M1 – D2HP

TU09 - ANALYTICAL SCIENCE

Analysis of chiral substances

Macromolecules → TU 08 (QC biopharmaceuticals)

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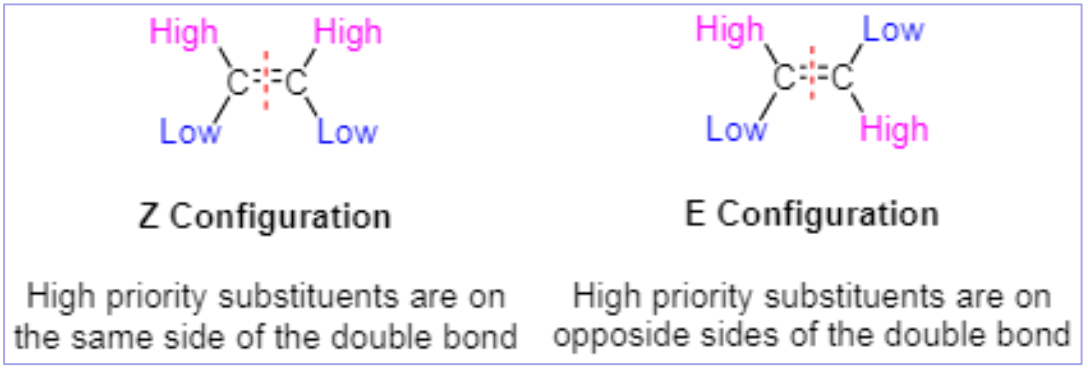
CHIRALITY: definitions

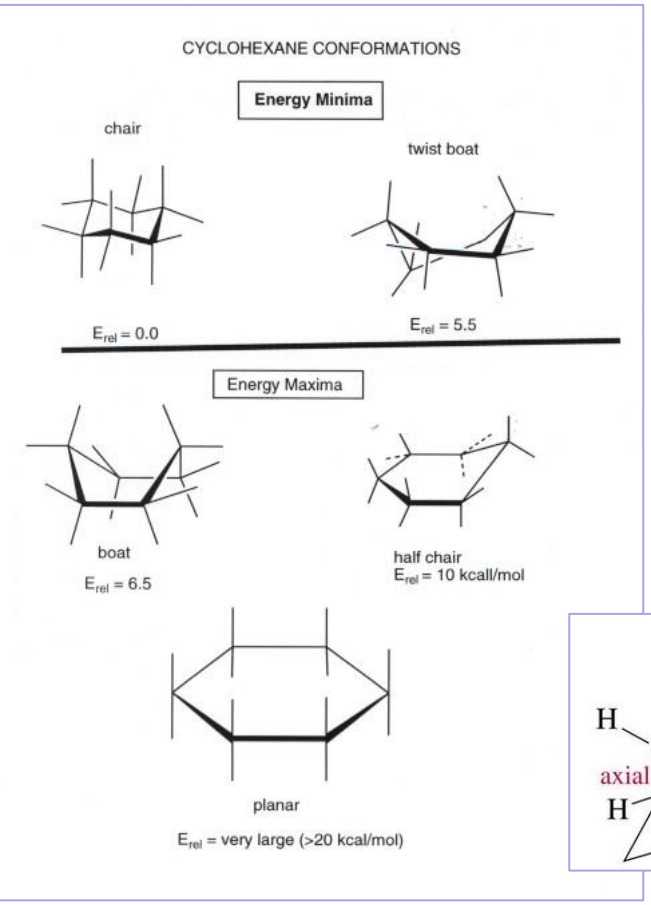
- **Isomers**

  - Same chemical structure

- **Stereoisomers / steric isomers**
  - Different spatial arrangement of atomes (Z/E, chair /boat, ax/eq)

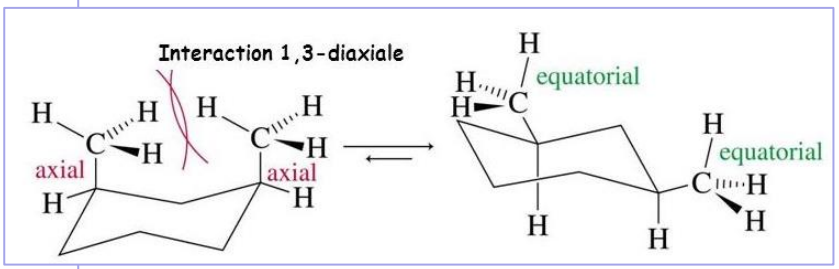


Alkene



Cyclic molecule

More stable



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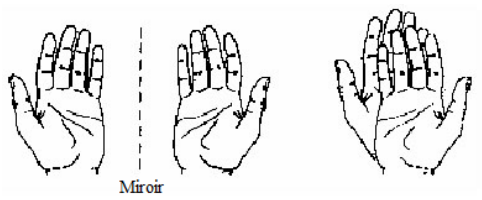
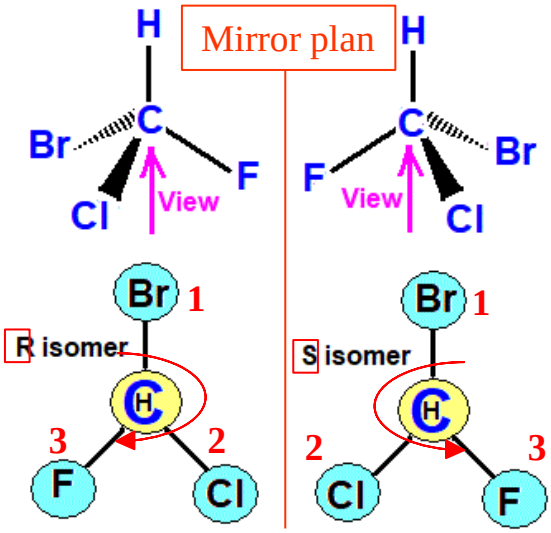
  - Different spatial arrangement of atomes (Z/E, seat /boat, ax/eq)
- **Enantiomers / optical isomers**
  - Not superimposed but a relation image / mirror





  - Identical physico-chemical properties (melting, boiling, solubility, density…)
  - BUT deviation of the polarisation plan of the light in opposite directions => **Optically active molecules**

1 asymmetric center (C, S, N, P, Si…) → Two 3D arrangements :
- optical rotation: dextro d (+) / levo l (-)
- convention: D / L
- absolute configuration : R / S

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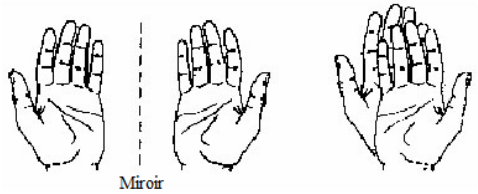

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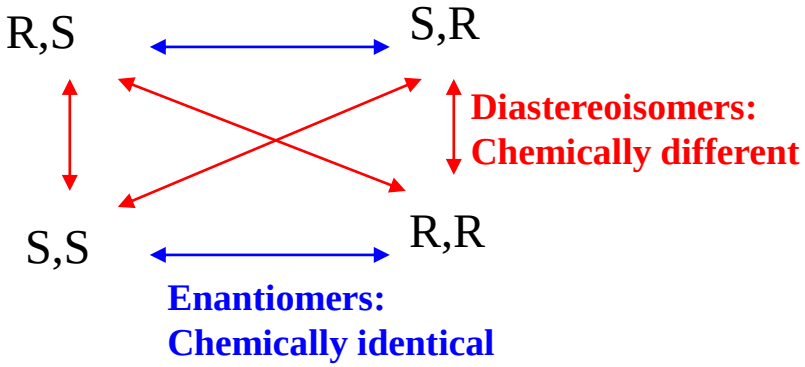
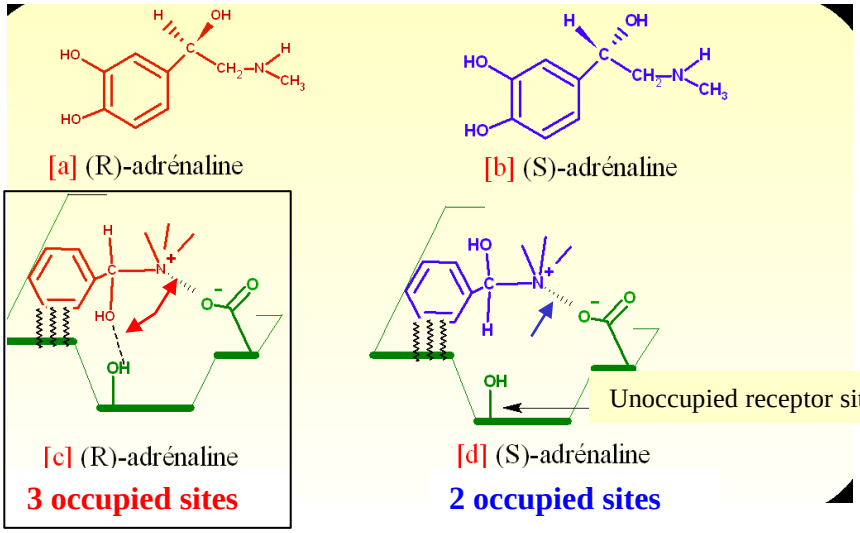
- optical rotation: dextro d (+) / levo l (-)
- convention: D / L
- absolute configuration : R / S

- **Diastereoisomers:**
  - At least 2 asymmetric centers → 4 combinations (2^n)
  - Different physico-chemical properties



Stereoisomerism and Drugs

Eutomer (E) : Enantiomer
- The highest pharmacological activity or
- The strongest affinity to a receptor /enzyme



Developpement / marketing: enantiopure / racemic??

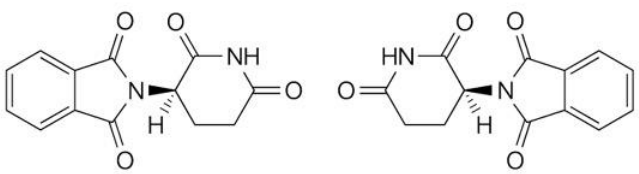
1. Identical activity of both enantiomers: Promethazine ➔ racemic
2. Identical activity but different efficiency : propranolol, adrenaline
3. 1 enantiomer presents one activity: ibuprofène
4. Different activities and perhaps toxic: barbiturate, thalidomide

Composés	Forme S	Forme R
Propranolol	b-blockers	100x less powerful
Adrenaline	less powerful	Vasoconstrictor
ibuprofen	Analgesic,	No activity
Barbiturate	Convulsant	Anesthetic

➔ Low production cost / increased drug doses ?

➔ High investments R&D, Prod, QC

Awareness of the chirality importance



***R*-(+)-Thalidomide**
sedative

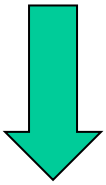
***S*-(–)-Thalidomide**
Teratogenic
(metabolite-OH of S isomer)

Severe fetal malformations of limbs

Separation techniques

Differentiate enantiomers

- **HPLC**
- ***GC***
- ***Supercritical Chromatography***
- **CE**



- **Purification monitoring (preparative)**
- **Quality control (analytical)**
  - **Purity**
  - **Assay**

•Only physico-chemical techniques :

- NMR

- Polarimetry

- Calorimetry

Enantiomeric excess:

$$\textbf{ee\%} = \frac{[R]-[S]}{[R]+[S]} \times 100$$

R major form, S minor form

Different approaches for chiral separations

Enantiomers → diastereoisomers

Molecule dependant

Indirect mode

Chiral derivatization agent

1. Pre-column formation of diastereoiomers **using a pure chiral reagent**
   → HPLC, GC, CE

Direct mode

Chiral selectors

2. Formation of **labile** diastereoisomers in the **mobile phase** (addition of a chiral selector)
   → HPLC, CE

3. Formation of **labile** diastereoisomers in a **chiral stationary phase**
   → HPLC, GC, *CE (not frequent)*

Approach 1.

Pre-column formation of diastereoiomers with a pure chiral reagent

- Universal strategy
- Not very suitable for preparative chromatography

•Derivation of all enantiomers

Strategies for derivation: Covalent bond

Derivatizing chiral agent **:** **PURE** enantiomer

(R)-AX

(S)-AX

(S)-BY

(R)-AX---(S)-BY

(S)-AX---(S)-BY

Racemate

2 enantiomers

2 Diastereoisomers

Separation of amino acides derivatized with FLEC

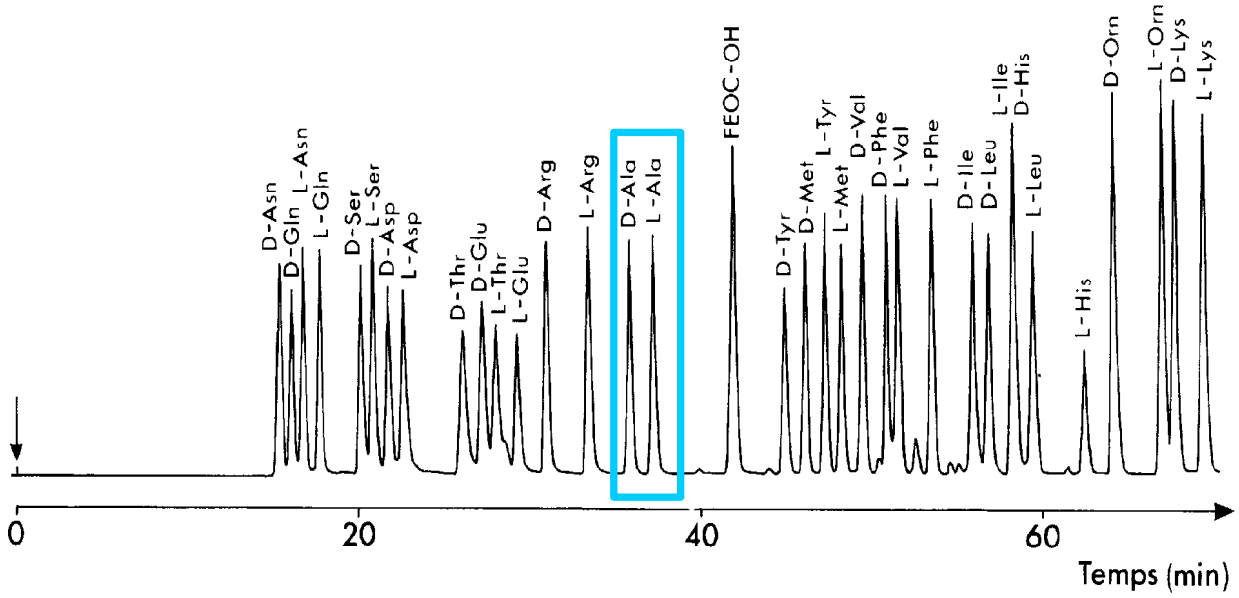
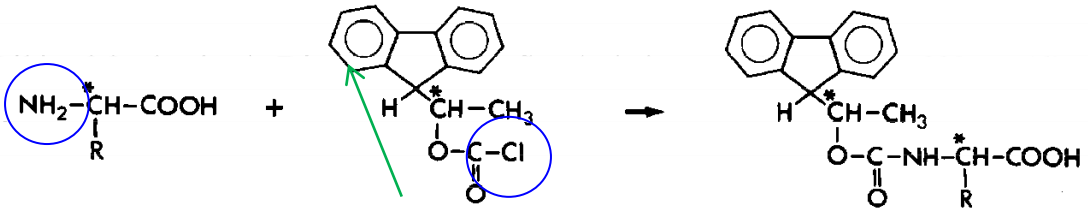


FIG. XVI.7. — Séparation d'un mélange étalon de 17 acides aminés racémates après réaction avec le (+)-(fluorényl-9)-1 éthylchloroformiate. Colonne : longueur : 15 cm ; diamètre intérieur : 4,6 mm. Phase stationnaire : silice greffée octyle Spherisorb 3 µm. Phase mobile : acétonitrile-tétrahydrofuranne-tampon acétate (*p*H = 4,35, 0,03 mol.l^{-1}).

FLEC reacts with amines I and II (stoechiometry 1:1)



hydrophobic

FLEC (+) fluorenyl-9)-1ethylchloroformiate = Good candidate:

- Stable
- Fast Reaction (4min)
- **Ratio of enantiomers not modified => quantification**
- Formed diastereoisomers : stable, fluorescent
- D always eluted before L (stereoselectivity of FLEC)

Selection criteria

- **Stationary phase**
  - Achiral (NP, RP, CEX)
  - Affinity for derivatized isomers (diastereoisomers)

- **Chiral reagent**
  - Optically pure
  - Fast reaction
  - Reaction without racemisation (the same % of enantiomers)
  - Stable derivatized isomers (no secondary product)
  - Adjust chromatography proprerties (nature of the chiral reagent)
  - Give properties (fluo, UV, ECD) favorable for detection

For information

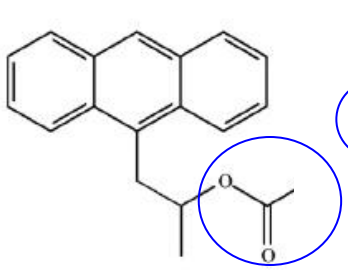
Derivatizing agents

Groups on analytes

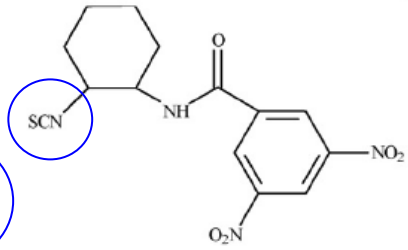
- **Amines**
  - Chloroformates
  - Isocyanates or isothiocyanates
  - Acids, chlorydrate acids
  - Anhydrides
  - Orthophtaldehydes +thiols

- **Alcohols**
  - Chloroformiates
  - Isocyanates
  - Acids, chlorydrate acids, chlorydrate
  - Anhydrides

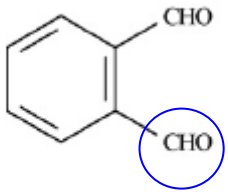
Reag



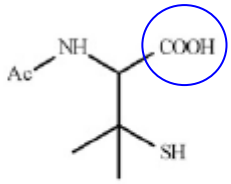
APOC



DDITC



OPA



NAP

- **Carboxyles**
  - Hydrazines
  - Diols
  - Hydroxylamine

or convert acids to amines or alcohols

Different approaches for chiral separations

Molecule dependant

Enantiomers → diastereoisomers

Indirect mode

Chiral derivatization agent

1. Pre-column formation of diastereoiomers **using a pure chiral reagent**
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Direct mode

Chiral selectors

2. Formation of labile diastereoisomers in the **mobile phase** (addition of a chiral selector)
   → HPLC, CE

3. Formation of labile diastereoisomers in a chiral **stationary phase**
   → HPLC, GC, *CE (not frequent)*

Approach 2.

Formation of labile diastereoisomers in the mobile phase (addition of a chiral reagent)

- Achiral stationary phase
- High quantities of chiral reagent
- Optical purity of the chiral reagent influences the selectivity (difficulty of the separation)
- Detection compatible with the chiral reagent (no optical properties…

It's the contrary of strategy 1 !!!
Indeed, if the chiral selector presents optical properties and as it is in the mobile phase, the baseline will be high and analytes won't be detected.

2.a) Formation of ternary complexes

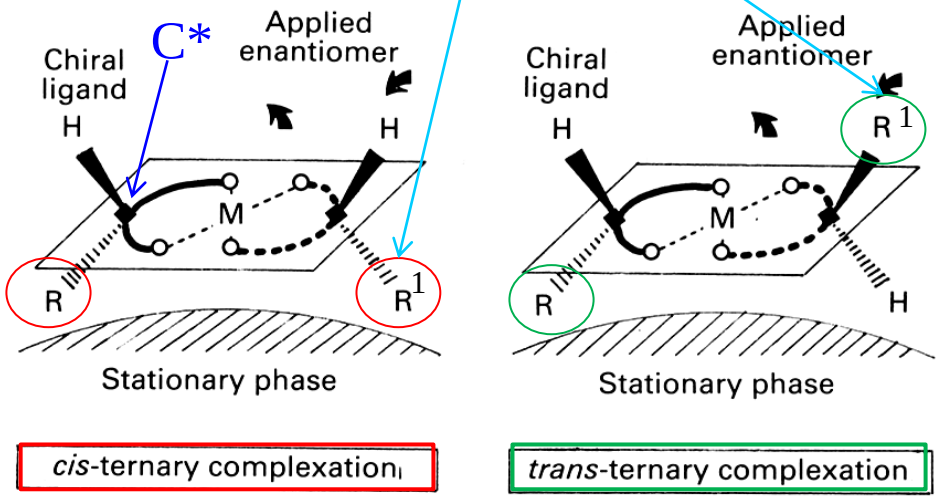
2.b) Enantio-separation with addition of inclusion selector

2.c) Enantio-separation using ion-pairing formation

Approche 2.a)
Formation of ternary complexes

Steric hindrance (nature of group R^1)
→ Inhibits the retention on the stationary phase
=> Plays on the selectivity

Coordination bond: stable



example:
-chiral ligand : C* + hydrophobic chain (group R)
-transition metal (Cu, Ni, Co, Fe, Zn, Cd) complexes the ligand

Formation of a ternary complexe
Chiral Selector – Metal – Enantiomer

Separations generally on alkylated stationary phases

Selection criteria of the chiral ligand

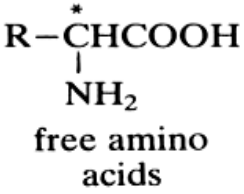
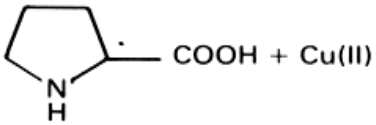
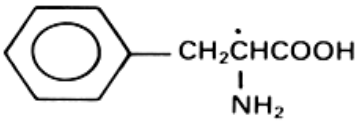
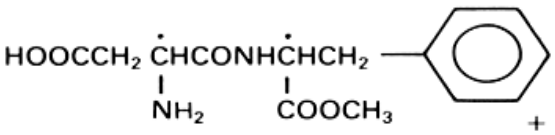
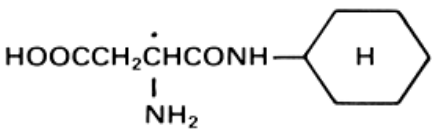
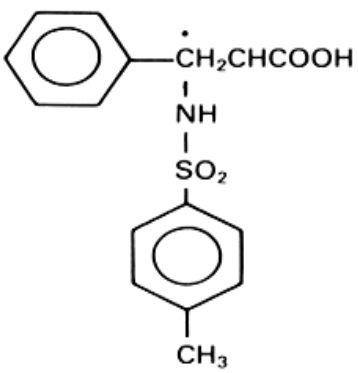
- At least 2 chelating functions at the proximity of C* (facilitate the recognition)
- One large group : steric repulsion (play on the selectivity)
- Retention on the stationary phase
- Optically pure

Parameters to be optimized:

- Concentration of the chiral additive
- Molar ratio metal / reagent
- pH of the mobile phase

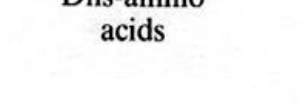
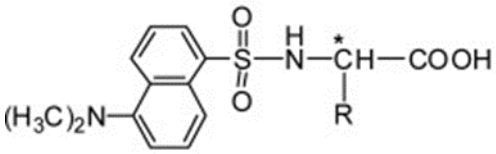
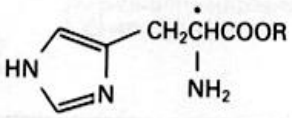
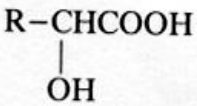
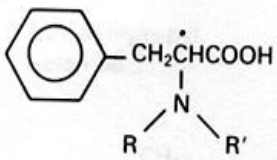
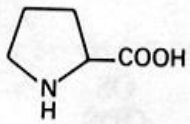
For information

Chiral additives

Applied enantiomers	Chiral additive	Column packing
 free amino acids	 + Cu(II) L- or D-proline	cation exchanger OS
	 + Cu(II) L-phenylalanine	ODS
	 + Cu(II) L-aspartyl-L-phenyl-alanine methyl ester	ODS
	 + Cu(II) L-aspartyl cyclohexyl amide	OS
	 + Cu(II) *N*-(p-toluenesulphonyl) L-phenylalanine	ODS

For information

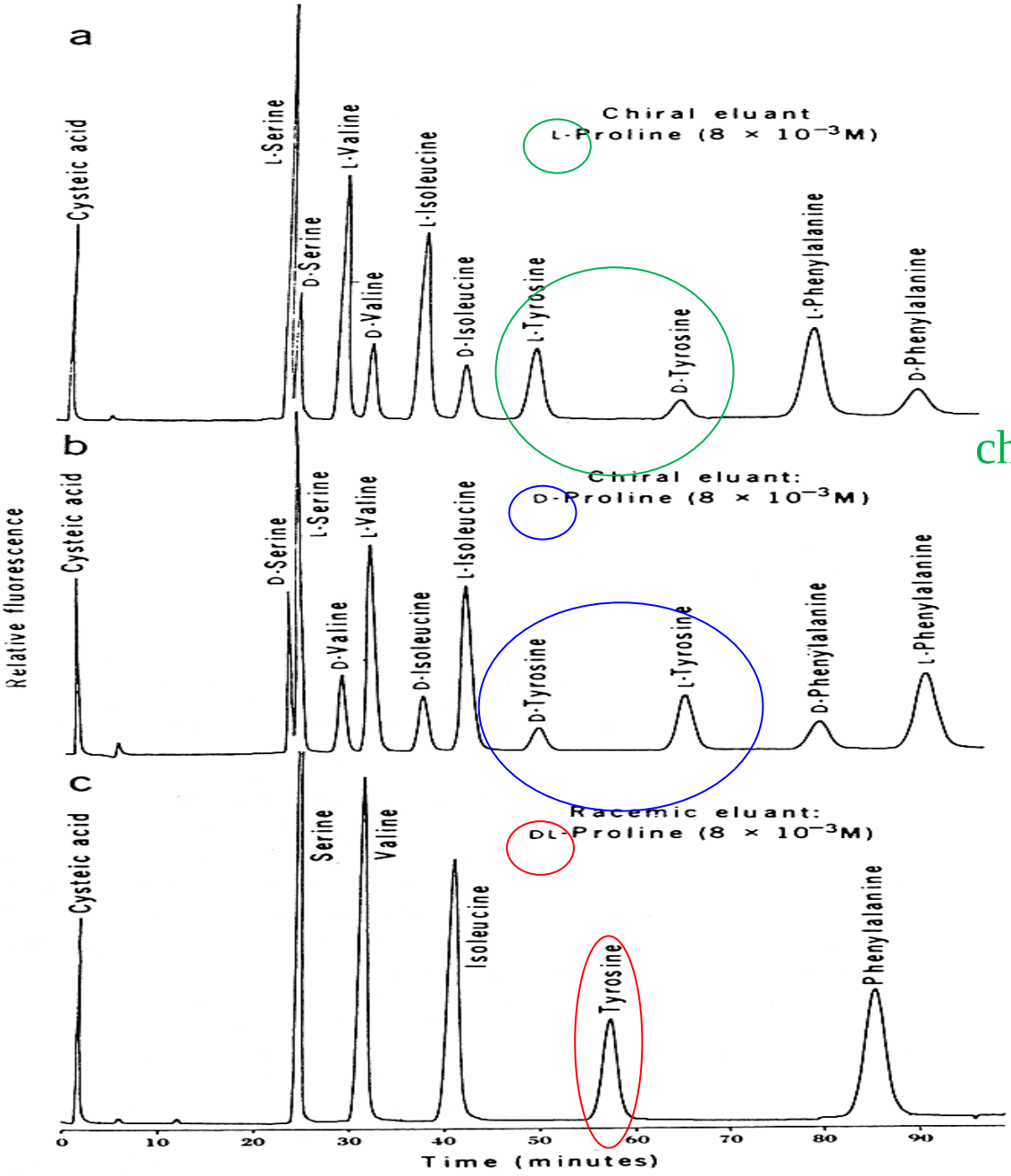
Chiral additives

Applied enantiomers	Chiral additive		Column packing
Dns-amino acids	 L-arginine	+ Cu(II)	OS
	 L-histidine (R=H)	+ Cu(II)	OS
	L-histidine methyl ester (R=CH_3)		ODS
 hydroxy acids	 L-phenylalanine (R=R′=H)	+ Cu(II)	ODS
	N-methyl-L-phenylalanine (R=CH_3, R=H) *N,N*-dimethyl-L-phenylalanine (R=R′=CH_3)		ODS
	 L-proline	+ Cu(II)	

Separations of amino acides derivatized with *l*-proline-Cu(II)

Stationary Phase : cation exchanger
Mobile Phase : chiral ligand L-proline + Cu II

Influence of the optical purity of the chiral eluent



Pure chiral selector

⬇

Enantio-separation

Racemic selector

⬇

No Enantio-separation

Approach 2.

Formation of labile diastereoisomers in the mobile phase (addition of a chiral reagent)

- Achiral stationary phase
- High quantities of chiral reagent
- Detection compatible with the chiral reagent (no optical properties…)
- Optical purity of the chiral reagent influences the selectivity (difficulty of the separation)

2.a) Formation of ternary complexes

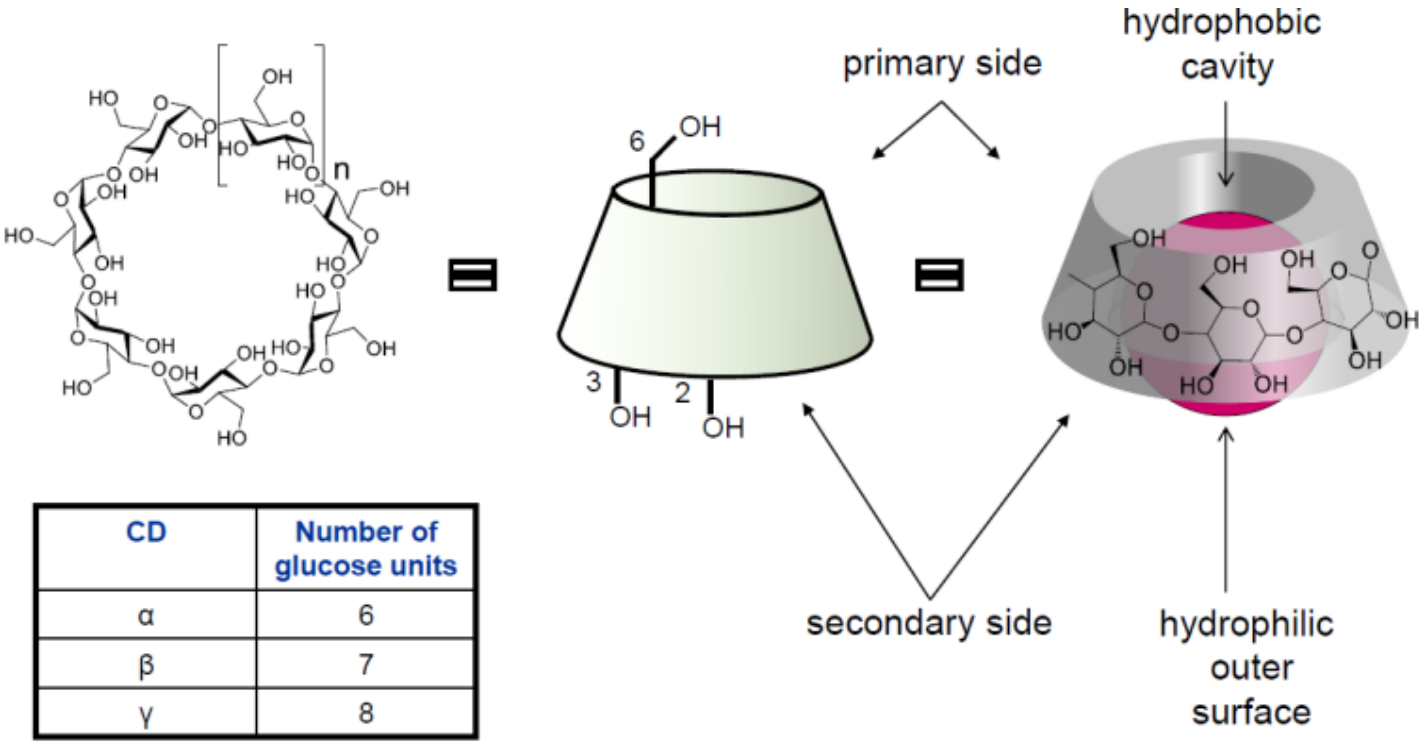
2.b) Enantio-separation with addition of inclusion selector

2.c) Enantio-separation using ion-pairing formation

Approche 2.b)

Enantio-separation with addition of inclusion selector

Cyclodextrins



CD	Number of glucose units
α	6
β	7
γ	8

- Aliphatic or aromatic no substitution → α
- Subtituted aromatic or naphtyl → β
- Subtituted naphtyl → γ

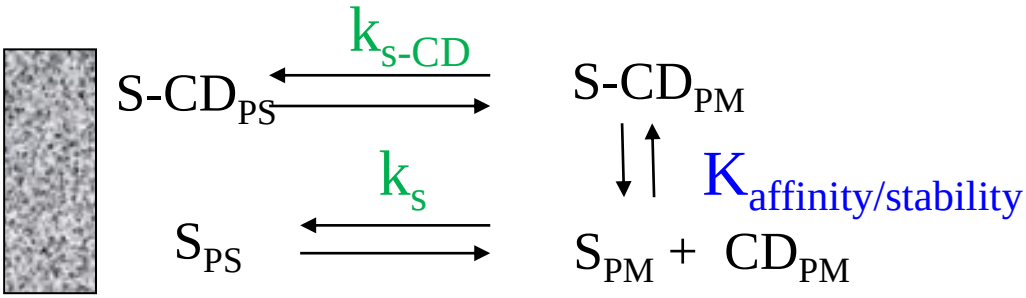
Chiral recognition mechanism:

- **inclusion** of hydrophobic group of analyte into the CD cavity → **selectivity**
- Formation of **H bonds** : polar groups neighboring the C* of analyte & OH on the CD mouth → **stability**

Approche 2.b)
Enantio-separation with addition of inclusion selector

Case of cyclodextrins:

PS: stationnary phase; PM: mobile phase



Enantio-separation based on:

-Difference in affinity (K) of enantiomer 1 or 2 for the CD

→ **selectivity**

-Difference in retention of complexes ($k_{S\text{-}CD}$) or free enatiomer (k_S) on the stationnary phase

Retention:

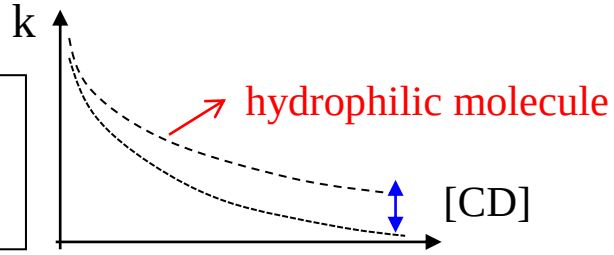
- Free-CD adsorption on the stationnary phase minimized
- Retention mechanism depends on the alkylation degree of the CD

TMCD > MCD > CD

tri-O-methyl O-methyl

- Apparent retention factor k

$$k = \frac{k_s - k}{K_s \cdot [CD]} + k_{S-CD}$$



Approach 2.

Formation of labile diastereoisomers in the mobile phase (addition of a chiral reagent)

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2.c) Enantio-separation using ion-pairing formation

Approche 2.c)
Enantio-separation using ion-pairing formation

Chiral counter-ion B^- + racemate

→ **2 ion-pairing diastereoisomers**

which interact differently with the stationnary phase

$$(+)B^- + (+/-)HA^+ \rightarrow (+)B^-\text{--}(+)HA^+$$
$$(+)B^-\text{--}(-)HA^+$$

Selectivity depends on :

*Nature, purity of the enantiomer (ion pairing agent)

*High concentration of the counter-ion ➔ favors ion-pairing

*Mobile phase composition

- high ionic strength, low dielectric constant : low dissociating effect on ion pair

- pH : favors ionisation ➔ favors ion-pairing

*Nature of the stationary phase …**retention of diastereoisomers but not/less the free enantiomer**

examples of counter-ions:

Camphor sulfonic acid

Quinine and analogs

Derivatized proline (benzoxycarbonyl group)

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1. Pre-column formation of diastereoiomers with a pure chiral reagent

→ HPLC, GC, CE

2. Formation of labile diastereoisomers in the mobile phase (addition of a chiral reagent)

→ HPLC, CE

3. Formation of labile diastereoisomers in a chiral stationary phase

→ HPLC, GC, CE (not frequent)

- **Normal mobile phase**
- **Different classes of chiral sationnary phase**

a. Donor-acceptor Type (Pirkle) TYPE IA
b. Ligand exchange Columns TYPE IB
c. Chiral phases containing cavity TYPE II
d. Polymer phases TYPE III
e. Proteins TYPE IV
f. New classes (MIP, glycopeptides, nucleic acids)

Approche 3. Different types of chiral stationnary phase in HPLC

•1. Donor-acceptor Type (Pirkle) TYPE IA

•2. Ligand exchange columns TYPE IB

Chiral selector linked to the silica surface

* proline treated with copper salts –> Complex formation with variable stability depending on the enantiomer

•3. Cavity-containing chiral phases TYPE II

* Cyclodextrins or crown ethers

•4. Polymer phases TYPE III

* Polymers are impregnated on silica or grafted onto stationary phase

* The chirality of polymers comes from their helicity (creates chiral cavities) or from the presence of asymmetry centers (optically active monomer).

•5. Proteins TYPE IV

Protein grafted onto silica surface → arge number of interaction sites

Complexation mechanism: protein structure varies according to mobile phase composition

•6. New classes

* Molecular Imprinted Polymer
* Glycopeptides
* Nucleic acids

The most frequently used

Approach 3 a.
Pirkle stationnary phase

- Selectors : covalently linked / ionic interactions to the stationnary phase support.
- At least 3 possible anchorage sites for interaction with the analyte :

- Hydrogen bond
- dipole-dipole interactions
- ionic interactions, dative bonds
- π-π interactions (charge transfert)

Dagliesh law:

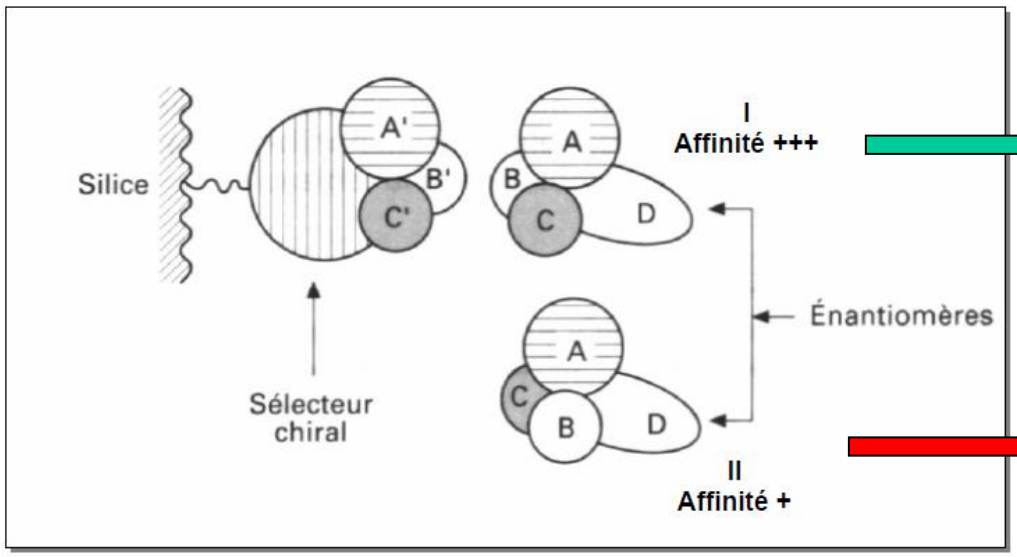
- 2 enantiomers are separated if at least 3 simultaneous interactions take place with one of the 2 enantiomers
- One of the 3 interactions must be stereoselective
- The interaction energies must be in the same magnitude

$E_{electrostat} > 10 \times E_{H\ bond}$

Does not consider the conformational states of the stationary phase or of the analyte

Chiral recognition:

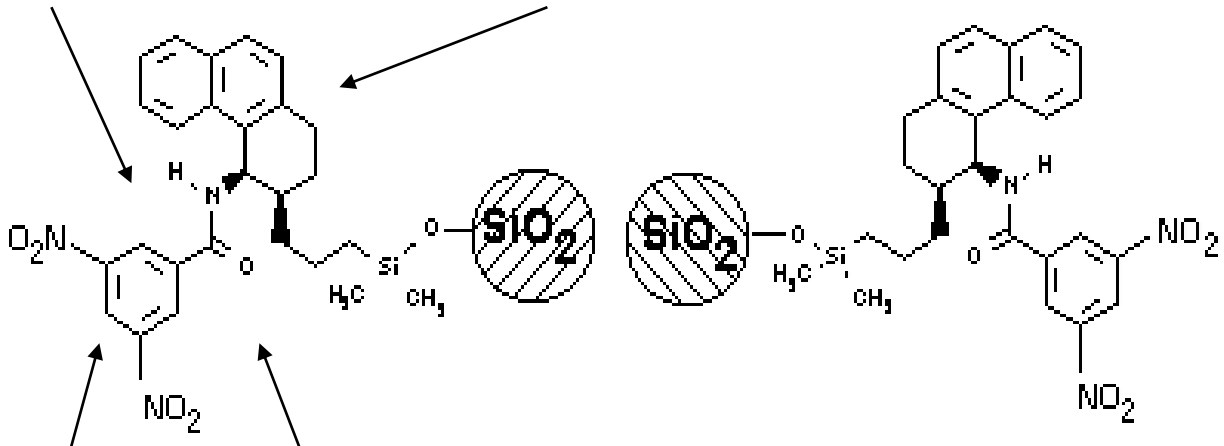
- No loss of one of the interactions with the less retained enantiomer
- only a decrease in overall energy



Les phases stationnaires de type Pirkle

Acidic site

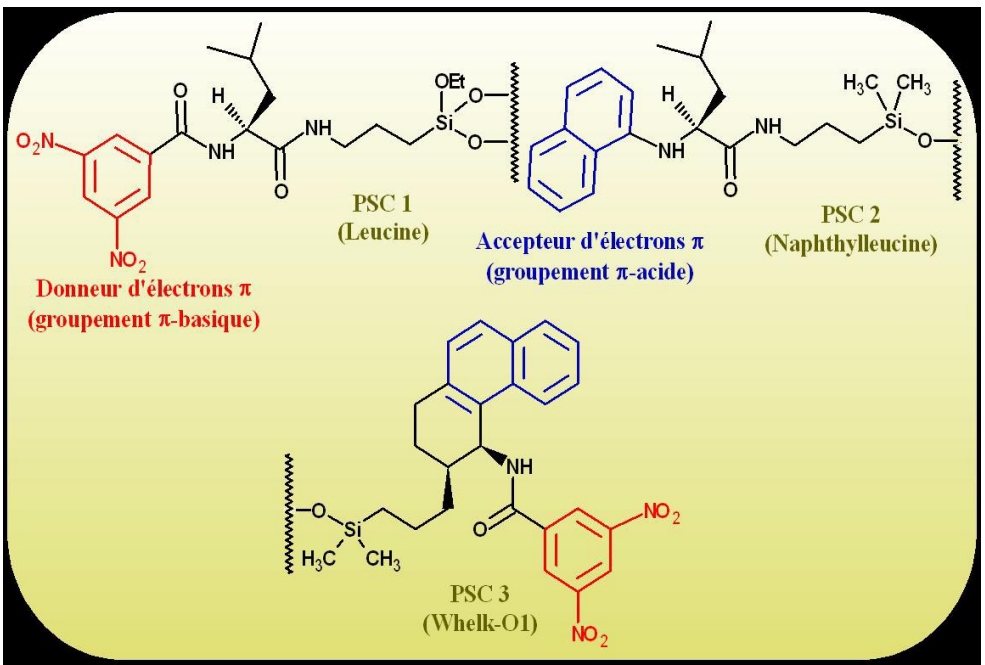
Steric interraction site



Basic site

Π Accepteur site

noyau aromatique ou donneur d ’électrons Π



Chiral capillary electrophoresis

Different approaches :

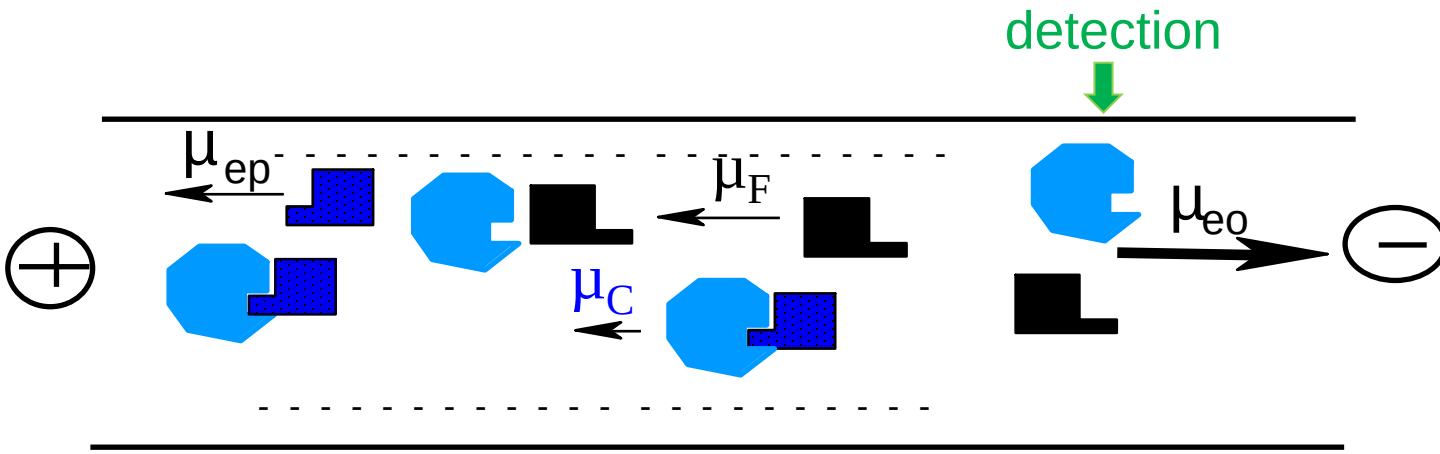
1. Pre-capillary formation of diastereoisomers (not frequent)

2. **Formation of labile diastereoisomers in the electrolyte**
   - **Selectors with cavity**
   - Ligand exchange
   - Others: polymers, proteins, chiral surfactants

3. Chiral stationary phase (not frequent)

Avantages of CE compared to LC:

- Small sample volumes
- Small selector quantity
- Faster new method development / optimisation
- Simpler and low cost development



Chiral selector

Enantiomers

μ_{ep}: electrophoretic mobility

μ_{eo}: electroosmotic mobility

μ_F: mobility of the free enantiomer

μ_c: mobility of the complex enantiomer–chiral selector

K: affinity constant enantiomer / chiral selector

$$\mu_1 = \frac{\mu_F + K_1 \times \mu_{C1} \times [CD]}{1 + K_1 \times [CD]}$$

Separation of enantiomers 1 and 2:

$$\Delta\mu = \mu_2 - \mu_1 = \frac{\mu_F + K_2 \times \mu_{C2} \times [CD]}{1 + K_2 \times [CD]} - \frac{\mu_F + K_1 \times \mu_{C1} \times [CD]}{1 + K_1 \times [CD]}$$

$\Delta\mu \neq 0$ if $\mu_F \neq \mu_C$

$K_1 \neq K_2$

$\mu_{C1} \neq \mu_{C2}$

Selection criteria of the chiral selector

- Optically pur
- Soluble in electrolytes
- Charged or neutral (depends on the analyte)
- $\mu_c \neq \mu_F$
- Kc (+) ≠ Kc (-)

Constraints:

- Low conductivity
- Properties : low absorbance or low fluorescence

Chankvetadze et al,, Anal Chem 2004

Chiral selectors employed in CE

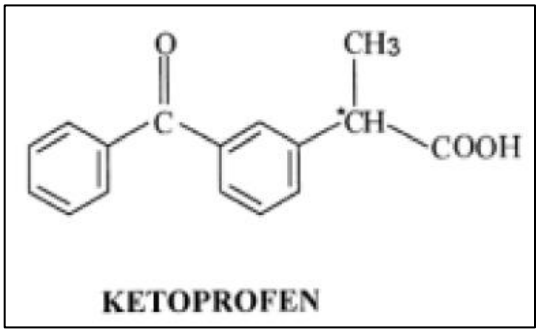
In capillary zone electrophoresis

In micellar electrokinetic chromatography

Types	**Names**
Cyclodextrins	native, neutral, anionic or cationic substituted
Antibiotics	Clarithromycin, Norvancomycin, Rifampicin, Clindamycinphosphate
Polysaccharides	Linéaires, branchés, cycliques (Cyclofurans)
Surfactants	•2,3-O-dibenzyl-6-Osulfobutyl-βCD •Sodium cholate •Polysodium N-undecenoylL,L,leucyl-alaninate
Ionic liquids	•TMA-LA
Oligonucleotides	•hpDNA_31

Stavrou, Electrophoresis , 2017

Influence of the CD size



KETOPROFEN

- Aliphatic or aromatic no substitution → α
- Subtituted aromatic or naphtyl → β
- Subtituted naphtyl → γ

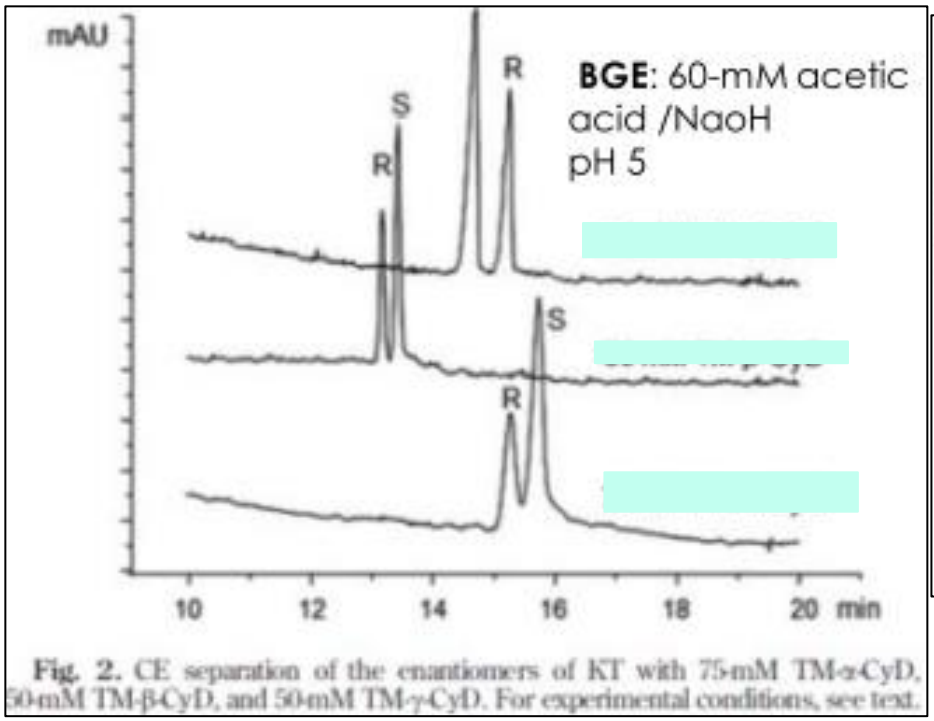
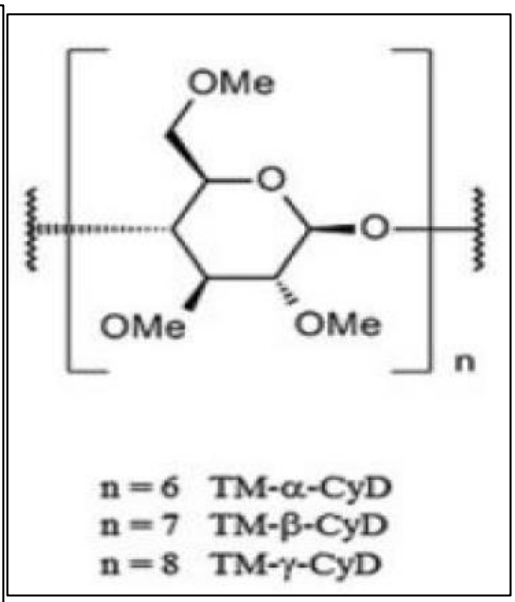
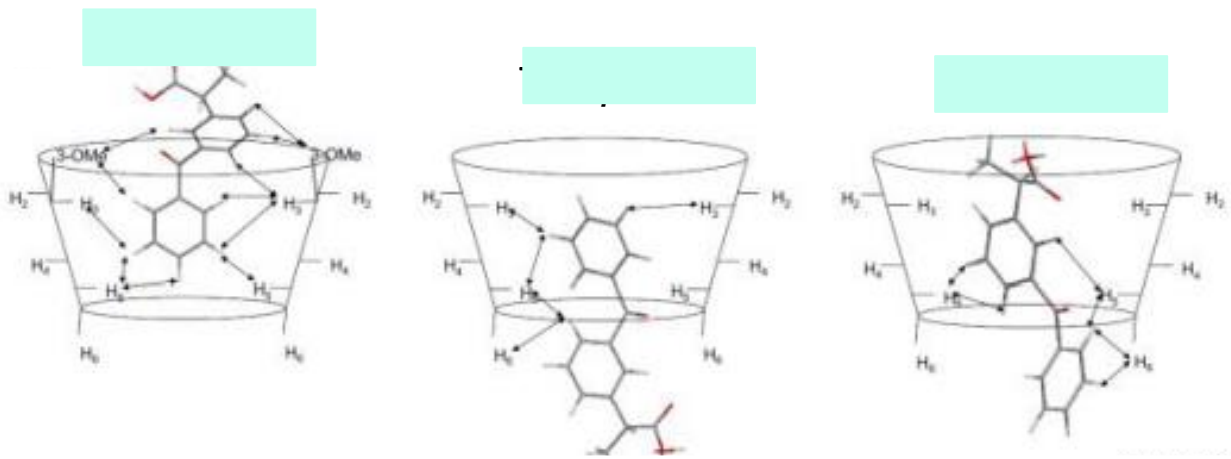


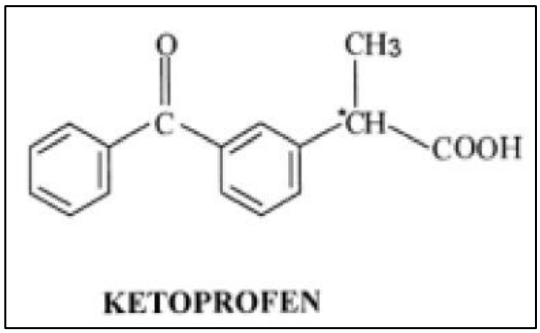
Fig. 2. CE separation of the enantiomers of KT with 75-mM TM-α-CyD, 50-mM TM-β-CyD, and 50-mM TM-γ-CyD. For experimental conditions, see text.





Samakashvili, Chiralty (2013) 25, 79–88

Influence of the CD size



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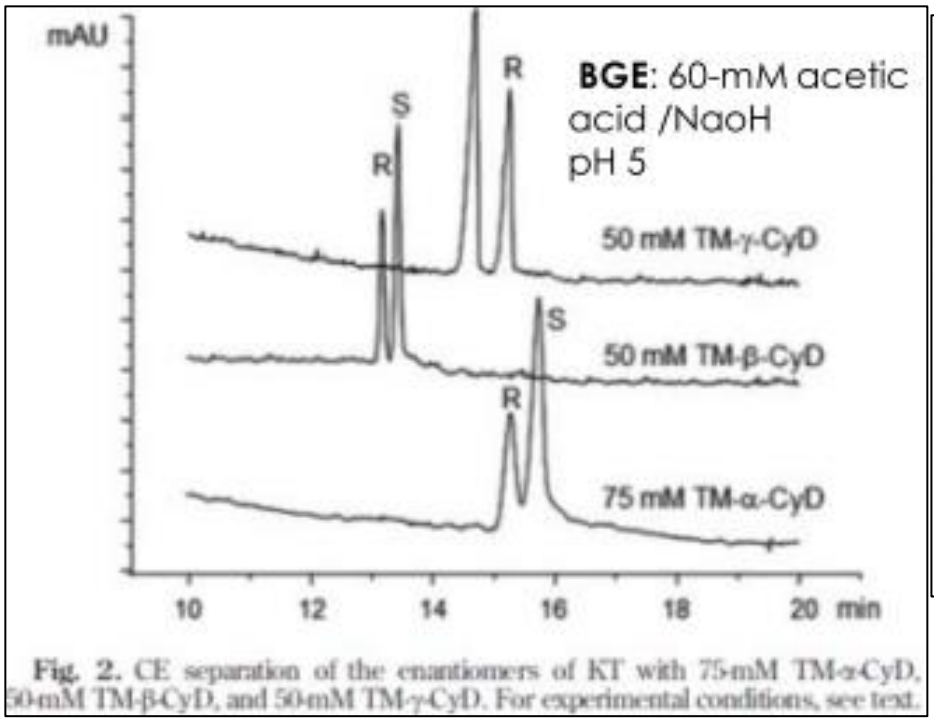
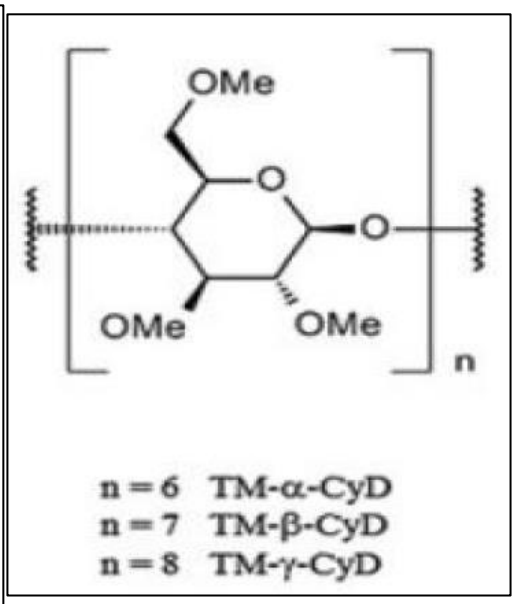
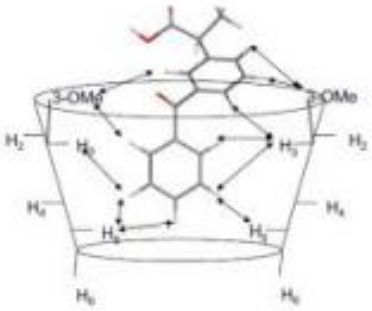


Fig. 2. CE separation of the enantiomers of KT with 75-mM TM-α-CyD, 50-mM TM-β-CyD, and 50-mM TM-γ-CyD. For experimental conditions, see text.

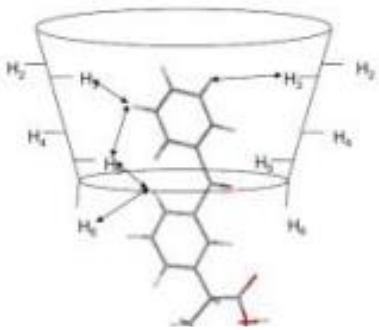


stabilization

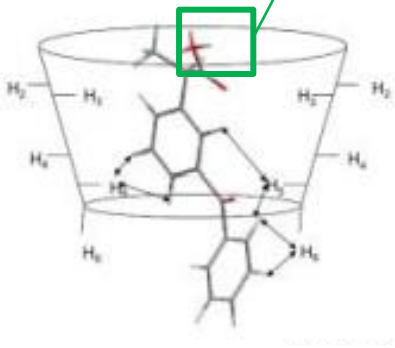
TM-α-CD



TM-β-CD



TM-γ-CD



Samakashvili, Chiralty (2013) 25, 79–88

Importance of the CD nature

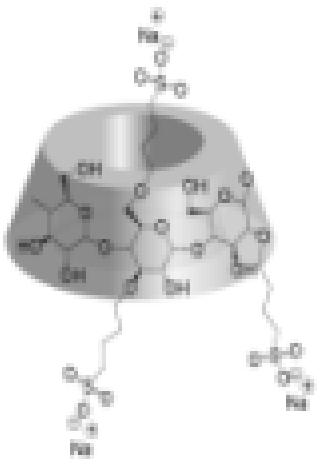
Neutral CDs:
- Size
- Nature and position substituent
- Mode (random, single) and degree of substitution

→ Charged analytes

Charged CDs:
- Nature of the ionized group
- Substitution degree
- Homogeneity of the substitution degree

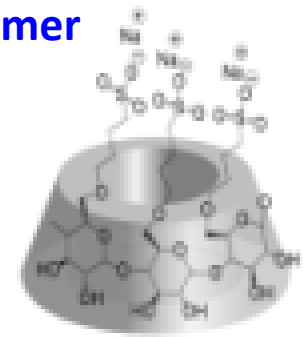
Neg → neutral + basic analytes
Pos → neutral + acidic analytes

Randomly substituted

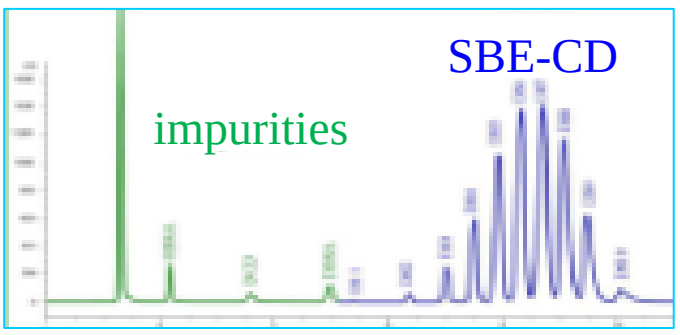


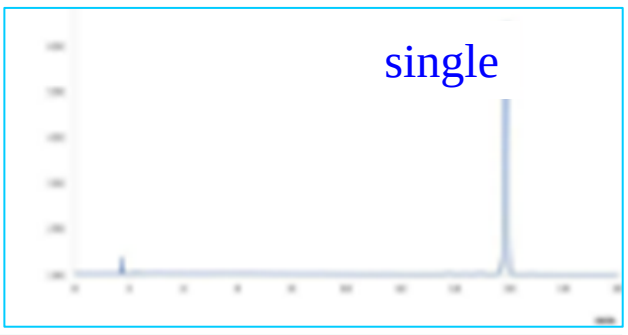
Sulfobutylated β-CD (DS=6.4)
(Dexolve™, Captisol™)

Single isomer



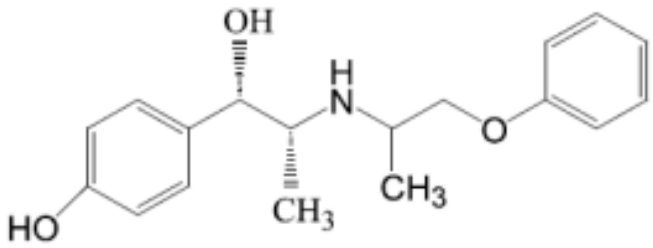
Heptakis-(6-*O*-sulfobutyl)-β-CD (DS=7.0)
6-(SB)$_7$-β-CD





Purtiy profile of substituted CDs by HPLC IEC, gradient, ELS detection

Enantio-separation of isoxsuprine



vasodilator

Di-O-Me CD

50 mg/mL

Tri-O-Me CD

70 mg/mL

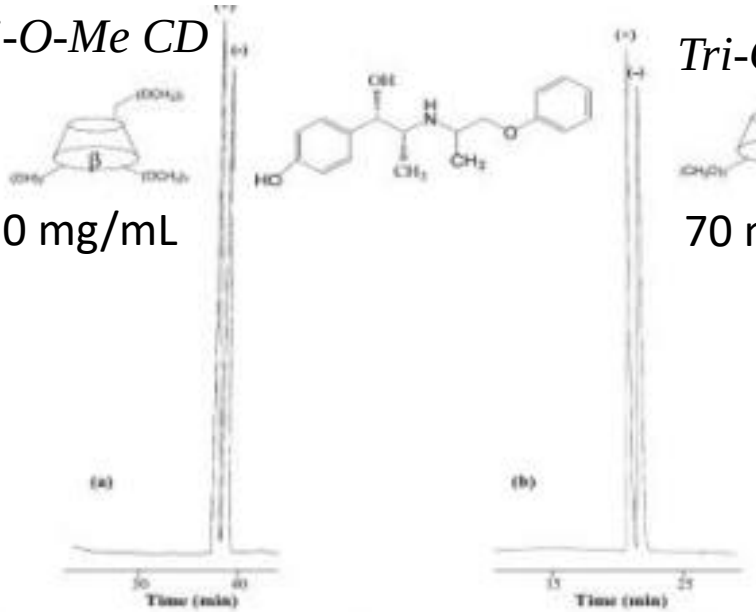
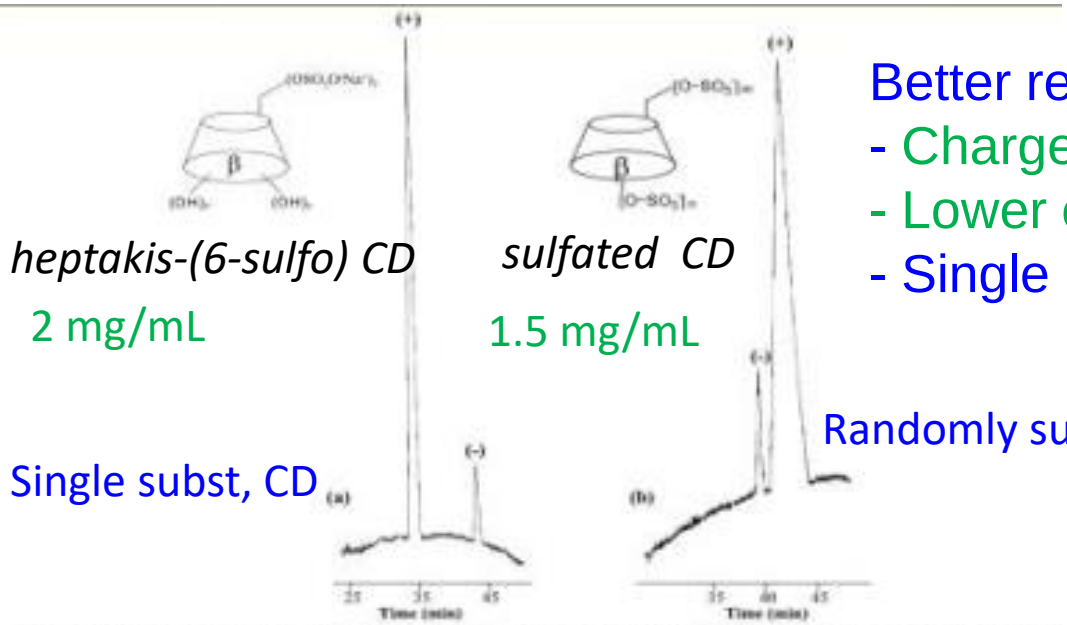


Fig. 5. CE enantioseparation of (±)-ISP using 50 mg/ml DM-β-CD (a) and 70 mg/ml TM-β-CD (b). Other conditions were as in the experiment shown in Fig. 4.

No difference in separation between both neutral alkylated CDs



heptakis-(6-sulfo) CD

2 mg/mL

sulfated CD

1.5 mg/mL

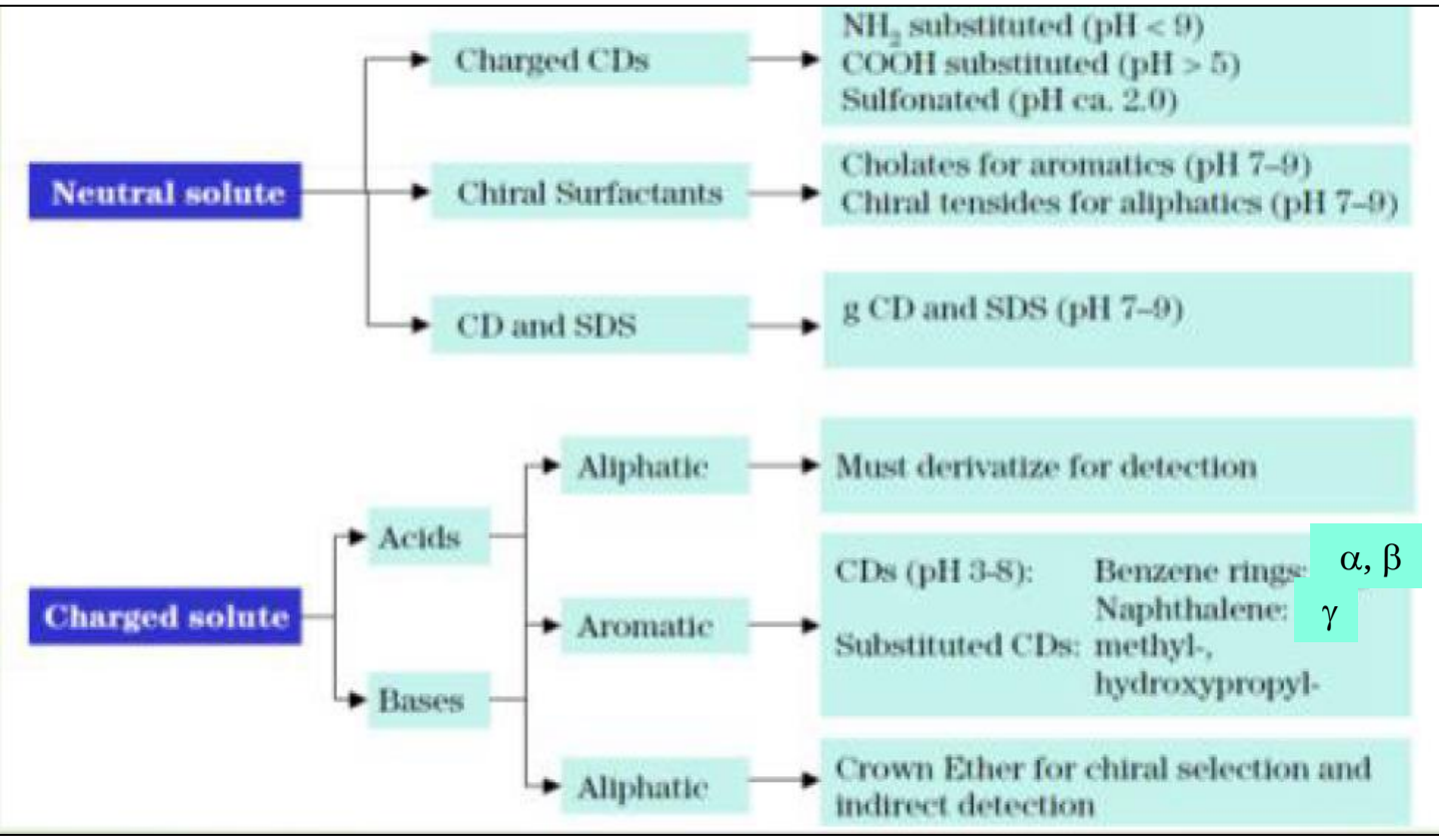
Single subst, CD

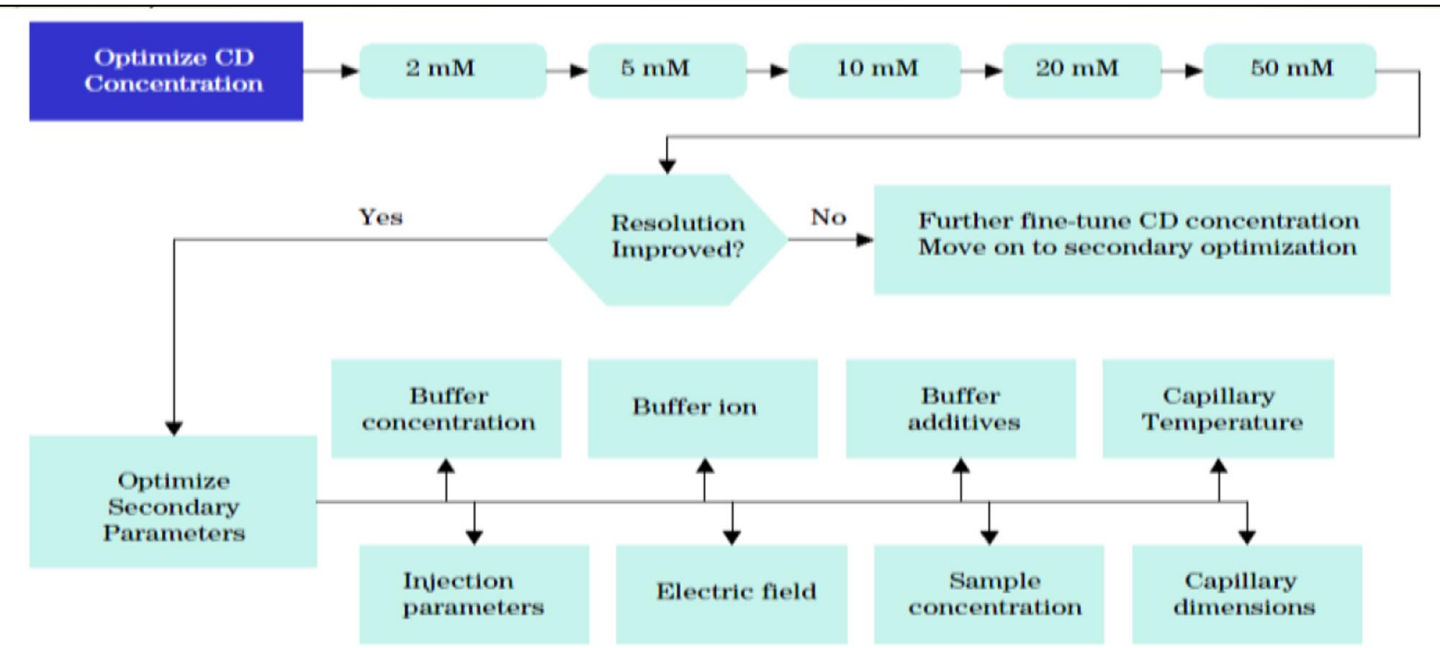
Randomly subst. CD

Fig. 6. CE enantioseparation of (±)-ISP spiked with (+)-ISP using 2 mg/ml HS-β-CD (a) and 1.5 mg/ml SU-β-CD (b). Other conditions were as in the experiment shown in Fig. 4.

Better resolution with:
- Charged substitution
- Lower concentration
- Single CD isomer

Strategy for the development of a chiral CE method





General strategy for the development of a chiral separation method

- **Choice of the technique**
  - Quantification / separation?
  - Analytical / preparative?

  } It depends on your goal

  - Analyte concentration
  - Nature of the analytes

  } The method is analyte dependent

  - Cost, time
  - Bibliography

- **Criteria linked to the analytes**
  - Solubility
  - Volatility…
  - Easy to derivatize ….
  - Detectability …. Ex: sugars
  - Chromatographic / electrophoretic behaviour

No unique solution!