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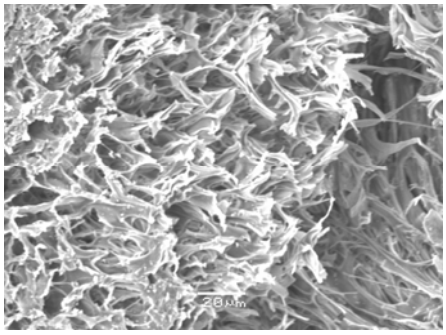

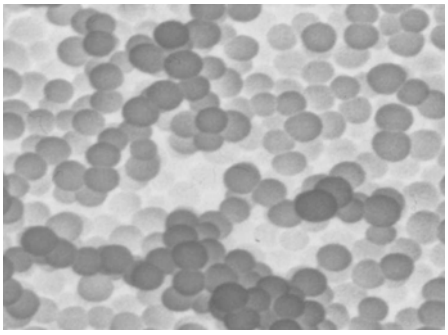

Removal of 17β-estradiol from Water using Molecularly Imprinted Polyethersulfone Microspheres

Paula Sequeira dos Ramos Silva

Dissertação para a obtenção do Grau de Mestre em

Engenharia Biológica

Júri

Presidente:	Professor Luís Joaquim Pina da Fonseca
Orientadores:	Professora Helena Maria Rodrigues Vasconcelos Pinheiro
	Professor Bo Mattiasson
	Doutor Mathieu Le Noir
Vogal:	Professora Marília Clemente Velez Mateus

Setembro de 2007

Resumo

Por separação líquido-líquido foram sintetizadas microesferas de polietersulfona (PES) e dióxido de titânio (TiO_2) obtidas por *imprinting* molecular (MIM), tendo por objectivo a remoção de 17β-estradiol (E2) de soluções aquosas. As novas microesferas apresentaram uma estrutura interna porosa e uma camada externa opaca. As MIM, sintetizadas tendo E2 como "molde", foram comparadas com partículas sintetizadas pelo mesmo método mas não sujeitas à técnica de *imprinting* (NIM). Foram realizados estudos de adsorção em *batch* com MIM e NIM sujeitando as partículas esféricas ao contacto com várias soluções aquosas contendo E2. Com o aumento do volume de solução, evidenciou-se a especificidade das MIM relativamente às NIM. Prepararam-se duas colunas de adsorção usando MIM e NIM como leito, para a remoção de E2 presente em amostras de água residual sintética e real. Após seis passagens na coluna com MIM, foram obtidas percentagens totais de remoção de 47% e 85% de soluções aquosas com 0,5 mg E2/L, em recirculação a 15 mL/min e 1 mL/min, respectivamente. Em comparação, obtiveram-se remoções de 27% e 76% usando a coluna com NIM nas mesmas condições experimentais. Ao melhorar-se a mistura na coluna contendo MIM, foi alcançada uma remoção de 60% de uma solução aquosa 0,5 mg E2/L a recircular no sistema a 15 mL/min. Este arranjo foi depois aplicado no tratamento de amostras de água residual real, suplementada ou não com 1 μg E2/L. Não foi possível quantificar o total de E2 removido nestas amostras. Foi, contudo, confirmada a sua presença natural na amostra não suplementada. Entre cada experiência MIM e NIM foram sujeitas a regeneração utilizando para o efeito uma mistura de metanol:ácido acético (4:1 v/v). Estes dois tipos de partículas foram facilmente regeneradas e reutilizadas.

Palavras-chave: disruptores endócrinos; 17β-estradiol; *imprinting* molecular; separação líquido-líquido microesferas; extracção em fase sólida

Abstract

Molecularly imprinted polyethersulfone (PES): titanium dioxide (TiO_2) microspheres (MIM) were synthesised by a liquid–liquid phase separation technique for the removal of 17β-estradiol (E2) from aqueous solutions. The original imprinted microspheres had a porous structure with a opaque layer. MIM synthesised with E2 as template were compared to non-imprinted polyethersulfone (PES): titanium dioxide (TiO_2) microspheres (NIM) synthesised in the same conditions but without the template. Removal experiments in batch with MIM and NIM were carried out by applying the microspheres to various E2 aqueous solutions. With increasing volumes the specificity of MIM to E2, when compared with NIM, was accentuated. MIM and NIM were used to prepare adsorption columns for the extraction of E2 from synthetic and wastewater samples. After six runs, removals of 47% and 85% were reached by a MIM packed column from a 0,5 mg E2/L aqueous solutions with flows of 15 mL/min and 1 mL/min, respectively. By comparison, E2 recoveries of 27% and 76% were achieved using NIM under the same experimental conditions. When mixing was improved through the MIM column, a removal of 60% was attained from a 0,5 mg E2/L aqueous solution and a flow of 15 mL/min. This system was then used for the recovery of E2 from wastewater samples non-spiked and spiked with 1 µg E2/L. It was not possible to quantify the amount of E2 recovered. However the natural presence of this contaminant in non-spiked samples was confirmed. MIM and NIM were easily regenerated after each experiment with MeOH:Aa (4:1 v/v) solvent and reused.

Keywords: endocrine disruptors; 17β-estradiol; molecular imprinting; liquid-liquid phase separation; microspheres; solid phase extraction

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List of Abbreviations

A

Aa	Acetic acid

B

BET	Brunauer-Emmett-Teller
BPA	Bisphenol A

C

C18	Carbon-18

D

DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DET	Dichlordiphenylethylene
DMAc	Dimethyl acetamide

E

E2	17β-estradiol
EDCs	Endocrine disrupting chemicals
EGDMA	Ethylene glycol dimethacrylate
EPA	Environmental Protection Agency

G

GAC	Activated carbon
GC	Gas chromatography

H

H_c	Henry's law constant
HPLC	High performance liquid chromatography

K

K_{ow}	Partition coefficient

L

LC	Liquid chromatography

M

MAA	Methacrylic acid
MDAA	N-methyl-N,N-diallylamine
MeOH	Methanol
MF	Microfiltration

MIM	Molecularly imprinted microspheres
MIP	Molecularly imprinted polymers
MS	Mass spectrophotometry

N

NIN	Non-molecularly imprinted microspheres
NIP	Non-molecularly imprinted polymers
NF	Nanofiltration

P

PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PES	Polyethersulfone

R

RO	Reverse osmosis

S

SEM	Scanning Electron Micrograph
SFM	Scanning force microscopy
SPE	Solid phase extraction

T

TBT	Tributyl tin
TiO_2	Titanium dioxide

U

UF	Ultrafiltration
UV	Ultraviolet

W

WWTP	Wastewater treatment plant

1. Introduction

Many environmental pollutants, coming from various sources, can act as endocrine disrupting chemicals (EDCs) and may affect the normal functions of the endocrine system causing adverse effects on exposed wildlife and humans. The impacts restricted to individuals may subsequently extend to effects on whole populations and the community.

The primary cases of endocrine disruption in humans appeared during the 1950s and 1960s. At that time the pesticide Dichloro-Diphenyl-Trichloroethane (DDT), a very persistent compound used worldwide after the Second World War, was shown to be estrogenic and accumulating at the top of the food chain affecting the reproductive system in mammals and birds (Birkett, 2003). Another synthetic estrogen, the drug diethylstilbestrol (DES), was prescribed to five million of pregnant women to block spontaneous abortion before being banned in the early 1970s. It was discovered that DES affected the development of the reproductive system and cause vaginal cancer to the born children (Birkett, 2003). About 40 years later, Kauffman et al. got more conclusions about DES effects looking at the births of the so-called DES daughters: women were more likely to have premature births and spontaneous abortions than unexposed women. Their daughters and sons were affected with severe reproductive tract deformities, declines in sperm count, alterations in behaviour and a greatly elevated risk of cancer (Kaufman et al., 2000). Those conclusions suggest the long-term reproductive effects in humans exposed *in utero*. However, the evidence of human impacts of EDCs also appear from several industrial accidents involving relatively high exposures, food contamination or exposure to pesticides and pollutants released in the environment (Colborn et al., 1997). For instance, recent studies have shown that semen quality is decreasing in men exposed to pesticides and traffic pollutants (Swan et al., 2003) and that there is a strong correlation between the exposure to household pesticides and the risk of childhood leukemia (Ma et al., 2002). Also intakes of iodine, a food additive, were related with increased prevalence of autoimmune thyroiditis (the most common thyroid disease in Norway).

In wildlife, the endocrine disrupters' effects are also mostly related with reproductive and developmental abnormalities. Perhaps the most widely cited example comes from the use of tributyl tin (TBT) as a component of antifouling paint on ships' hulls: the evidences of female mollusks masculinisation and shell thickening in oysters of French waters in the 1980s imposed restrictions in the use of organotin compounds (Alzeiu, 1991). The increase of plasma levels of vitellogenin (an estrogen dependent plasma protein) in British fish living below wastewater treatment plants was induced by the presence of steroids estrogens and alkyphenols in effluent, leading to the feminization of male fish (Jobling et al., 1998). Further studies have shown that the natural hormone, 17β-estradiol, and the synthetic birth control pharmaceutical, 17α-ethynylestradiol, are the most potent estrogens and induce changes in fish reproduction at low concentrations present in some wastewater treatment plants (Jobling et al., 1998; Routledge et al., 1998). Indeed, a study by Lahnsteiner et al. (2006) with male rainbow trout exposed to 0.5–2.5 ng l^{-1} 17β-estradiol during the spawning season showed that concentrations ≥1 ng l^{-1} were significantly reducing the semen volume obtained per male. In addition, Larsson et al. (1999) and Routledge et al. (1998) observed that 17α-ethynylestradiol can be a potential danger to fish and other aquatic organisms, even present at concentrations of 0.1–10 ng/L.

In fact, with the large number of chemicals introduced in the market every year, it will be impossible to recognize every emerging pollutant and to understand their environmental fates and effects. Consequently, new removal methods that can target a great diversity of potential toxic substances at very low concentrations are now needed.

In this perspective, this project presents a new method for the removal of trace contaminants by exploring a novel technique of molecular imprinting. The main objectives of this work included the synthesis of 17β-estradiol (E2) molecularly imprinted polyethersulfone (PES): titanium dioxide (TiO_2) microspheres (MIM) by a liquid–liquid phase separation technique followed by studies of 17β-estradiol (E2) removal from aqueous solutions in a column loaded with the molecularly imprinted particles by using a fluidized bed concept. The idea was to create a set-up without pressure or clogging problems and potential to be designed in a larger scale for industrial applications. Also some batch studies were

carried out prior to the column experiments, for the characterization of the novel molecularly imprinted microspheres (MIM). 17β-estradiol was chosen as model contaminant for being one of the most potent endocrine disrupters commonly found in wastewater (Jobling et al., 1998).

2. Endocrine Disrupters

2.1. The Endocrine System

With the nervous system and the immune system, the endocrine system is one of the three most important regulatory systems of an organism. It is crucial to both plants and animals because it is responsible for growth, reproduction, metabolism and thus helps maintain the individual's physiological balance (EPA, 1997). The endocrine system consists of several glands in different areas of the body (Figure 1) that produce chemical messengers, with different functions, called hormones. The glands release the hormones directly into the bloodstream, where they are transported to the target organs throughout the entire body and used to invoke a natural response (Greenstein, 1994).

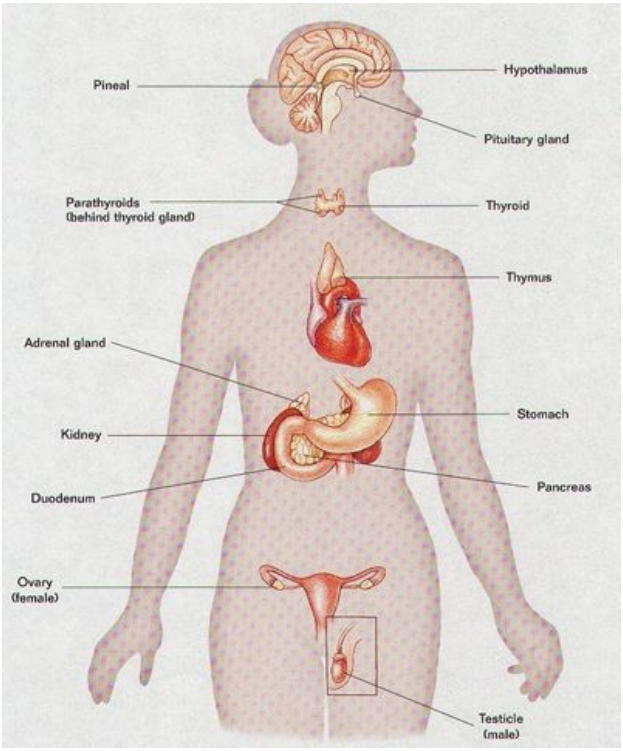


Figure 1 - Endocrine glands and their location in the human body. Source: www.epa.gov/scipoly/oscpendo/images/endocrine

Hormones are grouped into two major classes based on their structure: Non steroids and steroids. Non steroids are amino-acid-derived hormones, hydrophilic and, therefore, cannot cross the cell membrane. They act by binding to receptors on the surface of cells. The binding alerts a second messenger molecule inside the cell that activates enzymes and other cellular proteins or influences gene expression. Steroid hormones are hydrophobic molecules made from cholesterol. They are secreted by three “steroid glands” — the adrenal cortex, testicles, and ovaries (Figure 1) — and during pregnancy by the placenta.They can directly diffuse through the plasma membrane of a cell to bind to special receptors in the cytoplasm and/or the nucleus. Once the steroid hormones and receptors are bound together, the hormone receptor complex binds to hormone response elements in the DNA and acts as a transcription factor activating or inhibiting the transcription of the target genes (Kimball, 2005) (Figure 2).

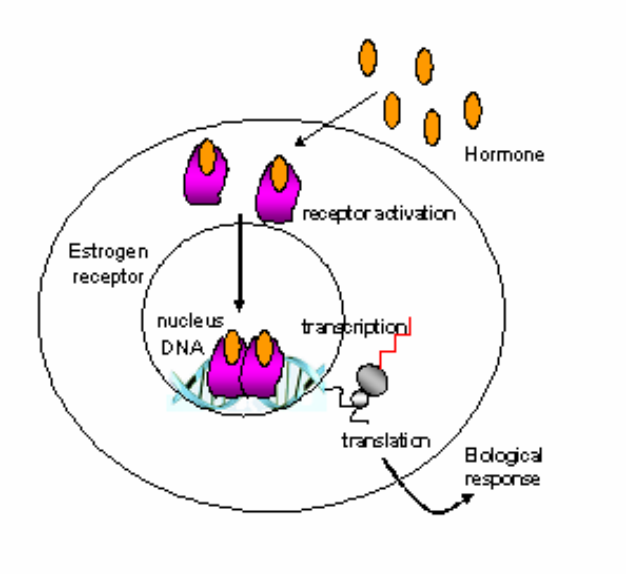


Figure 2 - Mode of action on the cellular level of steroid hormones

The three main classes of steroid hormones are androgens, estrogens and progestagens with the most important human examples from each class being testosterone, estradiol and progesterone. The androgens (testosterone, dehydroepiandrosterone and androstenedione) are male sex hormones primarily responsible for the development and maintenance of reproductive function and stimulation of the secondary sex characteristics in male. Estrogens (estradiol, estrone and estriol) are one of the two types of female sex hormones. They are secreted mainly by the ovaries and in smaller amounts by the adrenal glands and (in men) by the testicles. Functioning similarly to androgens, the estrogens promote the development of the primary and secondary female sex characteristics; they also stimulate linear growth and skeletal maturation. Finally, progestagens (progesterone) are the other type of female sex hormone and are named for their role in maintaining pregnancy (supports gestation), female menstrual cycle and embryogenesis of humans and other species. They can be thought of as a hormone balancer, particularly of estrogens (Ying et al., 2002).

2.2. Endocrine Disrupters

The endocrine disrupters are compounds that interfere with the function of the endocrine system and may cause adverse health effects such as obesity, diabetes and decalcification of the bones. They can be natural (hormones) or artificial substances and may disturb directly or indirectly the hormonal system of organisms. Table 1 presents their main mechanisms of action. Nevertheless, the specific mechanisms by which substances disrupt endocrine systems are very complex, and not yet entirely understood (Pocar et al., 2003).

Table 1 - Endocrine disrupters mechanisms of action

Type	Mechanism	Examples
Agonistic effect	The compound acts as a natural estrogen, binding and activating the estrogen receptor, (Figure 3 A)	e.g., imidazole and triazole fungicides on oocyte maturation in rainbow trout (*Oncorhynchus mykiss*) (Monod et al., 2004)
Antagonistic effect	The compound binds to an estrogen-receptor but no activation occurs (therefore acting as a hormone blocker), (Figure 3 B)	e.g., dichlordiphenylethylene (DDE) (byproduct of dichlorodiphenyltrichloroethane (DDT)) causes developmental problems in male by blocking testosterone (Birkett, 2003)
Abnormal response	Disruption of production, transport, metabolism, or secretion of natural hormones and disruption of production and function of receptors, (Figure 3 C)	e.g., dioxins and furans: presents in food, act through a hormone-like process interfering in the natural response neither by mimicking nor blocking

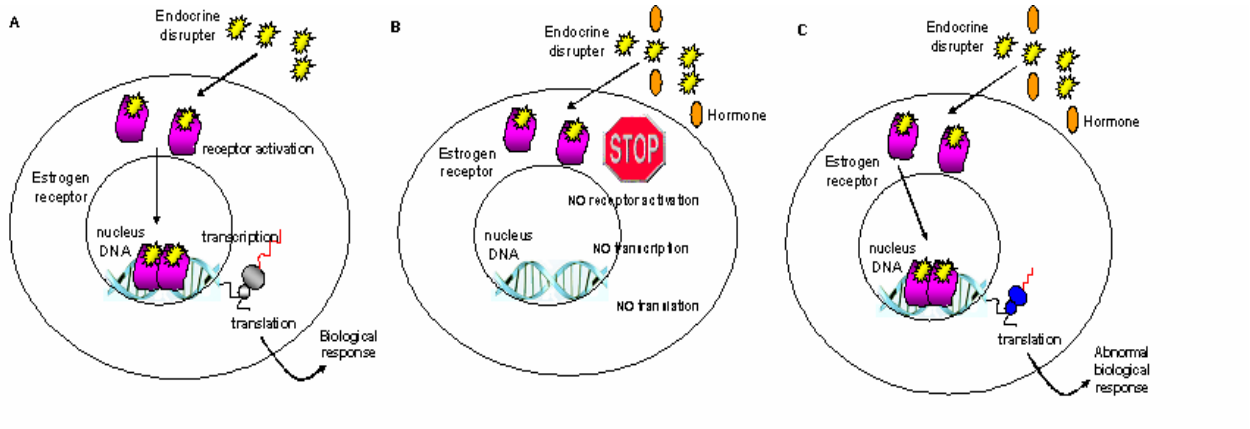


Figure 3 – Mode of action of endocrine disrupters. *Agonistic effect*: the mimics elicit the same chemical reaction as natural hormones (A). *Antagonistic effect*: the blockers bind to the cell receptors and prevent naturally occurring hormones from affecting cells in the usual way (B). The activation elicit unusual or abnormal reactions in the cells (C)

The effects of endocrine disrupters are not limited to male or female sex hormones; they also affect other endocrine glands that play a role in growth, development and reproduction (Environment Canada, 2000).

According to a European Union study, 118 substances were classified as potential endocrine disrupters including both natural and man-made substances (COM, 2001).

Natural substances are natural hormones which include:

- Steroid hormones (e.g., estrone, progesterone and testosterone found naturally in the body of animals and humans),
- phytoestrogens, chemicals produced by plants that act like estrogens in animal/human cells and bodies,

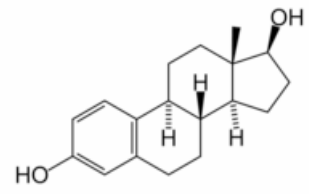
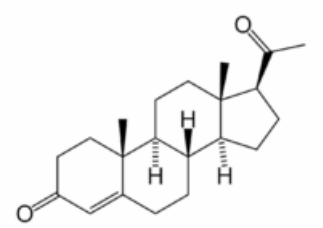
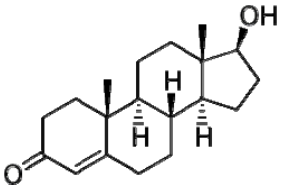
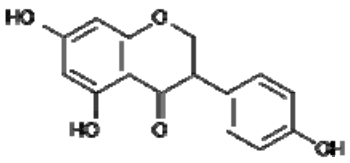
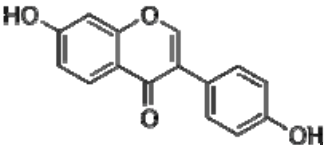
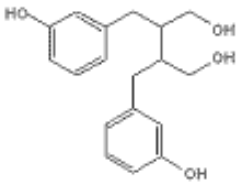
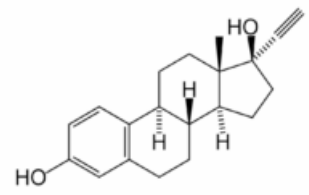
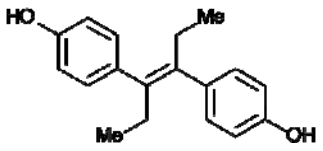
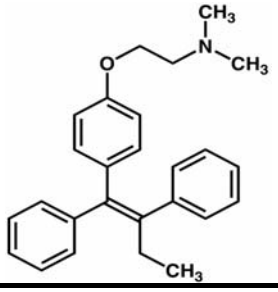
Man-made substances include:

- synthetically-produced hormones, that are identical to the natural hormones and have been designed to intentionally interfere and modulate the endocrine system (e.g., oral contraceptive, some animal feed additives, hormonal replacement treatment),

- chemicals designed for uses in industry and agriculture (e.g., surfactants (nonylphenol), pesticides (dieldrin, DDT), polyaromatic compounds (PAHs, PCBs), organic oxygen compounds (bisphenol A)).

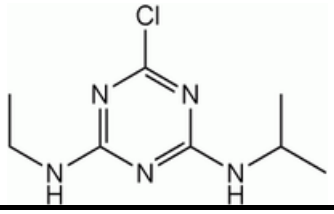
Considering the complexity of the endocrine system, it is not surprising that the number of substances believed to act as endocrine disrupters is wide and varied. Some suspected endocrine disrupters belonging to the long list of chemicals classified as potential EDCs are presented in the Table 2, which shows the diversity of their molecular structure.

Table 2 - Structural diversity of some chemicals classified as potential endocrine disrupters

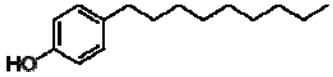
Steroid Hormones:		
17β-estradiol (E2) 	Progesterone 	Testosterone 
Phytoestrogens:		
Genistein 	Daidzein 	Enterodiol 
Synthetic Hormones:		
17α-ethynilestradiol (EE2) 	Diethylstilbestrol (DES) 	Tamoxifen 

Continuation:		
Pesticide:	Surfactant:	Dioxin:

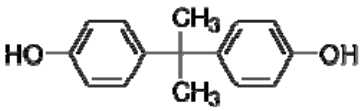
Atrazine



Nonylphenol



Bisphenol A



Therefore, it is impossible to determine whether a chemical is an endocrine disrupter or not by merely looking at its chemical structure. Currently, the process for deciding if a chemical is an environmental endocrine disruptor is to determine its effects on the endocrine systems of humans and other animals (Chapter 2.3). However, about 1000-2000 new chemicals are introduced into the market place every year and most of them with unknown effects on health (Younes, 1999). Therefore, monitoring EDCs in the environment is depending on effective methodologies, which will accurately and reproducibly quantify EDCs at the concentrations occurring in the different environmental media.

2.3. Methods for determination of Endocrine Disrupters

The emerging of endocrine disrupting substances released in the environment has stimulated the development of methods to determinate EDCs in the recent years. These methods include bioanalytical and chemical techniques with the required sensitivity to measure estrogenic activity. To select between them, it is necessary to consider what is to be determined and for what purpose the analysis is undertaken (Birkett, 2003).

2.3.1. Bioanalytical Methods

As EDCs are potent at extremely low concentrations (Auriol et al., 2006; Birkett, 2003; Ternes et al., 1999a; Ternes et al., 1999b), high sensitivity is required in measurement of estrogenic activity and this has led to the development of bioassays to detect estrogenic activity below the present limits of detection. *In vivo* methods are used to evaluate the impact of EDCs directly on the endocrine system. The most widely used assay to determine the estrogenicity of a compound is the rodent uterotrophic assay which is based on the ability of chemicals to stimulate uterine growth of immature female rats or mice (Beresford et al., 2000). *In vitro* assays have been widely used in order to measure the estrogenic activity of single compounds or complex environmental samples (Nelson et al., 2007; Soto et al., 2004). These assays include reporter gene assays which use yeast or cell lines to measure transcriptional activity of a "reporter" gene upstream of a hormone responsive gene (Gaido et al., 1997), cell proliferation assays such as the E-screen assay (Soto et al., 1995) and competitive binding assays that assess binding of a chemical with hormone receptors (Zacharewski, 1997). The advantage of *in vitro* assays over *in vivo* assays include lower costs and time consumption as well as avoiding ethical issues associated with studies in animals (Katzenellenbogen, 1995; Snyder et al., 2003). In addition, *in vitro* tests are more often used to assess the estrogenic potency of complex environmental matrices such as effluents of wastewater treatment plants, surface waters or industrial effluents (Desbrow et al., 1998; Witters et al., 2001). However, *in vitro* assays do not always reliably predict the results of *in vivo* assays because they represent only a part of the metabolic system present in all animals and may not take into account the effect of bioconcentration (Routledge and Sumpter, 1997). Furthermore, to perform in vitro tests, an extraction procedure of a real sample is usually required, introducing additional sources of error into the measurements (Le Noir et al., 2007a), and *in vivo* tests were shown to be 10-100 times more sensitive (Van den Belt et al., 2004). Therefore a combination of "*in vitro-in vivo*" studies seems to be a more precise screening and testing strategy (EDSTAC, 2000). Environmental samples should therefore also be tested in *in vivo* studies, which are moreover essential for linking exposure to biologically relevant effects, in order to accurately assess

the potential estrogenic activity of any compound or complex mixture (Birkett, 2003; Zacharewski, 1997).

2.3.2. Chemical Methods

One of the reasons why EDCs is of recent concern is that the levels present in the environment are extremely low, down to the range of ng/l or µg/l, and this was earlier below the detection limit for analysers. However, modern techniques make it possible to detect these low levels and a vast collection of chemical methods is available because the structure and the physicochemical properties of EDCs are so diverse. Also, the numerous interfering compounds in wastewater treatment plants (WWTP) effluents, the major source of EDCs, make the trace quantification of a particular compound delicate. Consequently, extraction techniques (Chapter 3.6), cleanup and sophisticated instrumentation are required to analyze EDCs (Birkett, 2003), although a direct quantification of these compounds is possible. Most of the chemical methods use solid phase extraction (SPE) followed by instrumental analyses using mainly liquid chromatography with mass spectrophotometric detection (LC-MS), gas chromatography with mass spectrophotometric detection (GC-MS), high performance liquid chromatography with fluorescence detection (HPLC-fluorescence) or ultraviolet detection (HPLC-UV) and immunoassays. However, with the numerous broad varieties of chemicals released every year into the environment, it is economically impossible to identify and quantify all these components. In addition, chemical methods do not give any information about the estrogenic potency of those compounds. Bioanalytical and chemical methods possess advantages and disadvantages specific to each approach (Figure 4). Hence, the assessment of estrogenic activity in complex environmental mixtures requires a combination of chemical analysis and specific bioassays (Petrovic et al., 2003).

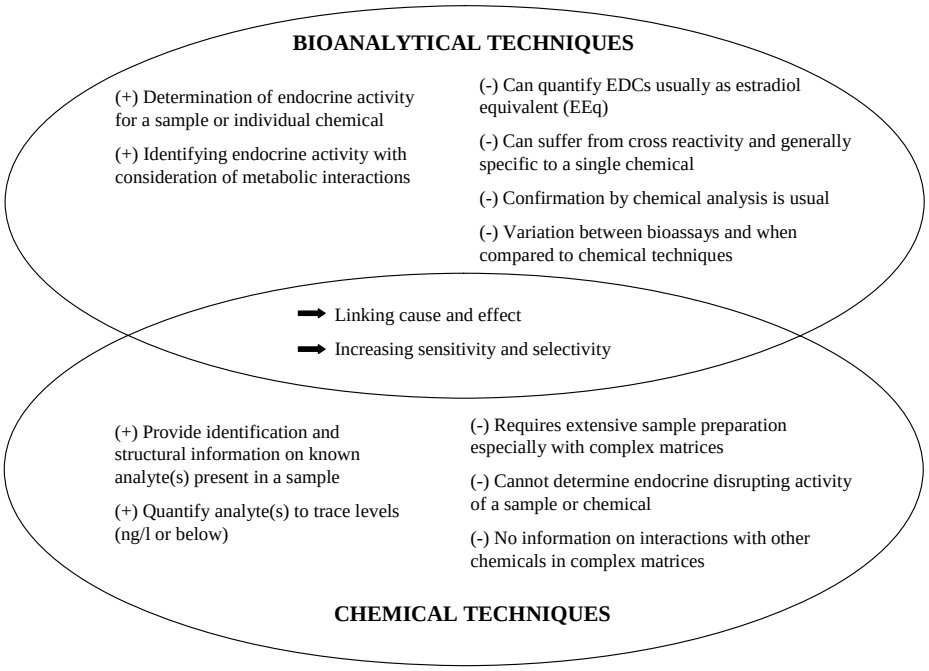


Figure 4 - Main advantages and disadvantages to EDCs determination using bioanalytical and chemical techniques (adapted from Gomes, R.L., Scrimshaw, M.D. and Lester, J.N., 2003. Determination of endocrine disrupters in sewage treatment and receiving waters. TrAC Trends in Analytical Chemistry, 22(10): 697-707)

2.4. 17β-estradiol

2.4.1. The Compound

E2 is a natural hormone that influences the development and maintenance of the female sex characteristics and the maturation and function of the sex organs. This hormone is also present in males, but in smaller amounts. E2 can be also connected to the neuroendocrine system and bone structure and may promote cancer in target tissues (Lee and Lee, 1996). Like all steroids, E2 has the same cyclopentane-*o*-perhydrophenanthrene ring as their parent compound, cholesterol (Birkett, 2003). The basic ring structure consists of three hexagonal rings and one pentagonal ring. Like other steroids, E2 is characterized by a phenolic ring. Additionally, it has also a hydroxyl group placed above the plane of the molecule i.e., in the "β" position on the C17 (Figure 5).

E2 was chosen as a model contaminant for being considered as the most active estrogen and its relative potency is even 10^4 to 10^6 times that of some alkylphenols and pesticides (Bonefeld-

Jorgensen et al., 2005; Jobling et al., 1998). Indeed, estrogenic activity for other compounds is measured having E2 as reference (Zava et al., 1997).

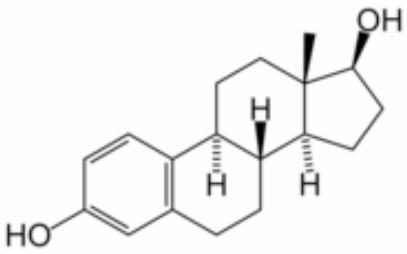
Molecular Formula: $C_{18}H_{24}O_2$	
Molecular weight: 272.38	
Chemical Standard Conditions: white powder	
Solubility in Water: 3,6 mg/L[(a)]	
Solubility in organic solvents: soluble in most organic solvents	
pKa: 10.4[(b)]	
Partition Coefficient (Log K_{ow}): 4,01[(c)]	
Boiling point: 180 ºC[(d)]	
Henry's Law Constant: 6.3×10^{-12} atm. m^3/mol[(e)]	
Vapor pressure: $2,3x10^{-10}$ mmHg[(f)]	

Figure 5 – Structure of E2 and physicochemical properties. a- Hakk et al., 2005;b- Nghiem et al., 2004; c- Lai et al., 2000; d – Grady et al., 1973; e- Lai et al., 2002b; f- Lai et al., 2000

2.4.2. Origin, Occurrence and Fate

All humans as well as animals can excrete steroid hormones from their bodies, which end up in the environment through sewage discharge and animal waste disposal. Like other EDCs, E2 is mostly detected in wastewater (Ying and Kookana, 2005) (Table 3) and is precisely their potency at extremely low level that makes their presence in the environment so troublesome.

The E2 excretion in the urine represent its main natural source in the environment: a woman can excrete 2-12 µg E2/day while a men excrete 1,6 µg E2/day. For pregnant women the daily excretion can go up to 259 µg (Johnson et al., 2000). Even if other xenoestrogens can be found in much higher concentration they are not as estrogenically active as 17β-estradiol (Korner et al., 2000). Estrone, a metabolite of 17β-estradiol, is observed as having the highest effluent concentration, explained by the fact that is the first metabolite of 17β-estradiol degradation (Johnson and Sumpter, 2001).

After a wastewater treatment plant process, the effluent is discharged to the aquatic environment including the surface water (streams, rivers and estuaries) and groundwater. The fate and transport of a contaminant in aquatic environment is controlled by its physical and chemical properties. Two of the most important properties to be considered are the octanol/water partition coefficient (K_{ow}) and aqueous solubility which influence the sorption and the partitioning of a compound. A log K_{ow} below 2.5 shows a low sorption potential, whereas a log K_{ow} greater than 4 illustrates a high sorption potential (Birkett, 2003). The Henry's law constant (H_c) gives the volatilization potential of a compound and it has been suggested that compound with an H_c above 10^{-3} atm.m^3/mol can be removed by volatilization (Wild and Jones, 1989). From the physicochemical properties of E2 (Figure 5), we conclude that E2 is a hydrophobic organic compound of low volatility and low aqueous solubility. Therefore it is expected that natural processes of sorption on soil or sediment will be important factors in reducing E2 aqueous phase concentrations. The compound does not bioaccumulate but is, from the sources mentioned above, constantly entering the aquatic environment (Tyler et al., 1998). Besides the physicochemical properties, the changes of temperature, microbial activity and climate, can also affect the concentration of EDCs in surface waters across the seasons (Birkett, 2003). In Table 3 some examples of E2 concentrations in wastewater effluents are given:

Table 3 - Concentration of E2 in effluent of WWTP

Compound	Concentration (ng/L), location, reference
17β-estradiol	2.7-48, UK (Desbrow et al., 1998); 0.44-3.3, Italy (Baronti et al., 2000); 3.2-55, Japan (Nasu et al., 2001); 4.5-8.6, France (Cargouet et al., 2004); 1.1, Sweden (Larsson et al., 1999); 6, Canada (Ternes et al., 1999); 0.2-4.1, USA (Huang and Sedlak, 2001); 0.3-2.5, Japan (Isobe et al., 2003); 3-8, Italy (Lagana et al., 2004); 1.6-7.4, UK (Xiao et al., 2001)

2.5. Removal of Endocrine Disruptors in Wastewater Treatment Plants

The removal of EDCs in wastewater treatment processes depends on the inherent physicochemical properties of the pollutants and on the nature of the involved treatment process (Birkett, 2003). In addition to adsorption and volatilization, the biodegradation and the chemical degradation constitute two other main removal pathways for organic compounds in the conventional wastewater treatment (Birkett, 2003). Such removal pathways can happen at different steps in the wastewater treatment process as illustrated in Figure 6.

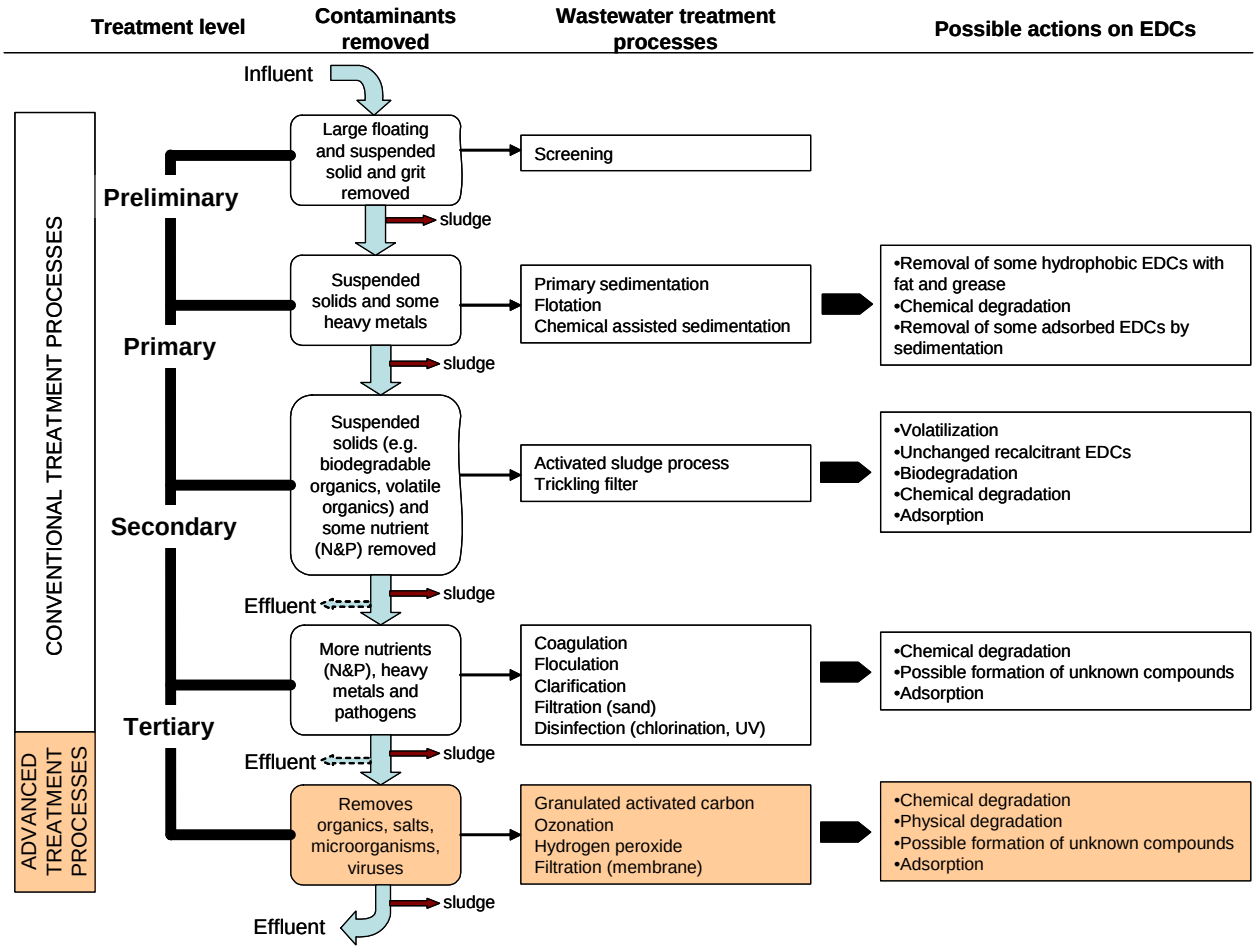


Figure 6 - Schematic diagram of a wastewater treatment plant and the possible actions on endocrine disrupting compounds (EDCs)

2.5.1. Conventional Treatment Processes

The presence of endocrine disruptors (EDCs) in water resources shows that conventional water treatment methods are inefficient to remove them (Jones et al., 2005; Ternes et al., 2002). Conventional wastewater treatment plants were normally, designed for carbon, nitrogen and phosphorous removal but not for emerging contaminants like EDCs that are present, moreover, in low concentration (Filali-Meknassi et al., 2004; Nghiem et al., 2004). Removal rates published diverge greatly due to local conditions, the nature of the contaminant and the plant design (Johnson and Sumpter, 2001). As an example, in a wastewater treatment plant in Netherlands, 94% of 17β-estradiol was removed in October, whereas the removal efficiency dropped to 75% in December (Johnson et al., 2000). Salinity and pH also change the removal of EDCs as higher sorption rate was observed for estrogens at neutral pH and at high salinity (Lai et al., 2000). Although the hydrophobic nature of most of EDCs suggests high binding with sediment/soil particles (Ying et al., 2002), these compounds have been widely reported in the aquatic environment, proving that their removal pathways are not well understood yet.

Biodegradability of EDCs is variable depending on factors such as climate, microbial activity and nature of EDCs (Ternes et al., 1999a). In fact, the latest research into wastewater treatment plant reduction capabilities indicates that naturally occuring compounds are more susceptible to breakdown and removal. For instance, 17β-estradiol disappears rapidly in approximately 10 minutes from sludge forming estrone (Birkett, 2003). With the man-made chemicals, very little reduction may occur through the wastewater treatment plant because to the fact that microorganisms lack the necessary enzymes required for transformation, therefore demanding a longer acclimation period (Birkett, 2003).

Tertiary treatment provides a final step to raise the quality of the effluent, which is often designated to drinking water industry including mainly sand filtration and chlorination. Huang et al. (Huang and Sedlak, 2001) showed that sand filtration removes 70% of 17β-estradiol from the secondary effluent, but does not have any effect on the removal of atrazine (Foster et al., 1991). Chlorination is completely effective on the removal of 17β-estradiol and 17α-ethynylestradiol but

several chlorianted by-products are formed, whith some of them having been reported to be carcenogenic and/or mutagenic (Hu et al., 2003; Moriyama et al., 2004). Anyhow, these latter treatments are costly and pose maintenance problems (Birkett, 2003).

Table 4 shows the variation of removal efficiency among the countries, process type and nature of the contaminant.

Table 4 - Treatment types and removal efficiencies from influent for some EDCs

Country	Compound	Process type	Removal efficiency	Reference
Brazil	Estrone	Trickling filter	67 %	(Ternes et al., 1999b)
Brazil	Estrone	Activated sludge	83%	(Ternes et al., 1999b)
Italy	Estrone	Activated sludge	74%	(Johnson et al., 2000)
Italy	Estrone	Activated sludge	61%	(Baronti et al., 2000)
Italy	17β-estradiol	Activated sludge	87%	(Baronti et al., 2000)
Switzerland	Nonylphenol	High loading/nitrifying	37%	(Ahel et al., 1994)
Switzerland	Nonylphenol	Low loading/non-nitrifying	77%	(Ahel et al., 1994)
USA	17β-estradiol	Sand filtration	70%	(Huang and Sedlak, 2001)
England	Triazines	Conventional 2 stage	<40%	(Meakins et al., 1994)

Since conventional treatment processes are not able to eliminate EDCs to a satisfactory level, more advanced treatment processes become wanted.

2.5.2. Advanced Treatment Processes

Numerous advanced treatment processes have been developed in order to minimize the discharge of EDCs (Figure 6). When EDCs are in the form of soluble or associated to particles or colloidal organic matter, they get easily transported through wastewater treatment systems (Schafer et al., 2002). Therefore, membrane systems are a treatment for wastewater which has grown in recent years and has shown to achieve high removals of pollutants (Coors et al., 2003; Khan et al., 2005). Indeed, nanofiltration (NF) and reverse osmosis (RO) can remove some EDCs (Kimura et al., 2003; Wintgens et al., 2002). For instance, 99.8%, 99.6% and 99.3% of removal of estradiol, estrone and nonylphenol, respectively, was achieved using a nanofiltration membrane+and also, complete removal of mestranol, progesterone and diethylstilbestrol was obtained (Weber et al., 2004). Although those techniques are efficient for most EDCs, they are not specific, require high energy demands (Duin et al., 2000) and the release of EDCs may be possible during membrane cleaning or erratic pH variation during operation (Nghiem and Schafer, 2006). Besides to the high pressure membrane filtrations, microfiltration (MF) and ultrafiltration (UF), operated at low pressure, are less effective and are not expected to remove dissolved EDCs after initial adsorption (Birkett, 2003; Schafer et al., 2002).

Recently, the scope of advanced oxidation processes (AOPs) such as photocatalytic oxidation ($UV–TiO_2$), ozonation, Fenton oxidation (H_2O_2/Fe^{2+}) has increased in regard to effective organic destruction from wastewater (Gogate and Pandit, 2004). The principle of photocatalytic oxidation and Fenton chemistry is the generation of free radicals and subsequent attack of the same on the organic contaminant molecules whereas ozonation oxidize the contaminants directly, or via formation of free radicals, if UV irradiation is present (Thiruvenkatachari et al., 2007). To reach economically high efficiency of removal of EDCs and high degree of energy efficiency in wastewater treatment plant, the combination of two AOPs processes is more often preferable (Gogate and Pandit, 2004). For instance, bisphenol A, 17α-ethynylestradiol and 17β-estradiol were more effectively degraded utilizing UV/H_2O_2 advanced oxidation as compared to direct UV photolysis treatment (Rosenfeldt and Linden, 2004). However, removal efficiency is a function of the contaminant structure and oxidant dose. For instance,

Zwiener and Frimmel (2000) reported that 5 mg/L of O_3 were necessary to remove 2 mg/L of nonylphenol in river water, which is more than 800 times the amount theoretically required to fully mineralize this compound. Although advanced oxidation processes are efficient in removing many pollutants (Nakagawa et al., 2002; Ternes et al., 2002), this technology can sometimes generate by-products, often with unknown toxicological properties and in most of the case, even potent than their parent compounds (Nghiem and Schafer, 2006). Thus, as an alternative, the combination of molecular imprinting technique and UV photolysis brought interesting results on the removal of 17β-estradiol. Indeed, 90% of 17β-estradiol was photodegraded by UV-light, after 10 hours, from a spiked solution at 3 µg/l extracted by molecularly imprinted polymers (Chapter 3) (Fernandéz et al., 2007).

Finally, adsorption processes is one of the most used technology in wastewater treatment with a purpose of getting a “polished” water that has already received normal biological treatment. Because granular activated carbon (GAC) is cheaper than other adsorbents (e.g. silica-based adsorbents (C18) and synthetic polymer), it is the most used commonly in advanced wastewater treatment for the removal of organic contaminants (Tchobanoglous, 4th ed., 2003). In fact, GAC is able to adsorb pesticides, PAHs, PCBs (Tchobanoglous, 4th ed., 2003), but also estrogens (Le Noir et al., 2006 and 2007a). The adsorbance efficiency was similar to that of the C18 silica adsorbents (Le Noir et al., 2006 and 2007a). However, GAC is difficult to regenerate; needing conditions of high pressure and/or temperature, and tends to saturate (Fukuhara et al., 2006; Tchobanoglous, 4th ed., 2003; Zhang, 2002). Hence, the lack of specificity of previous methods considerably reduces their efficiency at trace concentration as most of the adsorption or oxidation capacity is wasted in the removal of other, often harmless, compounds. Therefore, the use of molecular imprinting for the removal of endocrine disrupters at trace concentrations seems to be promising (Le Noir et al., 2006 and 2007a; Chapuis et al., 2006; Prasad et al., 2007).

3. Molecular Imprinting

3.1. General Principle

The structures, reactions and interactions of the molecular structures of living organisms are based on the ability of molecules to distinguish between the structures which they encounter. This ability is called the molecular recognition. Scientists have been trying to understand its underlying mechanism and thus to elucidate the interactions such as antibody-antigen, enzyme-substrate, hormone- receptor, DNA and RNA (Andersson, 1999). The ligand selectivity natures of protein and nucleic acid structures as well as their stability, pose a limit to their utility in abiotic environments. This has stimulated the development of synthetic recognition systems capable of displaying the same high selectivity and efficiency as their natural counterparts (Andersson, 1999). One synthetic approach to molecular recognition that has been developed in the recent decades is the molecular imprinting whose concept is rather simple. Indeed, it relies upon the formation of complexes between a molecular template (the structure for which recognition is desired) and functionalized monomers, which are subsequently fixed throw a polymerisation reaction. Removal of the template species reveals “imprints” complementary in shape and functionality to the template (Figure 7) (Andersson, 1999).

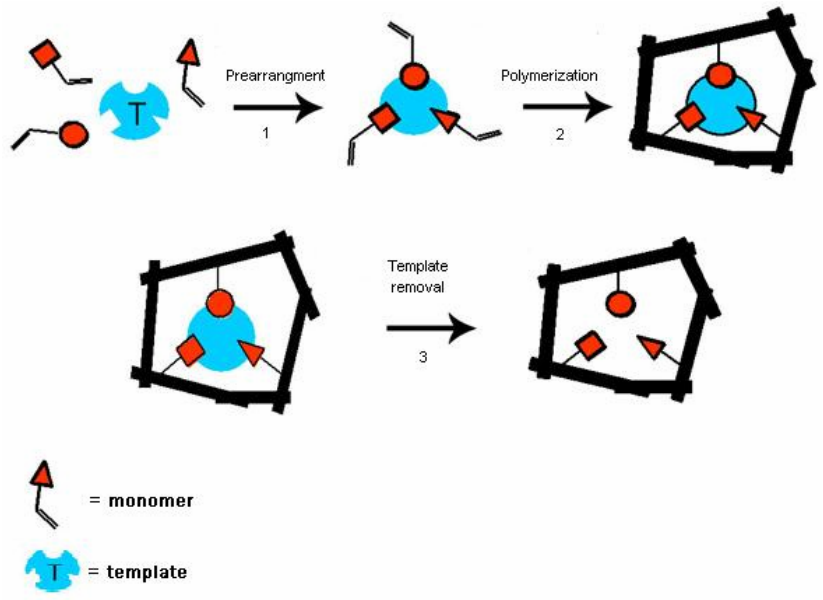


Figure 7- Schematic illustration of molecular imprinting principle: (1) preparation of covalent conjugate or non-covalent adduct between a functional monomer and a template molecule, (2) polymerisation of this monomer-template conjugate and (3) removal of the template from the polymer (Adapted from: www.infu.uni-dortmund.de)

In the first step, monomers, carrying suitable chemical functions, form a complex with the template through covalent or non-covalent interaction (Belmont, 2006). During the second step the polymer chains start to form, they are still flexible in the beginning, but as the polymerisation proceeds, they become cross-linked and the functional groups of the interacting monomers are held in place and topographically complementary to the template. In the third step, the templates are removed from the polymer; binding sites complementary to the target molecule in size, shapes, and position of functional groups are left as cavities (Komiyama et al., 2003). Their evidence is preserved by the cross linked structure as a memory imprinted on the polymer which is now capable of selectively rebinding the target (or its analogue) (Komiyama et al., 2003). Thus, molecular imprinted polymers (MIP) possess two of the most important features of biological receptors: the ability to recognize and bind specific target molecules (Belmont, 2006). Their obtainment is easy and inexpensive and they can be easily adapted to different chemistry fields (*i.e.* chromatographic stationary phases, sensors, catalysis,

immunoassays and adsorption). Accordingly, the use of MIP as selective sorbents seems extremely promising (Martin-Esteban, 2001).

3.2. Historical Perspective

The focus on molecular imprinting technique and its applications is relatively recent but the concept itself has a long history. The first recognized examples of molecular imprinting were achieved by the Soviet chemist Polyakov. He prepared rigid matrices made of silica by acidification of sodium silicate solutions followed by drying; the effects on the pore structure of the rigid matrix were studied by adding compounds such as benzene and toluene during the drying and then washing them off. In a published paper in 1931 it was demonstrated that under certain conditions the silica matrix was re-adsorbing slightly more of the different additive in whose presence it was prepared (Polyakov, 1931).

Later, in 1942, Linus Pauling tried to prepare antibodies *in vitro* by non-covalent modification of serum globulins using different compounds as antigen templates: methylene blue, an arsenic acid derivative and a polysaccharide (Pauling et al., 1942). The results indicated that the renatured antibody could precipitate the template more efficiently than structurally related compounds; this apparent success led Pauling to apply the imprinting in an abiotic system, silica. These experiments used a similar method as that of Polyakov with the difference that the template was introduced prior to the polymerisation, whereas Polyakov introduced during the drying process. The results were clear: the silica prepared in the presence of any of these “pattern molecules”, bound the pattern molecule in preference to the others. Shortly after these results several research groups started to use this method. In 1955, Dickey and Haldeman and Emmett, (1955) presented concurring and more detailed hypotheses to explain the observed template dependent selectivity of the silica. This “footprint” theory was later supported by Beckett and Youssef (1963) with the most important findings being the recognition properties of the imprinted silica adversely dependent on the amount of remaining template. Moreover, Bartels (1967) used UV spectroscopy to demonstrate a specific pre-organization

of silica acids around the template (1,10-phenantroline) in the polymerisation structure. During all this period the main applications for the silica imprinting were as chromatographic stationary phases in column or thin layer format. Several other examples of practical uses with molecularly imprinted silica have been addressed, however the interest in silica experienced a decline by the end of 1960's mostly due to limitations in stability and reproducibility of imprinted silica materials.

The year of 1972 marked the start of molecular imprinting as we know it today, when the laboratories of Wulff (Wulff and Sarhan, 1972) and Klotz (Takagishi and Klotz, 1972) independently reported the preparation of organic polymers with predetermined ligand selectivities. Template molecules, or derivatives, which were present during polymerisation, were recognized by the resultant molecularly imprinted polymers (MIP). The groups of Wulff, Mosbach and Shea were the pioneers for the current molecular imprinting techniques.

Klotz et al. reported that poly(ethyleneimine), cross-linked by disulfide bridges in the presence of methyl orange, exhibited enhanced adsorption capacity for this template in aqueous media as related to polymers cross-linked in the absence of template, whereas Wulff et al. (1995) reported chiral recognition of D-glyceric acid in a divinylbenzene polymer prepared in the presence of D-glyceric- (*p*-vinylanilide)-2,3-O-*p*-vinylphenylboronate, which was covalently incorporated in the polymer and subsequently hydrolyzed. This was the first example of reversible covalent interactions as the basis for recognition; later on, many other MIP protocols on covalent imprinting were developed during the 1970's and early 1980's.

The first steps on the non-covalent approach were taken by Arshady & Mosbach who prepared N-methyl-N,N-diallylamine (MDAA) MIP based on electrostatic prearrangement in water/DMF between two template dyes (rhodanile blue and safranine O) and the functional monomers N,N'-phenylenediacrylamide and 3,5-bis(acryloylamido) benzoic acid. Selectivity was demonstrated by batchwise ligand binding studies and chromatographic evaluations (Arshady and Mosbach, 1981; Norrlow et al., 1984). This non-covalent approach was then refined by the use of acetonitrile as a polymerisation solvent and ethylene glycol dimethacrylate (EGDMA) as the cross-linker. These were important leading steps to the more versatile methodology employed in recent years. Extensive

chromatographic investigations and direct physical studies of the pre-polymerisation mixture established the non-covalent approach as a general method for achieving pre-determined recognition; it is still by far the most widely used approach. In parallel, the metal coordination approach was further developed by the groups of Fujii (Fugii et al., 1985), Mosbach (Robinson et al., 1989) and Arnold (Dhal et al., 1991; Dhal et al., 1992)

During the past few years an exponential interest on the molecular imprinting was generated by the increase of studies based on systems similar to the one developed by the group of Mosbach, the general demonstrations of the versatility of the non-covalent approach, as well as the use of molecular imprinting in various applications. For example, the work published by Vlatakis et al., (1993), which involved the use of theophylline and diazepam MIP in an assay format, was the first demonstrating that MIP system may rival the selectivity of polyclonal antibodies. This work received much attention and represents one of the most cited works in the area.

3.3. Approaches to Molecular Imprinting

Although the non-covalent imprinting is the most commonly used, there are many other approaches that can be differentiated by the polymer framework constituents (*i.e.* organic matrix/inorganic matrix) or by the interactions involved in the prearrangement and in the rebinding process (*i.e.* reversible covalent, metal ion coordination or non-covalent). Despite these distinctions many parallels may be drawn on aspects of molecular recognition and in the applications and methods for evaluation of the MIP (Andersson, 1999).

Depending on the nature of the interactions between the monomers and the templates, they can be placed via a covalent linkage (in covalent imprinting), placed nearby through non-covalent interactions (in non-covalent imprinting) or by metal ion coordination. In either case, these bonds should be reversible in order to enable template extraction after polymerisation and rebinding of the target molecule (Komiyama et al., 2003).

3.3.1. Covalent Imprinting

In covalent imprinting, the monomers and the template are covalently linked during the polymerisation process. After the polymerisation, the template is removed from the polymeric matrix by chemical cleavage and during the rebinding process the same covalent bonds will be formed. These covalent linkages lead to a very stable and stoichiometric template-monomer or guest-monomer complex. The main disadvantages include a troublesome and less economical synthesis of the monomer-template complex, a possible diminution of the imprinting effect since the template removal step requires severe conditions, and a slow binding kinetics. This last disadvantage would be a serious problem if the polymer is to be used in rapid chromatography, columns for adsorption or in a biosensor (Belmont, 2006) (Table 5). Binding kinetics is thus an important factor to take into account when choosing the functional monomers which, with the limited choice of solvents and applications, are the main reasons why the covalent approach has played a small role in the molecular imprinting research (Belmont, 2006).

3.3.2. Non-covalent Imprinting

In non-covalent imprinting the functional monomers connect with the template by non-covalent interactions (ionic interactions, hydrogen bonds and hydrophobic interactions), for that reason the connection strength is weak (Komiyama et al., 2003). After polymerisation the template is removed from the polymer by a simple solvent extraction with an appropriate solvent. The rebinding to the polymer cavities by a guest molecule occurs based on the same non-covalent interactions. In general the non-covalent approach is less clear-cut (the monomer-template is labile and not strictly stoichiometric) (Komiyama et al., 2003). The interactions are weaker and habitually forbid the use of polar protic solvents, in particular water, which would interfere in complex formation (Belmont, 2006). The monomers are added in considerable excess in order to ensure an effective imprinting by a

completely degree of complexation with the functional groups of the template. As a result, the functional groups in the polymer are not exclusively situated in the recognition sites, which inevitably lead to non specific binding (Komiyama et al., 2003). Despite these drawbacks, the non-covalent method has key advantages that led to an advance in the field of molecular imprinting (Table 5) it is easier to attain and applicable to a wider spectrum of templates, which are simply removed from the polymer under very mild conditions as well as the target binding/release, avoiding the problem of slow binding kinetics (Komiyama et al., 2003). In biology, molecular recognition is mostly based on non-covalent interactions. Also the polymerisation method selected for this project is based on these connections. Mosbach and his co-workers were the first showing that non-covalent linkages between functional monomer and template work sufficiently for molecular imprinting (Arshady and Mosbach, 1981). For instance in the imprinting of methacrylic acid with theophylline (drug), a non-covalent monomer-template adduct was formed through hydrogen bonding and electrostatic interaction (Arshady and Mosbach, 1981). Indeed, imprints against molecules such as drugs, hormones, pesticides, amino acids, peptides, proteins, co-enzymes and nucleotide bases have been generated in this way, making this approach the most common applied on molecular imprinting in the later years.

Table 5 - Advantages and disadvantages of the covalent and the non-covalent imprinting

	Covalent	Non-covalent
Monomer- template stability	+	-
Monomer-template stoichiometry	+	-
Polymerisation conditions	-	+
Removal of the template	-	+
Guest-binding and guest-release kinetics	-	+
Economical	-	+

3.3.3. Others Approaches

As an alternative to use covalent imprinting to solve the above-mentioned problems is the use of metal containing monomers. The complex used for imprinting generally consists of polymerizable ligand(s) to complex the metal ion (generally a transition metal ion) which in turn coordinates to the template. These coordination bonds are often stable in polar solvents including water, which is a substantial advantage when using biomacromolecules as templates (Belmont, 2006). Another advantage is the possibility of using stoichiometric template-monomer ratios and increasing the binding site homogeneity and selectivity of the polymer (Belmont, 2006). This approach was first reported by Fujii et al., (1985) in the imprinting of amino acids. The group of Arnold investigated different metals and polymerizable ligands for the imprinting of templates including bis-imidazoles in organic matrixes and on solid supports (Mallik et al., 1994a). Recently it was shown that imprinted polymers prepared for cholesterol using Cu^{2+} acrylate, in place of acrylic acid, resulted in MIP with higher capacities (Sreenivasan, 2001) and a ferric acrylate-containing MIP for cholesterol has also been reported (Sreenivasan and Sivakumar, 2003).

Another approach is the semi-covalent imprinting introduced by Whitcombe and co-workers in 1995 (Whitcombe et al., 1995), where the advantage of covalent imprinting (clear-cut nature) and that of non-covalent imprinting (fast guest binding) were combined to address the problem of slow binding kinetics. In this hybrid method the polymers were prepared as in covalent imprinting whereas the guest binding employed non-covalent interactions. This was achieved by using carbonate esters which after hydrolysis leave a phenol residue in the matrix capable of rebinding the template.

Molecular imprinting by the use of inorganic materials, such as sol-gel made of silica or TiO_2 were also applied. Some bulk materials have been effectively imprinted this way using the non-covalent (Glad et al., 1985; Dai et al., 1997) or covalent (Katz et al., 2000) approaches or films (Makote et al., 1998). The resultant imprint left in the gel by the template has been used with a wide range of compounds such as ion (Dai et al., 1997) , organic molecules (Makote et al., 1998) and enzyme recognition (Glad et al., 1985) , or even as shape-selective catalysts (Katz et al., 2000). TiO_2

gel works as an organic receptor since a liberated hydroxyl group (Ti-OH) in the gel can be a binding site for the target molecule (Ichinose et al., 2002; Lee et al., 1998).

3.4. Reagents and Experimental Procedures

In general, the chemicals required for a standard imprinting process are (1) functional monomers, (2) templates, (3) crosslinking agents, (4) solvents for the polymerisation and (5) solvents (or bond-cleaving agents) to remove the templates from the polymers. They are the key elements and their proper selection will ensure that polymers with appropriate properties are obtained to a particular application (Martin-Esteban, 2001). All kinds of polymerisation (radial, anion, cation and condensation) can be employed for molecular imprinting (Komiyama et al., 2003). In the case of this project a different approach is proposed by means of a liquid-liquid phase separation technique. Therefore some of the features that will be described in this Chapter will not be applied in our method. Examples of reagents and procedures applied so far are given to permit an overall on molecular imprinting techniques.

3.4.1. Template and Monomers

For a good specificity and selectivity of the polymer, the cavities formed during the polymerisation should retain their conformation even after the template removal and should possess a good accessibility for later rebinding of the target molecule (Belmont, 2006).

The first step in the preparation of imprinted polymers consists of an arrangement with the template and the monomer(s) in a solvent. The selection of the monomer is dependent upon the template characteristics. The template has to contain in its structure functional chemical groups capable of interacting with the monomer(s) with sufficient strength to form a stable complex. Until now,

the most frequently employed monomers have been acrylic or vinylic. There is a wide range of monomers available for non-covalent imprinting that can be basic (vinylpyridines) or acid (Methacrylic acid (MAA)), hydrogen bonding (acrylamides), hydrophobic (styrene) and others. With MAA the used templates have been mainly restricted to those able to interact by hydrogen bonding (Esteban, 2001). In fact, steroids such as Progesterone, Testosterone, β-Estradiol and Estrone were used as templates for MAA-containing imprinted polymers (Rachkov et al., 1998). This study showed that molecules with a relatively rigid structure and the OH group at the C-17 position (e.g. Testosterone, 17-β-estradiol) are better succeed as templates for MAA. Also 4-vinylpyridine was successfully used for 17β-estadiol as template (Le Noir et al., 2006, 2007 (a) and (b)). During the recent years, other polymers like polyphenols and polyurethanes (Belmont, 2001), and commercially available polymers such as polyvinyl chloride (PVC), polysulfone, polystyrene and polyacrylonitrile ((Kobayashi et al., 2002)) have been studied into specific applications or for being easier to synthesize the imprinted polymers in a desired shape. Resembling this previous study (Kobayashi et al., 2002) a commercial polymer was used in this work.

Concerning the relative amounts of the template-monomers, it is important to point out that since the template–monomer interactions are governed by an equilibrium process, a high amount of monomer is used in order to assure the equilibrium to form the template–monomer complex. The main disadvantage is that the excess of free monomers leads to the formation of non-specific binding sites. The solvent used during the pre-polymerisation step is also of prime importance since it also has a direct influence on the strength of the template–monomer interaction. Finally, the template size and shape has a strong influence on the selectivity of the obtained polymers. In general, even small structural differences near the functional group responsible for the interaction with the monomer can lead to the formation of highly selective polymers preventing the binding of structurally related compounds. In some cases, however, the absence or presence of groups far from the functional groups has allowed formation of highly selective imprinted polymers (Martin-Esteban, 2001).

3.4.2. Solvents

Apart from its influence on the template–monomer strength interactions mentioned previously, the solvent used in the pre-polymerisation structure plays an important role in the morphology of the obtained polymer in terms of specific surface area and pore diameter (Martin-Esteban, 2001). Generally, a low surface area and low porosity leads to low template recognition in the subsequent rebinding experiments owing to slow diffusion of the target molecule through the porous structure. It is quite difficult to predict in advance the right solvent for the successful production of polymer because even when using a solvent capable of stabilizing the template–monomer complex during the pre-polymerisation step it is possible to obtain a polymer with an inadequate morphology, which decreases the template recognition (Martin-Esteban, 2001).

Solvents with a low dielectric constant (poor solvents), such as chloroform and toluene, are thus preferred to good ones since they offer an adequate medium to stabilize hydrogen bonding and/or electrostatic interactions between monomer(s) and templates leading to precipitation of growing chains during the polymerisation process (Martin-Esteban, 2001). When solvents with higher dielectric constants (ex. acetonitrile) were used, the obtained polymers usually showed a lower affinity to rebind the template. Protic solvents, such as water and methanol, are not recommended since they not only hinder polymerisation but also disrupt the template–monomer hydrogen-bonding interactions (Martin-Esteban, 2001). The solvent used in this work for the pre-polymerisation mixture was dimethyl acetamide (DMAc) since it has been reported as a good solvent for the polymer chosen (Zhao et al., 2004).

When the molecular imprinted polymer is prepared it is necessary to remove the template in order to obtain free binding sites. This step is usually carried out by washing the polymer repeatedly with a solvent capable of disrupting the template–monomer interactions or by Soxhlet Extraction. Concerning this work the removal of the template from the template–monomer complex was based on a Soxhlet Extraction. For the removal experiments during the project, the recovery of the target molecule was carried on with the same solvent used in the Soxhlet Extraction but using different

procedures in order to study the elution behaviour and find the optimum way to recover E2 from the microspheres.

3.4.3. Crosslinking agents

In order to guarantee the stability of the template–monomer complex during polymerisation and to increase polymer porosity, a high degree of cross-linking is necessary. The cross-linkers make the imprinted polymers insoluble in solvents and facilitate their practical applications (Komiyama et al., 2003). The mole ratio of cross linking agent to functional monomer is also important. The presence of a cross-linker not only preserves the binding sites but also has a direct influence on the physical and chemical properties of the polymeric matrix. If the ratios are too small, the guest-binding sites are located so closely to each other that they cannot work independently (Komiyama et al., 2003). Ethylene glycol dimethacrylate (EGDMA) is the cross-linker often used in methacrylate-based systems, since it provides mechanical and thermal stability, good wettability and rapid mass transfer. For instance, it has been reported that at least 50% of the total monomer in a MAA-ethylene glycol dimethacrylate (EGDMA) system has to be EGDMA, otherwise no recognition can take place (Sellergren, 1989).

3.5. Characterization of Molecularly Imprinted Polymers

The imprinting concept may suggest a homogeneous binding site distribution, however experimental work has demonstrated that a heterogeneous distribution is the most common situation (Garcia-Calzon and Diaz-Garcia, 2007). Several reasons have been suggested for the heterogeneous binding sites in MIPs: amorphous nature of the polymer and the low number of templated sites; dissociation of the template-functional monomer aggregate in solution; clusters formation by

interaction of the templates during the recognition process and collapse the binding sites by template solvent extraction (Garcia-Calzon and Diaz-Garcia, 2007). Therefore it is of great importance to characterize the binding sites of the MIPs. For instance, to characterize the shape by means of the specific surface area, specific pore volume and pore size distribution, a BET method combined with nitrogen adsorption can be used (Le Noir et al., 2007a and b). To characterize the rebinding properties and selectivity of MIPs, experimental methods such as batch rebinding approaches and frontal chromatography can be applied (Garcia-Calzon and Diaz-Garcia, 2007). Another option is to use radiometric assays (Le Noir et al., 2006, 2007 (a) and (b)). In this project only the shape and specificity were characterized by Scanning Electron Micrograph (SEM) and batch rebinding approaches, respectively.

A more quantitative approach to characterize the imprinted polymer heterogeneity includes analytical and numerical methods used for calculating the adsorption isotherms and the adsorption energy distribution. During the last 5 years, several models have been proposed to describe the heterogeneity of binding sites in MIPs making use of this methods (Garcia-Calzon and Diaz-Garcia, 2007).

3.6. Applications by Molecular Imprinted Polymers

3.6.1. Solid Phase Extraction

Solid phase extraction (SPE) consists of the selective pre-concentration (enrichment) of a compound to be analysed on a stationary phase, followed by elution and collection of the fractions containing the analyte. These fractions are then analyzed by chromatography in order to quantify their content. This pre-concentration step allows analysis of samples with very low concentrations. The first indication of the MIP potential technique in SPE was given by Sellergren with pentamidine MIP (Sellergren, 1994).

The fact that not all the template can be extracted after MIP preparation is a potential problem for application of MIP as solid-phase extraction materials. Several reports indicate that residues of template molecules interfere with the analyte response (Andersson, 1999). To solve this problem, the use of a “dummy” template can be an alternative (Matsui et al., 2000). Another limiting aspect relates to the extraction step, where the proper choice of extraction solvent is essential for the degree of pre-concentration. For instance, Mullett and Lai, (1998) demonstrated that a small pulse (20 µl) of the appropriate solvent may be used to disrupt the electrostatic interactions between the MIP and the template. Despite of these drawbacks, the use of MIP in SPE matrices appears as one of the most promising application areas for molecular imprinting.

For example, a non-covalent molecularly imprinted polymer (MIP) of cholesterol, prepared by UV initiated polymerisation, was used as SPE sorbent for direct extraction of cholesterol from different biological samples (Shi et al., 2006). Under the optimal conditions, high recoveries of spiked human serum (91.1%), yolk (80.4%), cow milk (86.6%), shrimp (78.2%), pork (81.4%) and beef (80.1%) were obtained (Shi et al., 2006). Another example is the MIP prepared with caffeine as template, by thermal polymerisation (60°C) (Theodoridis et al., 2002). This MIP was used as a SPE sorbent for selective trapping and pre-concentration of caffeine. Recoveries of 81.0, 83.4 and 82.6% were obtained from two beverages extracts and spiked human plasma, respectively (Theodoridis et al., 2002).

Even more recently, MIP solid phases for extraction of cocaine metabolites directly from water were designed by Zurutuza and co-workers by using a synthetic analogue of benzoylecgonine as the template. Successfully detection of clinically relevant concentrations (in the µg/ml range) were reported (Zurutuza et al., 2005).

Presently, most of the methods developed are based on polymers prepared by bulk polymerisation. In the traditional bulk polymerisation the polymer is ground and sieved until the desired particle size (Theodoridis et al., 2002, Zurutuza et al., 2005 and Shi et al., 2006). This leads to heterogeneous particles which limits scale-up and commercialization of SPE methods. New polymerisation strategies are now being developed for the obtainment of homogenous imprinted particles of the desired size and shape (Chapter 3.7). In addition, the preparation of polymers with

capacity to recognize analytes in aqueous samples has to be improved for different types of molecules with environmental impact.

3.6.2. Liquid Chromatography and Chromatography-based Methods

MIP have been employed as stationary phases for column chromatography ever since the development of the first truly functional silica-derived imprinted polymer systems. This method is very common to analyze the selectivity of MIP systems. Nevertheless these MIP - stationary phases have not yet been developed with the aim of future commercial applications. Some examples of MIP-based stationary phases for HPLC and related chromatographic techniques are presented here.

For example, Kempe's group described that S-naproxen MIP utilized as HPLC stationary phase were giving excellent results comparable with direct resolution of naxopren on conventional chiral stationary phases (Kempe and Mosbach, 1994). These authors verified that this new type of stationary phase was able to distinguish between naxopren and related compounds such as ibuprofen and ketoprofen. This consisted in a significant improvement in comparison with conventional stationary phases since none of these two molecules could be resolved.

The interest in developing imprinted polymer matrices selective for biologically active substances has also induced the production of a significant number of chromatographic stationary phases. Many of these studies were carried out to produce analytical procedures for substances of clinical or environmental concern. Synthetic and endogenous substances have been used as templates: a number of antibiotics such as vancomycin, cefazolin and phenethicillin were imprinted on cyclodextrin molecules (Asanuma et al., 2001); the anticancer drugs such as piritrexim (Lai et al., 2003), trimethoprim (Lai et al., 2002a), harmine and harmaline (Xie et al., 2002) and also β-blockers (Sanbe and Haginaka, 2003a), chloramphenicol (Suarez-Rodriguez and Diaz-Garcia, 2001), and nicotinamide (Fu et al., 2001).

For example, cortisol (Baggiani et al., 2000), estradiol (Haginaka, 2001), and cholesterol (Hwang and Lee, 2002) are also interesting templates for molecular imprinting in chromatography approaches, both from a fundamental perspective and on account of their biological activities, because of the rigidity of their steroid ring system and their inherently well-defined placement of functionality. Estrogenic substance imprinted polymers have also been used for simulated drug screening (Ye et al., 2001). Finally, both the D1 protein binding herbicide atrazine (Matsui et al., 1995) and bisphenol A (Sanbe and Haginaka, 2003b) have been used to develop chromatographic stationary phases selective for these substances, on account of interest in their hazardous influence on the environment.

MIP-based chromatographic stationary phases have and might continue to play a pivotal role in the development of our understanding of the molecular level events underlying ligand—molecularly imprinted polymer recognition. Furthermore, MIP still offers potential for the development of tailor-made HPLC stationary phases for specific separation problems.

3.6.3. Equlibrium Ligand-Binding Assays – Antibody and Receptor Mimics

MIP can also serve as artificial antibodies and be used as recognition elements in immunoassays (Belmont, 2006). It was the published work by Mosbach et al. on radioimmunoassays for theophylline and diapezam (Vlatakis et al., 1993) that opened a new application field and an exponential rise in the number of publications on MIP per year.

More recently, such assays have been done in competitive mode using radioligands, enzymes or fluorophores as labels for detection. An example using fluorophores is the work developed by Surugiu et al., 2001 who developed competitive enzyme immunoassays based on chemiluminescence imaging and polymer microspheres imprinted against the herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) as recognition element. With the 2,4-D imprinted polymer microspheres detection limits of 34 nM were obtained in buffer with a useful concentration range from 68 nM to 680 μM. These range was

only slightly narrower than comparable antibody-based assays, which had, however lower detection limits. Cross-reactivities of related compounds were also studied and results were similar to those reported for monoclonal antibodies raised against 2,4-D, and sometimes even better (Surugiu et al., 2001). From this example we can conclude that the use of antibody and receptor binding mimics is of particular interest, especially for small, non-immunogenic molecules where it is impossible to raise antibodies without conjugating the antigen to a carrier protein, or for immunosuppressive drugs. Further advantages of such systems are their low cost and the elimination of the need for laboratory animals in antibody production.

3.6.4. Sensor and Membrane Systems

The development of sensor systems is a demanding task. Ideally, the sensing tools should be selective, provide a rapid response, have a detection limit as low as possible and be stable for a long time and under different conditions. The first steps in the investigation of several ways to develop MIP sensors were done by the group of Mosbach however the sensor response times were generally too long (Hedborg et al., 1993). In recent years much effort has been dedicated to find general signalling mechanisms for MIP sensors (Andersson, 1999). The incorporation of signalling elements directly into the polymer structure is an attractive approach which has a lot of attention in recent times. The most successful example are the luminescent sensor systems developed for detection of the chemical warfare nerve gases Sarin and Soman via imprinting of the Soman hydrolysis product pinaconyl methyl phosphonate (PMP) (Jenkins et al., 1997). A portable model of the sensor had been developed and characteristics of this system were excellent as compared to previous works (Jenkins et al., 1997).

MIP membranes can also be used as selective adsorbents for solid-phase extraction and thin films of imprinted material deposited on solid surfaces are intended to act as sensing elements (Andersson, 1999). For instance, a recent study for MIP membranes were SPE is applied was made

by Ulbricht and Malaisamy, 2005; it consisted in the preparation of porous membranes by immersion precipitation phase inversion of cellulose acetate-sulfonated polysulfone (CA-SPS) blends with different compositions with the fluorescent dye Rhodamine B (RhB) as template. The MIP were analysed by scanning force microscopy (SFM), scanning electron microscopy (SEM) and gas adsorption isotherm method (BET) and binding tests obtained by SPE gave very positive results for the CA-SPS in a 95 : 5 blend.

3.6.5. Synthesis and Catalysis

The exact location and orientation of functional groups in the binding sites of imprinted polymers has also allowed for their application as microreactors for synthesis or catalysis (Belmont, 2006). In the case of synthesis the geometry of the binding sites is used to influence the stereochemical course of a reaction by maintaining the reactants in a certain position. This principle was demonstrated when producing specific enantiomeric cyclopropane derivatives (Shea et al., 1980): the hydroxyl groups in the binding sites of a MIP imprinted with cyclobutane dicarboxylic ester were esterified with fumaryl chloride. Then, after alkylation, they generated the desired cyclopropane derivative.

Binding sites can be also used for catalysis in the way that the template can be a transition state or coenzyme analogues. After the template removal one or more guest molecules are introduced in the sites. The interaction between these molecules and the functional groups located in the three dimensional scaffold permits the precise arrangement of chemical functionality between the guest molecule and the functional groups and to obtain defined reaction mechanisms. Examples for catalysis are carbon-carbon bond formation (Matsui et al., 1996), elimination (Beach and Shea, 1994) or metal mediated reactions using MIP as enzyme mimics or coenzymes (Gamez et al., 1995). The use of MIP as enzyme mimics is commonly regarded as the most challenging with respect to applications in chemical synthesis, but also other applications for MIP in synthetic chemistry, like

equilibrium shifting (Ye et al., 1999) or protecting groups (Alexander et al., 1999), have been focus of interest.

3.7. The Selected Technique for this Project: Molecularly Imprinted Microspheres by Liquid–Liquid Phase Inversion

Molecularly imprinted polymers have been prepared in a variety of forms. The first and most common technique used consists in the preparation of bulk polymers that have to be ground and sieved to the desired particle size (25 – 50 μm). In this process the particles obtained are irregular in size and shape. In addition, some binding sites are partially destroyed during the grinding, which leads to considerable loss of loading capacity (Martin-Esteban, 2001). Due to its great simplicity, this technique is still widely used, however different polymerisation strategies have been proposed by several authors leading to homogeneous, spherical particles or polymer films. The need for homogenous particles has been first induced with the use of MIP as stationary phase in chromatography, as the solvent flow and the quality of the peaks is very dependent on particles shape, size and homogeneity (Belmont, 2006).

Recently, a simple non-covalent approach for MIP preparation was developed using the phase inversion technique, which is a common method to prepare membranes (Ulbricht and Malaisamy, 2005) and was applied to an environmental estrogenic compound dibenzofuran (DBF) (Kobayashi et al., 2002). The DBF recognition by phase inversion imprinted polymers, made of commercially available polymers, was studied. DBF-imprinted polysulfone (PSf) membranes were prepared and showed the possibility of being used as an imprinted matrix (Kobayashi et al., 2002).

In the present work, liquid-liquid phase separation by immersion precipitation is employed to prepare 17β-estradiol-imprinted polymer microspheres (E2-MIM) made of polyethersulfone (PES) and titanium dioxide (TiO_2). In a first step, polymerisation occurs in a solution formed by the imprinting matrix (PES-TiO_2), the solvent (DMAc) and the template (E2). After homogenization, the polymeric

solution is dropped in a non-solvent (water) and solidification takes place. This method was firstly applied to prepare imprinted microspheres by Yang et al. (2005) which work consisted on the preparation of bisphenol A-imprinted PES microspheres for the binding and recognition of bisphenol A (BPA). It is rather complex, and detailed mechanisms of structure formation are still not fully understood. However it should have advantages for molecular imprinting since the microspheres can be easily prepared at room temperatures and used to load columns.

Therefore, exploring its potential advantages to produce new molecularly imprinted polymers is still in a very early stage. In particular, all previous studies had their clear focus either on porous membrane formation (Mulder, 1996) or on the imprinting effect (Kobayashi et al., 2002 and Wang et al., 1996).

To prepare the novel 17β-estradiol-imprinted PES microspheres, the liquid-liquid phase separation technique is applied and their potential for recognition and removal of 17β-estradiol from water is investigated.

4. Materials and Methods

4.1. Reagents

Polyethersulfone (PES, Ultrason E 6020P, CAS No.: 25608-63-3) was a gift from BASF Chemical Company (Germany), and was used to prepare the porous particles. 17β-estradiol ≥ 98% and Titanium (IV) oxide, powder, 99.8% were purchased from Sigma-Aldrich (Germany). Dimethyl acetamide (DMAc), methanol, acetonitrile, acetone and acid acetic were obtained from Fischer Scientific GTF (Sweden). All chemicals were HPLC grade, except acetic acid, which was analytical grade. All chemicals were used without further purification, unless otherwise described. The aqueous solutions were prepared with distilled water, except for the HPLC mobile phases and the coagulation medium for the solidification process, which were prepared with ultrapure water.

4.2. Preparation of the Imprinted Microspheres

Molecularly imprinted microspheres (MIM) were prepared by a modified procedure of the phase inversion method based on Yang et al. (2005). Polyethersulfone (PES) must be dried before processing in order to prevent bubbles/foaming caused by the water it takes up from the air (at 23°C 50rH ca. 0,7%). The drying was performed at 140°C for 4 hours. A polymer solution was prepared with 1,8 g of PES and 0,375 g of 17β-estradiol (E2) in 10 mL of DMAc, which correspond to the same ratios used by Yang et al., (2005). Additionally, titanium dioxide (TiO_2) (10% of the weight of PES) was added to the polymeric solutions to ensure that the density of the resultant MIM was enough to support the water flow coming from the bottom of the column. Scanning electron micrographs (SEM) of the produced MIM were taken and analysed.

All polymeric solutions were shaken approximately during 1 hour until homogeneity was obtained. The resulting solution was dropped into pure water from a syringe needle with an inner diameter of 0,4 mm to prepare microporous microspheres at room temperature.

These microspheres were incubated in water for over 24 hours to elute the remaining DMAc. Then, the extraction of the template molecule from the PES-TiO_2 matrix was carried out by washing the MIM with methanol for 3 times, using a soxhlet extraction method. To confirm the extraction, MIM were set in a glass column and a 50 mL solution of MeOH: Acetic acid (4:1 v/v) was passed through. The last 2 mL from the solvent were taken and analysed. Since there was no E2 present, the MIM were washed with 100 mL of distilled water and stored under room conditions until further experiments. With the same protocol, polymeric solutions without the template were made to prepare non-imprinted microspheres (NIM).

4.3. Scanning Electron Micrograph (SEM)

For the SEM study, the 17β-estradiol-imprinted PES microspheres samples were fixed in 2,5% glutaraldehyde in 0,12M sodium phosphate buffer, pH 7,2 overnight, post-fixed in 1% osmium tetroxide for 1 h. Then the samples were dehydrated in ethanol (0–50–75–99.5%) and critical point dried and coated with gold/palladium (40/60) and examined using a JEOL JSM-5600LV scanning electron microscope (Plieva et al*.,* 2004). To observe the inner part, the dried sample was diametrically cut with a single-edged razor blade before being coated.

4.4. Quantification of 17β-estradiol

E2 was quantified by high performance liquid chromatography (HPLC) using a high performance liquid chromatograph (Waters 2690) equipped with a fluorescence detector (Waters 474) and a Supelcosil C18 column (15 cm x 4,6 mm) (Supelco). For the elution a mixture of acetonitrile (50%) and pure water (50%) was used pumped with a flow rate of 1 mL/min. The excitation and emission wavelengths were set at 230 and 290 nm, respectively. The sample injection volume was 20 µL.

4.5. Removal Batch Experiments

The preparation of the molecularly imprinted microspheres was based on Yang et al. (2005) however TiO_2 was added in our system. TiO_2 was added in order to get heavier and more stable microspheres to the applied flows. NIM and MIM were previously tested in a glass column passing distilled water with flow rates of 15 and 20 mL/min to make sure that the system would have the expected behaviour. Since the density of the MIM and NIM with TiO_2 was enough to keep them stable

all along the column without clogging on the top (Figure 8), further experiments were carried out for a better characterization of these new microspheres.

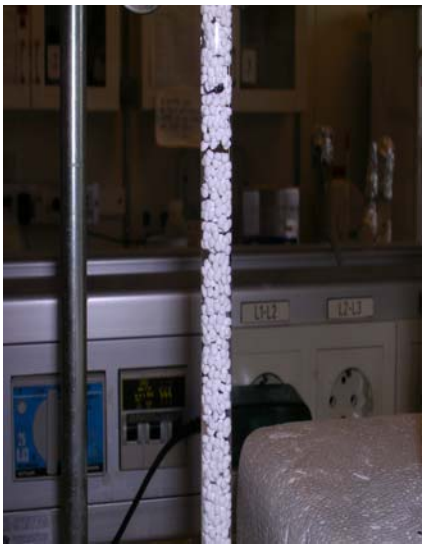


Figure 8 - Effect of 15 mL/min water flow in a column packed with MIM containing 10% of TiO_2 in PES (w/w)

In order to study the binding efficiency and ensure that TiO_2 was not affecting the microspheres binding properties, or specificity, batch experiments were made as removal test steps. The elution step performance was also studied.

For the first batch, 2 L of a distilled water solution containing 2 mg E2/L was prepared. Four flasks were filled with this solution (500 mL in each), two of them contained the same amount of MIM (~7 g in non-dried weight) while the other two were used in control experiments (the same conditions without MIM). The batches were stirred for 1,5 hours at room temperature. Samples were taken over time: 0, 5, 10, 15, 30, 60 and 90 min. The elution step was made with 500 mL of methanol: Acetic acid (MeOH:Aa) (4:1 v/v) in the same conditions during 30 min. After elution, several 25 mL portions of solvent were percolated through a glass column until no E2 was detected in the extract. The same experiment was repeated for the NIM.

Four more batch studies were performed during 2 hours with stirring at room temperature. Different volumes of water solution containing 2 mg E2/L: 100, 200, 300 and 400 mL were used. All the experiments were done in duplicates. Four flasks were set simultaneously, two filled with ~6 g of

MIM and the same amount of NIM in the other two. Samples were taken over time: 0, 10, 30 min, 1 and 2h.

The same volumes were used for the correspondent elution in the same conditions. The elution batch was performed during 1 hour and samples were taken over time: 0, 1, 5, 10, 15, 30 and 60 min. After each batch elution, MIM and NIM were placed in a glass column and eluted from three to six times with 15 mL of MeOH:Aa (4:1 v/v) until no E2 was retained in the particles. The concentration of E2 in the samples was quantified by HPLC.

4.6. Semi-continuous Removal Experiments

A glass column was prepared and packed with MIM and/or NIM. Different flow rates and the effect of mixing by aeration on the adsorption capacity of the system were studied and optimised for E2 aqueous solutions (synthetic wastewater).

The set-up was built with a glass column covered with plastic on top and base including a filter on the base (glass cylinder dimensions: volume 5 mL, external diameter 2,8 cm, height 15 cm). To avoid the particles to escape through the top, Teflon frits were used to cover the bed of particles. The water solutions were pumped using peristaltic pumps (Alitea, Stockholm, Sweden) through PharMed tubing (Saint Gobain Performance Plastics, Charny, France; D_{int}=3,2 mm, D_{out}=6,4 mm, Wall=1,6 mm). This system was made in duplicate in order to perform simultaneous experiments with NIM and MIM packed reactors.

To apply mixing through the system, an air pump (OPTIMA, Malaysia) was used to pump air through Tigon tubing (Saint Gobain Performance Plastics, Charny, France; D_{int}=4,8 mm, D_{out}=8,0 mm, Wall=1,6 mm). Also a glass tubing connector and compressors type tubing clamp to control the air flow were used (Figure 9).

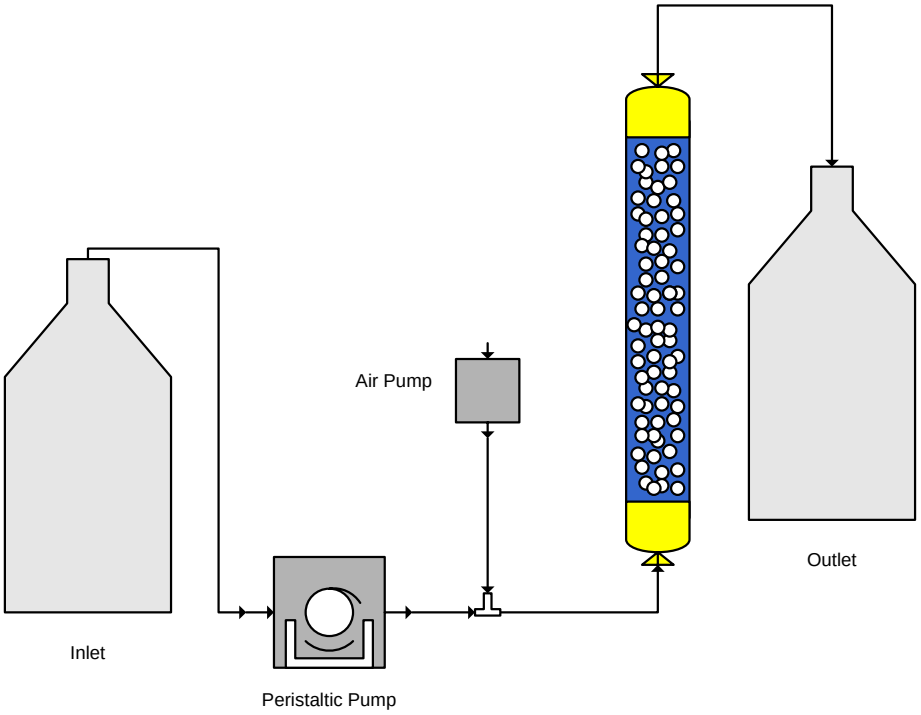


Figure 9 - Schematic presentation of the reactor set-up with mixing

For the experiments with synthetic wastewater 82% of column volume (5 mL) was loaded with MIM and NIM, (4,1 mL) which corresponds to ~3 g of non-dried particles. The water samples consisted of 1 L of 0,5 mg E2/L aqueous solutions. The elution steps after each performance were made in batch using different volumes of MeOH:Aa (4:1 (v/v)) and changing the contact times.

Previous to the described procedures a tubing adsorption test was performed. As E2 has affinity to hydrophobic polymers it was essential to check if there was pollutant being adsorbed by the tubing system. 1 L of a 1 mg E2 /L aqueous solution was passed through the system at 1 mL/min with and without mixing.

4.7. Extraction of Spiked and Non-spiked Wastewater

Wastewater effluent (40 L taken after secondary treatment from the wastewater treatment plant in Lund, Sweden) was autoclaved at 121 ºC for 1 hour (10 L each time). 20 L were spiked with 1 µg E2/L while the remaining volume was not. Spiked and non-spiked 20 L volumes were passed through the adsorption columns with mixing filled with MIM as described above (Figure 9). There was no available amount of MIM enough to fill 82% in both columns. So in this case, 72% of column volume was loaded with MIM (3,6 mL), corresponding to ~2,5 g of non-dried MIM in each system.

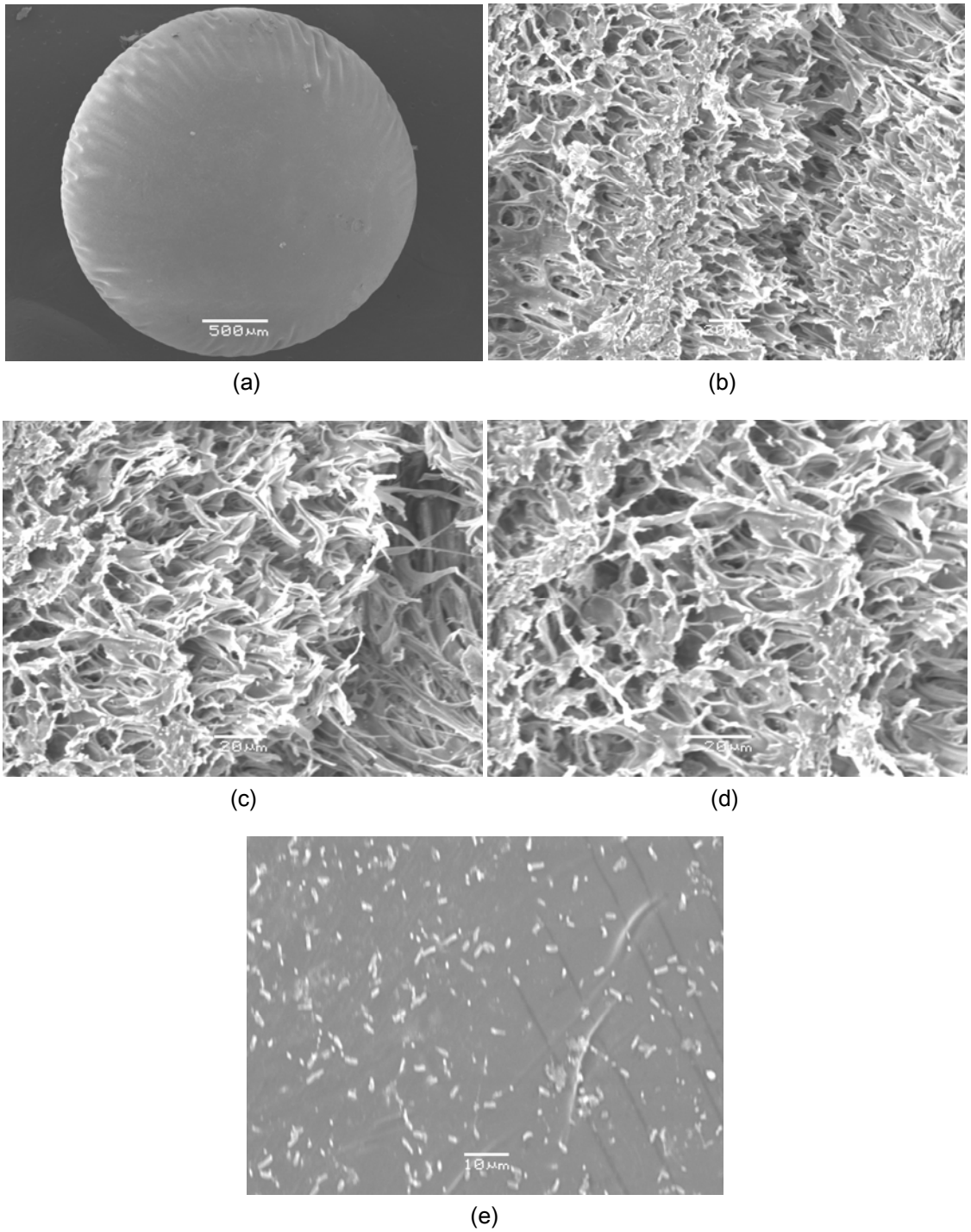
The captured E2 was extracted from the MIM in batches with 20 mL of MeOH:Aa (4:1 v/v) (4 times) and the concentration of E2 in each extract was quantified by HPLC analysis.

5. Results and Discussion

5.1. Characterization of the MIM

The preparation of E2-imprinted microspheres made of PES and TiO_2 was preformed by a liquid-liquid phase inversion technique by immersion precipitation of a PES-TiO_2-DMAc-E2 polymeric solution. Pure water was selected as the coagulation medium, which is a medium commonly used to prepare PES membranes (Yang et al., 2005). Water is a non-solvent for PES and it showed high solubility for DMAc but not for PES, TiO_2 and 17β-estradiol. Therefore, when the polymeric solutions were dropped into water, a liquid–liquid phase separation caused by the rapid exchange between the solvent (DMAc) and the non-solvent (water) occurred. Simultaneously, imprinting happened immediately during PES-TiO_2 solidification, and the 17β-estradiol template was retained in the recently formed microspheres. With completion of the exchange between the solvent and the non-solvent the

microspheres were produced. After the extraction of the template molecules, E2-MIM were prepared and observed by SEM (Figure 10).



(a) (b)

(c) (d)

(e)

Figure 10 -SEM photographs of the bead low – 50 x (a); outer part at high magnification – 1200 x (e) and inner part at different magnifications - 550 x (b), 700 x (c) and 900 x (d)

The prepared microspheres have a homogenous spherical shape, and they are opaque on their surface (Figure 10 (a) and (e)). SEM photographs were taken as well for the inner parts (Figure 10 (b), (c) and (d)) which show many small pores inside the microspheres. The morphology shown by SEM suggests that the PES transformation from polymer solution to the solid state quickly occurred in water by instantaneous PES precipitation. Because of the poor solubility of 17β-estradiol in water, the template remained in the solid PES microspheres while the solvent exchange between DMAc and water proceeded. With the extraction of 17β-estradiol from the solid microspheres, imprinting sites of the template were left as cavities and no change in particles size was verified. In fact, after the soxhlet extraction the imprinted and the non-imprinted microspheres seemed to have similar sizes. Therefore, it is expected that the surface area, which is a significant factor related to the binding (higher surface area: volume ratios give higher binding potential) (Yang et al., 2005) was not affecting the binding differences between MIM and NIM.

The diameter and the porosity of the microspheres depend on the diameter of the syringe needle and the polymer solution (Zhao et al., 2004). Here, the diameter of the syringe, 0,4 mm, was selected based on a previous comparison between the potential of microspheres made with syringes of different diameters: 0,4, 0,5 and 0,6 mm (Le Noir., 2007). The results showed that smaller particles are more appropriate since the smaller diameter, the greater surface area they have relative to their volume, improving the binding kinetics to the target molecule. Also, the injection speed and the air gap strongly affect the shape of the microspheres due to the surface tension of water, the polymer solution and the viscoelasticity of the polymer. These parameters were taken into account while dropping the polymeric solution. The solution was dropped maintaining always the same distance from the water surface and injection speed.

5.2. Removal Batch Experiments

I. For the first batch, four flasks were filled with a water solution containing 2 mg E2/L (500 mL in each), two of them contained ~7 g (in non-dried weight) of MIM while the other two were controls. The batches were stirred for 1,5 hours at room temperature. The concentration of E2 was measured over time. The same experiment was repeated for the NIM and the results compared (Figure 11).

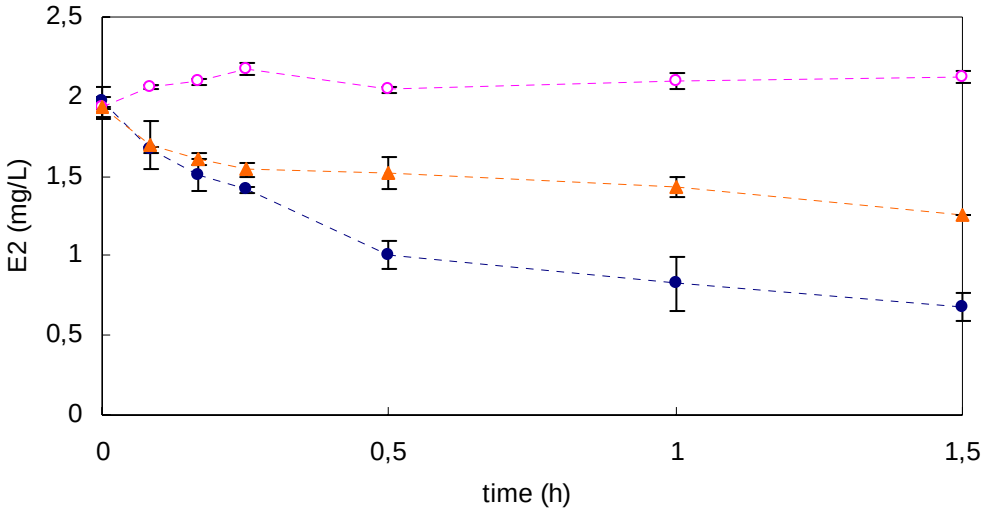


Figure 11 - Concentration of E2 in the aqueous phase for the MIM (●), NIM (▲) and control (○). Vertical bars represent the standard deviation on duplicates

Table 6 - Total %Removal and after the first 1/2 hour

time (h)	%Removal	
	NIM	MIM
0,5	21 ± 2%	49 ± 7%
1,5	33	66 ± 3%

The concentration of E2 in the control flasks for the MIM and NIM was stable which confirm that the experimental conditions like the stirring, room temperature and light were not affecting the E2 degradation during the batches (Figure 11). The total removal with the MIM (66 ± 3%) was

considerably higher than with the NIM (33%) (Table 6). The higher efficiency of the MIM is presumably due to the specific binding sites. The non-specific binding between NIM and E2 occurs due to the hydrophobic interactions and the porosity of the microspheres (Yang et al., 2005). For MIM, the specific recognition sites left by the template play a major role on the binding amounts. However, the imprinted microspheres have also non-specific binding sites. Previous studies showed that the hydrophobic and porosity effects of PES microspheres can act as non-selective binding forces in the template uptake (Yang et al., 2005). In fact, during the first 15 min a similar reduction of E2 concentration occurs for both MIM and NIM and the specificity of MIM only starts to accentuate between the 15 and 30 min (Figure 11). These experimental data suggest that the non-specific binding kinetics is faster and consequently the removal occurred during the first 15 min for MIM, is mostly by non-specific binding, as for NIM. In general, the greater part of the removal was verified in the first 30 min (Table 6) and after that, a reduction in the removal rate is verified. This probably occurs because the more accessible specific and non-specific binding sites are already filled, decelerating the binding kinetics.

Because the total removal of E2 was much higher for MIM than for NIM, we can expect that the presence of TiO_2 in the polymeric solution does not affect the adsorption specificity given by the molecular imprinting.

In fact, TiO_2 has been used in molecular imprinting, generally in sol-gel approaches (Lee et al., 1998 and Lahav et al., 2001). TiO_2 gel works as an organic receptor since a liberated hydroxyl group (Ti-OH) in the gel can be a binding site for the target molecule. For instance Lee et al. (1998) used the sol-gel process to the preparation of ultrathin films synthesised from TiO_2. The substrate selectivity-shape, size, and functionality was readily attained, adsorption and desorption of guest molecules were rapid, either by covalent linkage or through non-covalent hydrogen bonding, and imprinted sites could be created in high density (Lee et al., 1998).

Previous studies with molecularly imprinted and non-imprinted polymers (MIP and NIP) showed that regardless of the amount of polymer used the difference on the binding %, i.e. the specificity, was always constant between MIP and NIP (Le Noir, et al., 2006 and 2007a). Therefore, as

TiO_2 gets its function as an organic receptor, the effect of adding TiO_2 in 10% of the PES weight is probably the same as increasing the polymer concentration: the unspecific binding for both imprinted and non-imprinted polymers increases; however the difference between them is always preserved. In this case TiO_2 was chosen because it is denser than PES [(ρ (TiO_2)=4,23 g/cm^3 ; ρ (PES)=1,37 g/cm^3 (www.basf.de/ultrason)] and so the effect of making heavier and more stable microspheres, keeping the same binding specificity, is attained.

II. The influence of different polymer concentrations to MIP and NIP has been previously studied (Le Noir, *et al.,* 2006 and 2007a and b). Also, diverse binding experiments were carried out by changing the target molecules and their concentrations, the template amounts on the polymeric solution and the media composition for the binding/removal of the pollutants (Yang et al., 2005). In order to study the influence of the increasing aqueous phase volume, and therefore the amount of pollutant, in the binding differences between a certain amount of MIM and NIM 4 more batch experiments were set with increasing volumes of 2 mg E2/L aqueous solutions: 100, 200, 300 and 400 mL and then compared to the removal results for 500 mL shown above (Figure 11). The same volumes were used for the correspondent elution in the same conditions. The flasks were at room temperature with the same stirring speed. Samples were taken during the removal and the elution steps to analyze the uptake and extraction performance of both imprinted and non-imprinted microspheres.

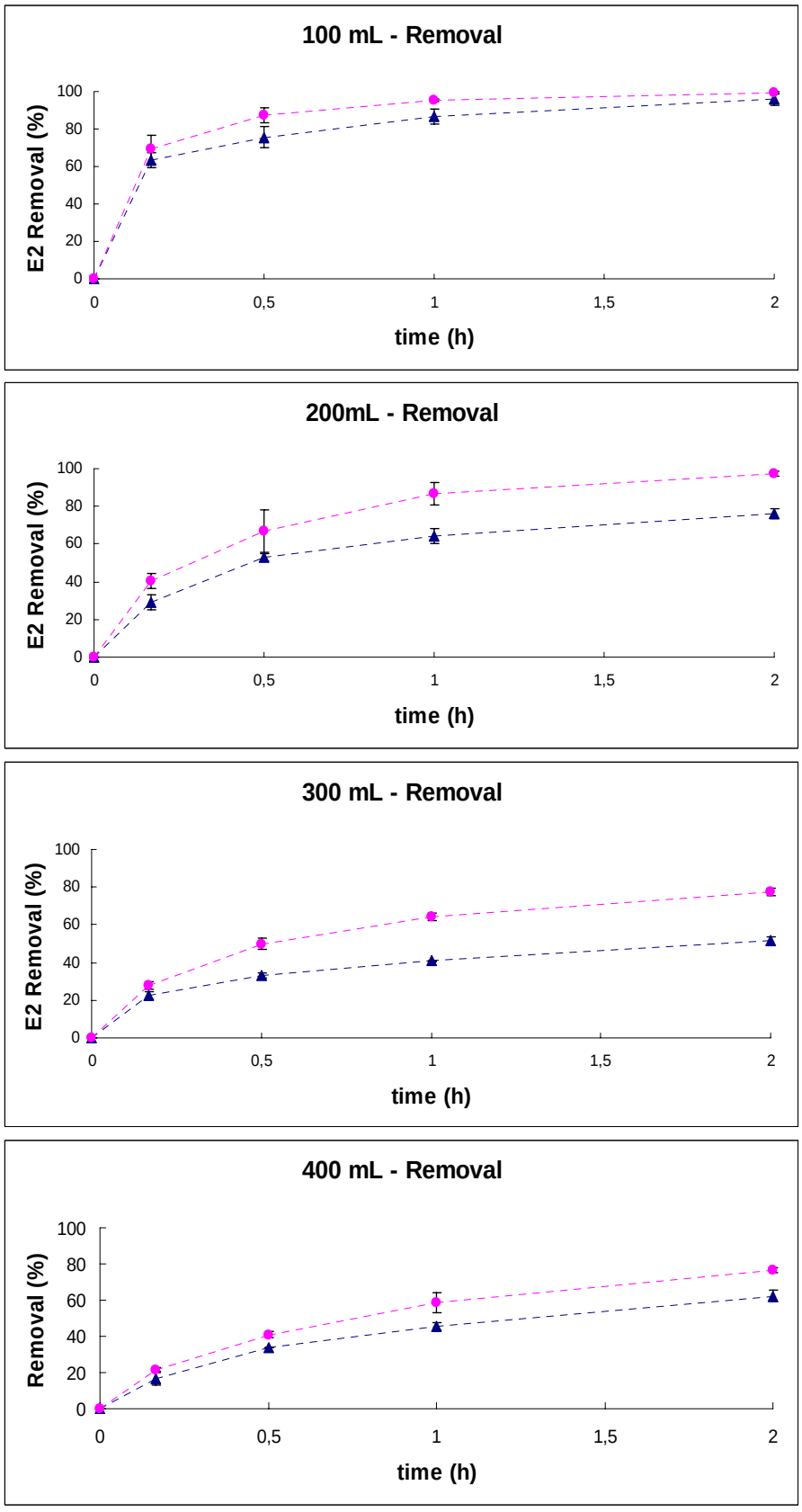


Figure 12 - Removal of E2 in the aqueous phase for the MIM (●) and NIM (▲). Vertical bars represent the standard deviation on duplicates

Observing the removal graphs from the Figure 12 it is evident a quite similar removal for MIM and for NIM during the first 10 min. This is mostly due to the non-specific bindings that probably occur easier than the specific ones. Therefore the binding kinetics seems to be synchronized for MIM and NIM during the first period of batch. At 30 min and after, regardless the volume treated, all the experimental points show a higher removal for MIM than for NIM. As the time goes by, the removal rate decreases for MIM and NIM. This decrease was more accentuated for the small treated volumes, 100 and 200 mL than for the larger ones. During the batches the major removal occurred during the first 30 min while after this time until the end of the experiment, the removal rate decreased. The decline in the removal rate suggests that the binding sites closer to the surface, specific and non-specific binding sites, are already taken as mentioned above. So, this performance is in agreement with uptake behaviour analyzed on Figure 11 for both MIM and NIM.

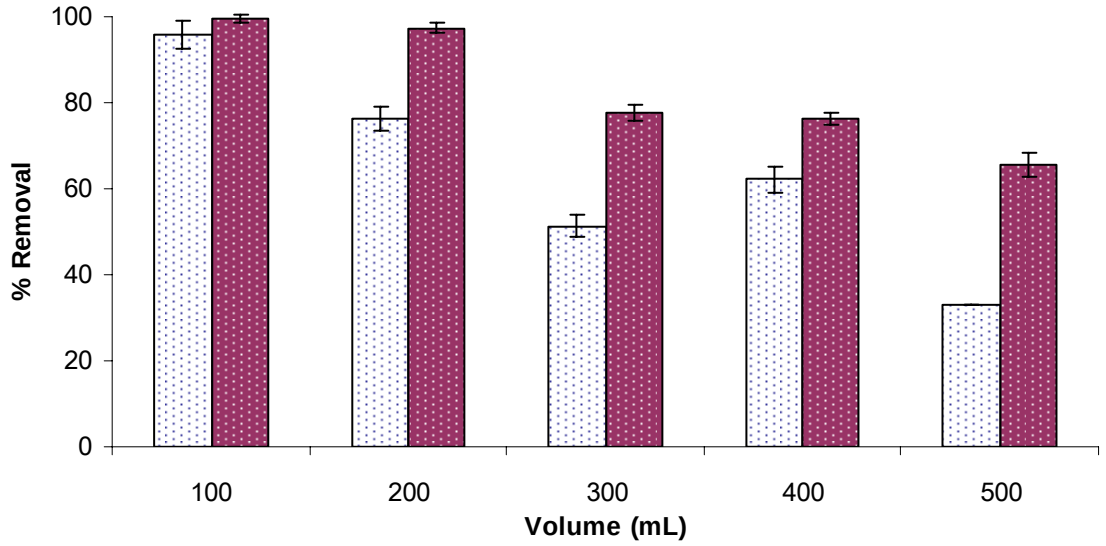


Figure 13 - %Removal for MIM ( ) and NIM ( ) at different volumes of aqueous media of 2 mg E2/L. Vertical bars represent the standard deviation on duplicates

For higher volumes, the percentage of removal decreases for MIM and NIM, however the effect of the specificity of MIM increases (Figure 13). For 100 mL, 75% and 87% of E2 were removed in 30 min and after 2 hours E2 was almost completely removed for MIM (99,4 ±0,8%) and NIM (96 ±3%). This small difference shows that the non-specific binding sites were sufficient to capture the

total amount of E2 contained in the solution. As the volume added in the flasks increased, the total removal of E2 decreased for both MIM and NIM. With larger volumes the specific binding effect becomes more evident and for 200mL the E2 uptake was of 76 ±3% for NIM and 97 ±1% for MIM. For 300 and 500 mL the difference between MIM and NIM removal is accentuated, for the treated 300 mL a total removal of 51 ±3% and 78 ±2% were obtained for NIM and MIM respectively and for 500 mL, 66 ±3% was considerably higher than the 33% with the NIM as mentioned before. This corresponds to a difference between MIM and NIM of 26 and 32% in the removal from 300 and 500 mL, respectively. For 400 mL, we also got a difference between the particles (14%) however it was expected to be between the values obtained from the experiments with 300 and 500 mL. A first possible explanation to this value is that the particles had been regenerated and reused after each batch experiment. Even with full regeneration confirmed, some saturation effect by trace contamination or even some damage of the specific cavities might occur. The 400 mL experiment corresponded to the sixth use of the microspheres after the fifth regeneration (1 aborted batch of 500 mL is included). Similar results were obtained when we repeated the batch in the same conditions (data not shown). The higher removal results for the 400 mL when compared to 300 mL might be also due to an error procedure while preparing the E2 solution. The aqueous solubility of E2 is low (3,6 mg/L) (Hakk et al., 2005). To prepare 2 mg E2/L synthetic solutions, a stirrer and a sonicator were used alternatively until the E2 was completely dissolved in water. Nevertheless it was sometimes difficult to guarantee that the 2 mg of E2 were totally dissolved. Thus, these higher removal percentage obtained for 400 mL, is probably due to a less amount dissolved, which means less concentration with inherently a higher percentage of removal.

After each removal study, a batch elution with MeOH:Aa (4:1 v/v) solution in the same experimental conditions was carried out. The main goal of this step was to regenerate the particles and recover E2. To have also an idea of how efficient the disruption of 17β-estadiol-PES complex is, different volumes were applied and samples taken over time.

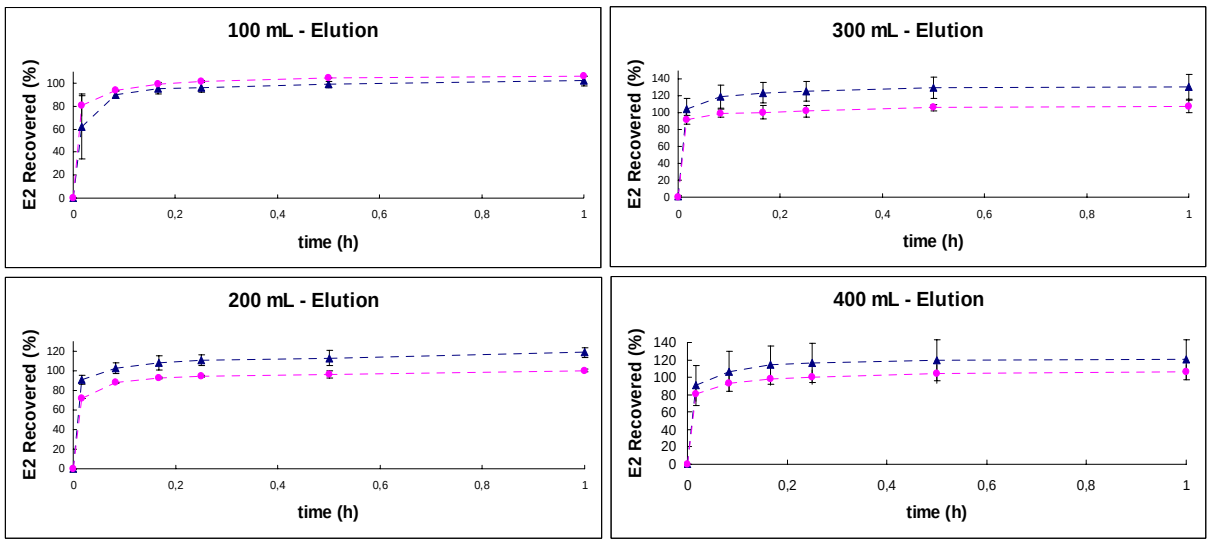


Figure 14 - Recovery of E2 from MIM (●) and NIM (▲) with MeOH:Aa (4:1 v/v). Vertical bars represent the standard deviation on duplicates

With respect to the Figure 14 the major part of the pollutant is removed from the binding sites in the first minute. After that, E2 concentration gets progressively stable in the solvent. Generally, after 5 min of elution, 100% of recovery was reached for all the NIM but not for MIM which total recovery was reached later. It also must be noted that concentrations in the solvent are higher for NIM than for MIM, which means that a more efficient extraction from NIM is obtained earlier. It is easier to extract from NIM than from MIM because the non-specific bounds are weaker making their kinetics release faster. The only exception was for 100 mL. Since this volume is small and approximately 100% of removal was reached for MIM and NIM, there was no saturation of the binding sites and the non-specific binding sites from NIM were sufficient to capture the amount of E2 contained in the solution. Since the kinetic of non-specific binding seems to be faster, it is quite predictable that mostly non-specific binding occurred for the 100 mL E2 solutions treated with MIM, therefore the elution is as easy as for NIM.

The total recovery for NIM was sometimes much higher than expected, indeed, irregular average recoveries of 130 ±15% and 120 ±22% were obtained from 300 mL and 400 mL, respectively. In both cases, one of the duplicates was much higher than predictable while the second one was in the range of expected values. This is possibly due to an error procedure or to the accumulated effect of trace contamination, not detected in the earlier regenerations.

Even with 100% of recovery it is important to check if there is no trace contamination on MIM and NIM. The particles were transferred to a glass column and eluted with 15 mL of MeOH:Aa (4:1 v/v). No E2 was found in the NIM extracts while in some cases for MIM there was a trace amount of E2. In this case the polymers were eluted more 6 times with 15mL of solvent until no pollutant was detected. Furthermore, even if the concentrations in the extraction solutions correspond to 100% of recovery or even more, and the HPLC does not detect the presence of pollutant in the column extracts, there is always an intrinsic saturation effect of the specific binding sites due to the presence of 17β-estradiol at a trace level not detectable by the HPLC. A possible damage of some specific cavities can also have occurred during the removal and elution batches because of the stirring. These two effects, as well as a possible error procedure while preparing E2 aqueous solution, are the possible factors for the 400 mL–removal difference between MIM and NIM was lower than expected. To minimize the saturation effect, elutions will be performed during longer periods before the reuse of the MIM and NIM for the semi-continuous column experiments. Also smaller volumes will be used in order to reduce the use of solvent elution and render the elution step more cost-effective.

The microspheres can be easily regenerated and such characteristic is a great advantage since it allows the polymer reuse. By comparison, activated carbon (GAC) regeneration is generally very hard to achieve and must be conducted under conditions of high pressure and/or temperature (Zhang, 2002). Prior studies showed that only a small fraction of the amount retained with such adsorbent could be extracted by solvent elution which shows how hard is to regenerate GAC and its tendency to saturate (Le Noir et al., 2007a).

Even with the greatest elution period being in the first instants of contact between the particles and the solvent, the elution step can be optimised by using smaller volumes of solvent for a longer time as it will be seen in the Chapter 5.3.

5.3. Semi-continuous Removal Experiments

The main goal of this project was to study the capture of E2 from the aqueous phase in a column packed with MIM without pressure or clogging problems. The idea was to create a set-up with the potential to be designed in a larger scale for industrial applications. Therefore the influence of two different flow rates and mixing in the adsorption capacity of the system was studied for synthetic wastewater samples all with the same concentration (0,5 mg E2/L).

5.3.1. Tubing Adsorption Test

A tubing adsorption test was performed with 1 L of a 1 mg E2/L aqueous solution passed through the system at 1 mL/min with and without mixing. The concentration of E2 in the effluent was determined after each run[a]. To confirm the results, three runs were made (Figure 15).

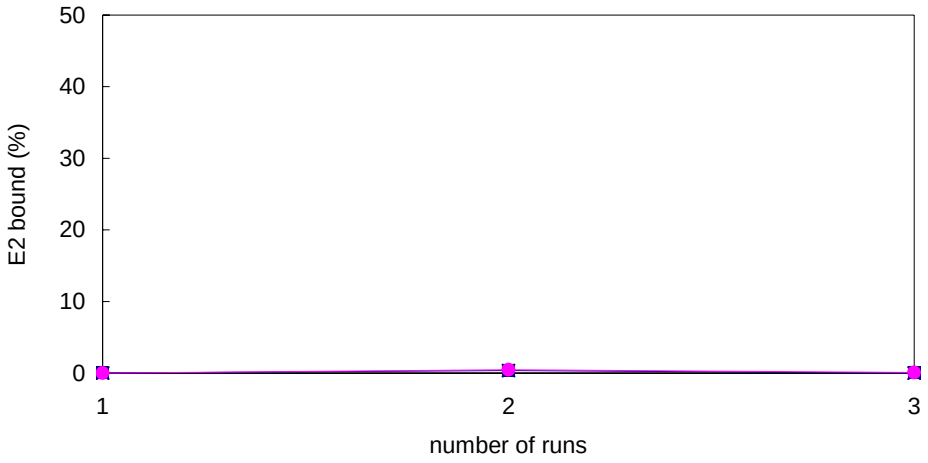


Figure 15 - E2 bound (%) in 3 runs for the system with air mixing (●) and no air mixing effect (■)

[a] One run corresponds to one circulation of the sample through the system.

After the first run, 2 samples were taken from the outlet and since there was no change in the E2 concentration it was assured that no adsorption by the system would mask the removal results. Two recirculations at 1 mL/min were made to confirm the result and again no adsorption was noticed.

5.3.2. Column Experiments with Synthetic Wastewater Samples

I. The first test was made with 1 L of 0,5 mg E2/L aqueous solution percolated through the columns packed with MIM and NIM at 15 mL/min (Figure 16).

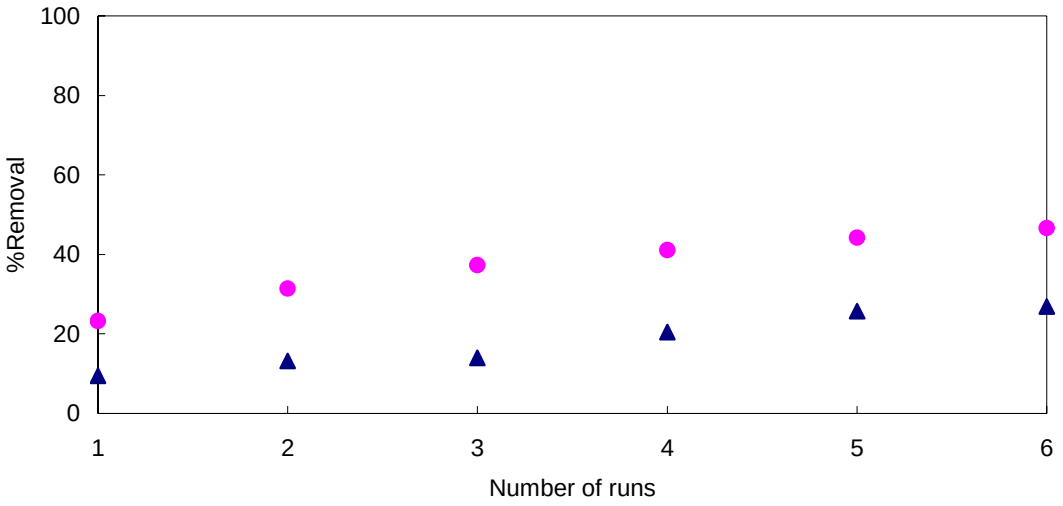


Figure 16 – Total E2 removed after 6 runs with 1 L of 0,5 mg E2/L aqueous solution through the column for MIM (●) and NIM (▲)

After the first run the removal was 9,5 and 23% for NIM and MIM respectively. Then, in order to reach a higher removal, the solution was recirculated 5 times. In each run the removal rate decreased but the total removal was always higher for MIM than for NIM. An average difference of 19%, between MIM and NIM, was verified in the accumulated removal after each run. At the sixth run the total removal was 47% for MIM and 27% for NIM, this difference emphasizes what was previously concluded with the batch experiments about the MIM specificity. Nevertheless, the removal was less

than expected, the flow rate was enough to make the particles in suspension moving slightly inside the column but the retention time (20 sec) was too small for a good binding efficiency.

II. In order to increase the retention time to 5 min and reduce the influence of binding kinetics, a flow rate of 1 mL/min was applied. 1 L of 0,5 mg E2/L aqueous solution was percolated through the columns packed with MIM and NIM (Figure 17).

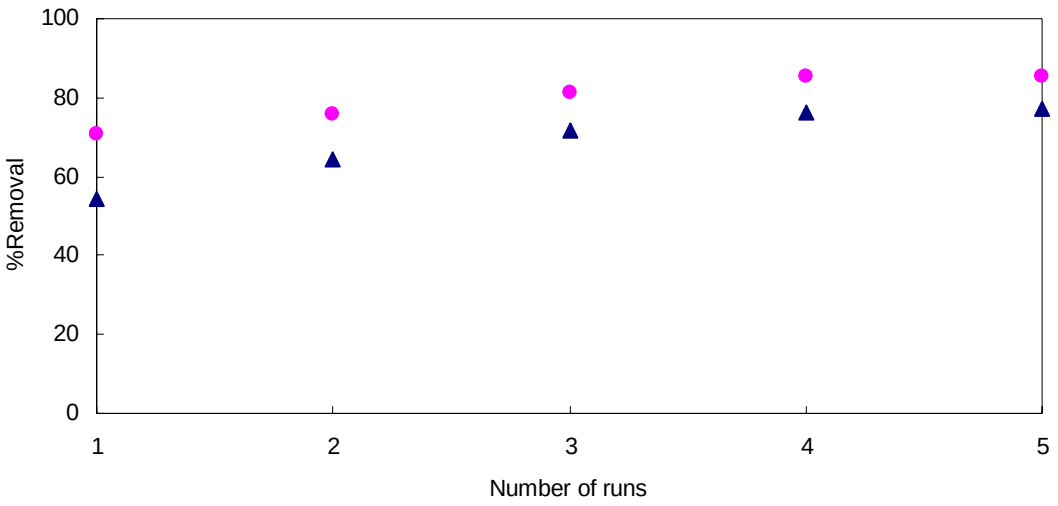


Figure 17 – Total E2 removed after 5 runs with 1L of 0,5 mg E2/L aqueous solution through the columns for MIM (●) and NIM (▲)

This flow was not enough to put the microspheres in suspension and moving, i.e., they remained fixed in the 4,1 mL occupied in the column. In general the efficiency was much higher with 54% of removal for the NIM and 71% for the MIM after the first run. After 4 runs the total removal was 85% for MIM and 76% for NIM. This result shows that the total removals of 47% for MIM and 27% for NIM obtained at 15 mL/min (Figure 16) were not due to a saturation effect but to an insufficient retention time for the highest binding rate to occur.

In this case (Figure 17), the removals were significantly higher than for a flow of 15 mL/min, however the flow was too small to consider this removal efficient. It was not performed a sixth run after the fifth since no additional removal for MIM was detected in this last recirculation (Figure 17).

III. In order to increase the particles movement, aeration was applied to the column packed with MIM. 1 L of 0,5 mg E2/L aqueous solution was circulated through the system keeping the same flow and then compared to the results from Figure 16 (Figure 18).

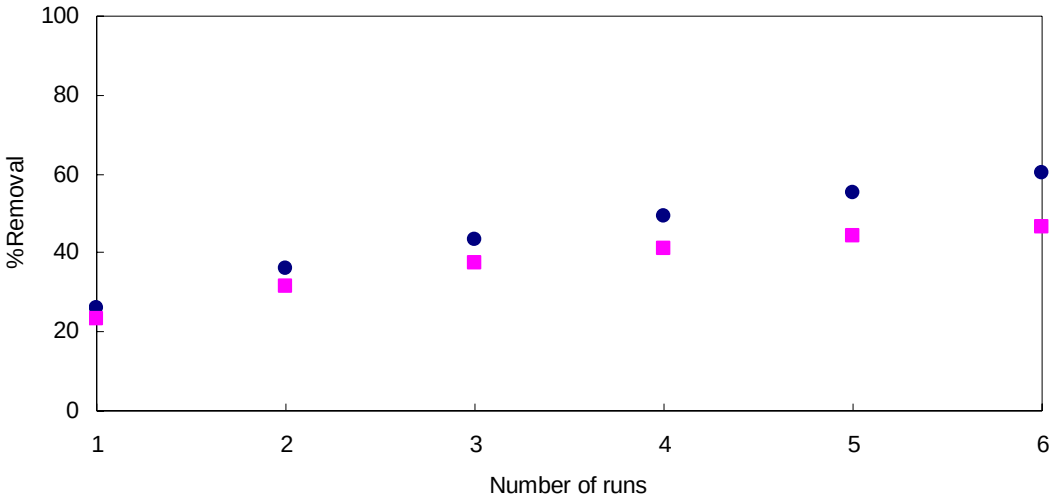


Figure 18 - Total of E2 removed during 6 runs with 1 L of 0,5 mg E2/L aqueous solution through the column for MIM with mixing (●) and no mixing (■)

Table 7 - %Removal for 6 runs at 15 ml/min with and without aeration.

	% Removal	
Runs	MIM-mixing	MIM-no mixing
1	26	23
2	14	11
3	11	9
4	10	6
5	12	5
6	11	4

The removal after the first run with aeration was 26% while in the absence of air it was 23% (Table 7). Five more runs were made and after the last recirculation the total removal of the effluent was 60% with mixing against 47% without mixing. Based on the results in Table 7 we can conclude

that the mixing has some positive effect in the adsorption capacity of the system. There was an increase on the accumulated removal difference between operating with air and without air, after each run. Even with the same retention time (20 sec) the mixing permits a better fluid-solid contact (Trambouze & Euzen, 2004). The aeration induces the microspheres to move more, making faster the renewal of the particle surface in contact with water. This can explain why the removal efficiency does not decline so much in each run in the aerated system. Although the removal after each run decreases, it was kept higher in the presence of air.

5.3.3. Elution Steps Column

Until establishing the set-up described above (Figure 9), other systems were built without successful outcomes due to various factors such as clogging problems, high flows, inadequate column dimensions or tops, tubing adsorption, leakage of water in the system etc. In these systems an alternated sequence procedure to study the removal and recovery capacities was applied:

1.

- Washing step with 500 mL of distilled water
- Removal process circulating 1 L of 2 mg E2/L aqueous solution

2.

- Washing step with 500 mL of distilled water
- Elution circulating 1 L of MeOH:Aa (4:1 v/v) through the system

The main advantage of this method is that the microspheres were kept inside the column, i.e., there was no need to unpack the system making possible a continuous operating process. However, washing steps were necessary after each adsorption and elution in order to clean the system from residual particles of contaminated water and from the solvent solution, respectively. On the other hand, the volume of MeOH:Aa (4:1 v/v) needed to completely recover the E2 retained in the

microspheres was much higher. Indeed, after several elutions with 1 L of solvent it was concluded that was necessary at least 2 L until no trace of E2 was detected in the bed of particles (data not shown).

To avoid the use of large volumes of MeOH:Aa (4:1 v/v), which can make this treatment not economically feasible, the elution steps after each performance with the definitive set-up were made in batch, using different volumes of MeOH:Aa (4:1 v/v) and changing the contact times (Table 8).

Table 8 - Elution step conditions after the experiments with synthetic water and respective elution effectiveness

Experiment		Elution	Volume of solvent (mL)	time (h)	E2	%Eluted
I	NIM	1st	250	1	detected	-
		2nd	100	1	0	100
	MIM	1st	500	1	detected	-
		2nd	150	1	0	100
II	NIM	1st	100	2	0	100
	MIM	1st	100	24	detected	-
		2nd	50	24	0	100
III	MIM	1st	100	24	detected	-
		2nd	100	24	0	100

From Table 8 and taking into account the removal efficiency achieved in each experiment (I, II and III) a recovery of 100% of E2 is possible with smaller volumes of solvent if the contact time is increased to 24 hours. To assure that no E2 was retained, MIM and NIM were eluted with 5 mL of MeOH:Aa (4:1 v/v) percolated through a glass column.

To avoid the unload, another solution was to percolate MeOH:Aa (4:1 v/v) through the top of the column, closing the bottom entrance as much as possible to retain the solvent inside for a longer

period. However it was concluded that the recovery was not as efficient as the ones performed with the stirred batch.

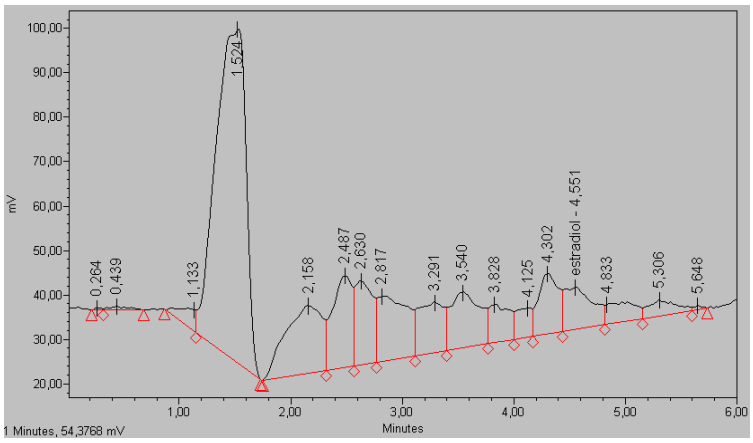
5.3.4. Experiment with Wastewater Samples

Spiked and non-spiked 20 L of wastewater were passed through the adsorption columns packed with MIM with a flow of 15 mL/min. Mixing was applied for a better removal. To determine the E2 recovery from the wastewater samples, MIM were removed from the columns and eluted in batch with 20 mL of MeOH:Aa (4:1 v/v), until no E2 was detected on the solvent. After eluting 4 times with 20 mL E2 was no more detected on the MIM. The results obtained by HPLC are presented on Table 9.

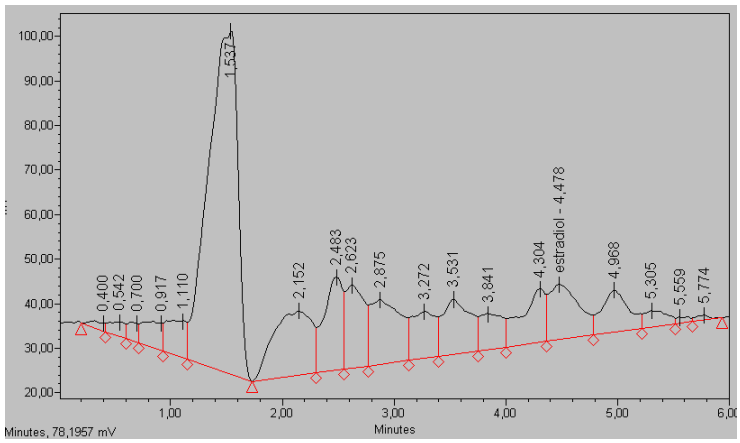
Table 9- Detection of E2 in each extract and respective extraction periods

		E2	
Elution (20 mL MeOH:Aa (4:1))	Time (h)	Spiked	Non-spiked
1	1	not determined	not determined
2	4	detected	detected
3	4	detected	detected
4	4	b.d.l[a]	b.d.l

[a] b.d.l. – below the detection limit



(a)



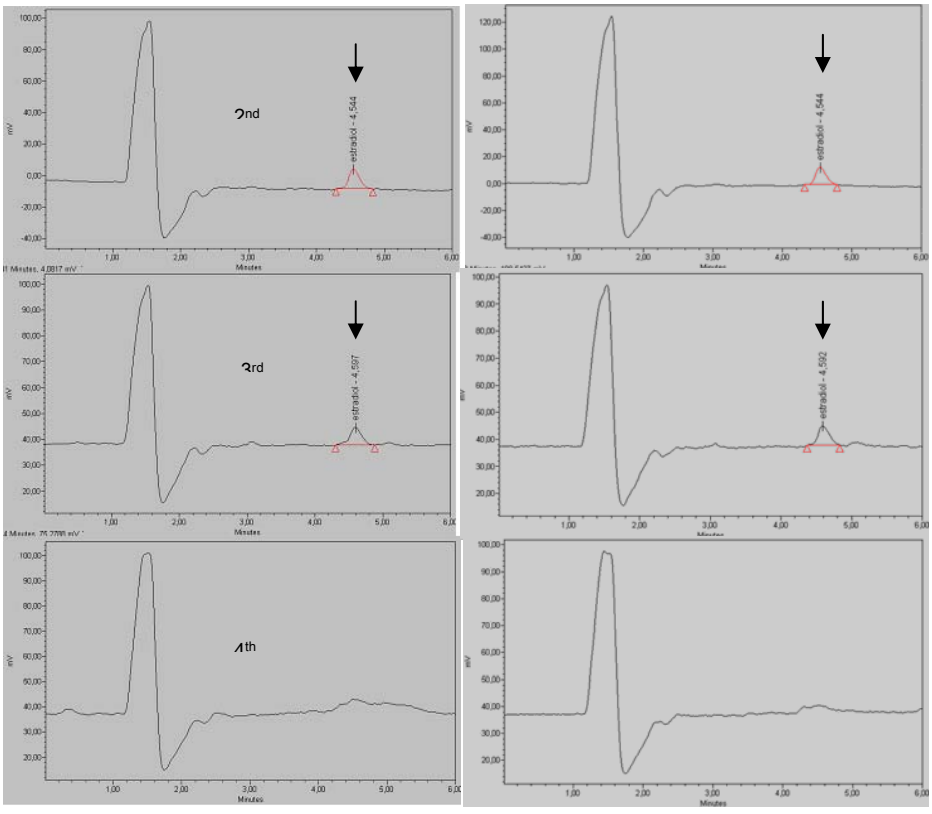
(b)

Figure 19 - Chromatographic data from the fluorescence detector for the first recovery from Non-spiked (a) and spiked (b) samples

Wastewater samples are a very complex media and may have diverse organic compounds with possible affinity to bind non-specifically to MIM. Consequently, the chromatograms obtained from the first 20 mL of solvent recovery, showed varied peaks with some intensity at different retention times. Figure 19 shows the same peaks for spiked and non-spike except the one at 4,968 min. These peaks may correspond to hydrophobic compounds present in wastewater which have a molecular structure with affinity for MIM binding sites. In fact, it was not possible to quantify the amount of E2 recovered during the first elution, neither for spiked nor non-spiked samples, because another compound was eluted with approximately the same retention time (~4,3 min) thus interfering with E2 peak. It was not possible to separate these components with the mobile phase in use (Acetonitrile:

Water (50:50)). The samples were tested with different ratios of Acetonitrile: Water: 15:85, 30:70 and 40:60, in order to optimize the separation and retention time of the compounds, however it was not possible to separate the peaks (data not shown). To avoid this problem in further experiments, a previous wash step with a solvent with less affinity for E2 should be performed to remove non-specifically bound compounds. A further improvement for the extract analysis can be made by using a HPLC-MS/MS method. It permits a multi-compound analysis with possible quantification and qualification at low detection levels.

Three more elutions were made until no E2 was detected in the solvent. These three elutions were performed during 4 hours in order to maximize the recovery. The respective chromatograms are shown on Figure 20.



(a) Spiked (b) Non-spiked

Figure 20 - Chromatographic data from Spiked (a) and Non-spiked (b) samples recoveries

Figure 20 confirm the retention time of E2 at ~4,5 min. It is also clear that contaminants recovered by non-specific bonds to MIM were rapidly eluted in the first 20 mL of solvent (after 1 hour of elution), with E2 being the only compound recovered on the second and third elutions. The determination of E2 concentration with this data was not possible because it would give an underestimation of this contaminant in both spiked and non-spiked samples. However E2 was detected in the non-spiked samples confirming the occurrence of this compound in the effluent coming from secondary treatment.

6. Conclusions and Perspectives

E2-molecular imprinted PES-TiO_2 microspheres were prepared for recognition of 17β-estradiol (E2) using a liquid-liquid phase separation technique that can also be regarded as a macromolecular self-organization method. It was easy to prepare the imprinted microspheres using this method. The E2-MIM morphology was analyzed by SEM suggesting an opaque and homogenous surface with many small pores inside.

The novel E2-PES-TiO_2 synthesised particles were having the expected behaviour since the column system was performing without clogging problems and the microspheres remained quite stable along the column. Moreover, it was confirmed that PES provides excellent mechanical, thermal and chemical stability. The removal batch experiments confirmed that MIM prepared by the new method are more efficient and specific adsorbents than NIM. The addition of TiO_2 was therefore not interfering with the MIM specificity. Indeed, it was verified that as the treated volume becomes larger the specificity of the MIM becomes more evident. Hence, MIM prepared by the method described in this work could be used to load columns to perform a SPE method for selective removal and pre-concentration of E2.

The polymers were easy to regenerate by washing with a solvent under normal conditions of temperature and pressure. Different elution procedures were made leading to the conclusion that the

best way to regenerate the particles is by elution steps in batch. It was also confirmed that a complete regeneration of the particles was possible with less volume of solvent for longer periods. The drawbacks of this procedure are the need to unload the microspheres from the column, designing this process to operate in a semi-continuous mode, and a longer elution period. However, this step becomes more economical and more efficient; also, a higher pre-enrichment of trace contaminants can be reached to help further destruction by biological or chemical treatment.

The MIM were successfully operating in the adsorption column under normal conditions without pressure or clogging problems. With water influent percolated at 15 mL/min and a column of 5 mL packed with MIM, 47% and 60% (with mixing) of removal were obtained from synthetic water highly concentrated in 17β-estradiol. In fact, 6 runs (circulations) through the system were needed to get these extraction rates. The removal percentages reached were still well below the necessary to carry out a scale-up based on this type of system. It must be pointed out that it was the first usage of the liquid-liquid phase separation technique to produce molecularly imprinted PES microspheres with E2 as template. Thus, the investigation on molecularly imprinted PES microspheres for the removal of water contaminants such as E2 is still at an early stage. More studies in order to optimise the ratios of the imprinting matrix components/template/solvent need to be done in order to get higher binding potential and efficient removals. Nevertheless, the set-up established was operating well, readily automated by minimum sample manipulation.

With a previous optimisation of new microspheres well succeed and confirmed with several recognition, binding and removal tests, a scale-up of the established system during this project, for a pilot study evaluation, can be made. Subsequently, treatment tests with larger volumes of synthetic water and wastewater, at higher flow rates, will be possible to carry out. In the case of successful removal and enrichment of contaminants, it will be feasible to project this kind of system in a full scale. It is also important to notice that a further essential development in molecular imprinting is to develop preparation methods which extend the specificity of the imprinted polymers to bind a larger range of toxic compounds and be used for advanced wastewater treatment purposes.

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