UNIVERSITY OF VALENCIA
FACULTY OF CHEMISTRY
Department of analytical chemistry

CHARLES UNIVERSITY
FACULTY OF PHARMACY
Department of analytical chemistry

DIPLOMA THESIS

Chiral pesticides separation using cyclodextrins by capillary electrophoresis

Barbora Denková

Supervisor: Dr. Salvador Sagrado Vives

Valencia 2009

UNIVERSITA VE VALENCII
FAKULTA CHEMIE
Katedra analytické chemie

UNIVERSITA KARLOVA
FARMACEUTICKÁ FAKULTA
Katedra analytické chemie

DIPLOMOVÁ PRÁCE

Separace chirálních pesticidů pomocí cyklodextrinů kapilární elektroforézou

Barbora Denková

Školitel: Dr. Salvador Sagrado Vives

Valencie 2009

Hereby I affirm in lieu of an oath, that I made the present thesis autonomously and without other than the indicated auxiliary means. The data used indirectly or from other sources, and concepts are characterized with lists of sources.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

……..

ACKNOWLEDGEMENT

I would like to acknowledge Maria José Medina-Hernández, Salvador Sagrado-Vives, Laura Escuder-Gilabert, Yolanda Martin-Biosca and all colleagues from the department of analytical chemistry at the University of Valencia for their professional help, valuable advices, patience and assistance with my work, many thanks for providing me with pleasant conditions during working on this thesis.

I would like to express my thanks to Mgr. Pavel Jáč PhD. for his assistance during completing my thesis.

My thanks are also expressed to LLP/Erasmus project for the financial support.

ABREVIATIONS

ACE	Affinity Capillary Electrophoresis
ACN	Acetonitrile
AM-β-CD	Aminomethyl-β-cyclodextrin
2-AHP-β-CD	2-O-acetonyl-2-O-hydroxypropyl-β-cyclodextrin
BGE	Background Eletrolyte
Bt	Bacillus thuringiensis
CDs	Cyclodextrins
CE	Capillary Electrophoresis
CE-MS	Capillary Electrophoresis-Mass Spectrometry
CEC	Capillary Electrochromatography
CGE	Capillary Gel Electrophoresis
CIEF	Capillary Isoelectric Focusing
CM-β-CD	Carboxymethylated-β-cyclodextrin
CZE	Capillary Zone Electrophoresis
CZE-UV	Capillary Zone Electrophoresis-Ultraviolet
DAD	Diode Array Detection
DDT	Dichlorodiphenyltrichloroethane
DM-β-CD	Dodecylmethyl-β-cyclodextrin
DNA	Deoxyribonucleic Acid
DS	Degree of Substitution
EKC	Electrokinetic Chromatography
EOF	Electroosmotic Flow
ESA	Ethane Sulfonic Acid
ESI-MS	Electrospray Ionization-Mass Spectrometry
FTPFACE	Flow-through Partial Filling Affinity Capillary Electrophoresis
HP-β-CD	Hydroxypropyl-β-cyclodextrin
HPLC	High Performance Liquid Chromatography
ITP	Isotachophoresis

LC	Liquid Chromatography
MEKC	Micellar Electrokinetic Chromatography
MeOH	Methanol
MES	2-(N-Morpholino)-ethane-sulfonic acid
MS	Mass Spectrometry
NACE	Non-aqueous Capillary Electrophoresis
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OPs	Organophosphorus Pesticides
OT-CEC	Open-tubular Capillary Electrochromatography
OXA	Oxalinic Acid
P-CEC	Packed-Capillary Electrochromatography
cis-PA	cis-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylic acid
PFACE	Partial Filling Affinity Capillary Electrophoresis
PFCC	Partial Filling Counter Current mode
PFT	Partial Filling Technique
pI	Isoelectric Point
PIPs	Plant-Incorporated Protectants
PME	1-phenyl-2-(p-methoxyphenyl)ethylamine
PPA	2- phenoxypropionic acid
PPAHs	Phenoxy Propionic Acid Herbicides
PTE	1-phenyl-2-(p-tolyl)ethylamine
SBE-β-CD	Sulfobutylether-β-cyclodextrin
SDS	Sodium Dodecyl Sulphate
THCMH	cysteamine-bridged hemispherodextrin
TM-β-CD	Trimethyl-β-cyclodextrin
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet

CONTENTS

1. INTRODUCTION

1.1 ELECTROPHORESIS

1.1.1 General aspects of electrophoresis

The process of electrophoresis is a movement of electrically charged particles or molecules in a conductive liquid medium under the influence of an electric field. In practical terms, a positive (anode) and negative (cathode) electrode are placed in a solution containing background electrolyte (BGE). When a voltage is applied between the electrodes, across the capillary, solute ions of different charge, *i.e.*, anions (negative) and cations (positive), will move through the solution towards the electrode of opposite charge. Capillary electrophoresis, then, is the technique of performing electrophoresis in buffer-filled, narrow-bore capillaries, normally from 25 to 100 µm in internal diameter [1].

1.1.2 Capillary electrophoresis system

The instrumentation required for CE is remarkably simple in design, as Fig. 1 illustrates.

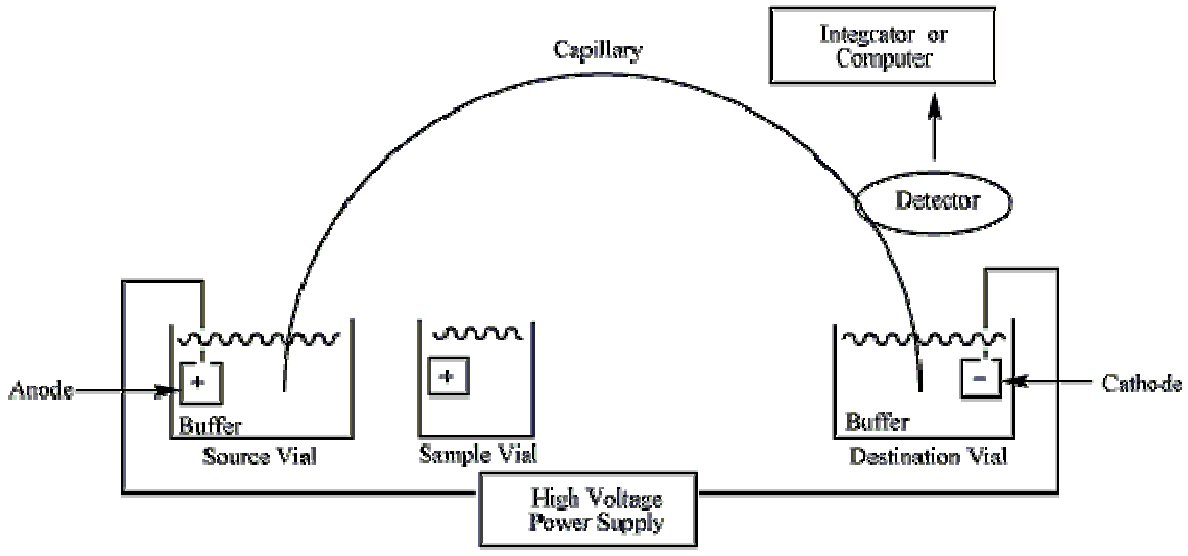


Fig. 1. A schematic representation of the arrangement of the main components of a typical CE instrument [2].

The ends of a capillary are placed in separate buffer reservoirs, each containing an electrode connected to a high-voltage power supply. The sample is injected onto the capillary by temporarily replacing one of the buffer reservoirs (normally at the anode) with a sample

reservoir and applying either an electric potential or external pressure for a few seconds. Afterwards, an electric potential is applied across the capillary and the separation is performed. Detection of separated analytes is achieved directly on the capillary [1].

Basic features of a CE instrument include an autosampler, a detection module, a high-voltage power supply, the capillary and a computer [1].

Sample injection

One of the main advantages of CE is its ability to inject extremely small volumes of sample. There are two commonly used injection modes for CE: hydrodynamic and electrokinetic. Hydrodynamic injection is accomplished by the application of a pressure difference between the two ends of a capillary. Electrokinetic injection is performed by simply turning on the voltage for a certain period of time [3].

Capillary column

The capillary column is a key element of the CE separation. Fused silica is by far the most frequently used material, although columns have been made from teflon and borosilicate glass. The widespread use of fused silica is due to its intrinsic properties, which include transparency over a wide range of the electromagnetic spectrum and a high thermal conductance. Fused silica is also easy to manufacture into capillaries with diameters of a few micrometers. Neutral or hydrophilic substituents can be covalently attached to the inner wall of the capillary in order to reduce electroosmotic flow and prevent adsorption of the analyte [3].

An uncoated fused silica capillary is prepared for its first use in electrophoresis by rinsing it with 10 to 15 column volumes of 0.1M NaOH followed by 10 to 15 column volumes of water and 5 to 10 column volumes of the separation buffer. For a coated capillary, the preparation procedure is the same except that 0.1M NaOH is replaced with methanol. In commercial instruments, the carrier fluid is forced through the capillary by either applying pressure to the inlet reservoir or reducing pressure at the outlet reservoir [3].

Detectors

With some modifications, most HPLC detection modes can be applied to CE, including: UV/VIS absorbance, fluorescence, laser-induced fluorescence, mass spectrometry, conductivity and amperometry. The most widely used are UV/VIS absorbance detectors. Sometimes, more detectors can be connected in series [3].

Table 1 contains some examples of detection methods with their respective limits of detection.

Table1: CE detection modes and limits of detection [3]:

Methods	Concentration detection limit (mol/l)	Mass detection limit (mol)
UV/VIS	10^{-5}-10^{-8}	10^{-13}-10^{-15}
Fluorescence	10^{-7}-10^{-9}	10^{-11}-10^{-15}
Laser induced fluorescence	10^{-14}-10^{-16}	10^{-18}-10^{-20}
Mass spectrometry	10^{-8}-10^{-9}	10^{-16}-10^{-17}
Amperometry	10^{-10}-10^{-11}	10^{-18}-10^{-19}

1.1.3 Basic principles of electrophoretic separation

1.1.3.1 Electrophoretic mobility [3]

As mentioned earlier, electrophoresis is the movement or migration of ions or solutes under the influence of an electric field. Separation is based on differences in solute velocity. The velocity of an ion can be given by the following equation:

$$\upsilon = \mu_e E \qquad \text{(eq.1)}$$

where υ is ion migration velocity (m s^{-1}), μ_e is electrophoretic mobility (m^2 V^{-1} s^{-1}) and E is electric field intensity (Vm^{-1}).

The electric field intensity is a function of the applied voltage divided by the total capillary length.

Electrophoretic mobility is a factor that indicates how fast a given ion or solute may move through a given medium (such as a buffer solution). It is an expression of the balance of forces acting on each individual ion; the electrical force acts in favor of motion and the frictional force acts against motion. Since these forces are in a steady state during electrophoresis, electrophoretic mobility is a constant (for a given ion under a given set of conditions).

The equation that describes the mobility in terms of physical parameters of solute is:

$$\mu_e = \frac{q}{6\pi\eta r} \qquad \text{(eq.2)}$$

where q is the charge of the ion, η is the BGE viscosity and r is the ion radius. The charge of the ion (q) is fixed for fully dissociated ions, such as strong acids or small ions, but can be affected by pH changes in the case of weak acids or bases.
From this equation it is evident that small, highly charged species have high mobilities whereas large, minimally charged species have low mobilities.

1.1.3.2 Electroosmotic flow [3]

A vitally important constituent of CE operation is the electroosmotic, or electroendosmotic flow (EOF). EOF is the bulk flow of liquid in the capillary and is a consequence of the surface charge on the inner capillary wall. An uncoated fused-silica capillary tube is typically used for CE. The inner wall of the capillary possesses ionisable silanol groups, which are in contact with the buffer during CE. These silanol groups readily dissociate, giving the capillary wall a negative charge. When the capillary is filled with buffer, the negatively charged capillary wall attracts positively charged ions from the buffer solution, creating an electrical double layer and a potential difference (zeta potential) close to the capillary wall, as described according to Stern's model. Stern's model for an electrical double layer includes a rigid layer of adsorbed ions and a diffuse layer, in which ion diffusion may occur by thermal motion. When a voltage is applied on the capillary, cations in the diffuse layer can migrate towards the cathode, carrying the bulk solution with them.

The result is a flow, which velocity is described by the following equation:

$$\upsilon_{EOF} = \left(\frac{\varepsilon_0 \varepsilon \xi}{4\pi\eta} \right) E \qquad \text{(eq.3)}$$

where ε_0 is the dielectric constant of vacuum, ε is the dielectric constant of the buffer, ζ is the zeta potential, η is the viscosity of the buffer and E is the electric field intensity. The terms enclosed in brackets equate to the mobility of the EOF (μEOF).

1.1.3.3 EOF control [3]

As it can be seen in eq. 3, the main variables affecting EOF mobility are the dielectric constant and viscosity of the buffer and the zeta potential. The dielectric constant and viscosity of the buffer can be modified by the use of buffer additives and/or other modifications of the buffer composition. Buffer viscosity will also depend on the separation temperature.

The zeta potential is proportional to the charge density on the capillary wall, which itself is pH dependent. Therefore, EOF mobility will vary according to the buffer pH, such that at high pH the EOF mobility will be significantly greater than at low pH. For a typical fused-silica capillary, silanols are completely ionized above pH 9 providing the greatest EOF mobility. Below pH 4, the ionization of silanols is low and the EOF mobility is negligible.

The zeta potential also depends on the buffer ionic strength. As ionic strength increases, the double layer becomes compressed, decreasing zeta potential and reducing EOF mobility. At pH ~7, the EOF mobility is sufficient to ensure the net migration of most ions towards the cathode, regardless of their charge.

1.1.3.4 Flow profile in CE [3]

An important feature in EOF is flat flow profile (see Fig. 2). For the flow to be almost uniform throughout the capillary, the driving force must be distributed uniformly along the walls. In contrast, a pressure-driven flow (as used for HPLC) is parabolic or laminar.

The importance of the flat profile consists in the minimalization of zone broadening, leading to high separation efficiencies.

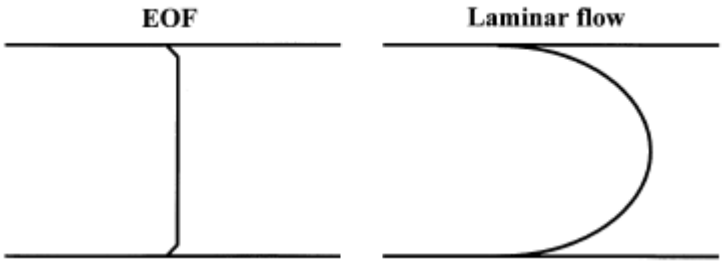


Fig. 2: Flow profiles of EOF [3].

1.1.4 Capillary electrophoresis modes

Capillary electrophoresis (CE) encompasses a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. Sample introduction is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Depending on the types of capillary and electrolytes used, the technology of CE can be segmented into several separation techniques. Examples of these include [4]:

-Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis, due to its simplicity of operation, is the most commonly used electrophoretic mode. The capillary is only filled with buffer and separation occurs because solutes migrate in discrete zones and at different velocities. Both cationic and anionic solutes can be separated due to the existence of EOF. At the contrary, neutral solutes migrate unseparated together with EOF [3].

-Capillary gel electrophoresis (CGE)

CGE is the adaptation of traditional gel electrophoresis into the capillary using polymers in solution to create a molecular sieve also known as replaceable physical gel. This allows analytes having similar charge-to-mass ratios to be resolved by size. This technique is

commonly employed in SDS-Gel molecular weight analysis of proteins and the sizing of applications of DNA sequencing and genotyping [4].

-Capillary isoelectric focusing (CIEF)

CIEF allows amphoteric molecules, such as proteins, to be separated by electrophoresis in a pH gradient generated between the cathode and anode. A solute will migrate to a point where its net charge is zero. At the solutes isoelectric point (pI), migration stops and the analyte is focused into a tight zone. In CIEF, once a solute has focused at its pI, the zone is mobilized past the detector by either pressure or chemical means. This technique is commonly employed in protein characterization as a mechanism to determine a protein's isoelectric point [4].

-Isotachophoresis (ITP)

ITP is a focusing technique based on the migration of the sample components between leading and terminating electrolytes. Solutes having mobilities intermediate to those of the leading and terminating electrolytes stack into sharp, focused zones. Although it is used as a mode of separation, transient ITP has been used primarily as a sample concentration technique [4].

-Electrokinetic chromatography (EKC)

EKC is a family of electrophoresis techniques named after electrokinetic phenomena. In the example of cyclodextrin-mediated EKC, the differential interaction of enantiomers with the cyclodextrins allows the separation of chiral compounds. This approach to enantiomer analysis has made significant impact on the pharmaceutical industry's approach to assessing drugs containing enantiomers [4].

-Micellar electrokinetic chromatography (MEKC)

MEKC is a mode of electrokinetic chromatography. In this case, surfactants are added to the buffer solution at concentrations exceeding the concentration at which micelles are formed. The separation principle of MEKC is based on a differential partition between the micelle and the solvent. This principle can be employed with charged or neutral solutes and may involve stationary or mobile micelles. MEKC has great utility in separating mixtures that contain both

ionic and neutral species, and has become valuable in the separation of very hydrophobic drugs from their very polar metabolites [4].

-Capillary electrochromatography (CEC)

CEC is a hybrid separation method that couples the high separation efficiency of CZE with HPLC and uses an electric field rather than hydraulic pressure to propel the mobile phase through a packed bed. Because there is minimal backpressure, it is possible to use small-diameter packings and achieve very high efficiencies. Its most useful application appears to be in the form of on-line analyte concentration that can be used to concentrate a given sample prior to separation by CZE [4].

1.2 CAPILLARY ELECTROPHORESIS USED FOR ENANTIOMERS SEPARATION

CE can be considered as complementary to other analytical techniques such as LC for enantioseparation, which presents a number of advantages: (1) the amounts of samples and separation buffer are much smaller than those used in HPLC; (2) usually the chiral selectors are dissolved in the background electrolyte (BGE) and thus the expensive chiral columns are not required; (3) compared to HPLC, higher efficiencies and shorter analysis times are obtained. CE techniques can be classified in two ways, either indirect CE using chiral derivatization agents or direct CE using chiral selectors as additives to the electrolyte [5].

Nowadays many chiral selectors are available. This fact makes challenging the selection of proper analytical conditions to obtain an acceptable result for a given analyte. Often the search for suitable conditions is a trial-and-error approach which costs a lot of time, money and work [6].

The most frequently used chiral selectors in CE are cyclodextrins, but among others belong crown ethers, macrocyclic antibiotics, proteins, chiral surfactants, linear polysaccharides, chiral metal complexes, etc [6].

1.3 CYCLODEXTRINS

Cyclodextrins (CDs) are nonreducing oligosaccarides which originate from amylose by the action of glucosyltransferase. The cyclic polymers formed by six, seven and eight glucopyranose units are named α-, β- and γ-CD, respectively; they have the shape of truncated cones with hydrophilic outside surfaces, whereas their inner cavities are hydrophobic. They can thus form inclusion complexes with hydrophobic molecules, or with hydrophilic molecules which possess hydrophobic moieties. Due to the optical activity of their structural monomer, CDs are optically active, therefore they can, in principle, distinguish between the optical antipodes of chiral molecules. Thanks to this capability, CDs are among the most widely used chiral selectors. The enantioselectivity of complexation depends on several factors, the dimensions of the CD cavity and the presence and properties of functional groups being among the most relevant, but no general rule for linking the stereoselectivity of the CDs with their chemical structure has been discovered as yet [7].

Cyclodextrins (CDs) are the most frequently used chiral selectors in CE. A great variety of neutral and charged CD derivatives are available. Inclusion of bulky hydrophobic groups into the cavity supported by interactions of the hydroxy groups at C2 and C3 at the mouth of the cavity with hydrophilic groups of the analyte is the proposed chiral recognition mechanism [8].

Fig. 3 shows a schema of a cyclodextrin:

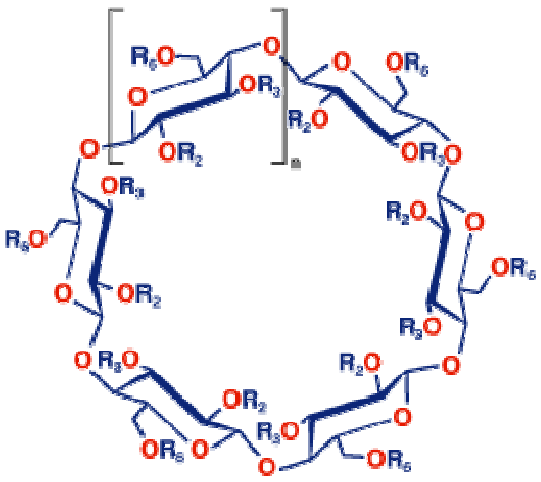


Fig. 3: Schematic representation of a cyclodextrin [9].

1.3.1 Native and neutral cyclodextrins

A broad spectrum of neutral CD derivatives is commercially available. A new highly water soluble CD derivative, 2-*O*-acetonyl-2-*O*-hydroxypropyl-β-CD (2-AHP-β-CD) was prepared by Lin et al. [10]. It represents a mixture of isomers with an average degree of substitution (DS) of about 1.0 for the acetonyl group and 3.8 for the hydroxypropyl group. This CD derivative showed improved resolution properties compared to β-CD, dodecylmethyl-β-CD (DM-β-CD) and hydroxypropyl-β-CD (HP-β-CD) for a broad spectrum of basic and acidic compounds.

Since most of the CD-derivatives represent mixtures of different products showing different substitution patterns, separations are often difficult to reproduce. A recent trend is to synthesize selectively substituted derivatives or single isomers. Schmitt et al. [11] compared the reproducibility of resolution of single-isomers, heptakis(2,3,6-tri-*O*-methyl)-β-CD, heptakis(2,6-di-*O*-methyl)-β-CD and heptakis(2,3-di-*O*-acetyl)-β-CD with that of the corresponding randomly substituted CDs using 1,1′-binaphthyl-2,2′-diyl hydrogen phosphate as a model compound. The authors found out that the batch to batch variation of randomly substituted CDs was remarkable and there was no relation with the DS of the molecule.

Wistuba et al. [12] investigated δ-CD as chiral selector and checked its enantiodiscrimination properties by means of 5 dimethylammonaphthalene-1-sulfonyl (Dns)-amino acids, 2,4-dinitrophenyl (Dnp)-amino acids, Fmoc amino acids, flavones and three positively charged drugs, carvedilol, mefloquine and clidinium bromide. The authors compared the separation properties of δ-CD with those of α- ,β-, and γ-CD and observed that in many cases an increase of enantioresolution was obtained with increasing degree of oligomerization.

1.3.2 Negatively charged cyclodextrin derivatives

Yang et al. described a systematic study of a broad spectrum of drugs using sulfated β-CD at pH 2.5 in the reversed polarity mode. Among 50 drugs investigated 37 were resolved [13]. A multivariate optimization approach based on an overlapping resolution scheme for the chiral separation of arylalcohols using a sulfated CD was presented by Zhang et al. [14]. In seven preexperiments the critical parameters such as CD-concentration, phosphate concentration

and pH were checked. Further seven experiments were necessary for optimization of the separation conditions. As a final result the authors achieved baseline resolution within 30 min using a selector concentration of 5.4% (v/v), a phosphate concentration of 28 mM and a pH of 5.0.

A series of single isomers of negatively charged CDs have been synthesized by Vigh's group and applied to a broad spectrum of neutral, basic, as well as acidic and zwitterionic drugs [15], [16], [17], [18] and [19].

1.3.3 Positively charged cyclodextrin derivatives

A new highly water soluble derivative, prepared by Lin et al., is 2-*O*-(2-aminoethyl-imino-propyl)-β-*O*-hydroxypropyl-CD [20].The authors compared the separation power of this selector with that of β-CD, HP-β-CD and DM-β-CD by means of some acidic analytes such as hydroxy acids and anti-inflammatory drugs of the profen type (Fig. 4). Improved enantioseparations were obtained with this selector. To avoid adsorption of the selector onto the capillary wall, coating of the capillary with polyacrylamide was necessary.

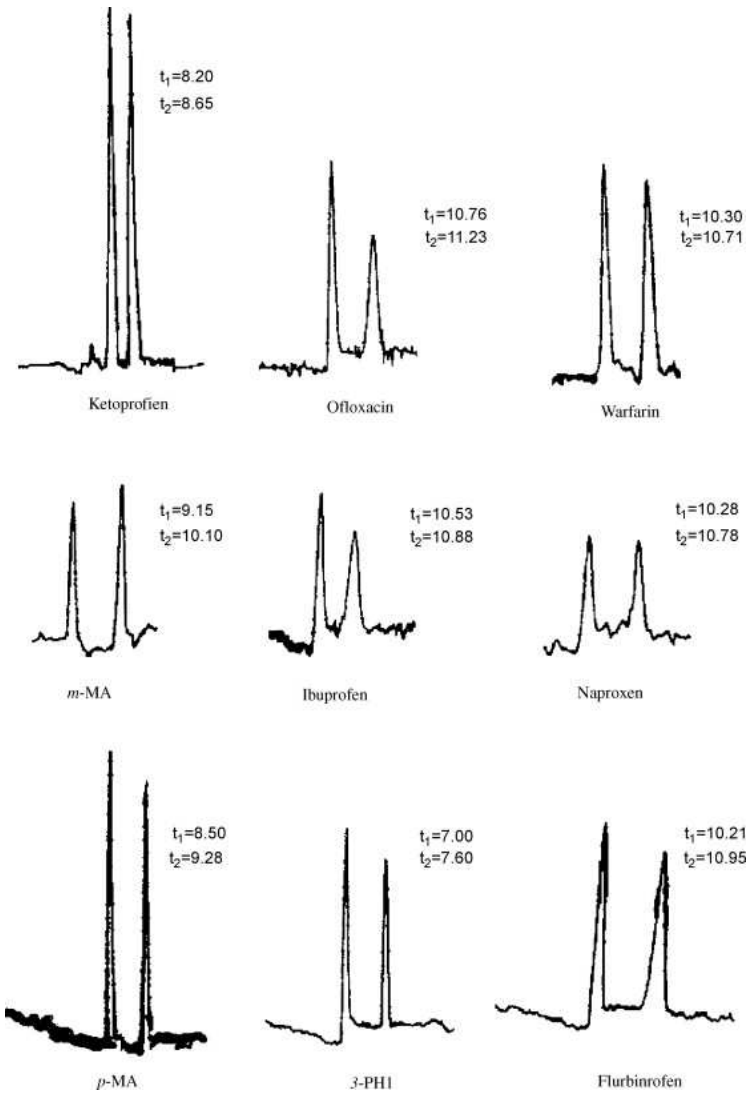


Fig. 4: Electropherograms of different analytes using 2-*O*-(2-aminoethyl-imino-propyl)-β-*O*-hydroxypropyl-CD as chiral selector in 50 mM NaH_2PO_4 [20].

Recently, Cucinotta et al. [21] reported on the synthesis of two new cylodextrin derivatives, an ethylenediamine derivative substituted in primary position (CDen) and a cysteamine-bridged hemispherodextrin (THCMH) (Fig. 5). The authors checked the applicability of these selectors by means of 11 amino acids and achieved good separations. The effect of alkylimidazolium substituents on chiral recognition ability was studied by Tang et al. [22]. A series of single isomer 6-mono (3-alkylimidazolium)-β-cyclodextrins were prepared and applied to the chiral separation of amino acids. The authors found out that derivatives with shorter alkylchains showed higher chiral recognition power. A pH of 5.0 with a selector concentration not less than 3 mM was found to be optimal.

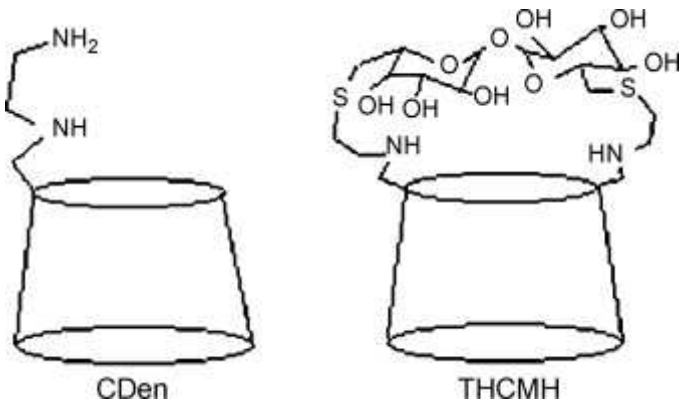


Fig. 5: Schematic formula of CDen and THCMH [21].

CDs containing quaternary ammonium groups show some advantages, because they are strong bases and therefore the electrophoretic mobility is pH-independent. Furthermore, only very low selector concentrations are required to resolve acidic enantiomers. A reversal of the EOF is observed with quaternary ammonium compounds [8].

1.4 PARTIAL FILLING MODE IN CAPILLARY ELECTROPHORESIS

The partial filling technique (PFT) was first introduced by Valtcheva et al. [23] and successfully applied in CZE to increase the detection sensitivity when using chiral selector with strong UV absorption. In PFT only part of the capillary is filled with the chiral selector that is never reaching the detection path [22].
There are many methods using the partial filling technique. Some examples are:

1.4.1 Partial filling counter current mode (PFCC)

In the counter current mode a chiral selector of opposite charge to the analyte is used, therefore, they migrate in opposite direction, offering a powerful enantioselective capability at low concentration. As reviewed [24], [25] and [26], PFCC is particularly advantageous in CE–MS to perform chiral separations avoiding MS detector contamination [22].

1.4.2 Partial filling affinity capillary electrophoresis (PFACE)

Here, the capillary is partially filled with ligand and the sample plug of receptor is introduced into the capillary and electrophoresed. During electrophoresis the zones of samples overlap within the capillary and equilibrium is established prior to the point of detection. PFACE reduces the amount of sample required for the assay and expedites the speed of analysis [27].

1.4.3 Flow-through partial filling affinity capillary electrophoresis (FTPFACE)

In FTPFACE a similar procedure as that used for PFACE is used except a smaller zone of ligand is partially filled into the capillary column. Upon electrophoresis, the sample plug flows through the partially filled zone. Subsequent analysis of the change in migration time of the receptor realizes a value for the binding constant [28].

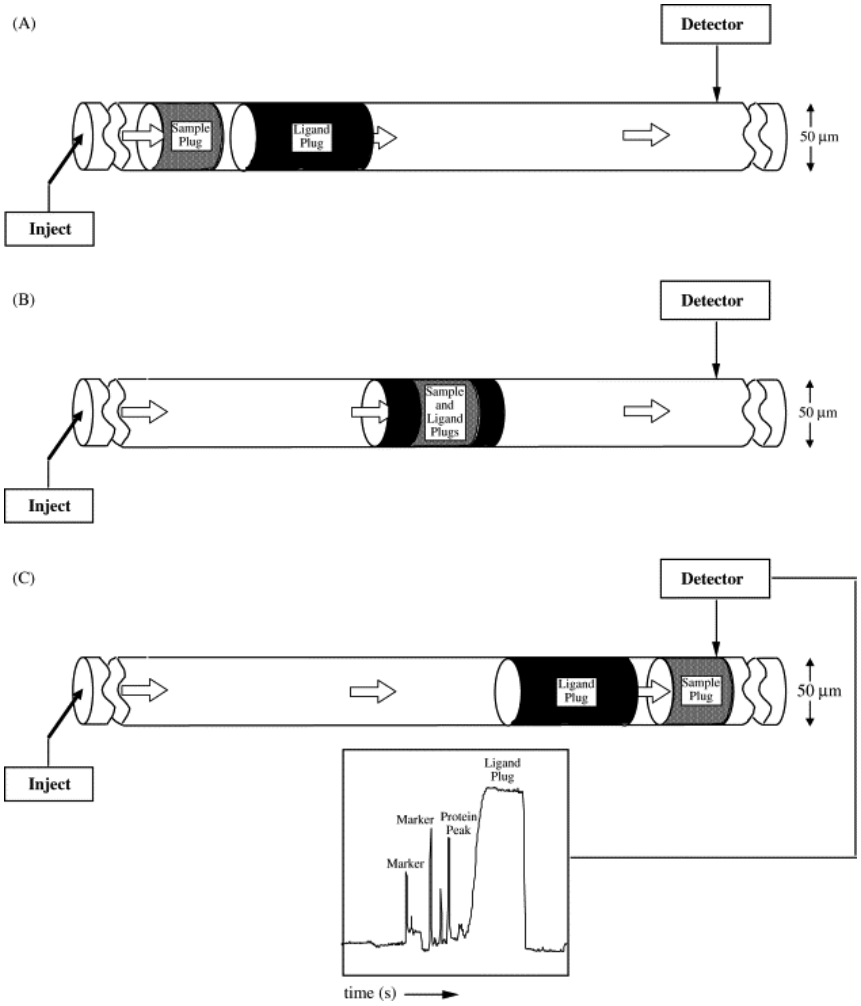


Fig. 6: Schema of a flow-through partial-filling affinity capillary electrophoresis (FTPFACE) experiment [28].

In Table 2 are shown some examples where cyclodextrins were used as chiral selectors, with the partial filling technique.

Table 2. Examples of using the partial filling technique with cyclodextrins as chiral selectors:

Analyte Chiral Drugs (or metabolites)	**CE-mode**	**Cyclodextrins**	**References**
baclofen	CE-MS (on-line UV detection)	sulfobutylether-β-CD (SBE-β-CD)	[29]
methadone	CE/ESI-MS	SBE-β-CD, CM-β-CD, HP-β-CD	[30]
basic adrenoreceptor antagonists enantiomers	CE-MS	HP-β-CD	[31]
amphetamines, methadone, venlafaxine and selected tropane alkaloids	CE/ESI-MS	neutral and negatively charged cyclodextrins	[32]
tramadol and its main phase I metabolites	CE/ESI-MS	SBE-β-CD	[33]
prilocaine, bupivacaine, mepivacaine	CZE	α-CD, methyl-β-CD	[34]

1.5 PESTICIDES

A pesticide is a substance or mixture of substances used to kill a pest. Although there are benefits to the use of pesticides, there are also drawbacks, such as potential toxicity to humans and other animals [35].

1.5.1 Types of pesticides

A classification of pesticides considers chemical pesticides or those derived from a common source or production method. Other categories include biopesticides, antimicrobials, and pest control devices. However, they are usually classified according to the type of pest they control [35].

1.5.1.1 Chemical pesticides

The most common groups of chemical pesticides are [35]:

Organophosphate Pesticides - These pesticides affect the nervous system by disrupting acetylcholinesterase. Most of them are insecticides. They were developed during the early 19th century. In 1932, their effects on insects, which are similar to their effects on humans, were discovered. Although some of them are very toxic, they usually are not persistent in the environment.

Example: malathion, phenthoate, phenamiphos, isomalathion

Carbamate Pesticides also affect the nervous system by disrupting acetylcholinesterase. The enzyme effects are usually reversible.

Example: carbaryl, benomyl, carbendazim

Organochlorine Insecticides were commonly used in the past, but many have been removed from the market due to their health and environmental effects and their persistence.

Ex: dichlorodiphenyltrichloroethane (DDT), aldrin, dieldrin, heptachlor

Pyrethroid Pesticides were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system.

Ex: bifenthrin, cypermethrin, deltamethrin

1.5.1.2 Biopesticides

Biopesticides are derived from animals, plants, bacteria or certain minerals (for example, baking soda is considered as biopesticide). They fall into three major classes [35]:

(1) **Microbial pesticides.** A microorganism (e.g., a bacterium, fungus, virus or protozoan) is the active ingredient. Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest[s]. For example, there are fungi that control certain weeds, and other fungi that kill specific insects.

The most widely used microbial pesticides are subspecies and strains of Bacillus thuringiensis (Bt). Each strain of this bacterium produces a different mix of proteins that specifically kills one or a few related species of insect larvae. While some Bt's control moth larvae found on plants, other Bt's are specific for larvae of flies and mosquitoes. The target insect species depends on the specific protein produced by the particular Bt, which binds to a larval gut receptor causing the insect larva to starve.

(2) **Plant-Incorporated-Protectants (PIPs)** are pesticidal substances produced by genes that have been incorporated to a plant genetic material. For example, the gene for the Bt pesticidal protein can be introduced to the plant, which then produces the pesticide instead of Bt.

(3) **Biochemical pesticides** are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are generally synthetic materials that

directly kill or inactivate the pest. Biochemical pesticides include substances, such as insect sex pheromones, that interfere with mating, as well as various scented plant extracts that attract insect pests to traps.

1.5.1.3 Pest types

According to the pest type, pesticides can be classified in various groups, for example [35]:

Algicides	Control algae in lakes, canals, swimming pools, etc.
Antimicrobials	Kill microorganisms (such as bacteria and viruses).
Attractants	Attract pests, for example to lure an insect or rodent to a trap.
Disinfectants and sanitizers	Kill or inactivate disease-producing microorganisms on inanimate objects.
Fungicides	Kill fungi (including blights, mildews, molds, and rusts).
Herbicides	Kill weeds and other plants that grow where they are not wanted.
Insecticides	Kill insects and other arthropods.
Miticides (or acaricides)	Kill mites that feed on plants and animals.
Nematicides	Kill nematodes, that feed on plant roots.
Ovicides	Kill eggs of insects and mites.
Pheromones	Biochemicals used to disrupt the mating behavior of insects.
Repellents	Repel pests, including insects (such as mosquitoes) and birds.
Rodenticides	Control mice and other rodents.

1.5.2 Chiral pesticides

Many compounds, including some pesticides, contain structural centres of asymmetry, which convey the property of a type of stereoisomerism known as chirality. Such compounds can exist in two or more forms, depending on the number of chiral atoms and are termed

stereoisomers or enantiomers, and can exhibit different bioactivity [36]. Enantiomers usually differ in their biological properties as a result of their interaction with enzymes or other naturally occurring chiral molecules. This difference may lead to variations in microbial degradation rates and would mean that one enantiomer is more persistent in the environment than the other. In addition, enantiomers often exhibit different effects or toxicity: the "active" enantiomer of a chiral pesticide would have the desired effect on a target species, whereas the other enantiomer may not. Moreover, one or both enantiomers may have adverse effects on some nontarget species [37]. Therefore, this has led to increased research on enantioselectivity [38].

Upwards of 25% of pesticides are chiral [39]. However, the great majority of chiral pesticides are produced and marketed as racemates. In the past 5–10 years several single- or enriched-enantiomer pesticide formulations have been developed and promoted in North America and in Europe to protect the environment from unintended effects [37].

To obtain information about the differential biological behavior of chiral pesticides it is necessary to develop suitable methods for the enantioresolution of such compounds.

1.5.2.1 Chiral pesticides separation using capillary electrophoresis with cyclodextrins as chiral selectors

Cyclodextrins are the most frequently used chiral selectors in capillary electrophoresis enantioseparation. Some examples of chiral pesticides separation using capillary electrophoresis with cyclodextrins as chiral selectors are summarized in Table 3.

Table 3. Examples of chiral pesticides separations using capillary electrophoresis with cyclodextrins as chiral selectors:

Analytes Chiral Pesticides (or metabolites)	**CE-mode**	**Cyclodextrins**	**Buffer & Aditives**	**References**
chiral pesticides	MEKC		sodium dodecyl sulphate (SDS) (neutral analytes)	[40]
profenofos, prothiofos, sulprofos, pyraclofos	ACE, NACE	sodium cholate:γ-CD	methanol:ACN (4:1 v/v) or MeOH:H_2O:ACN (5:4:1 v/v/v).	[41]
stereoisomers of metolachlor and its two polar metabolites: ethane sulfonic acid (ESA) and oxalinic acid (OXA)	CZE	γ-CD for ESA and OXA	borate buffer (pH 9.0) containing 20% methanol (v/v) and 2.5% γ-CD (w/v).	[42]

Analytes Chiral Pesticides (or metabolites)	**CE-mode**	**Cyclodextrins**	**Buffer & Aditives**	**References**
metalaxyl	CZE-UV	native CDs and modified β-CD	55 mM succynyl-β-cyclodextrin in 50 mM sodium tetraborate buffer (pH 9.3)	[43]
metalaxyl, imazaquin, fonofos (dyfonate), ruelene (cruformate) and dichlorprop	CE			[44]
organophosphorus pesticides (OPs): malathion, phenthoate, phenamiphos, isomalathion	EKC	CM-β-CD	25 mM Tris buffer (pH 7.0), 20 mM CM-β-CD solution	[45]
pesticides containing asymmetric N or P besides carbon	CE	β-CD, macrocyclic glycopeptides, DM-β-CD		[46]
chiral pesticides, chiral metabolites of achiral pesticides	EKC-UV MEKC-UV			[47]
2-phenoxypropionic acid (PPA), cis-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylic acid (cis-PA), 1-phenyl-2-(p-tolyl)ethylamine (PTE), 1-phenyl-2-(p-methoxyphenyl)ethylamine (PME)	CZE	β-CD	14 g/L CD solution; 30 mM tris-HCl , pH 6.0 (PPA and cis-PA); pH 2.5 and pH 3.0 for PTE and PME respectively	[48]
NSAIDs, aminoacids, phenoxypropionic acid herbicides	CE, CD-MEKC, CEC, OT-CEC, P-CEC, combination of flow injection with CE	-neutral CD-derivatives (basic analytes, lower CD conc.) -negatively charged CDs (basic and neutral drugs) -positively charged CDs (acidic and neutral compouds) -amphoteric CDs (Glu-β-CD and AM-β-CD) -CDs and non-chiral additives		[49]
triazole fungicides: bitertanol, cyproconazole, difenoconazole, diniconazole, flutriafol, hexaconazole, myclobutanil, paclobutrazol, penconazole, propiconazole, tebuconazole, tetraconazole, triadimefon, triadimenol	CE	sulphated-β-CD		[50]
s-triazines, phenoxy acids	partial-filling MEKC, NACE		-MEKC: 80 mmol/l boric acid solution + 14% (v/v) methanol -NACE: acetonitrile, methanol, glacial acetic acid, ammonium acetate (pH 4.7)	[51]
organochlorine pesticides	CEC	HP-β-CD		[52]
4 enantiomers of mecoprop and dichlorprop	CZE	ethylcarbonate derivative of β-CD with 3 substituents per molecule, HP-β-CD and native α-CD		[53]
ESA and OXA (derivatives of acetanilide herbicides)	CZE	γ-CD		[54]
propiconazole, bioallethrin, fenpropathrin, phenothrin, bitertanol, triadimenol, dimethomorph	CD-modified MEKC		sodium cholate in the running buffer	[55]

Analytes Chiral Pesticides (or metabolites)	**CE-mode**	**Cyclodextrins**	**Buffer & Aditives**	**References**
phenoxy acid herbicides and their enantiomers: ▪ 2-(3-chlorophenoxy)propionic acid ▪ 2-(2,4,5-trichlorophenoxy)propionic acid ▪ (2,4,5-trichlorophenoxy)acetic acid ▪ 2-(2-chlorophenoxy)propionic acid ▪ 2-phenoxypropionic acid ▪ 2-(2,4-dichlorophenoxy)propionic acid ▪ 2-(4-chloro-2-methylphenoxy)propionic acid ▪ (2,4-dichlorophenoxy)acetic acid ▪ 2-(4-chlorophenoxy)propionic acid	CZE	unmodified and selectively methylated α-CD, β-CD and γ-CD derivatives. Among the α-CD chiral selectors, hexakis (2,3-di-O-methyl)-α-CD (2,3-DM-α-CD)		[56]
PPAHs: ▪ (±)-2-(3-chlorophenoxy)propionic acid ▪ (±)-2-(2-chlorophenoxy)propionic acid ▪ (±)-2-(4-chlorophenoxy)propionic acid ▪ (±)-2-(2,4-dichlorophenoxy)propionic acid ▪ 2(2,4,5-trichlorophenoxy)propionic acid ▪ (±)-2(2-phenoxy)propionic acid	CE	cationic β-cyclodextrin derivative (hepta-substituted β-CD bearing the methoxyethylamine group linked to the upper CD rim		[57]
dichlorprop, (±)-2-(2,4-dichlorophenoxy)propionic acid (only the (+)-isomer is herbicidally active)	CZE	ethylcarbonate derivative of β-CD		[58]
dichlorprop, mecoprop, fenoprop	CD-CZE, ESI-MS	heptakis(2,3,6-tri-O-methyl)-β-CD (TM-β-CD)	negative-ionization mode, methanol-water-formic acid solution as sheath liquid, nitrogen as sheath gas, 20 mM TM-β-CD solution, 50 mM ammonium acetate buffer (pH 4.6)	[59]
▪ organophosphorus: ruelene, isofenphos, dialifor or dialifos, fenamifos, malathion ▪ phenoxy acid methyl esters: fenoprop-, mecoprop-, dichlorprop-methyl ester ▪ organochlorine: p,p′-DDT, p,p′-DDD, o,p′-DDT, o,p′-DDD, p,p′-DDE, o,p′-DDE ▪ acetamide: metolachlor	CD-MEKC	6 CD: α-, β-, γ-, HP-β-, DM-β- and TM-β-CD added to the borate SDS-buffer, with and without organic modifier (MeOH or ACN)	fenoprop-, mecoprop-, dichlorprop-methyl ester: γ-CD-methanol; organochlorine: γ-CD-acetonitrile; metolachlor: three of the enantiomers separated by γ-CD-methanol	[60]
imazaquin, diclofop, imazamethabenz	CD-CZE	various commercially available CDs	mixed CDs in the running buffer: three herbicides simultaneously separated in a single run	[61]
chlorophenoxy acid herbicides and their enantiomers: ▪ 2-(2,4,5-trichlorophenoxy)propionic acid ▪ 2-(2,4-dichlorophenoxy)propionic acid ▪ (2,4-dichlorophenoxy)acetic acid ▪ 4-(2,4-dichlorophenoxy)butyric acid ▪ 4-chloro-2-methylphenoxyacetic acid ▪ 4-(4-chloro-2-methylphenoxy)butyric acid ▪ 2,4,5-trichlorophenoxyacetic acid	CD-CE	4 mM α-CD and 1 mM β-CD solutions in the buffer		[62]
dichlorprop	CZE	heptakis(2,3,6-tri-O-methyl)-β-CD	acetate buffer (pH 4.7)	[63]

THE AIM

Today, up to 25% of pesticides are chiral molecules; however, almost all of them are manufactured and applied as racemic mixtures. The agrochemical industry and government regulators are beginning to take enantioselectivity into account to make a more accurate risk assessment of chiral pesticides. To obtain information on the toxicity and biotransformation of chiral pesticides it is necessary to develop suitable methods for their chiral resolution. In this sense, capillary electrophoresis in the modality of electrokinetic chromatography, EKC, adding a chiral selector to the background electrolyte, has become a powerful analytical technique for enantiomeric separations. Among the chiral selectors that have been used in EKC, cyclodextrins, CDs, and their derivatives are the most popular.

The aim of this Diploma Thesis is to perform preliminary studies on the applicability of EKC (with CD as chiral selectors) to the separation of chiral pesticides. Concretely, we are interested in the evaluation of enantioresolution of nine neutral and cationic chiral pesticides (benalaxyl, cyproconazole, hexaconazole, imazalil, myclobutanil, penconazole, propiconazole, tebuconazole and trichlorphon) (screening) using carboxymethylated-β-cyclodextrin (CM-β-CD) as chiral selector; a negatively charged CD widely used for enantioresolution of neutral and/or cationic compounds. Another point of interest is the possibility of using EKC in the partial filling mode, EKC-PF, in order to minimize the amount of CM-β-CD used, and then the costs.

Those compounds exhibiting adequate enantioresolution will be then extensively studied in terms of the effect of CM-β-CD injection time (selector plug length in the EKC-PF mode), voltage, temperature, pH and buffer nature and concentration on the enantioresolution.

The chiral separation methodologies developed in this Diploma Thesis could be used to perform further quality control of commercial formulations, as well as biological activity/toxicity enantiodifferentiation assessment of studied pesticides.

2. EXPERIMENTAL PART

2.1 INSTRUMENTATION

A Hewlett-Packard HP 3DCE capillary electrophoresis system (Agilent, AZ, USA) equipped with a diode array detection (DAD) system and HP 3DCE Chemstation software was used.
Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 µm I.D. and 363 µm O.D. with total and effective length of 48.5 and 40 cm, respectively, were used. The capillary cassette temperature was studied in the range 20-35°C and UV detection was performed at 220 nm. A running voltage in the 10-30 kV was applied. The current was 20 µA.
All solutions were degassed in an ultrasonic bath (JP Selecta, Barcelona, Spain) prior to use.
A Crison Micro pH 2000 pH meter from Crison Instruments (Barcelona, Spain) was employed to adjust the pH of the electrophoretic buffer.

2.2 CHEMICALS AND SOLUTIONS

Trichlorphon, benalaxyl, imazalil, tebuconazole, cyproconazole, hexaconazole, myclobutanil, penconazole and propiconazole were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).
Carboxymethylated-β-cyclodextrin (CM-β-CD s.d. ~3) was from Cyclolab (Budapest. Hungary). 30 mM solutions were prepared in the running buffer solution.
Tris-(hydroxymethyl)-aminomethane (Tris) buffer solutions (in the range 25-75 mM) at different pH values (in the range 5-9) were prepared by dissolving the appropriate amount of Tris purchased from Scharlau Chemie S.A. (Barcelona, Spain) in water and adjusting the pH with HCl 2 M.
2-(N-Morpholino)-ethane-sulfonic acid monohydrate (MES) buffer solutions (in the range 25-75 mM) were prepared by dissolving the appropriate amount of MES (Acros Organics, Geel, Belgium) in water and adjusting the pH to 7 with HCl 2 M.
Stock standard solutions of the pesticides (1000 ppm) were prepared in methanol.
Working solutions were obtained by dilution with buffer solution from the corresponding stock solution to a final concentration of 200 ppm.
Barnstead E-pure deionised water (Sybron, Boston, MA, USA) was used throughout.

2.3 PROCEDURES

2.3.1 Capillary conditioning

New capillary was activated for 10 min flush with 1M NaOH at 60°C. Then, it was rinsed for 5 min with water and 15 min with the running buffer at 25°C. In order to obtain good peak shapes and reproducible migration times, the capillary was conditioned at the beginning of the working session and between runs with the following sequence: (i) 1 min rinse with deionised water, (ii) 2 min rinse with 0.1M NaOH, (iii) 1 min rinse with deionised water, and (iv) 3 min rinse with running buffer. The whole conditioning was performed at 50 mbar.

2.3.2 Capillary filling conditions

Each run consisted in capillary conditioning (see previous paragraph), chiral selector (CM-β-CD solution) injection and pesticide solution injection.

The chiral selector injected solutions were 30 mM CM-β-CD solution applied at 50 mbar for different time periods (0-100 s).

The pesticide injected solutions were 200 ppm stock solutions applied at 50 mbar for 3s (benalaxyl, imazalil, penconazole, myclobutanil) or 5s (cyproconazole, hexaconazole, propiconazole, tebuconazole, trichlorphon). Initially the analytes were injected during 3 s. Injection time was increased to 5 s when no signal was observed.

The electropherogram was then obtained under the experimental conditions assayed (described in the previous sections).

2.3.3 Enantioresolution calculations

The enantioresolution was calculated using equation 4:

$$R_s = \frac{1.18.(t_2 - t_1)}{w_1 + w_2} \qquad \text{(eq. 4.)}$$

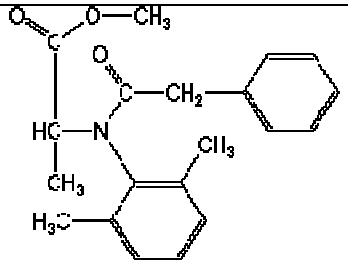
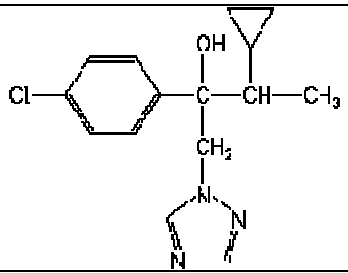
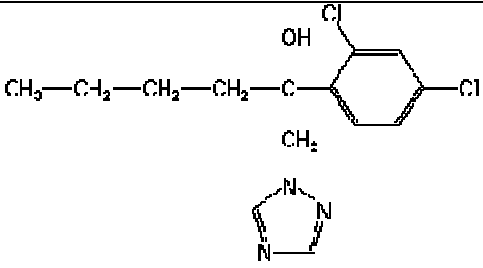
where t_1 and t_2 are migration times of each enantiomer and w_1 and w_2 represent peak width measured at half-height of the respective peak.

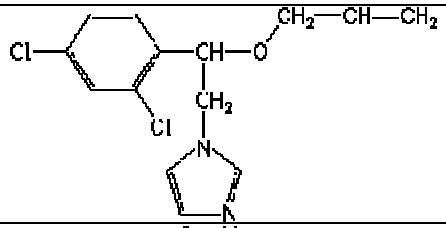
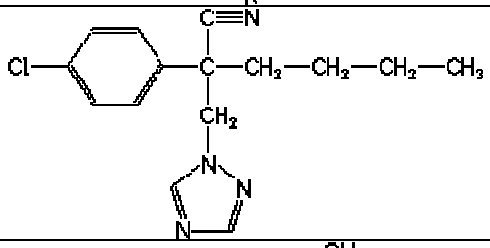
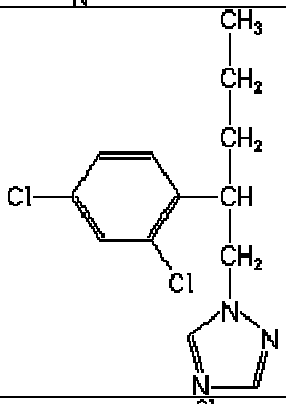
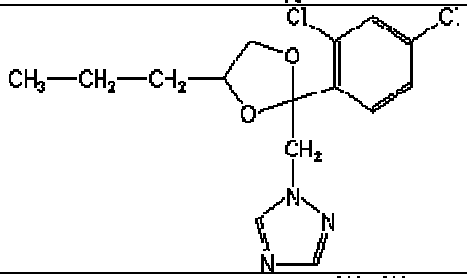
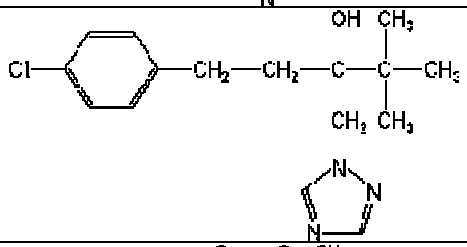
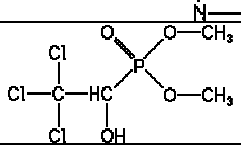
3. RESULTS AND DISCUSSION

-Selected compounds

Compounds selected in this work, which appears as accepted (or still non investigated) molecules in the European Directive 91/414/CE, are listed in Table 4. This table contains the molecular structure of the chiral pesticides studied, as well as their logP (octanol/water partition coefficient) and pK_a (negative logarithm of the acidity equilibrium constant) values. At can be observed, except trichlorphon (log P = 0.43), all the compounds exhibit high hydrophobicity (log P in the 3.09-3.82 range). On the other hand, all pesticides are neutral or weak basic compounds. However, except imazalil (pK_a = 6.53), all the analytes are unionized at the whole operating pH range in CE with uncoated silica capillaries (pH > 5). Therefore, since CM-β-CD has proven to be useful for the enantioresolution of neutral and cationic compounds, it could be a priori a suitable chiral selector for the enantioseparation of the selected pesticides.

Table 4. Selected compounds:

Name	**Structure**	**Log P** [64]	**pK_a** [64]
Benalaxyl		3.54	–
Cyproconazole		3.09	–
Hexaconazole		3.90	2.30

Name	Structure	Log P [64]	pK_a [64]
Imazalil		3.82	6.53
Myclobutanil		2.89	2.30
Penconazole		3.72	1.51
Propiconazole		3.72	1.09
Tebuconazole		3.70	_
Trichlorphon		0.43	_

-Choice of CM-β-CD

The reason for choosing CM-β-CD is the necessity of using a charged CD for the separation of neutral compounds. Cationic CD are more expensive than anionic ones, so CM-β-CD was

selected due to its availability. Obviously, other negatively charged CD could be also adequate, but for a preliminary study, starting with CM-β-CD was preferred.

-Choice of electrophoretic buffers

As buffers in EC, large molecules are more convenient in order to keep ionic strength at low values thus avoiding working with large current values. Especially Tris was chosed for its availability.

3.1 EVALUATION OF ENANTIORESOLUTION OF BENALAXYL USING CARBOXYMETHYL-BETA-CYCLODEXTRIN AS CHIRAL SELECTOR

3.1.1 The effect of CM-β-CD injection time on the enantioresolution of benalaxyl

In order to study the effect of the chiral selector plug length, experiments using 30 mM CM-β-CD solution applied at 50 mbar for variable times from 10 to 100 s before 200 ppm racemic benalaxyl solution injection were performed. The applied voltage was set at 15 kV. The detection wavelength was 220 nm. Afterwards, the enantioresolution (R_s) was calculated using equation 4 for each time of injection of the cyclodextrin.

The results are shown in Figures 7 and 8. As can be seen, the best enantioresolution occurred with a plug time of 40 s. Therefore applications of CM-β-CD solutions for 40 s were selected for further studies.

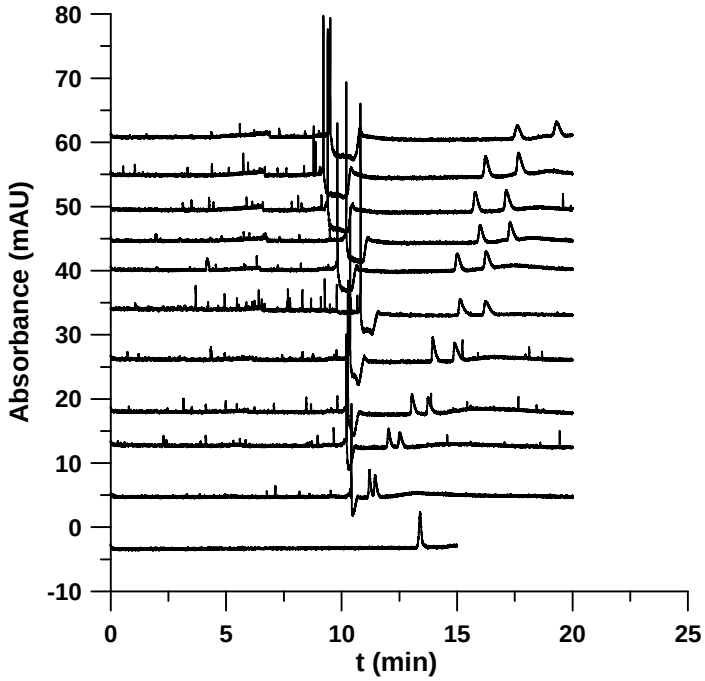


Fig. 7. Electropherograms obtained using 30 mM CM-β-CD solution applied at 50 mbar for (A) 0 s, (B) 10 s; (C) 20 s; (D) 30 s; (E) 40 s; (F) 50 s; (G) 60 s; (H) 70 s; (I) 80 s; (J) 90 s; (K) 100 s (from A to K in the ascendant order)

Experimental conditions: the injected solution was 200ppm benalaxyl stock solution in methanol-Tris buffer applied at 50 mbar for 3s, the electrophoretic buffer was 50mM Tris at pH 7, temperature 25°C. UV detection was performed at 220nm. Voltage 15 kV.

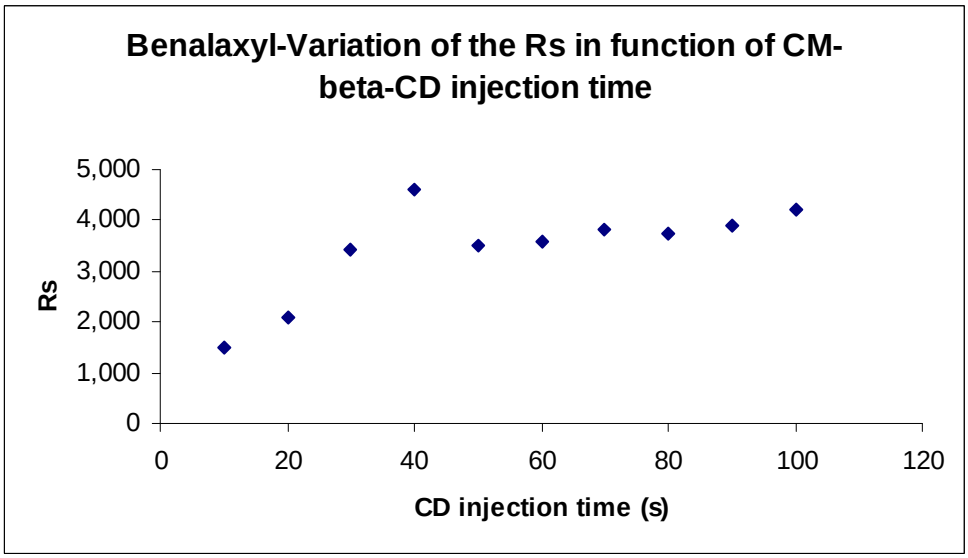


Fig. 8. Influence of the chiral selector plug length on the resolution of racemic benalaxyl. 30 mM CM-β-CD solution applied at 50 mbar for different times from 10 to 100 s.

3.1.2 The effect of voltage on the enantioresolution of benalaxyl

A study of the applied voltage from 10 to 30 kV was carried out. Migration times decreased from 22 to 6 min and resolution decreased from 2.145 to 1.974, with increasing voltage (see Table 5 and Fig. 9). An applied voltage of 15 kV was selected because a voltage of 10 kV produces longer migration times and a similar resolution (10 kV, Rs = 2.145; 15 kV, Rs = 2.112)

Voltage (kV)	t_1 (min)	t_2 (min)	Rs
10	21.02	22.305	2.145
15	13.78	14.623	2.112
20	14.034	14.852	2.014
30	6.084	6.421	1.974

Table 5. Influence of voltage applied on the migration times and resolution of benalaxyl enantiomers.

Experimental conditions: the injected solution was 200ppm benalaxyl stock solution in methanol-Tris buffer applied at 50 mbar for 3s; 50 mM Tris at pH 7 as electrophoretic buffer; 30 mM CM-β-CD solution applied at 50 mbar for 40 s; temperature 25°C. UV detection was performed at 220nm.

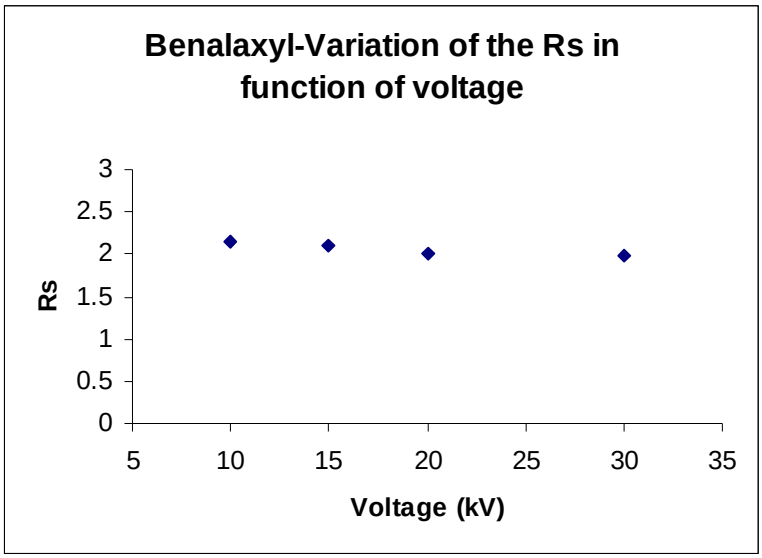


Fig. 9. Influence of applied voltage on the resolution of benalaxyl enantiomers.

3.1.3 The effect of temperature on the enantioresolution of benalaxyl

A study of the temperature from 20 to 35°C was made. Migration times decreased from 16 to 12 min and resolution increased from 2.207 to 2.625, with increasing temperature (see Table 6 and Fig. 10). A temperature of 30°C was selected because the use of a temperature of 35°C produces almost identic migration times and resolution.

Temperature (℃)	t_1 (min)	t_2 (min)	Rs
20	15.191	16.075	2.207
25	13.84	14.588	2.378
30	13.845	14.659	2.536
35	12.441	13.155	2.625

Table 6. Influence of temperature on the migration times and resolution of benalaxyl enantiomers.

Experimental conditions: the injected solution was 200ppm benalaxyl stock solution in methanol-Tris buffer applied at 50 mbar for 3s; 50 mM Tris at pH 7 as electrophoretic buffer; 30 mM CM-β-CD solution applied at 50 mbar for 40 s; voltage 15 kV. UV detection was performed at 220nm.

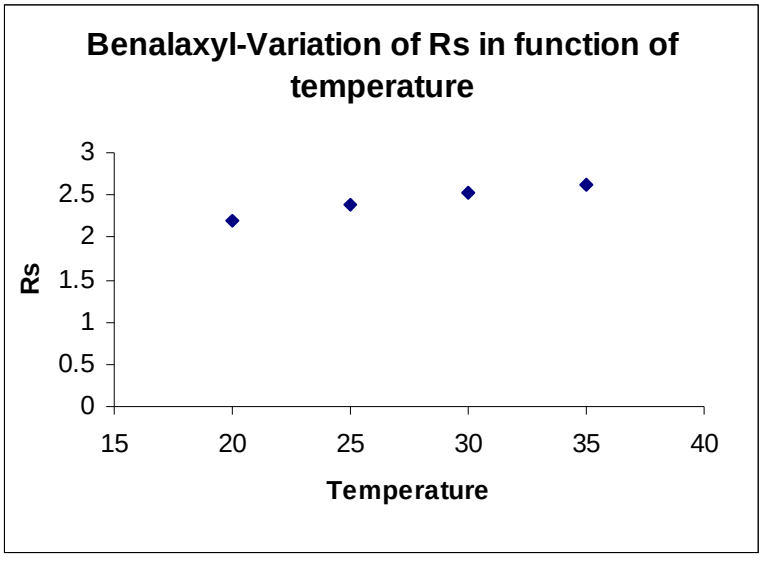


Fig. 10. Influence of temperature on the resolution of benalaxyl enantiomers.

3.1.4 The effect of pH on the enantioresolution of benalaxyl

In order to study the effect of pH, 50 mM Tris at pH 7, pH 8 and pH 9 was used as electrophoretic buffer. pH values lower than 7 were not studied to avoid migration times excessively long, due to the low EOF velocity. The results are shown in Fig. 11. As it can be seen, resolution decreased with increasing pH and no chiral recognition was obtained at pH 9. Therefore pH 7 was selected for further studies.

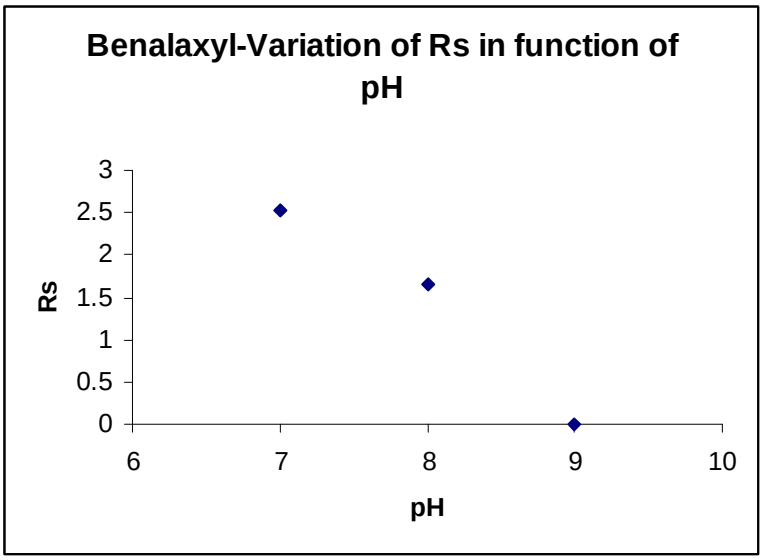


Fig. 11. Influence of pH on the resolution of benalaxyl enantiomers.
Experimental conditions: the injected solution was 200ppm benalaxyl stock solution in methanol-Tris buffer applied at 50 mbar for 3s; 30 mM CM-β-CD solution applied at 50 mbar for 40 s; 50 mM Tris as electrophoretic buffer; temperature 30°C; voltage 15 kV. UV detection was performed at 220nm.

3.1.5 The effect of Tris concentration on the enantioresolution of benalaxyl

The effect of Tris concentration in the electrophoretic buffer in the range 25 to 75 mM at pH 7 on the resolution was evaluated. Tris is a zwitterionic buffer, which may minimize interactions of solute with the capillary wall by shielding the capillary surface charge and by reducing sample adsorption [65]. The increase of Tris concentration produces more effective

charge shielding; therefore a decrease in EOF is observed. As can be observed in Table 7 and Fig. 11, the maximum resolution was achieved at 25 mM Tris buffer. The use of higher Tris concentrations produces a decrease in resolution, as a consequence of the increase in current and subsequent Joule heating that leads to peak broadening [65]. As it can be seen in Table 7 and Fig. 12, migration times increased from 12 to 16 min and resolution decreased from 2.171 to 2.075, with increasing concentration. Nevertheless, no significant differences were observed.

Buffer concentration (mM)	t_1 (min)	t_2 (min)	Rs
25	12.349	13.067	2.171
50	13.501	14.322	2.155
75	15.38	16.247	2.075

Table 7. Effect of Tris concentration on the migration times and resolution of benalaxyl enantiomers.

Experimental conditions: the injected solution was 200ppm benalaxyl stock solution in methanol-Tris buffer applied at 50 mbar for 3s; 30 mM CM-β-CD solution applied at 50 mbar for 40 s; Tris electrophoretic buffer at pH 7; temperature 30°C; voltage applied 15 kV; UV detection at 220 nm.

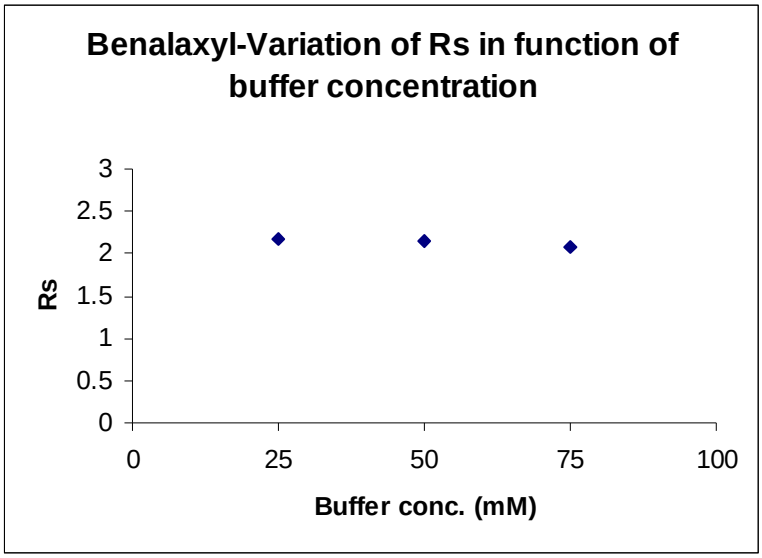


Fig. 12. Effect of Tris concentration on the resolution of benalaxyl enantiomers.

3.1.6 Enantioresolution of benalaxyl using MES as electrophoretic buffer and effect of MES concentration

The effect of MES monohydrate concentration in the electrophoretic buffer in the range 25 to 75 mM at pH 7 on the resolution was evaluated. Although the best resolution occurred with 50 mM MES, no significant differences were observed with increasing concentration.

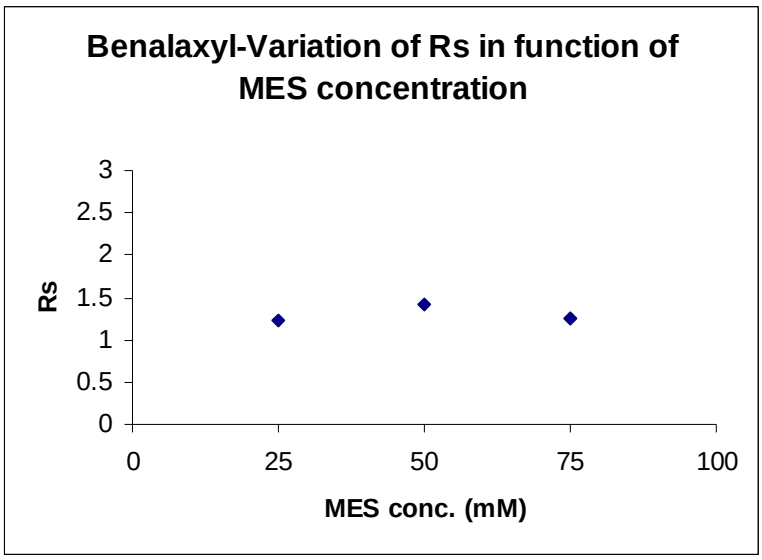


Fig. 13. Effect of MES concentration on the resolution of benalaxyl enantiomers. Experimental conditions: the injected solution was 200ppm benalaxyl stock solution in MES monohydrate buffer applied at 50 mbar for 3s; 30 mM CM-β-CD solution applied at 50 mbar for 40 s; MES electrophoretic buffer at pH 7; temperature 30°C; voltage applied 15 kV; UV detection at 220 nm.

3.1.7 Comparation of Tris and MES as electrophoretic buffer and their effects on the resolution of benalaxyl enantiomers

From the previous studies it is possible to compare the effects of two different electrophoretic buffers on the resolution. As can be seen in Table 8, the migration times were very similar in both cases, but the resolution was better in the case of Tris (MES, Rs = 1.409; Tris, Rs = 2.155). Therefore, Tris was selected as electrophoretic buffer for further studies.

	t_1 (min)	t_2 (min)	Rs
Tris	13.501	14.322	2.155
MES	13.007	13.664	1.409

Table 8. Comparation of Tris and MES and their effects on the migration times and resolution of benalaxyl enantiomers as running buffers.

Experimental conditions: the injected solution was 200ppm benalaxyl stock solution in methanol-buffer applied at 50 mbar for 3s; 50 mM buffer at pH 7; 30 mM CM-β-CD solution applied at 50 mbar for 40 s; temperature 30°C; voltage applied 15 kV; UV detection 220 nm.

3.2 EVALUATION OF ENANTIORESOLUTION OF IMAZALIL USING CARBOXYMETHYL-BETA-CYCLODEXTRIN AS CHIRAL SELECTOR

3.2.1 The effect of CM-β-CD injection time on the enantioresolution of imazalil

In order to study the effect of the chiral selector plug length, experiments using 30mM CM-β-CD solution applied at 50 mbar for variable times from 10 to 40 s before 200 ppm racemic imazalil solution injection were performed. Migration times increased from 11 to 16 min and resolution increased from 0.742 to 1.956 with increasing cyclodextrin plug length (Table 9 and Fig. 14)

t CD (s)	t_1 (min)	t_2 (min)	Rs
10	10.852	11.161	0.742
20	11.708	12.287	1.479
30	13.662	14.488	1.953
40	15.19	16.293	1.956

Table 9. Influence of the chiral selector plug length on the resolution of racemic imazalil. 30 mM CM-β-CD solution applied at 50 mbar for different times from 10 to 40 s.

Experimental conditions: the injected solution was 200ppm imazalil stock solution in methanol-Tris buffer applied at 50 mbar for 3s, the electrophoretic buffer was 50mM Tris at pH 7, temperature 30°C. UV detection was performed at 220nm. Voltage 15 kV.

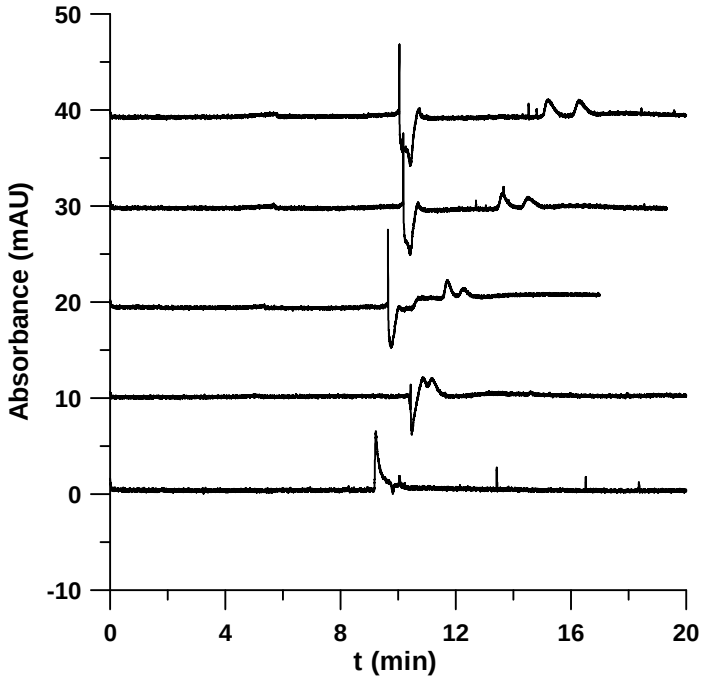


Fig. 14. Electropherograms obtained using 30 mM CM-β-CD solution applied at 50 mbar for (A) 0 s; (B) 10 s; (C) 20 s; (D) 30 s; (E) 40 s. (from A to E in the ascendant order)

3.2.2 The effect of pH on the enantioresolution of imazalil

Imazalil is a weak base with a pKa value of 6.53, therefore, at pH<6.53, the ionic form of the analyte is prevalent, while at pH>6.53 the neutral is the predominant form. Although running buffer pH values lower than 7 provides migration times excessively long, in this case, pH 5 and 6 (where the analyte molecule is positively charged) was included in the study to evaluate the effect of ionization on enantioresolution of imazalil. In order to study the effect of pH, 50 mM Tris at pH 5, pH 6, pH 7 and pH 8 was used as electrophoretic buffer. The results are shown in Table 10. In the case of pH 5 and 6 peaks were not observed after 30 min from the injection and the analysis was stopped. This run-time is too long for analysis purposes and it is also undesirable in EC since running buffer solution becomes electrolyzed. As can be observed, at pH 7 imazalil enantiomers were adequately separated (Rs > 1.5) with reasonable migration times.

pH	t_1 (min)	t_2 (min)	Rs
5	-	-	-
6	-	-	-
7	15.19	16.293	1.956
8	13.43	13.967	1.334

Table 10. Influence of pH on the migration times and resolution of imazalil enantiomers. Experimental conditions: the injected solution was 200ppm imazalil stock solution in methanol-Tris buffer applied at 50 mbar for 3s; 30 mM CM-β-CD solution applied at 50 mbar for 40 s; 50 mM Tris as electrophoretic buffer; temperature 30°C; voltage 15 kV. UV detection was performed at 220nm.

3.3 PRELIMINARY STUDIES OF ENANTIOSEPARATION OF OTHER PESTICIDES USING CM-BETA-CD AS CHIRAL SELECTOR

The potential of CM-β-CD as chiral selector for the enantioseparation of other neutral and weak basic pesticides was also studied. Compounds tested were trichlorphon, cyproconazol, hexaconazole, myclobutanil, penconazole, tebuconazole and propioconazol. As has been shown in the optimization studies for benalaxyl and imazalil, pH and selector plug length are the most critical experimental variables affecting enantioresolution with CM-β-CD. Regarding pH, all these compounds (see Table 4) are unionized (as benalaxyl) at the whole operating pH range in CE with uncoated silica capillaries (pH > 5). Therefore, to carry out the preliminary studies of these pesticides, in all experiments the buffer pH was set at the optimum value found for benalaxyl (pH 7) and different runs were performed at different selector plug lengths. Since the other experimental variables are not critical on enantioresolution, they were also kept constant at the optimum values found for benalaxyl.

Figures 15 and 16 show the electropherograms obtained for each compound at the different CM-β-CD injection times studied. As can be observed, in all cases migration times increased when increasing the selector plug length, which indicates that all compounds interact in a higher or lesser extent with CM-β-CD. Migration times of trichlorphon, the less hydrophobic compound (logP = 0.43), were scarcely modified when increasing CM-β-CD plug length. However, separation of enantiomers was not observed for myclobutanil, propiconazole, tebuconazole and trichlorfon (Fig. 15). This fact indicates that the interaction observed

between these compounds and CM-β-CD is not enantioselective or at least it is not enantioselective enough to achieve separation of enantiomers with the separation technique used and/or under the experimental conditions assayed.

On the other hand, for cyproconazole, hexaconazole and penconazole (Fig. 16) partial separation of enantiomers was observed at the highest selector plug lengths assayed. These results indicate that CM-β-CD can be a suitable chiral selector for these compounds. To achieve better enantioresolution, optimization of the other experimental variables (pH, nature and concentration of the running buffer, concentration of CM-β-CD, applied voltage and separation temperature) should be performed. It must be noted that cyproconazole has two centers of chirality and the separation of both pairs of enantiomers occurred. In the case of hexaconazole, migration times higher than 25 min were obtained with a selector plug length of 20 s. As indicated previously, these large migration times are undesirable for analysis purposes and can electrolyze running buffer solution.

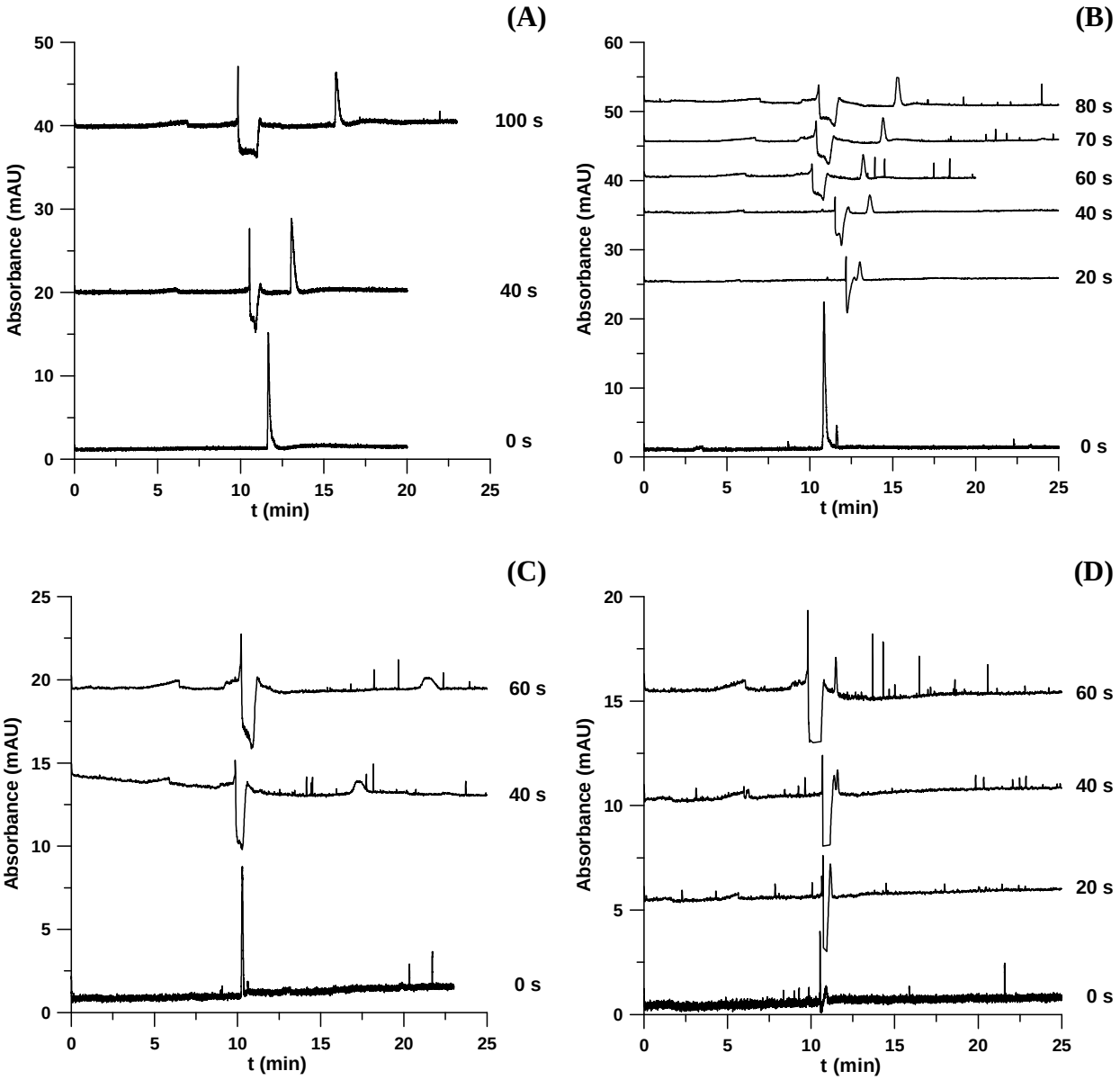


Fig. 15. Effect of CM-β-CD plug length on the enantioresolution of ***(A)*** *myclobutanil;* ***(B)*** *propiconazole;* ***(C)*** *tebuconazole and* ***(D)*** *trichlorphon. CM-β-CD injection times are indicated in the plots left side.*

Experimental conditions: the injected solution was 200 ppm compounds stock solution in methanol-Tris buffer applied at 50 mbar for 3 s (myclobutanil) or 5 s (propiconazole, tebuconazole and trichlorphon); 30 mM CM-β-CD solution applied at 50 mbar; 50 mM Tris as electrophoretic buffer at pH 7; temperature 30°C; voltage 15 kV. UV detection was performed at 220nm.

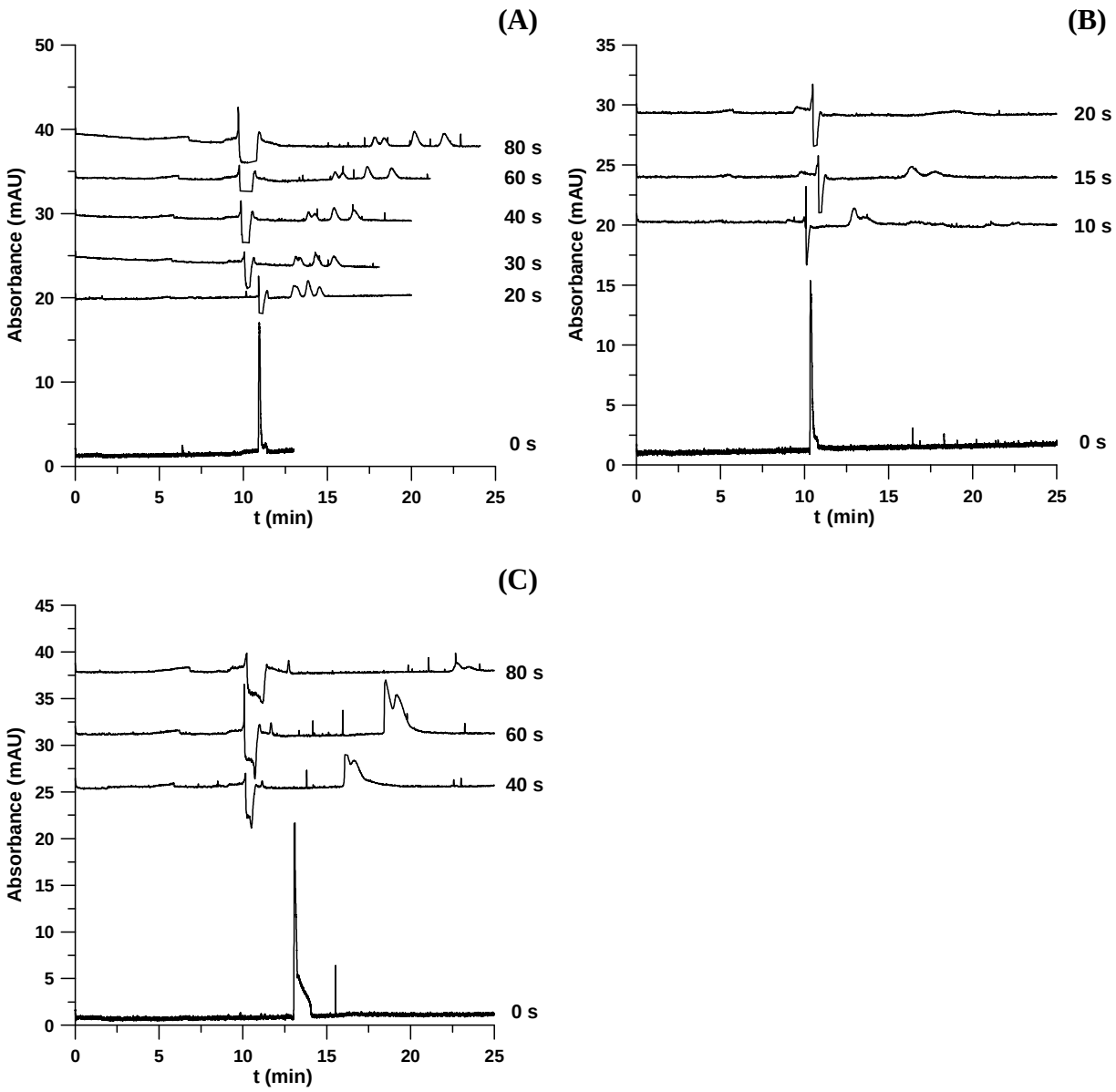


Fig. 16. Effect of CM-β-CD plug length on the enantioresolution of ***(A)*** *cyproconazole;* ***(B)*** *hexaconazole and* ***(C)*** *penconazole. CM-β-CD injection times are indicated in the plots left side.*

Experimental conditions: the injected solution was 200 ppm compounds stock solution in methanol-Tris buffer applied at 50 mbar for 3 s (penconazole) or 5 s (cyproconazole and hexaconazole); 30 mM CM-β-CD solution applied at 50 mbar; 50 mM Tris as electrophoretic buffer at pH 7; temperature 30°C; voltage 15 kV. UV detection was performed at 220 nm.

4. CONCLUSIONS

The preliminary studies on the applicability of EKC (with CD as chiral selectors) to the separation of chiral pesticides suggests that CM-β-CD used as chiral selector allows the enantioresolution of some pesticides like benalaxyl, imazalil, cyproconazole, hexaconazole and penconazole. However, it is not able to separate those from the rest of the studied chiral pesticides (trichlorphon, myclobutanil, tebuconazole, and propiconazole).

In the case of benalaxyl experimental condition to guarantee adequate enantioresolution are: 30 mM solution of CM-β-CD injected for 40 s at 50 mbar, 25 mM Tris buffer at pH 7.0, separation temperature of 30ºC applying a voltage of 15 kV. The most critical parameters affecting enantioresolution were chiral selector plug length and running buffer pH. The effect of other experimental variables can be neglected for further optimization studies of other chiral pesticides.

Those results suggest that EKC in the partial filling mode with CM-β-CD as chiral selector presents an efficient technique to perform further quality control studies of commercial formulations of racemic, enriched-enantiomer or single-enantiomer pesticides as well as in biological activity/toxicity enantiodifferentiation studies.

5. REFERENCES

[1]. www.rsc.org/pdf/books/capelectrosc.pdf;12.11.2008

[2]. http://www.answers.com/topic/capillary-electrophoresis;26.4.2009

[3]. http://www.chem.agilent.com/temp/radEB765/00000071.PDF;18.3.2009

[4]. http://www.beckman.com/resourcecenter/labresources/ce/cedefinitionmodes.asp; 12.11.2008

[5]. Z. Wang, J. Ouyang and W.R.G. Baeyens, J. Chromatogr. B, 2008. 862(1-2): p. 1-14

[6]. H. Ates, D. Mangelings and Y. Vander Heyden, Journal of Pharmaceutical and Biomedical Analysis, 2008. 48(2): p. 288-294

[7]. O. Zerbinati, F. Trotta, C. Giovannoli, C. Baggiani,G. Giraudi, A. Vanni, J. Chromatogr. A, 1998. 810(1-2): p. 193-200

[8]. G. Gübitz and M.G. Schmid, J. Chromatogr. A, 2008. 1204(2): p. 140-156

[9]. http://www.mn-net.com/GCStart/Enantiomers/HYDRODEXcyclodextrinphases/tabid/5719/language/en-US/Default.aspx;26.4.2009

[10]. X. Lin, C. Zhu and A. Ha, J. Chromatogr. A, 2004. 1059(1-2): p. 181-189

[11]. U. Schmitt, M. Ertan, U. Holzgrabe, Electrophoresis, 2004. 25(16): p. 2801–2807

[12]. D. Wistuba , A. Bogdanski , K.L. Larsen, V. Schurig, Electrophoresis, 2006. 27(21): p. 4359–4363

[13]. G.S. Yang, D.M. Chen, Y. Yang, B. Tang, J.J. Gao, H.Y. Aboul-Enein and B. Koppenhoefer, Chromatographia, 2005. 62(7-8): p. 441-445

[14]. Y.P. Zhang, H.J. Noh, S.H. Choi, J.J. Ryoo, K.P. Lee, K. Ohta, C. Fujimoto, J.Y. Jin, T. Takeuchi, Bulletin of the Korean Chemical Society, 2004. 25(3): p. 377-381

[15]. S. Li and G. Vigh, J. Chromatogr. A, 2004. 1051(1-2): p. 95-101

[16]. S. Li, G. Vigh, Electrophoresis, 2004. 25(16): p. 2657–2670

[17]. S. Sanchez-Vindas and G. Vigh, J. Chromatogr. A, 2005. 1068(1): p. 151-158

[18]. M. Brent Busby, G. Vigh, Electrophoresis, 2005. 26(20): p. 3849-3860

[19]. M. Brent Busby, G. Vigh, Electrophoresis, 2005. 26(10): p. 1978–1987

[20]. V. Cucinotta, A. Giuffrida, G. Grasso, G. Maccarrone, M. Messina and G. Vecchio, J. Chromatogr. A, 2007. 1155(2): p. 172-179

[21]. W. Tang, T.T. Ong, I. Wayan Muderawan and S. Choon Ng, Anal. Chim. Acta, 2007. 585(2): p. 227-233

[22]. C. Desiderio, D.V. Rossetti, F. Perri, B. Giardina, I. Messana and M. Castagnola, J. Chromatogr. B, 2008. 875(1): p. 280-287

[23]. L. Valtcheva, J. Mohammed, G. Pettersson, S. Hjerten, J. Chromatogr., 1993. 638(2): p. 263-267

[24]. Shahab A. Shamsi, Electrophoresis, 2002. 23(22-23): p. 4036–4051

[25]. Shahab A. Shamsi, Blair E. Miller, Electrophoresis, 2004. 25(23-24**)**: p. 3927–3961

[26]. B. Chankvetadze, TrAC Trends in Analytical Chemistry, 1999. 18(7): p. 485-498

[27]. A. Brown, C. Morales and F.A. Gomez, Talanta, 2008. 74(4): p. 605-612

[28]. A. Brown, R. Desharnais, B.C. Roy, S. Malik and F.A. Gomez, Anal. Chim. Acta, 2005. 540(2): p. 403-410

[29]. C. Desiderio, D.V. Rosseti, F. Perri, B. Giardina, I. Messana, M. Castagnola, J. Chromatogr. B, 2008

[30]. S. Rudaz, S. Cherkaoui, J.Y. Gauvrit, P. Lantéri, J.L. Veuthey, Electrophoresis, 2001. 22(15): p. 3316-3326

[31]. S. Grard, P. Morin, M. Dreux, J.P. Ribet, J. Chromatogr. A, 2001. 926 : p. 3-10

[32]. S. Cherkaoui, S. Rudaz, E. Varesio, J.L. Veuthey, Electrophoresis, 2001. 22(15) : p. 3308-3315

[33]. S. Rudaz, S. Cherkaoui, P. Dayer, S. Fanali, J.L. Veuthey, J. Chromatogr. A, 1999. 868(2000) : p. 295-303

[34]. A. Amini, U. Paulsen-Sörman, Electrophoresis, 1997. 18(6): p. 1019-1025

[35]. http://www.epa.gov/pesticides/about/types.htm;22.11.2008

[36]. J.M. Battershill, P.M. Edwards, M.K. Jonhson, Food and Chemical Toxicology, 2004. 42(8): p. 1279-1285

[37]. A.W. Garrison, Environmental Science and Technology, 2006. 40(1): p. 16-23

[38]. C. Xu, J. Wang, W. Liu, G.D. Sheng, Y. Tu, and Y. Ma, Environmental Toxicology and Chemistry, 2008. 27(1): p. 174–181

[39]. K. Wiberg, T. Harner, J.L. Wideman, T.F. Bidleman, Chemosphere, 2001. 45(6-7): p. 843-848

[40]. A.W. Garrison, P. Schmitt-Kopplin, J.K. Avants, Methods in molecular biology (Clifton N.J.), 2008. 384: p. 157-170

[41]. L. Huang, J. Lin, L. Xu, G. Chen, Electrophoresis, 2007. 28(15): p. 2758-2764

[42]. C. Klein, R.J. Schneider, M.T. Meyer, D.S. Aga, Chemosphere, 2006. 62(10): p. 1591-1599

[43]. A. Santilio, M. D'Amato, L. Cataldi, A. Sorbo, R. Dommarco, Journal of Capillary Electrophoresis and Microchip Technology, 2005. 9(5-6): p. 79-84

[44]. J.L. Jarman, W.J. Jones, L.A. Howell, A.W. Garrison, Journal of Agricultural and Food Chemistry, 2005. 53(16): p. 6175-6182

[45]. C. García-Ruiz, G. Álvarez-Llamas, A. Puerta, E. Blanco, A. Sanz-Medel, Ma.L. Marina, Anal. Chim. Acta, 2005. 543(1-2): p. 77-83

[46]. R. Bhushan, Biomed. Chromatogr., 2005. 19: p. 413-414

[47]. J. Hernandez-Borges, Electrophoresis, 2005. 26: p. 3799-3813

[48]. X. Shi, P. Liang, D. Song, X. Gao, R. Fu, Fenxi Huaxue, 2004. 32(11): p. 1421-1425

[49]. G. Gübitz, M.G. Schmid, Biopharmaceutics and Drug Disposition, 2001. 22(7-8): p. 291-336

[50]. Y.S. Wu, H.K. Lee, S.F.Y. Li, J. Chromatogr. A, 2001. 912(1): p. 171-179

[51]. F. Mezinger, P. Schmitt-Kopplin, M. Frommberger, D. Freitag, A. Kettrup, Fresenius J. Anal. Chem., 2001. 371 : p. 25-34

[52]. M. Zhang, Z. El Rassi, Electrophoresis, 2000. 21(15) : p. 3126-3134

[53]. O. Zerbinati, F. Trotta, C. Giovannoli, J. Chromatogr. A, 2000. 875(1-2): p. 423-430

[54]. D.S. Aga, S. Heberle, D. Rentsch, R. Hany, S.R. Müller, Environmental Science and Technology, 1999. 33(19): p. 3462-3468

[55]. D. Shea, K.V. Penmetsa, R.B. Leidy, Journal of AOAC International, 1999. 82(6): p. 1550-1561

[56]. M. Miura, Y. Terashita, K. Funazo, M. Tanaka, J. Chromatogr. A, 1999. 846(1-2): p. 359-367

[57]. J.L. Haynes III, S.A. Shamsi, F. O'Keefe, R. Darcey, I.M. Warner, J. Chromatogr. A, 1998. 803(1-2): p. 261-271

[58]. O. Zerbinati, F. Trotta, C. Giovannoli, C. Baggiani, G. Giraudi, A.J. Vanni, J. Chromatogr. A, 1998. 810(1-2): p. 193-200

[59]. K. Otsuka, C.J. Smith, J. Grainger, J.R. Barr, D.G. Patterson Jr., N. Tanaka, S. Terabe, J. Chromatogr. A, 1998. 817(1-2): p. 75-81

[60]. P. Schmitt, A.W. Garrison, D. Freitag, A. Kettrup, J. Chromatogr. A, 1997. 792(1-2): p. 419-429

[61]. K.V. Penmetsa, R.B. Leidy, D. Shea, J. Chromatogr. A, 1997. 790(1-2): p. 225-234

[62]. Y.Z. Hsieh, H.Y. Huang, J. Chromatogr. A, 1996. 745(1-2): p. 217-223

[63]. A.W. Garrison, P. Schmitt, D. Martens, A. Kettrup, Environmental Science and Technology, 1996. 30(8): p. 2449-2455

[64]. http://sitem.herts.ac.uk/aeru/footprint/es/Reports/;18.3.2009

[65]. D.N. Heiger, *High Performance Capillary Electrophoresis: An Introduction, Hewlett-Packard Company, France*, 1992

Abstract

In this diploma thesis, a preliminary study on the applicability of EKC-PF (with CD as chiral selector) to the separation of chiral pesticides was performed. In introduction, theoretical informations are presented concerning electrophoresis instrumentation and theory as well as different electrophoresis modes. Then, enantiomeric separation is introduced through cyclodextrins and possibilities of partial filling mode to finally terminate with a definition of chiral pesticides and some information about their enantioseparation. Concretely, in the experimental part, the evaluation of enantioresolution of several chiral pesticides (trichlorphon, cyproconazole, hexaconazole, imazalil, myclobutanil, penconazole, tebuconazole, propiconazole, and benalaxyl) using CM-β-CD as chiral selector was performed. First of all, a univariate optimization of variables (CM-β-CD injection time, voltage, temperature, pH, and buffer nature and concentration) was carried out for benalaxyl. Finally a screening, involving the effect of the CM-β-CD injection time, was performed for the other pesticides to check the potential of this CD as chiral selector.

Abstrakt

V této diplomové práci je představena předběžná studie využití EKC-PF (s cyklodextrinem, jako chirálním selektorem) pro separaci chirálních pesticidů. Úvodem jsou prezentovány teoretické informace týkající se přístrojového vybavení, teorie elektroforézy jakož i různých elektroforetických technik. Dále je zmíněna problematika chirálních separací pomocí CE, využití cyklodextrinů jako chirálních selektorů v CE a možnosti techniky částečného plnění kapiláry. Nakonec jsou definovány chirální pesticidy a uvedeny informace o separaci jejich enantiomerů. Experimentální část se zabývá hodnocením separace enantiomerů některých pesticidů (trichlorphon, cyprokonazol, hexakonazol, imazalil, myklobutanil, penkonazol, tebukonazol, propikonazol, a benalaxyl) pomocí CM-β-CD, jako chirálního selektoru. Nejprve byla provedena univariantní optimalizace separačních podmínek (doba nástřiku CM-β-CD, napětí, teplota, pH, povaha a koncentrace elektrolytu) u benalaxylu. Dále byl realizován screening efektu nástřikového času CM-β-CD na rozlišení a migrační časy u ostatních pesticidů se záměrem zjistit potenciál tohoto CD jako chirálního selektoru pro enantioselektivní separaci tohoto typu analytů.