O$) and **glutathione peroxidase**

[‡] ROS are sometimes not accurately referred as "free radicals". ROS include both radical species (e.g. $OH^{\bullet}$) and non-radical species (e.g. H_2O_2)

(catalyzes reaction ROOH + 2GSH → ROH + H_2O + GSSG)[116]. Examples of low-molecular weight antioxidants which act as a free-radical scavengers are vitamin E (*α*-tocopherol), ascorbic acid, *β*-carotene and ubiquinol.[115]

The oxidative stress arises from an imbalance between generation and elimination of ROS. An increase in intracellular oxidants might result in damage[114] to various cell components, including nucleic acids (DNA, mitochondrial DNA and ribonucleic acid (RNA)), proteins and lipids[118].

However, ROS toxicity play also beneficial role in physiologically useful reactions. For example ROS are generated by activated macrophages, presumably to kill their target cells (bacteria, fungi, etc.) before phagocytosis. Moreover, ROS play role in the mechanisms of apoptosis[116].

Under the oxidative stress, ROS attack double bonds in polyunsaturated fatty acids in lipid membranes[115]. Non-enzymatic self-propagating chain of free radical reactions is called **lipid peroxidation** and gives complex products including hydroperoxides, aldehydes, isoprostanes (prostaglandin like compounds), alkenals and hydroxyalkenals, such as malondialdehyde and HNE. Many of these products are cytotoxic, probably because of their reactivity toward proteins. HNE is thought to be one of the most reactive product of these reactions and is an important mediator of free-radical damage[119].

Oxidative stress is implicated in aging and in the numerous diseases, including neurodegenerative diseases (Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease), stroke, atherosclerosis, diabetes, hepatitis, HIV[118] and cancer[115].

1.4.2 Measurement of oxidative stress

Detection of ROS

Free radicals are paramagnetic species directly detectable using **electron paramagnetic resonance (EPR)**. However, many free-radicals species are highly reactive, with relatively short half-lives, and the concentrations found in biochemical systems are usually too low for direct detection by EPR spectroscopy. This problem is overcome by **spin-trapping**. Spin traps are compounds that react covalently with highly transient free

radicals to form relatively stable, persistent spin adducts that possess paramagnetic resonance spectra detectable by EPR spectroscopy.[118] Hydroxyl radical detection by spin trapping and EPR procedures often has been done using 5,5-dimethylpyrroline *N*-oxide as the spin trap.[120]

Several **fluorescence probes**[121] to detect ROS are available, including 2',7'-dichlorodihydrofluorescein and dihydrorhodamine 123. Signal caused by reaction of ROS with fluorescence probe is detected by spectrofluorometer, fluorescence microscope or flow cytometer.

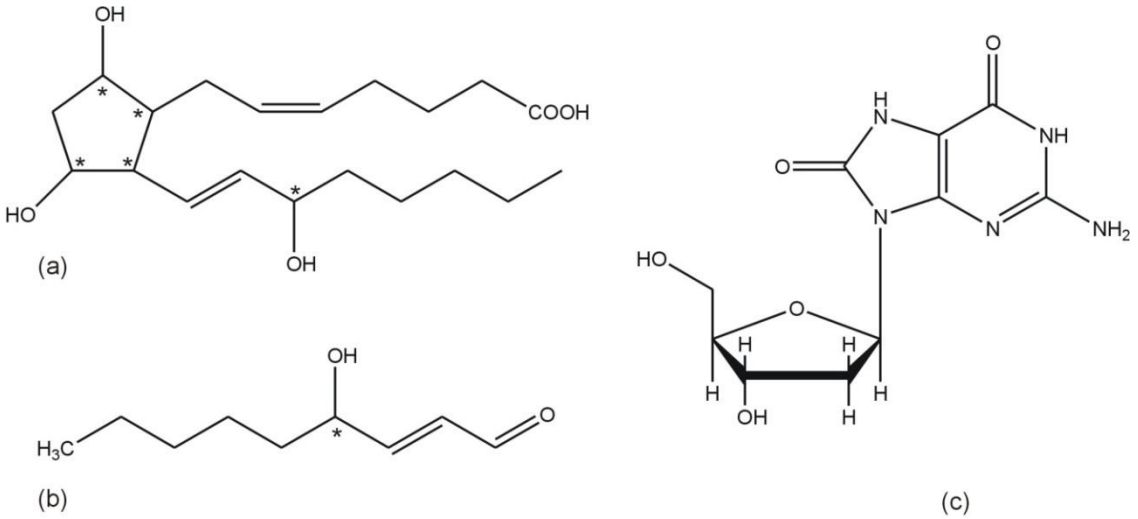


Figure 23. Markers of oxidative stress: (a) F2-isoprostane (15-serie), (b) HNE, (c) 8-hydroxy-2'-deoxyguanosine

Detection of oxidative damage to lipids (lipid peroxidation)

F2-isoprostanes are prostaglandin like compounds derived from the nonezymatic oxidation of arachidonic acid. F2-isoprostanes provides an accurate assessment of oxidative stress both in vitro and in vivo[122] and are measured in tissues, urine and plasma using LC-MS/MS[123] and GC-MS[122, 124].

Hydroperoxides, primary products in lipid peroxidation, are measured using HPLC-chemiluminiscence method[125]. Lipid peroxidation is also assessed by thiobarbituric acid reacting substances assay. The formed products are measured by fluorimetry. However, it is well known that the assay is nonspecific[126].

Aldehyde markers of lipid peroxidation[127] malondialdehyde[126] and HNE are detected by GC-MS[128, 129] and LC-MS[130, 131] (see **IV**).

Detection of oxidative damage to nucleic acids

The 8-hydroxy-2'-deoxyguanosin lesion is often used in the assessment of oxidative DNA damage and has become marker for oxidative damage to DNA[118]. 8-hydroxy-2'-deoxyguanosine is measured in urine using HPLC with electrochemical detection or by LC-MS[132].

Detection of oxidative damage to proteins

Protein oxidation is the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary products of oxidative stress. With so many different potential reaction products, there is no single universal marker for protein oxidation.[133] For example, 3-hydroxyvaline, 5-hydroxyleucine and 3-hydroxylysine might be measured using HPLC-UV or N^{ε}-(carboxymethyl)lysine is analyzed using GC-MS[120]. Detection of total protein carbonyls levels might be performed after reaction with dinitrophenylhydrazine followed by UV spectroscopy[133]. However, it is unclear whether the measurement of such materials accurately reflects damage to proteins[120].

1.4.3 *trans*-4-hydroxy-2-nonenal (HNE) and its detoxification

HNE[112] is a cytotoxic α,β-unsaturated aldehyde implicated in the pathology of multiple diseases involving oxidative damage[113]. HNE formation results from the oxidation of ω-6 polyunsaturated fatty acids such as arachidonic acid and linoleic acid by hydroxyl radicals.[112] The chemical basis of HNE toxicity is related to its electrophilic character and the ability to alkylate cellular nucleophilic groups and to deplete glutathione.[134]

HNE may be detoxified via numerous mechanisms, including adduction of the C-3 electrophilic center with GSH via glutathione S-transferases (GSTs), oxidation of the aldehyde by aldehyde dehydrogenases (ALDH) to form *trans*-4-hydroxy-2-nonenoic acid (HNEA), or reduction of the aldehyde to an alcohol by aldo-keto reductases (AKRs) or alcohol dehydrogenases (ADHs) to form 1,4-dihydroxynonene.[135] Oxidation of HNE to

HNEA via ALDHs is a major route of metabolism in many systems including smooth muscle cells, hepatocytes, astrocytes, and mitochondria from liver and brain.[136, 137]

HNE possesses a chiral center at C4 and exists as two enantiomers, (*R*)-HNE and (*S*)-HNE, with potentially different biochemical reactivities. Recent studies have shown that the chirality of HNE effects its detoxification in liver cells[138, 139] and that the protein adducts of (*R*)-HNE differ in localization from those of (*S*)-HNE protein adducts in cellular models[140].

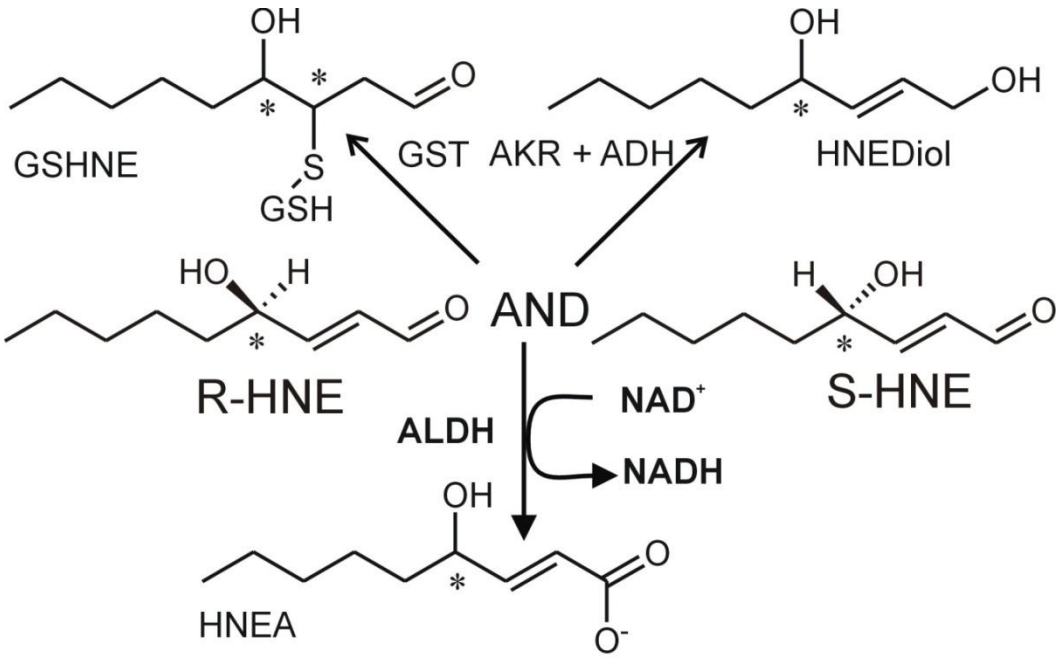


Figure 24. HNE detoxification.

2. AIMS OF THIS THESIS

Chirality plays important role in various areas of biomedicine and biotechnology.

Enantiomers might differ significantly in their biochemical reactivities when exposed to a chiral environment of a living organism. Consequently, pharmacological and toxicological effects and metabolism of individual enantiomers should be studied.

Furthermore, preparation of optically pure chemicals using enantioselective enzymes might be cheap and an environmental friendly alternative to classical organic synthesis.

Obviously, analysis of chiral compounds is an integral part of this research. For this reason, it is necessary develop methods for chiral separation and analysis of chemicals of contemporary interest.

This thesis can be divided into several objectives:

First objective was to synthesize chemicals necessary for further studies. To obtain enantiomers of HNE, kinetic resolution by enantioselective enzymes was employed. Then, enantiomers of HNEA were prepared by oxidation of the optically pure HNE enantiomers. The identity and purity of prepared chemicals was verified by various analytical techniques.

Second objective was to study the basis of enantioselective oxidation of HNE by brain mitochondria. We tested hypothesis, that various rat brain mitochondrial ALDHs differ in the ability to oxidize HNE enantiomers. Enzyme kinetics parameters were measured using rat brain mitochondrial lysate, recombinant ALDH5A, and recombinant ALDH2. To test whether the absolute configuration of the HNE C4 carbon was retained during the enzymatic oxidation of HNE, it was necessary to develop NP-HPLC chiral separation method for determination of HNEA enantiomers.

We showed in **II.** that HNE is detoxified *(R)*-enantioselectively with retention of stereoconfiguration to HNEA by rat brain mitochondria lysate. Moreover, HNEA was reported as marker of lipid peroxidation[141]. However, no RP-HPLC method was available

for the enantioseparation of HNEA. Therefore, third objective was focused on the development of convenient RP-HPLC methods. Direct and indirect RP-HPLC methods for separation of HNEA enantiomers were developed, validated and compared. The indirect separation method was successfully applied for the determination of *ER* of HNEA in rat brain mitochondrial lysate.

Fourth objective was to optimize ESI-MS/MS parameters for analysis of HNE enantiomers and its metabolites. First, MS/M optics parameters were optimized using direct infusion of each analyte in MeOH-ammonium acetate buffer. Afterwards, electrospray ionization (ESI) parameters were established.

Chiral epoxides can serve as valuable building blocks in an organic synthesis. This is due to the versatility of the oxirane ring, which can be chemically transformed by reaction with nucleophiles into numerous enantiopure intermediates[142].

The goal of this study was to find microorganisms which produce novel enantioselective epoxide hydrolases and apply these enzymes to enzyme kinetic resolution of epoxide racemates. Epoxide hydrolases are hydrolytic enzymes that catalyze the hydration of epoxides to their corresponding diol products.[143] Epoxide intermediates often occur in microorganisms due to the degradation pathways. Therefore, we tested hypothesis, that microorganisms isolated from a harsh industrial environment (biofiltration* and bioremediation† sites) are adapted to high concentrations of organic and toxic chemicals and might be promising source of novel enantioselective epoxide hydrolases.

In order to measure an enantioselectivity, it was necessary to develop and/or optimize methods for analyses of chiral epoxides and diols using a chiral GC. Based on the screening performed, most promising strains were selected and *E* was determined using a chiral GC.

* Biofiltration – removing of volatile organic contaminants from industrial exhaust gasses by microorganisms inherent in the biofilter medium.

† Bioremediation – using of microorganisms to clean up contaminated soil or water. E.g. decontamination of petroleum-polluted soils using hydrocarbon-degrading microorganisms.

3. CONTRIBUTION TO THE CONTEMPORARY SCIENCE AND GENERAL RESULTS

This thesis describes development and optimization of the analytical methods for determination of various chiral compounds. All separation methods presented in this work were successfully applied for analysis of analytes in various biological matrixes, including bacteria, filamentous fungi, yeasts, rat brain mitochondria and recombinant enzymes. Moreover, experiments beyond scope of analytical chemistry were performed, including measurement of ALDHs enzyme kinetics. All these measurements were employed to study enzyme enantioselectivity. Overall conclusions and contribution to the contemporary science are summarized in following chapters corresponding to the publications enclosed as appendix section.

3.1 PAPER I: Enantioselective metabolism of *trans*-4-hydroxy-2-nonenal by brain mitochondria

Racemic HNE[144] and racemic HNEA[145] were synthesized as described previously. In house synthesis provided sufficient amount of HNE racemate[144] for preparation of individual HNE and HNEA enantiomers (see Figure 25). These chemicals were necessary for experiments described in **II**, **III** and **IV**.

The identity and purity of products were determined using MS direct infusion, NMR, polarimetry, HPLC with UV detection and/or TLC.

- Racemic HNE was synthesized starting from fumaraldehyde bis(dimethyl acetal). In the first step of the synthesis, the fumaraldehyde dimethyl acetal was partially hydrolyzed using amberlyst catalyst to obtain monoacetal. HNE was synthesized by the Grignard reaction of the fumaraldehyde monoacetal with 1-bromopentane.[144] Crude mixture was filtrated through celite and purified by flash chromatography on silicagel using hexane – ethylacetate (75:25, v/v) as a mobile phase.

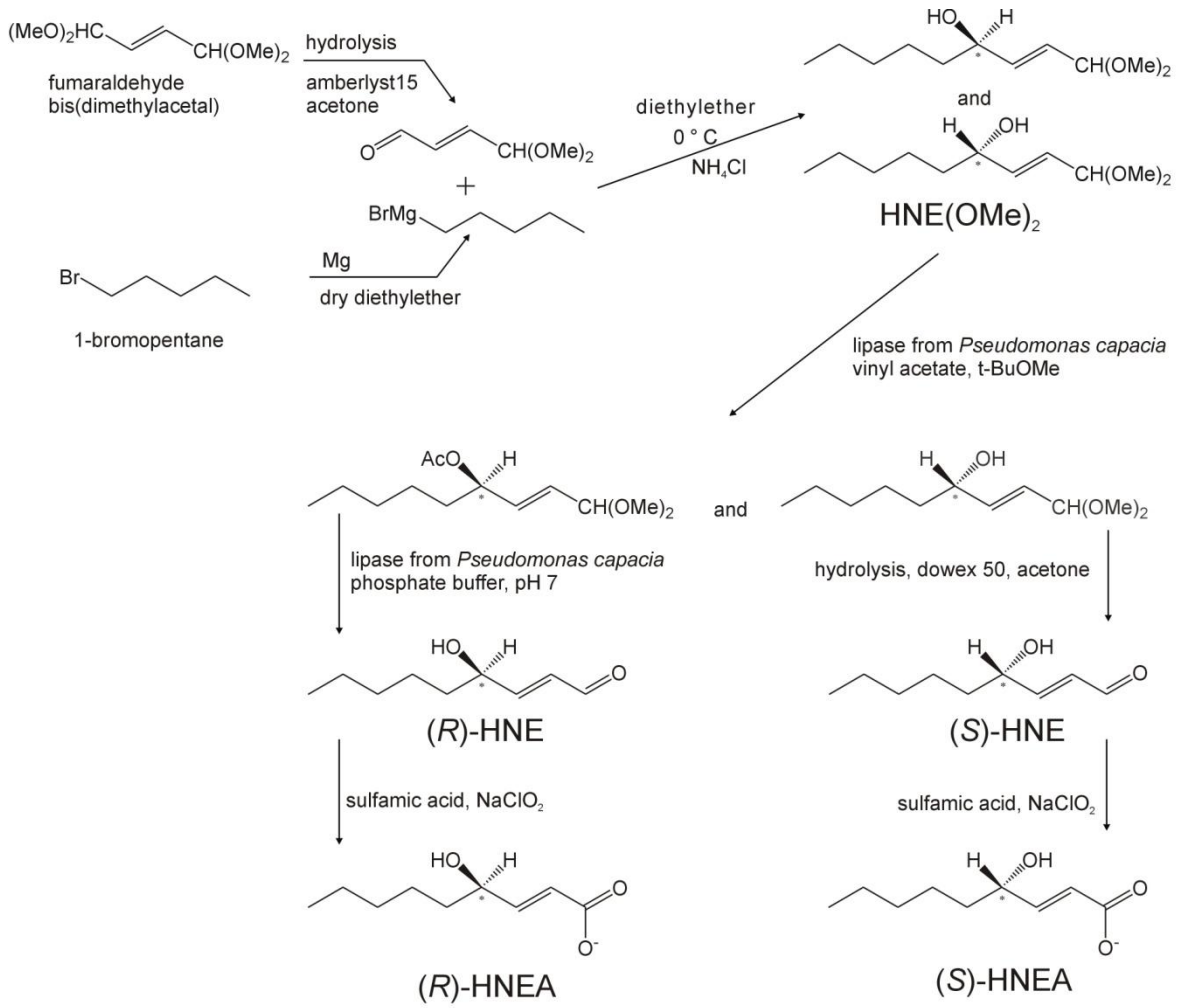


Figure 25. Synthesis of HNE and HNEA enantiomers. Note, that unless kinetic resolution was applied using enantioselective lipase from *Pseudomonas cepacia*, racemate (*ER* 50:50) is obtained by achiral organic synthesis.

- Individual enantiomers of HNE were prepared by enzymatic resolution of racemic HNE dimethylacetal with a lipase from *Pseudomonas cepacia* bound to a ceramic support according to the procedure described previously with a lipase from *Pseudomonas fluorescencis*[146]. The *Pseudomonas cepacia* lipase was bound to ceramic beads that allowed for easier extraction of the sample and the enzyme could be used multiple times. (*R*)-HNE was enantioselectively acetylated and resulting mixture of acetyl-(*R*)-HNE$(OMe)_2$ and (*S*)-HNE$(OMe)_2$ was separated by flash chromatography using silicagel and

hexane/ethyl acetate as an eluent. The purity of underivatized enantiomers was measured by HPLC on a chiral column, Chiracel OB in mobile phase consisting of 2-propanol/hexane[139] with >96% purity for (*S*)-HNE and >98% for (*R*)-HNE.

- Racemic HNEA was prepared by sodium chlorite oxidation of HNE in the presence of sulfamic acid[145]. For purification of HNEA, SPE procedure was developed and applied (see **II**). HNEA enantiomers were synthesized by oxidation of the individual HNE enantiomers (see **III**).

3.2 PAPER II: Enantioselective oxidation of *trans*-4-hydroxy-2-nonenal is aldehyde dehydrogenase isozyme and Mg^{2+}-dependent

This section describes measurement of kinetic properties of enantioselective oxidation of HNE by rat brain mitochondrial ALDHs. This work demonstrates that chirality plays an important role in the metabolism of HNE and that the enantiomers of HNE serve as useful tools for studying enzyme-substrate interaction. The results of this study can be summarized as follows:

- HPLC method for enantioseparation of HNEA enantiomers was developed. Prior to the HPLC separation of HNEA enantiomers, the samples were purified by SPE using Oasis HLB extraction cartridges. Oasis HLB is a RP sorbent, prepared from two monomers, the hydrophilic *N*-vinylpyrrolidone and the lipophilic divinylbenzene. HNE, which interfered during enantioseparation of HNEA, was removed using the SPE method. HNEA enantiomers were separated by HPLC-UV using a chiral column Chiralcel OB and a mobile phase hexane-2-propanol (97:3, v/v). Chiralcel OB is polysaccharide type CSP and contains cellulose tribenzoate coated on a silicagel. The method was used for the determination of the HNEA enantiomeric ratio *ER* only, achiral RP-HPLC was used for quantification of HNEA. Baseline resolution was achieved (R_s = 1.83), and the retention time was 11.0 min for (*S*)-HNEA and 12.9 min for (*R*)-HNEA. The limit of detection (LOD) was 4.8 μM (192 pmol) for (*S*)-HNEA and 5.4 μM (216 pmol) for (*R*)-HNEA. It was shown by using this method that oxidation of HNE enantiomers to HNEA by rat brain mitochondria occurs with retention of stereoconfiguration.

- The apparent kinetic parameters of HNEA formation by rat brain mitochondrial lysate were determined using HPLC-UV. Using enantiomeric ratio of the kinetic resolution *E* (a comparison of V_{max}/K_M) as a measure of efficiency, these data show that the ALDHs present in rat brain mitochondria oxidize (*R*)-HNE 3.7-fold more efficiently than (*S*)-HNE. There was no consumption of (*R*)-HNEA or (*S*)-HNEA by lysed mitochondria supplemented with nicotinamide adenine dinucleotide (NAD^+), demonstrating that selective formation of (*R*)-HNEA was not an artifact resulting from enantioselective metabolism of (*S*)-HNEA.

- Recombinant rat ALDH5A and ALDH2 containing *N*-terminal polyhistidine tags were prepared as described previously[147] with modification. Enzymes were expressed in Rosetta 2 (DE3) *Escherichia coli* cells and purified using immobilized-metal affinity chromatography and dialysis. During the affinity chromatography, 6 histidine tagged proteins interact with nickel immobilized on nitrilotriacetic acid agarose stationary phase. Proteins of just over 50 kDa and of >95% purity were obtained as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

- Kinetic parameters of oxidation of individual HNE enantiomers by rat ALDH5A were measured by monitoring NADH formation at 340 nm under the initial rate conditions. Assays were performed with dialyzed protein on a microplate spectrophotometer. Because of the low K_M of ALDH2 for HNE, the enzyme activity was measured via monitoring the formation of HNEA by LC-MS/MS as described previously with some modifications (see **I**). Instead a Beta Basic C18 stationary phase, chromatographic column Luna 3 μm C8(2) was used. The limit of quantitation (LOQ) for HNEA was below 30 fmol.

- Based on kinetic parameters of purified recombinant enzymes, it was concluded, that rat ALDH5A enantioselectively oxidized (*R*)-HNE, whereas rat ALDH2 was not enantioselective. However, enantioselectivity of ALDH5A and ALDH2 is altered by presence of Mg^{2+} ions. Interestingly, *trans*-2-nonenal, a substrate which lacks the hydroxyl group at the C4 position, had kinetic characteristics similar to those of (*S*)-HNE when oxidized by ALDH5A.

3.3 PAPER III: Direct and indirect high-performance liquid chromatography enantioseparation of *trans*-4-hydroxy-2-nonenoic acid

In this section, development and validation of two RP-HPLC methods for enantioseparation of HNEA is described. Results obtained from these experimental steps are summarized into the following conclusion:

- The indirect separation method is based on the derivatization of HNEA enantiomers with the chiral derivatization agent (1*S*,2*S*)-(+)-2-amino-1-(4-nitrophenyl)-1,3-propanediol (ANPAD) in a phosphate buffer–MeOH mixture in the presence of the coupling agent *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and the additive 1-hydroxybenzotriazole (HBT). The derivatization conditions for generation of HNEA-ANPAD were optimized, including pH of reaction, concentration of phosphate buffer and concentration of derivatization agent and additives. The identity of formed HNEA-ANPAD diastereomers was confirmed by ESI MS/MS in positive mode using direct infusion. To best of our knowledge, no method allowing derivatization of carboxylic acid by chiral amine directly in buffer-based biological matrix has been reported previously.

- Various RPs were screened for efficiency to separate HNEA-ANPAD diastereomers. It was showed, that separation factor α was in linear correlation with hydrophobicity of RP reported by Euerby and Petersson[148]. Mobile phase and temperature suitable for the separation of HNEA-ANPAD were optimized using the RP Spherisorb ODS2. Optimal separation conditions were achieved using a mobile phase MeOH–5mM ammonium acetate, pH 7.0 (39:61, v/v). (*R*)- and (*S*)-HNEA-ANPAD eluted at 40 and 43 min, respectively, with resolution R_s = 2.26.

- Direct separation of HNEA was performed on a CSP Chiralpak AD-RH. The Chiralpak AD-RH is a chiral column prepared by coating silica with a derivatized amylose. The hydroxyl groups on the amylose are derivatized to give the *tris*(3,5-dimethylphenylcarbamate). An application of ternary mobile phase MeOH–ACN–5mM ammonium acetate, pH 3.5 (58:4:38, v/v/v) led to the shortest analysis time and no interference of HNE. Resolution R_s was as follows: (*R*)- and (*S*)-HNEA 1.78, (*S*)-HNEA and (*S*)-HNE 2.81 and (*S*)-HNE and (*R*)-HNE 1.07. The separation factor α of HNEA enantiomers was 1.23.

- Validation parameters of both direct and indirect separation methods were determined, including linear range, LOQ, precision and stability. The accuracy of *ER* was estimated by analyzing enantiomerically enriched samples containing various *ER* of HNEA. Difference in *ER* obtained by direct and indirect separation method was less than 0.7%, showing excellent accuracy. These data clearly demonstrate, that kinetic resolution (for example ANPAD preferentially reacted with one enantiomer) or racemization did not occur during derivatization procedure, and that the influence of chiral impurities in the derivatization agent was negligible.

- SPE preconcentration and purification method for HNEA enantiomers was optimized. The LOQ of HNEA enantiomers was below 10 pmol, when SPE and direct separation method on Chiralpak AD-RH was used.

- The indirect separation method was successfully applied for the determination of *ER* of HNEA formed in rat brain mitochondria lysate by enzymatic oxidation of HNE. HNEA-ANPAD was separated and no interfering compounds were observed in the blank. Because of the long analysis time and no internal standard available, the chiral separation was used for determination of ER only. The total amount of HNEA formed by oxidation was quantified by a non-chiral RP-HPLC method routinely used in our laboratory.[149] Oxidation of 160 μM racemic HNE by ALDHs present in rat brain mitochondria led to (*R*)-enantioselective formation of HNEA.

3.4 PAPER IV: Quantification of *trans*-4-hydroxy-2-nonenal enantiomers and metabolites by LC-ESI-MS/MS

ESI-MS/MS parameters were optimized for analysis of HNE enantiomers; HNEA; 4-hydroxy-1-oxononan-3-yl glutathione; 1,4-dihydroxynonan-3-yl glutathione and their corresponding *d*-11 internal standard. Prior the analysis, HNE was derivatized using (*S*)-carbidopa to form *(R,S)*-HNE-*(S)*-carbidoba diastereomers.

For optimization of MS/MS parameters, approximately 0.5 μM concentrations of each analyte were prepared in MeOH:ammonium acetate buffer (50:50, v/v). MS/MS system was used in multiple reaction monitoring (MRM) mode. Optimal parent ion/daughter ion pairs were selected and corresponding parameters of ion optics were established. Subsequently, ESI parameters were optimized using flow injection analysis, including curtain, nebuliser and collision gas flow rate, ion spray voltage and temperature. ESI interface was used at a temperature of 500 °C with nitrogen as a nebulizer gas at a flow rate of 12 L/min. Samples were analyzed in a negative ion mode with an ion spray voltage of −4200 kV.

3.5 PAPER V: Novel microbial epoxide hydrolases for biohydrolysis of glycidyl derivatives

This section describes determination of enantioselectivity of microbial epoxide hydrolases using chiral GC with flame ionization detector (FID). Chiral GC methods were developed for determination of various epoxides and diols. Conditions of analyses were optimized (data not shown), including selection of CSP and column temperature program. Highly polar diols were derivatized to their corresponding acetonides using 2,2-dimethoxypropane as a derivatization agent (see Figure 26). Various derivatization conditions were tested (data not shown), including composition of derivatization mixture, time of derivatization reaction and stability of analytes during derivatization procedure. Microbial isolates from biofilters and petroleum-polluted bioremediation sites were screened for the presence of enantioselective epoxide hydrolases active towards *tert*-butyl glycidyl ether (TBE), benzyl glycidyl ether (BGE), and allyl glycidyl ether (AGE).

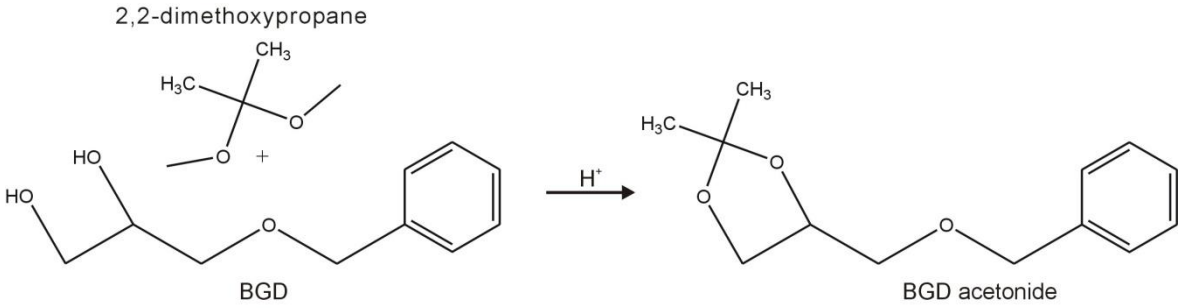


Figure 26. Derivatization of BGD to its corresponding acetonide using 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid (see **V** for experimental details). Derivatization was performed to decrease the polarity of diols before GC analysis.

Results are summarized into the following conclusions:

- Enantioseparation of AGE and 3-allyloxy-1,2-propanediol (AGD) after derivatization to its acetonide was performed using chiral column *β*-DEX 225 (see Figure 27). *β*-DEX 225 contains as a CS 2,3-di-*O*-acetyl-6-*O*-TBDMS-*β*-cyclodextrin, which is embedded in SPB-20 poly(20% phenyl/80% dimethylsiloxane). To best of our knowledge, no method for chiral determination of AGE and AGD was reported previously.

- Determination of TBE enantiomers and its hydrolytic product 3-*tert*-butoxy-1,2-propanediol (TBD) was performed using CSP Chirasil-DEX CB (25 m, 0.25mm i.d., 0.25 μm film thickness). This CSP is based on *β*-cyclodextrin chemically bonded to a dimethylpolysiloxane. Column temperature was set up for 50 °C for 5 min, followed by a gradient of 5 °C.min^{-1}.

- Analysis of BGE and 3-benzyloxy-1,2-propanediol (BGD) were reported previously. However, BGE and BGD after derivatization to its acetonide were separated in two independent runs using chiral GC on *β*-DEX 120 column[150, 151]. In this study (see **V**), we separated enantiomers of BGE and BGD acetonide in single run using different column *β*-DEX 225 column at an isothermal temperature of 107 °C. Extraction procedure was also modified and analytes were extracted directly into derivatization agent 2,2-dimethoxypropane.

- Screening of activity toward epoxides of more than 30 microbial strains was performed using chiral GC-FID methods described above. Microorganisms producing the most promising epoxide hydrolases were selected and conversion curves of enzymatic reactions were measured using GC-FID. *E* was determined by linear (see **V**.) and non-linear regression (see equation (1-10)). The enzyme of *Aspergillus niger* M200 preferentially hydrolyses (*S*)-TBE to (*S*)-TBD with a relatively high enantioselectivity (*E* is about 30 at a reaction temperature of 28 °C). Epoxide hydrolases of *Rhodotorula mucilaginosa* M002 and *Rhodococcus fascians* M022 hydrolyze BGE with relatively low enantioselectivities, the former reacting predominantly with the (*S*)-enantiomer, the latter preferring the (*R*)-enantiomer. Enzymatic hydrolysis of AGE by *Cryptococcus laurentii* M001 proceeds with low enantioselectivity (*E* = 3). As far as we know, racemic AGE and racemic TBE have not been tested yet as substrates for epoxide hydrolases.

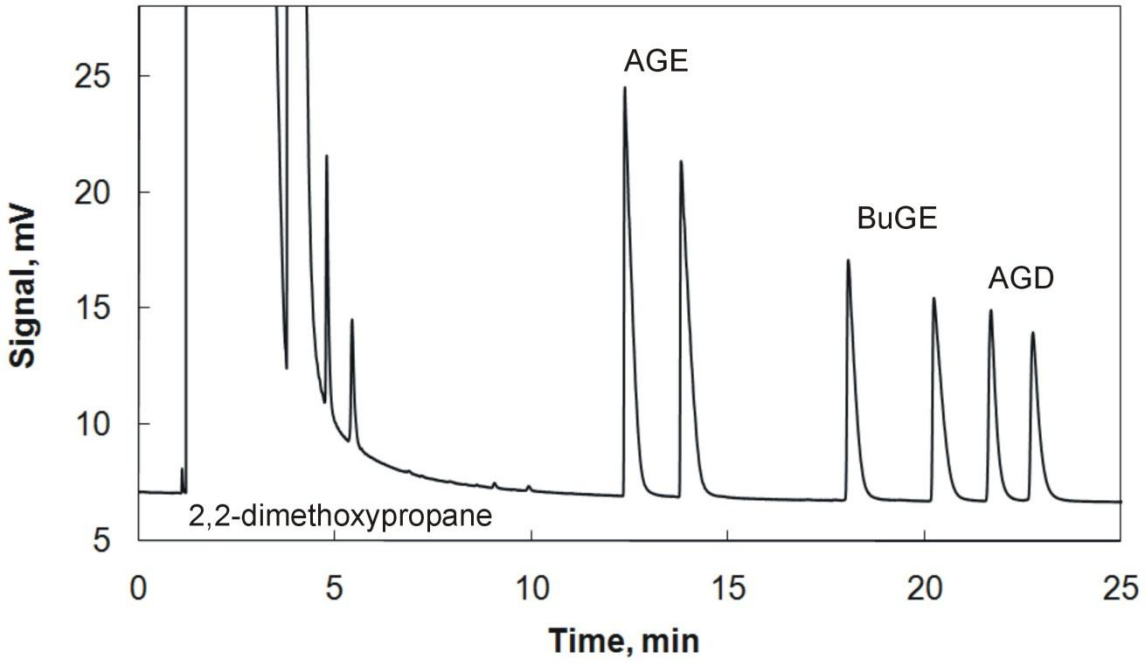


Figure 27. GC-FID enantioseparation of AGE and AGD (concentration of individual enantiomer c = 8 mM). AGD was derivatized to corresponding acetonide. Butyl glycidyl ether (BuGE) was used as an internal standard (concentration of individual enantiomer c = 3.5 mM). A *β*-DEX 225 column (30 m, 0.25mm i.d., 0.25 μm film thickness) at an isothermal temperature of 71 °C was used. H_2 was used as a carrier gas (85 kPa, split 1:25), temperature of injection port was 220°C. Elution order of the individual enantiomers was not determined, because optically pure AGE, AGD and BuGE were not available.

4. SUMMARY

Enantioselective enzymes play important role in metabolic pathways of chiral compounds. Enantioselective metabolism of lipid peroxidation product HNE was studied. For this reason, direct and indirect chiral HPLC methods were developed and enzyme kinetic was measured by various analytical methods. Difference in an ability to detoxify individual HNE enantiomers by rat brain mitochondrial ALDHs was found.

Enantioselective enzymes are useful biocatalyzators allowing cheap preparation of optically pure chemicals. Screening of epoxide hydrolases produced by various microorganisms was performed and enantioselective enzymes were found using chiral GC.

In conclusion, chiral separation methods were developed and used as a tool to answer emerging questions in biomedicine and biotechnology.

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