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Immunochemical Methods for Biomonitoring of Chlorophenols as Potential Biomarkers of Exposure

Memoria presentada para optar al título de Doctor en Química por

Mikaela Ivanova Nichkova

Върви народе възродени,
към светли бъднини върви...
... Напред, науката е слънце,
което във душите грей...!

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Abbreviations

Ab	Antibody
AcOH	Acetic acid
ACGIH	American Conference of Governmental Industrial Hygienists
ACN	Acetonitrile
ADI	Average Daily Intake
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
AE	Active ester
Ag	Antigen
Anti-IgG	Goat antibody generated against rabbit immunoglobulines
As	Antisera
ATSDR	Agency for Toxic Substances and Disease Registry
BAT	Biological Tolerance Values
BEI	Biological Exposure Indexes
BM	Biological Monitoring
BSA	Bovine Serum Albumin
BSTFA	*N,O*-Bis(trimetilSilyl)TriFluroAcetamide
CA	Coating antigen
$CDCl_3$	deuterated chloroform
CDR	Complementary Determing Regions
CONA	Conalbumin
CP	Chlorophenol
CR (%)	Cross-reactivity (%)
CV (%)	Coefficient of Variation (%)
1D	1 Dimension
2D	2 Dimension
DBP	Dibromophenol
DCC	Dicyclohexylcarbodiimide
DCP	Dichlorophenol
DIC	Diisopropyl carbodiimide
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EIA	Enzyme ImmunoAssay
ELISA	Enzyme Linked Immunosorbent Assay
EMIT	Enzyme-Multiplied Immunoassay Technique
EPA	Environmental Protection Agency
ET	Enzyme tracer
EtOH	Ethanol
EU	Eoropean Union
Fab	Antibody binding fraction
Fc	constant (crystallized) fraction
FIA	Fluorescence Immunoassay

FIIA	Flow Injection ImmunoAssay
FNH_2	Fluoresceinamine
Fv	Variable fraction
GC	Gas Chromatography
GC-ECD	Gas Chromatography-Electron Capture Detection
GC-FID	Gas Chromatography-Flame Ionization Detection
GC-MS	Gas Chromatography-Mass Spectrometry
HBM	Human Biological Monitoring
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
HS	Health surveillance
HTS	High Throughput Screening
IAC	Immunoaffinity Chromatography
IAE	Immunoaffinity Extraction
IgG	Immunoglobuline G
IS	Immunosorbent
ISt	Internal standard
KLH	Keyhole Limped Hemocyanin
LC	Liquid Chromatography
LIF	Laser Induced Fluorescence
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MA	Mixed anhydride
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry
MeOH	Methanol
MRL	Minimal Risk Level
NIOSH	National Institute for Occupational Safety and Health
NHS	*N*-Hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
OEL	Occupational exposure limits
OSHA	Occupational Safety and Health Administration
OVA	Ovalbumin
pAb	Polyclonal antibody
PAHs	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyls
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline solution Tween 20
PCDD	Polychlorinated dibenzo-p-dioxins
PCDF	Polychlorinated dibenzofurans
PCP	Pentachlorophenol
ppb	part per billion
QFIA	Quenching Fluorescence Imunoassay

RIA	RadioImmunoAssay
Rpm	revolutions per minute
scFv	single chain variable fraction
SD	Standard deviation
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
TBP	Tribromophenol
TCA	Trichloroanisole
TCP	Trichlorophenol
TCP-F	hapten 5-fluorescein
TCDD	tetrachlorodibenzo-p-dioxin
TCF	Total Chlorine Free
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLV	Threshold Limit Values
TMB	Tetramethylbenzidine
TtCP	Tetrachlorophenol
V_L	Light variable domains
V_H	Heavy variable domains
WHO	World Health Organization

1. Introduction

Nowadays environmental contamination is a recognized worldwide problem. Significant part of the environmental pollution is caused by the application of pesticides in agriculture, horticulture and forestry, by emissions of hazardous chemical substances formed during industrial processes and combustion reactions. A large number of these compounds and/or their degradation products are highly toxic and they have negative effects not only on the ecosystem, but also on human health. In this context it is necessary to control the presence of xenobiotics in the environment and at the same time to assess the risk to human health due to the presence of these chemicals at workplace or in the environment in order to prevent adverse effects.

1.1. Environmental and human biological monitoring

Environmental monitoring provides information about the use, the fate and the degradation of contaminants, and more important, about their concentration in air, water, soil, plants, and foods to which the population is exposed. These data can be used only as an estimation of the external human exposure to xenobiotics. Because of various environmental factors (e.g. temperature, humidity, and wind), biological factors (e.g. work habits, smoking, compliance with safety protocols, etc.), the adsorbed or internal dose estimates generated by environmental monitoring are frequently under suspect. Actual internal exposure can be evaluated only by ***biological monitoring (BM)*** (see **Figure 1.1**). Biological monitoring, defined as an evaluation of exposure through the measurement of parent compounds, metabolites, or DNA and protein adducts in biological samples, can confirm the absorbance of a chemical from the environment regardless of the route [2,3]. BM is a complement to environmental monitoring. It is an approach for early detection of exposure, and indirectly of risk, and prevention of future adverse health effects. Although BM was promoted as a valuable method for exposure assessment as early as 1954 [4], in recent years it is gaining increasing popularity in occupational and environmental health in order to assess individual chemical exposure [5-7].

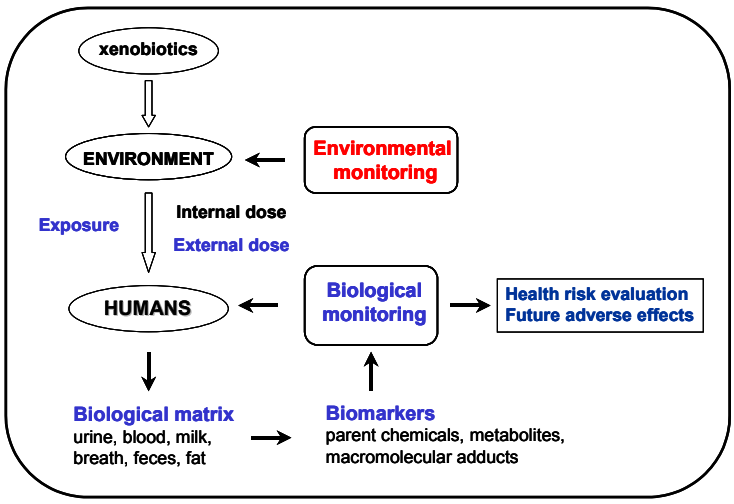


Figure 1.1. Environmental and biological monitoring

Biomonitoring usually involves the determination of an exposure marker (***biomarker*****)** such as parent chemical and/or its metabolite(s) in body fluids (blood, urine), tissues or exhaled breath [6]. The definition of biomarker varies slightly acrross scientific fields, but the most widely used by toxicologists, occupational hygienists and epidemiologists is an indicator of some change in a biological system that can be related to the exposure to, or the effect from, a specific chemical [8-10]. Biomarkers are classified into ***biomarkers of exposure*** to xenobiotics (include intact parent chemicals, metabolites, and macromolecular adducts), ***biomarkers of effect*** (may be an endogenous compound, a measure of functional capacity, or any indicator of actual or potential health impairment), and ***biomarkers of susceptibility*** (may be a genetic characteristic or a preexisting disease) [8].

Some of the desired characteristics of an ideal biomarker of exposure are: specificity in relation to the xenobitic, stability in vivo and in vitro, availability by non-invasive sampling, relevance to the dose of exposure, presence in concentrations detectable by current analytical methods at a reasonable cost in a reasonable time [9,11]. Recently Aitio outlined three types of biomarker specificity: analytical specificity (well known to analytical chemists and pertaining to the ability of the method to respond specifically to the analyte in a complex matrix), metabolic specificity (the extent to which the marker is the product of the chemical of interest), and source specificity (the extent to which the source of exposure can be attributed to the workplace, lifestyle factors, diet, ecological exposures, etc.) [12].

In the selection of a ***biological medium*** for monitoring are important the excretion patterns of the selected analyte, the complexity for obtaining the sample, the sampling time, and the availability of data relating excretion to exposure [13]. Urine is the most widely used biological medium, because is the route of excretion for many xenobiotics, it is easy to collect, and the measured concentration of the parent chemical or metabolite is proportional to the adsorbed dose for the majority of xenobiotics. To standardize results, analyte concentrations in urine usually are adjusted with respect to specific gravity,

osmolality, or creatinine concentration. Blood is also an excellent medium for biomonitoring, but practical considerations often limit its use for routine control. Thus, obtaining samples requires an invasive procedure and blood is frequently a troublesome matrix for many analytical methods. Expired breath is used to monitor volatile chemicals. Nails, hair, sweat, saliva, milk, feces, teeth, and fat have potential utility for biological monitoring, but each is accompanied by disadvantages, such as collection difficulties or problems related to contamination, storage, or analysis of samples.

1.1.1. Governmental regulations

In order to control environmental contamination and to protect the population from it, governmental agencies have established several directives. In 1976 the European Union (EU) set the "black list" of 132 dangerous substances (based on their toxicity, stability and bioaccumulation) that should be monitored in waters (Directive 76/464/CE). In the list are included organohalogen compounds, substances that can be converted to them and other carcinogenic compounds (pesticides, polycyclic aromatic hydrocarbons, etc.) (see **Table 1.1.**).

Table 1.1. Chlorophenols included in the lists of priority contaminants set by EU and by US-EPA

EU **Directive 76/464/CEE**	**US-EPA** **List of priority contaminants (EPA 8041)**
2-Amino-4-chlorophenol	Phenol
2-Chlorophenol	2-Chlorophenol
3-Chlorophenol	2,4-Dichlorophenol
4-Chlorophenol	4-Chloro-3-methylphenol
4-Chlorophenol-3-methylphenol	2,4,6-Trichlorophenol
3,4,5-Trichlorophenol	Pentachlorophenol
3,5,6-Trichlorophenol	
2,4,6-Trichlorophenol	
2,4,5-Trichlorophenol	
2,3,4-Trichlorophenol	
Pentachlorophenol	

Later the Directive 96/61/CE advised the establishment of monitoring programs to control the emission of industrial discharges from textile, pulp, and paper factories, refineries, etc. to air, water and soil. More recently, the Directive 2000/60/CE sets the general

strategies for water protection and the priority dangerous substances to be controlled during the following years. Among them are hexachlorobenzene (HCB), hexachlorocyclohexane (HCH, lindane), polycyclic aromatic hydrocarbons (PAHs), pentachlorobenzene, pentachlorhenol (PCP), etc. With the aim to protect the health of the general population the European Union has established the value of 0.1 μg/L as maximum individual concentration and 0.5 μg/L for total concentration of pesticides and related products in drinking water (Directive 80/778/CEE). In the US the Environmental Protection Agency (EPA) has established the maximum level for each pesticide or its transformation products according to their toxicity [14]. For the protection of the public against toxic effects of pesticides, regulatory agencies in many countries have established standards specifying the residue levels of each pesticide in various foodstuffs. Thus, WHO has evaluated and reviewed the acceptable daily intakes (ADI) of pesticides [15].

In occupational hygiene, biological monitoring is used as part of an array of techniques for evaluating the workers risk of health damage due to exposure to chemical agents. It is especially valuable when conducted to indicate exposure to potentially harmful chemicals at a time when preventive measures can be effective in reducing or eliminating the health risk. Many organizations worldwide have developed reference values for biological monitoring. The Biological Exposure Indexes (BEI) published by the American Conference of Governmental Industrial Hygienists (ACGIH) are reference values intended as guidelines for the evaluation of potential health hazards in the practice of industrial hygiene [16]. The ACGIH also establishes reference values for airborne chemical concentrations in the workplace, called Threshold Limit Values (TLV) and represent conditions under which nearly all workers may be exposed repeatedly over a working lifetime without adverse health effects. Each year ACGIH publishes a list of those chemicals for which there are BEIs. The list consists of reference values for chemicals and their metabolites in urine, exhaled air, and blood. The chemical contaminants included are inorganic compounds, organic solvents, gases, pesticides, and specific metabolites. Occupational exposure limits (OELs) issued in the US by the Occupational Safety and Health Administration (OSHA) require measurements of toxic substances in air rather than in biological samples [17]. Most of OSHA's limits were

adopted from the 1968 list of TLVs set by the ACGIH. Although there are no formal requirements to monitor exposures to these substances, it is implicit in the standards that air sampling will be performed. TLVs and in lesser extent the BEIs have been used by governmental agencies around the world as the basis for workplace environmental regulations. At this time, biological monitoring plays at most a supporting or complementary role in industrial hygiene practice in the US since BEIs have been established only for ~50 compounds, while about 700 have TLVs set [18].

BM has been used more widely in Europe than in the US as one of the several complementary tools for assessing worker exposure to chemicals. The most extensively used reference values for biological monitoring in Europe are the Biological Tolerance Values (BAT) of the German Research Society [19]. Reference and human biological monitoring (HBM) values for environmental toxins have also been recommended by the Commission on Human Biological Monitoring of the German Federal Environmental Agency [20]. Reference values are intended to indicate the upper margin of the current background exposure of the general population and used to identify subjects with an increased level of exposure. HBM values are derived from human toxicology and epidemiology studies and are intended to be used as a basis for a health-related evaluation of HBM data. Usually the commission recommends two different HBM values: HBM I, the concentration of an environmental toxin in a human biological material (usually blood, serum, plasma, or urine) bellow which there is no risk for adverse health effects in individuals of the general population; and HBM II, above which there is an increased risk for adverse health effects. Reference values and methods have been developed also in UK [21]. United Kingdom Control of Substances Hazardous to Health (COSH) uses two types of OEL – the occupational exposure standard (OES) and the maximum exposure limit (MEL). In many cases the reference values used worldwide are similar, but there are significant differences in both the approach to setting biological reference values and in their interpretation [19]. For example, the BAT are established as maximum tolerable levels, whereas in the US the BEI values established by ACGIH are generally linked to air concentrations and apply nearly all workers. A part from different approaches, geographic variation in background levels may also influence the reference values.

During the past decade the European Union has established its own procedure for setting OELs [22]. The Directive 98/24/EC on the protection of the health and safety of workers from the risks related to chemical agents at work lays down the minimum requirements regarding protection of workers. It defines the terms OELV, biological limit value (BLV), health surveillance (HS) and set employers` obligations to determine, assess and prevent risks arising from hazardous chemical agents. A key feature of the directive is the setting of exposure limits, on the one hand, in the form of indicative and binding OELVs (the concentration, in the air breathed in by a worker, of a chemical agent, over a certain period) and, on the other hand, in the form of binding BLV (the concentration, in the human body, of the relevant agent). The Directive 2000/39/EC establishes the first list of indicative OELVs containing limits for 63 substances and also places an obligation on member states to establish national OELVs taking into account the Community OELVs, determining its nature in accordance with national legislation and practice. The last directive must have been implemented by December 2001.

1.1.2. Biological monitoring methods

The classical procedure used to identify and quantify the biomarkers includes its extraction and separation from other potentially interfering substances of the biological sample and further quantification by instrumental analysis (GC, LC, MS), genetic toxic assays, enzymatic and bacterial assays, and immunoassays. The current techniques and methods applied for BM are reviewed in [7,23,24]. Although conventional analytical methods offer detection limits in the sub-ppb level, they are labor intensive, require specialized expensive equipment and sometimes suffer recovery losses. Techniques that are not laboratory-based (test kits, dipsticks, indicators, portable devices, and real-time monitors) are needed to reduce the cost, and to provide information in time to avoid hazardous chemical exposures. BM as part of the occupational medicine is seeking for techniques “that cost next to nothing per analysis” [12]. Immunochemical techniques are gaining application in the area of human exposure assessment [25,26]. Immunoassays have been developed for the detection of key urinary biomarkers of exposure to pesticides

and other environmental pollutants, such as triazines, organophosphorous insecticides, carbaryl, naphtalene, and PAHs [27-30].

1.2. Organochlorinated compounds

1.2.1. Production, use and application of chlorophenols

Chlorophenols have been used as anti-stain agents for wood products since the early 1930s. They have been widely used as anti-fungal agents (preservatives for adhesive, synthetic textiles, rubber, wood, paints) in the textile and leather industry and as anti-microbial agents in pulp and paper mill systems. Although the use of chlorophenols as preservatives has dropped down in many developed countries and bleaching procedures use alternative procedures other than chlorination processes, chlorophenols are also important synthetic intermediates in the manufacture of many industrial and agricultural chemicals, such as chlorophenoxy herbicides (2,4,5-T), dyes, fungicides (i.e. prochloraz) or bleaching agents (i.e. chloranile). The importance of this industry is evidenced by the great amount of phenoxy herbicides and chlorophenols produced (e.g. only one phenoxy herbicide manufacturing plant in Germany produced more than 9820 tones/year for the period 1965-1989) [31]. Chlorophenols, also appear in the environment when industrial wastewater or drinking water resources containing organic materials such as phenolic derivatives (i.e derived from lignin), or certain aromatic acids are disinfected. Wastewaters from pulp and paper mills have important levels of chlorinated organic compounds due to the bleaching processes used. Thus, between 0.6 to 1.6 mg/L adsorptive halogenated organic compounds (AOX) were estimated to be emitted daily, by the Finish industries in the late 80's [32]. In fact is worth noting that the amount of AOX found in several American and European pulp and paper samples was significantly high (6 to 1265 mg/L), even if in some occasions these were marketed as total chlorine free (TCF) or elemental chlorine free (ECF) products [33,34]. Chlorophenols are also formed during the combustion of organic matter in the presence of chlorine or chlorine containing compounds [35,36].

1.2.2. Physicochemical properties of 2,4,6- and 2,4,5-trichlorophenols

2,4,6-Trichlophenol (2,4,6-TCP) is yellow to pinkish-orange solid and 2,4,5-trichlophenol (2,4,5-TCP) is a colorless solid, both in the form of needles. The technical grade product of 2,4,6-TCP is 97% pure and of 2,4,5-TCP is 95% pure. All technical and formulated 2,4,6- and 2,4,5-TCP products are contaminated in varying degrees by 1,3,6,8-tetrachlorodibenzo-p-dioxin, 2,3,7,8-trichlorodibenzo-p-dioxin, and tetra-, penta, hexa-, and hepta-chlorodibenzofurans. Some of their physicochemical properties are presented in **Table 1.2**.

Table 1.2. Physicochemical properties of 2,4,6-TCP and 2,4,5-TCP

Chemical/Physical Property	2,4,6-TCP	2,4,5-TCP
Molecular formula	$C_6H_3Cl_3O$	$C_6H_3Cl_3O$
Molecular weight	197.45	197.45
Boiling point (760 mmHg)	246°C	247°C
Melting point	69°C	69°C
pKa	6.23	7.4
Log Kow (octanol/water)	3.69	3.72
Water solubility (25°C)	800 mg/L	1200 mg/L

The commercial production of 2,4,5- and 2,4,6-TCP in the US was first reported in 1950. In the 80s their production in US ceased. The present major use of 2,4,5-TCP is as an intermediate in the manufacture of the herbicide 245-T (2,4,5-trichlorophenoxyacetic acid), and the pesticides Silvex (2-(2,4,5-trichlophenoxy)propionic acid, 2,4,5-TP), Ronnel (o,o-dimethyl-o-2,4,5-trichlorophenyl phosphorothionate), and sodium 2,4,5-trichlorophenate [37]. 2,4,6-TCP is still used in the synthesis of the fungicide prochloraz and in the synthesis of chloranile.

1.2.3. Distribution and degradation of trichlorophenols in the environment

Even though the discharge of chlorophenols to the environment has decreased during the last decade in developed countries, their persistence has determined their widespread distribution in the environment still today. Chlorophenols have been identified as usual contaminants of surface waters. Particularly, 2,4,5-TCP and 2,4,6-TCP were detected in several rivers and lakes in Europe, Canada and South Africa, e.g. in the Isipingo river, South Africa, the concentrations were ranging from 0.41 to 6.51 μg/L for 2,4,5-TCP and from 1.6 to 26.6 μg/L for 2,4,6-TCP [38]; in the Peace and in the St. Maurice Rivers in Canada 2,4,6-TCP has been detected at concentrations up to 30 μg/L [39,40]; in a lake in Finland 2,4,5-TCP was found at concentrations 0.001-0.651 μg/L [41]. Similarly, trichlorophenols have been found to contaminate soils and sediments. They have been measured in sediments of Swedish riverine at low μg/g levels, 40-50 Km downstream from cellulose plant discharges [42]. Concentrations of 25-10000 μg/Kg have been also measured in the sediments of streams in British Columbia in Canada [43]. In the suspended particles and the sediment of Lake Saimaa in Finland, 2,4,6-TCP was detected at concentrations between 0.14 and 0.99 μg/g [44]. In New Zealand the sediments of the Tarawara and Waikato Rivers contained up to 7.5 and 9.4 ng/g of 2,4,6-TCP, respectively [45]. As a consequence significant levels of TCP accumulate in fish and seafood [40,46]. A bioconcentration factor of 250-310 has been measured in fish. Contamination of the groundwater near sawmills or waste sites has also been reported [47,48]. Drinking water may also contain TCPs among other chlorinated phenolic compounds at low levels. Thus, concentrations of 2,4,6-TCP varying from 0.03 to 0.7 μg/L have been detected in the drinking water of Janakka (Finland), Ville Mercier (Quebec, Canada), and in Utah (USA) [49-51]. 2,4,5-TCP has been detected at concentrations 35-59 μg/L in Finland [52] and at 28 ng/L in Taiwan [53].

The complete degradation of TCPs (to HCl, CO, CO_2) in the environment is slow. Various studies on aerobic and anaerobic degradation of TCPs were carried out [54-56]. Their abiotic degradation (photocatalytic and ozonation) was also described [57-59].

1.2.4. Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) of 2,4,6- and 2,4,5-trichlorophenols

Human exposure to chlorophenols takes place by skin absorption, inhalation or oral ingestion. As no rigorous toxicokinetic studies in humans are available, the information on their ADMET is based mainly on animal studies. ADMET studies on rats fed with 2,4,6-TCP revealed that the highest concentration of 2,4,6-TCP was found in kidney with biological half-times between 1.4 and 1.8 h in the blood, brain, fat, kidney, liver and muscle [60]. 82% of the dose is excreted in the urine and 22% in the feces. Binding capacity of chlorophenols to serum proteins is enhanced according to their molecular weight and level of chlorination [61,62]

Chlorophenols are excreted to the urine as such or in the form of glucuronide and sulfate conjugates, the amount of conjugation depending on the particular chlorophenol and its concentration in urine [63,64]. At low concentrations sulfate conjugation is dominant, but when chlorophenol concentration increases, acid conjugation becomes more important [64]. Tubular reabsorption has been observed. A study carried out with sawmill factory workers demonstrated that tri- and tetrachlorophenols were excreted totally conjugated (97-92.9% for 24h urine, 80.5-79.1% for morning urine and 86.4-81.6% for afternoon urine) and the extent of conjugation of PCP was lower (76.2% - 24h urine, 69% - afternoon urine) [64]. The urinary half-times for tri-, tetra-, and pentachlorophenol were 18h, 4.3 days and 16 days, respectively.

2,4,6-TCP and 2,4,5-TCP are within the five chlorophenols out from 19 considered to have significant ***toxicological effects*** and potential carcinogenicity. Although human studies have lead to inconclusive results, those carried out in animals have provided sufficient evidence of the 2,4,6-TCP carcinogenic effects and inadequate evidence of 2,4,5-TCP carcinogenicity [65]. CPs affect porphyry metabolism and have been confirmed to have mutagenic and immunosuppressive properties [66]. Their toxicity seems to be related to the dechlorination mechanism in tissues, which causes enzyme inactivation and liver dystrophy [67]. It was also demonstrated that 2,4,6-TCP induces

chromosome breakage and aneuploidy in vitro [68]. Long-term exposure of experimental animals fed with 2,4,6-TCP contaminated food results in changes in liver and spleen cells, in a decrease of the body weight and a decrease of the survival [69]. Test involving acute exposure of animals, such as the LD_{50} test in rats, have shown 2,4,6- and 2,4,5-TCPs to have moderate acute toxicity [70]. Chronic (long-term) ihnalation by humans is associated with respiratory effects such as cough, chronic bronchitis, chest wheezing, altered pulmonary function, and pulmonary lesions [69]. Acute intoxication produces irritation of the skin, eyes, nose, mucous membranes and upper respiratory tract [71]. Additional exposure symptoms may include skin rashes (chloracne), fever, convulsions, nausea, vomiting, etc.

An oral Minimal Risk Level (MRL) of 0.003 mg/Kg/day would be applicable to TCPs for intermediate exposure duration according to the updated toxicological profiles of chlorophenols of the Agency for Toxic Substances and Disease Registry (ATSDR) of the U.S. Department of Health and Human Services [70]. Similarly, in Finland an ADI of 50 µg/day has been established [48]. A recent study among U.S. men aged 30-60 demonstrated that the occupational exposure to chlorophenols is a risk factor for nasal and nasopharyngeal cancers closely associated with the duration of exposure [72]. On the other hand, long-term use of chlorophenol polluted household drinking water and consumption of contaminated fish have been correlated with certain symptoms, related to those occurred after chlorophenol occupational exposure, such as gastrointestinal and skin symptoms [73].

1.2.5. Trichlorophenols as potential biomarkers of exposure to orgarnochlorine compounds

2,4,5-TCP and 2,4,6-TCP are considered urinary pesticide residues. They are metabolic products of some organochlorine fungicides (hexachlorobenzene), organochlorine insecticides (lindane) [74], certain organophosphorous insecticides [75,76], and chlorophenoxyacid herbicides, [77]. Animal experiments demonstrated that chlorophenols (including 2,4,5-TCP and 2,4,6-TCP) are the main metabolites of HCH

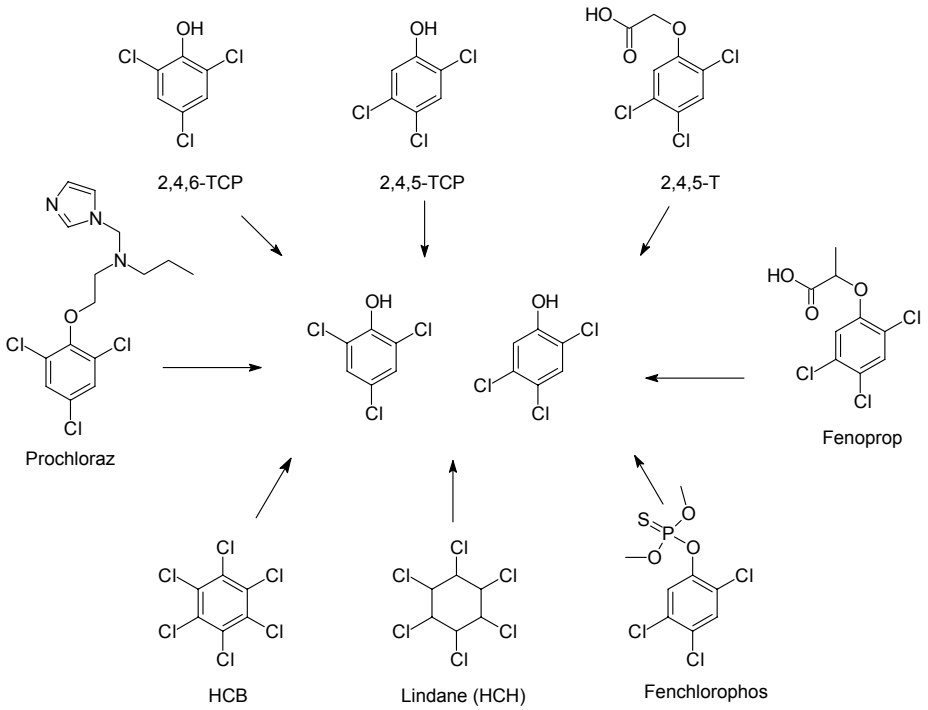


Figure 1.2 Trichlorophenols as potential biomarkers of exposure

[78,79]. In addition, 2,4,5-TCP was found to be the urinary metabolite of HCB [80]. **Figure 1.2.** gives an overview of the chlorinated substances which can be metabolised to 2,4,5- and 2,4,6-TCPs in the human body. The resulting TCPs are excreted with the urine as glucuronic conjugates or sulfates. A study on the excretion and conjugation of chlorophenols in saw mill workers demonstrated that the average apparent half-time for 2,4,6-TCP was 18h [64]. This has an important implication for biological monitoring of chlorophenol exposure: Specimen collection for analysis must take place rather soon after exposure; otherwise, the TCP that is rapidly excreted may go undetected [64]. Thus, the detection of 2,4,5-TCP and 2,4,6-TCP in urine may be an indicator of exposure to chlorophenols and to the above-mentioned organochlorine substances, frequently used in industry, commerce, agriculture and private households for a variety of reasons [66].

1.2.5.1. Chlorophenols as indicators of exposure to dioxins

Chlorophenols have been named ***predioxins*** and can be contemplated as indicators of the formation of dioxins [81]. In all combustion processes where chlorine or chlorine compounds are present, chlorobenzenes, PCBs, and CPs are formed, the last two groups recognized as the actual precursors for dioxin (PCDDs and PCDFs) formation [82]. Dioxins, including the most toxic congener 2,4,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are formed as unwanted impurities during the manufacturing of chlorophenols and their derivatives. Thus, in a German plant the estimated median concentration of TCDD in 1955-1970 was 5 ppm in 2,4,5-T products, but could reach values between 10 to 50 ppm in some processes. Measurements made in the 1970s, revealed that the median concentration of TCDD was about 1 ppm and lower than 0.1 ppm after the introduction of a new dioxin extraction procedure in 1981 [83]. Dioxins also form in the manufacture of pulp and paper following chlorination processes and are emitted from the metallurgical industry. They are ubiquitous environmental contaminants and have been found throughout the world in soil [84,85], sediment [86], food (fish, meat, cow's milk) [87-90], human adipose tissue [91,92] and mothers' milk [93,94]. The main source of exposure of the general population is through ingestion of contaminated food. It is important to note that animals´ exposure to PCP (cows fed PCP-treated wood and steers accidentally exposed to PCP-treated wood) results in high levels of dioxins in milk and beef products [87,88].

PCDDs/PCDFs have been recognized as hazardous highly toxic organochlorine compounds [95], since the Seveso accident in 1976 [96] and other accidents at different chlorophenol and phenoxy herbicide manufacturing plants [97-101],. Exposure to dioxins has been associated to an increased risk for all cancer combined and numerous acute and chronic health effects among humans (severe chloracne, porphyria cutanea tarda, endocrine disruption etc.) (For recent review on sources of exposure and toxicological effects see [102]. Thus, an increased risk for circulatory disease, especially ischemic heart disease and possibly diabetes was present among phenoxy herbicide and chlorophenol production workers and sprayers according to an IARC international cohort study [103].

The exposure to the TCDD-contaminated herbicide 2,4,5-trichloropheno1 (2,4,5-T) in an Austrian plant resulted in symptoms of chloracne and neurogical diseases in the production workers [104]. A morbidity study of former pentachlorophenol-production workers demonstrated that 17.8% of them had evidenced current or past chloracne [105].

In general, their excretion in urine can be regarded as an indirect indicator of current exposure to dioxins [81]. A recent study [106] demonstrates that 2,4,5-TCP and other chlorophenols are transformed in vitro to PCDDs/PCDFs by a biochemical-catalyzed oxidation. The experiments confirmed that a biochemical formation of dioxins from precursors such as chlorophenols can take place in the human body and that this metabolic pathway may lead to a higher inner exposure with PCDDs/PCDFs than is now assumed. **Figure 1.3** presents an overview of the possible relationship between CPs and dioxin exposure and their excretion in biological fluids.

There is such type of controversy in the literature dedicated to the discussion on the correlation between CPs and dioxin exposure and excretion and the resulting health

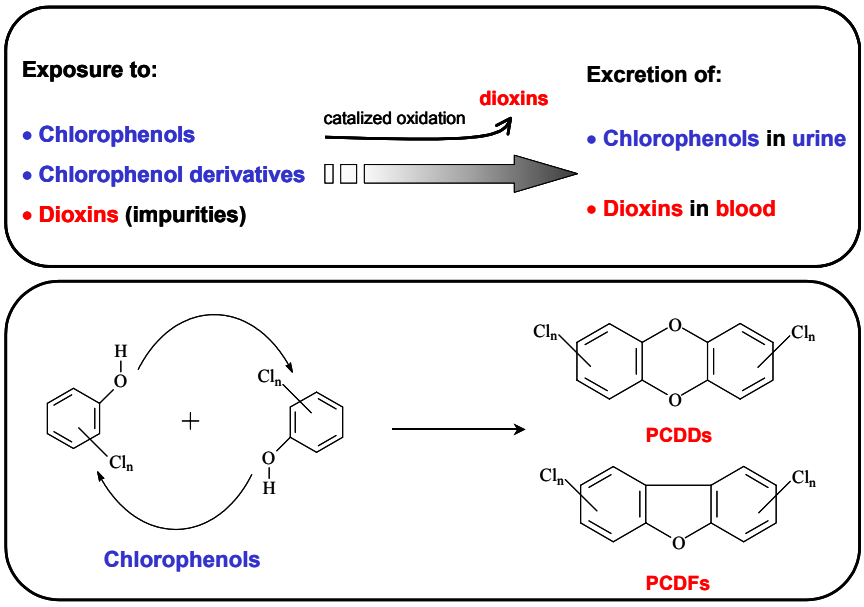


Figure 1.3. Relationship between chlorophenol and dioxins exposure and excretion

effects. However, no doubt exists on the fact that occupational exposure to CPs, chlorophenoxy herbicides, and other chlorophenol derivatives produces increased dioxin levels in serum. This observation has been extensively described for a variety of industrial sectors (waste incinerators, saw mills, chemical plants, etc.). A study performed on workers from three different incineration plants in Germany demonstrated that both levels of chlorophenols in urine and PCBs in serum were related to the exposure and they were higher for the plant with greater amounts of waste burned [35]. An association between PCDD/PCDF blood plasma concentration and past exposure to chlorophenols has been found for sawmill workers exposed to chlorophenol-containing anti-stain agents [107,108]. A study conducted with a group of 20 active employees from BASF exposed to phenoxy acid herbicides or/and chlorophenols revealed that although median PCDDs/PCDFs concentrations in blood were comparable to the background concentrations in the general population, median levels of 2,3,7,8-TCDD, the sum of hexachlorodibenzo-p-dioxin, pentachlorodibenzofurnas, and two dioxin toxicity equivalents values were higher in 7 employees assigned to synthesis operations than for 12 employees assigned to other operations [109]. Time of employment in certain production areas in chlorinated phenoxyacetic acid herbicides (2,4-D, 2,4,5-T) and chlorophenol-manufacturing plants was shown to be a good predictor of 2,3,7,8-TCDD levels in blood serum or adipose tissue [83]. Similarly, serum levels of 2,3,7,8-TCDD were positively correlated with overall duration of spraying for phenoxy herbicides sprayers [83]. They were considerably lower among workers employed after the implementation of government regulations to reduce dioxin content in phenoxy herbicides. Finally, it has been reported that chimney sweeps had higher levels of PCDDs/PCDFs and PCBs in blood, and chlorophenols in urine than those of the control group without occupational exposure [110]. However, this study does not present a clear evidence on the relationship between urinary CP levels and serum dioxin levels: low correlations between blood-fat PCB concentrations as well as urinary chlorophenol concentrations and the corresponding PCDDs/PCDs blood concentrations were found [110]. In this connection the authors pointed out that chlorophenols indicate actual exposure to chlorine compounds, whereas PCDDs/PCDFs and PCBs indicate chronic exposure. There are also some uncertainties, in the literature, on the correlation between

the presence of dioxin in serum and the resulting toxicological effects. Thus, no clear association to some non-cancer mortalities such as cerebrovascular disease was observed among phenoxyacid herbicide and chlorophenol production workers and sprayers [103]. It was also reported little evidence to support a relationship between the risk of childhood cancer and paternal occupational exposure to chlorophenate fungicides and their dioxin contaminants in British Columbian sawmills [111].

In conclusion, it is evident that further well-defined studies on the correlation between dioxin exposure and urinary chlorophenol excretion would be needed to establish if CPs, which are analytical easier to determine, are suitable as predicting parameters for the levels of internal PCDD/PCDDF concentration.

1.2.5.2. Occupational exposure to chlorophenols

Occupational exposure may occur through inhalation and dermal contact with this compound at workplaces where 2,4,6-TCP, 2,4,5-TCP or the above-mentioned substances are used or produced. The National Institute for Occupational Safety and Health (NIOSH) had statistically estimated that 851 workers were potentially exposed to 2,4,6- and 2,4,5-TCP during a three years period National Occupational Exposure Survey (NOES) [112]. Worker exposure has been reported in plants producing such kind of chlorinated pesticides or fungicides [83,105,109], in pesticide spray operators [83], in Finnish saw mills [108], industrial incinerator waste plants [36,113] and electrical utility linemen in contact with chlorophenols treated poles used in the electric line construction [114]. Some examples for urinary and serum chlorophenol levels for occupationally exposed persons are presented in **Table 1.3**. It can be observed that the levels are quite different depending on the industry sector. According to the literature found TCPs are mainly determined in urine, although they can be also detected in blood serum. As biological fluid, urine is more convenient for samples collection, treatment and analysis. On the other hand, the levels of TCPs in urine are higher than in serum [64].

Table 1.3. Cholorophenols levels in biological fluids from occupationally exposed persons (μg/L)

Industry	Clorophenol	Min-Max conc.	Mean conc.	Reference
Lindane plant	2,4,6-TCP	280-3900	850	[74]
	2,4,5- and 2,3,6-TCP	300-6900	2300	
Chemical plant	2,4,6-TCP	nd-168	27	[115]
	2,4,5-TCP	nd-1168	132	
	2,3,6-TCP	nd-284	52	
	2,3,5,6-TtCP	nd-528	69	
	2,3,4,5-TtCP	nd-800	111	
	PCP	nd-1266	154	
TtCP plant: synthesis	Total CPs	nr	2300	[83]
packers		nr	1900	
Saw-mills	2,4,6-TCP	nd-0.24		[116]
	2,3,4,6-TtCP	nd-0.13	nr	
	PCP	nd-0.08		
	2,4,6-TCP	nr	5.04 [d]	[64]
	2,3,4,6-TCP	nr	7.59 [d]	
	PCP	nr	0.34 [d]	
	2,4,6-TCP	nd-0.53 [c]	0.22 [c]	
	2,3,4,6-TCP	1.05-6.01 [c]	2.78 [c]	
	PCP	0.26-1.49 [c]	0.85 [c]	
	2,4,6-TCP	nd-3.81	nr	[117]
	2,3,4,6-TCP	1.35-16.36	nr	
	PCP	nd-19.56	nr	
Waste incinerator	2,4,6-TCP	nr	3.5 [a]	[118]
	2,4,5-TCP	nr	0.6 [a]	
	PCP	nr	1.9 [a]	
	2,4,6-TCP	0.16-28.3	2.91	[36]
	2,4,5-TCP	0.02-1.18	0.46	
Harbour mud	2,4,6-TCP	1.9 [b]	0.5	[119]
	2,4,5- and 2,3,5-TCP	0.7 [b]	0.7	
	2,3,4,6- and 2,3,5,6-TtCP	2.1 [b]	<0.5	
	PCP	11.5 [b]	1.6	
Electrical utility	2,4,6-TCP	2-5	3	[120]
	2,4,5-TCP	2-34	4	
	2,3,4,6-TtCP	1-9	2	
	2,3,4,5-TtCP	1-5	1	
	PCP			
	<15 years experience	nr	45.8 [a]	[114]
	>15 years experience	nr	18.9 [a]	

[a] The data is expressed in μg/g creatinine urine.
[b] The reported value is the 95% percentile.
[c] The data correspond to serum levels. The remaining data in the table correspond to urinary levels.
[d] The data is expressed in μmol/L.
nr – not reported; nd-not detected (below LOD);

Finally, it is important to note that in most of the studies reported the health risk assessment due to occupational exposure is evaluated on the basis of a comparison of the chlorophenol levels with those corresponding to control groups, such as administration workers, general population living close to the industry plant or reference values set for the country.

1.2.5.3. General population exposure to chlorophenols

Exposure of the general population has been noted via contaminated environment, textiles, leather goods, domestic preservatives, and edible products [121,122]. Several studies performed in Germany and the US demonstrate that 2,4,5-TCP and 2,4,6-TCP can be found in the urine of a high percentage of the population [121-124]. Some examples of the cholorophenols levels in urine and serum from non-occupationally exposed persons are presented in **Table 1.4**. Urinary 2,4,5-TCP and 2,4,6-TCP were found in 54% and 37%, respectively, of the population of Germany [121]. Besides, the levels of 2,4,5-TCP and 2,4,6-TCP in urine of refugees from the former USSR, Africa and Asia were significantly higher than those found in Germans (the medians differed by a factor of three) [124]. The analysis of the urine of adults in the US having not known occupational contact to chlorophenols or related substances revealed that 2,4,5-TCP and 2,4,6-TCP were present in 20 and 9.5% of the population [122]. In another study on the levels of Arkansas children 2,4,5-TCP was observed in 54% of the samples and 2,4,6-TCP in 11% of the samples [125].

Although exposure limit values (BEI/BAT) for some CPs (p-chlorophenol, pentachlorophenol) in biological fluids are established, there is still lack of information about TCPs (see **Table 1.5**).

Table 1.4. Cholorophenols levels in biological fluids from non-occupationally exposed persons (μg/L)

Country	Clorophenol	Min - Max	Median	95%	Reference
Germany	2,4,6-TCP	1.2-15	2	4.7	[121]
	2,4,5-TCP	0.8-75	1	4.5	
	2,3,4,6- / 2,3,5,6-TtCP	2.5-191	2	22.2	
	2,4,6-TCP	dl [a]-3.97	0.61	1.74	[123]
	2,4,5-TCP	dl-3.35	0.25	1.1	
	2,3,4,6-TtCP	dl-4.86	0.36	1.86	
	PCP	0.5-189	2.8	nr	[126]
	PCP	nr-59.3 [b]	1.7 [b]	6.1 [b]	[127]
Asia	2,4,6-TCP	<dl-3.80	0.60	2.37	[124]
	2,4,5-TCP	<dl-3.00	0.85	2.73	
	2,3,4,6- / 2,3,5,6TtCP	<dl-15.49	<dl	6.94	
Africa	2,4,6-TCP	nd-35.78	1.90	33.05	
	2,4,5-TCP	0.34-8.89	2.00	8.48	
	2,3,4,6- / 2,3,5,6TtCP	<dl-4.73	<dl	4.49	
former USSR	2,4,6-TCP	0.40-11.70	1.70	9.63	
	2,4,5-TCP	0.70-42.50	1.85	34.77	
	2,3,4,6- / 2,3,5,6TtCP	<dl-16.30	0.60	12.69	
Yugoslavia	2,4,6-TCP	0.50-19.32	1.71	16.32	
	2,4,5-TCP	0.50-14.4	1.80	12.05	
	2,3,4,6- / 2,3,5,6TtCP	<dl-5.40	0.60	5.25	
	2,4,6-TCP	nd-4.00	1.1	1.70 [d]	
	2,4,5-TCP	0.51-3.00	1.1	1.90 [d]	
	2,3,4,6- / 2,3,5,6TtCP	<dl-1.70	<dl	0.80 [d]	
Spain	TCPs	not detected	-	-	[128]
	2,3,4,6- / 2,3,5,6TtCP	nr	6.2	nr	
	PCP	4-136	25	nr	
	PCP	2.5-116.5	21.9 [b]	nr	
Spain	2,4,5-TCP	nr	0.4 [c]	nr	[118]
	2,4,6-TCP	nr	0.6 [c]	nr	
	PCP	nr	1.7 [c]	nr	
Canada	PCP	0.1-3.6	0.5	nr	[129]
Slovakia	2,4,6-TCP	nd-106	nd	nr	[115]
	2,4,5-TCP	nd-380	nd	nr	
	2,3,6-TCP	nd-116	17	nr	
	2,3,5,6-TtCP	nd-161	nd	nr	
	2,3,4,5-TtCP	nd-268	nd	nr	
	PCP	nd-300	19	nr	
USA	2,4,6-TCP	nd-41	<1	4	[125]
	2,4,5-TCP	nd-32	1	7	
	2,4,6-TCP	0.8-63	<1	3	[75]
	2,4,5-TCP	1.2-25	<2	3.3	

[a] The detection limit (dl) is 0.02 μg/L for urine samples.
[b] The data correspond to serum levels. The remaining data in the table correspond to urinary levels.
[c] The data is expressed in μg/g creatinine urine. [d] The data correspond to the 75th percentile.
nr – not reported; nd-not detected (below limit of determination)

Table 1.5. Reference values for biological monitoring

Parent chemical	Biomarker	Biological fluid	BEI[a] / BAT[b]	Reference values
PCP	PCP	urine	2 mg/g creat.[a]	8 μg/L [c] (6 μg/g creat.)
		serum/plasma	5 mg/L [a]	12 μg/L [c]
Lindane	Lindane	blood	20 μg/L [b]	0.3 μg/L [c]
		serum/plasma	25 μg/L [b]	-
	trichlorophenols	urine	nd	4.5 μg/L [d] (5 μg/g creat.)
HCB	HCB	serum/plasma	150 μg/L [b]	0.4 μg/L [c]
	trichlorophenols	urine	nd	4.5 μg/L [d] (5 μg/g creat.)
p-dichlorobenzene	2,5-dichlorophenol	urine	nd	5 μg/g creat. [d]
chlorobenzene	p-chlorophenol	urine	25 mg/g creat.[a]	-

[a] Biological Exposure Index (OSHA); [b] Biological Tolerance Value (GCIHHCCWA)
[c] Reference values of the Commission on Human Biological Monitoring of the German Federal Environmental Agency. The value reported for HCB corresponds to the age group 26-36 years [20]; [d] Background levels in the general population of Germany [36,81]; nd – not defined

At present, reference values based on the background level of the general population are used to evaluate whether occupational exposure to CPs or related organochlorine compounds has taken place or not. The reference values means that 95% of the non-occupationally exposed general population has concentrations lower than this level. In Germany the reference value of 4.5 μg/L (5 μg/g creatinine) for 2,4,6-TCP and 2,4,5-TCP is set [81]. In Finland, a biological reference limit of 0.5 μmol/L has been established for total urinary chlorophenols (as the sum of TCPs, TtCPS and PCP) for non-exposed persons [130].

1.2.6. Analytical methods for trichlorophenol determination in environmental and biological samples

The methods used for chlorophenol analysis are quite diverse and depend on the type of the matrix sample used (water, solid, air, urine, serum, etc.). Analytical techniques mainly used in the determination of chlorophenols in environmental and biological samples are gas chromatography with electron-capture (GC-ECD) [78,123,131], flame ionization

(GC-FID) [132,133] or mass spectrometer detectors (GC-MS) [117,121,134-136]. Liquid chromatography (LC or HPLC) in combination with ultraviolet (UV) [137], electrochemical detection [77,138-140] or capillary electrophoresis [141-143] has also been used. The standard and official methods for determination of TCPs in water are the EPA methods 604, 625 and 8041 [144-146]. They are based on chlorophenol liquid-liquid or solid phase extraction followed by derivatization with diazomethane, methylene chloride or pentafluorobenzyl bromide and GC-FID, GC-ECD or GC-MS detection. However, the LOD of these methods (for example, 0.64 μg/L for method 604-GC-FID, 0.58 μg/L for method 604-GC-ECD and 2.7 μg/L for method 625-GC-MS) cannot satisfy the requirements of the EU regarding pesticide levels in drinking water (Directive 80/778/CCE).

The most frequently employed analytical procedures for chlorophenols involve the use of solvent extraction [117,120,147], solid-phase extraction [36,120,123,131,136,148], solid-phase microextraction [116,149,150] or supercritical fluid extraction [151,152]. Different derivatization reactions are often required to increase the volatility of the analytes [117,121,135,153,154]. Although many of the methods mentioned above offer very low detection limits (at the low ppb range), they lack the necessary speed for rapid screening of large number of samples, such as those needed for biomonitoring. This is due to the extensive sample clean-up required prior to the analysis as well as the time needed for chromatographic measurements. Furthermore, the lipids present in the serum and in urine samples often cause interferences in the GC/LC analysis of these compounds, especially at levels close to the detection limit. Therefore, rapid, cost-effective, and reliable screening techniques with simple or no sample pre-treatment are essential for routine assessment of human and environmental exposure to hazardous chemicals.

1.3. Immunochemical techniques

1.3.1. Antibody - structure, properties and production

The key component in any immunochemical technique is the antibody. Antibodies are globular proteins produced by the immune system of the mammals as a defense against foreign agents (antigen, Ag). The structure of the antibody molecules varies depending on their isotype, but all possess common characteristics, typified by the immunoglobulin G (IgG) subclass, which is the one most used in immunochemical applications. The IgGs (Mw 150 kDa) are composed of four polypeptide chains: two identical heavy (H) and two identical light (L) chains (50 kDa and 23-25 kDa, respectively) interlinked by disulphide bridges (see **Figure 1.3**) [155].

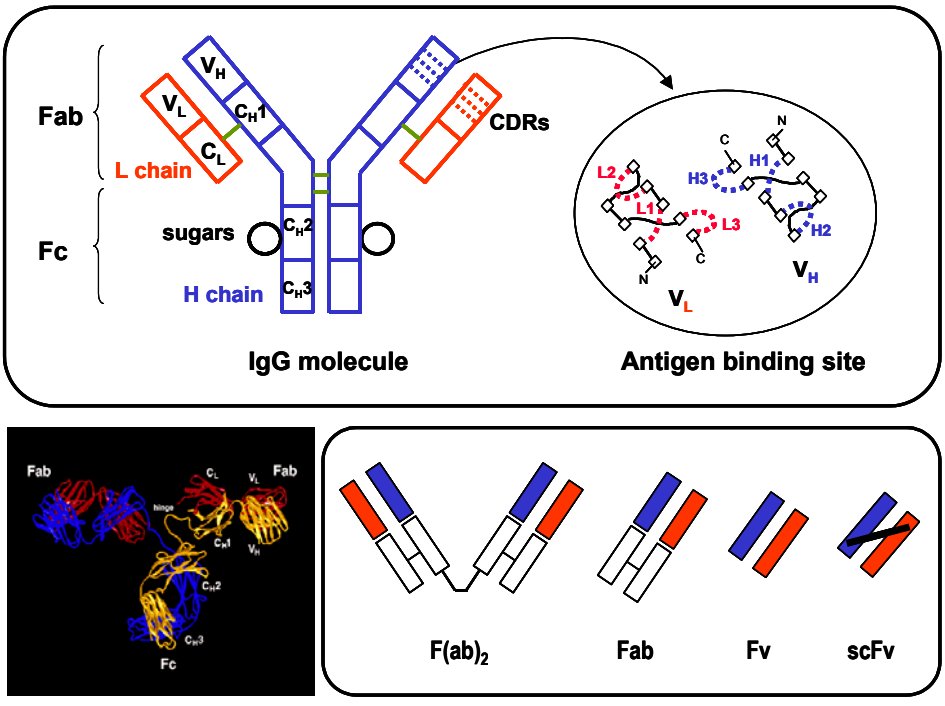


Figure 1.3. Antibody structure. Constant domains: C_L; C_H1, C_H2 and C_H3; variable domains: V_L and V_H. Antigen binding site: CDR (hypervariable regions): L1, L2, L3; H1, H2, H3; constant framework: black line.

The region that carry the *a*ntigen *b*inding sites is known as Fab fragment, and the constant (*c*rystallized) region that is involved in immune regulation is termed Fc. Both H and L chains are divided into constant (C) and variable (V) domains based on their amino acid sequence variability. The most important regions of the antibody with regard to the Ab-Ag binding interaction are the variable regions, consisting of the association of the V_H and V_L domains. Within each of these domains there are three distinct areas of even higher sequence variability, known as complementary determing regions (CDRs), bounded by four framework (FR) regions. The CDRs of the V_H and V_L domains formed the antigen-binding site. Their amino acid sequence and spatial conformation determine the binding specificity and binding strength (affinity) of the Ab molecule. The interaction between Ab and Ag is reversible and it is stabilized by electrostatic forces, hydrogen bonds, hydrophobic and Van der Waals interactions [156]. At equilibrium when the AbAg complex is formed, the affinity constant Ka is defined as :

$$Ka = \frac{[AbAg]}{[Ab]\cdot[Ag]}$$

Antibody fragments can be generated by enzymatic or chemical degradation and by genetic engineering [157]. These include the $F(ab)_2$, Fab, Fd fragments; Fv, the smallest fragment required for complete Ag binding; single-chain scFv fragments, in which Fv is stabilized by a flexible amino acid linker; etc. (see **Figure 1.3**).

Currently, antibodies can be produced by three different techniques, yielding ***polyclonal*** Abs (pAb, if purified from animal's antiserum), monoclonal Abs (mAb, if produced via hybridoma techniques), and recombinant Abs (if based on genetic engineering). Polyclonal antiserum contains several different populations of IgGs exhibiting binding properties to different epitopes (antigenic determinants) on the antigen. The activity of a polyclonal antiserum is a combination of the responses from different Abs having as well as different tolerance to solvent and matrix interferences, which is important for assay performance [155]. Many environmental immunoassays are based on pAbs, because they

can have a high affinity for a given analyte (average detection limit 0.01-0.1 ppb), robust nature for analysis in sample matrixes and their production is far simpler than monoclonal and recombinant Abs [158]. Polyclonal Abs can be difficult to obtain in large and reproducible quantities; however, large-scale production can be achieved using goats and sheeps.

Monoclonal Abs are produced by fusing antibody-producing spleen cells from an immunized animal (mouse) with mutant tumor cells derived from myelomas (cancerous murine plasma cells) [159]. Thus, mAbs contain a unique defined IgG molecule produced by a single cell clone. The advantages of the hybridoma technology are that, theoretically, it provides an unlimited amount of Abs with identical affinity for an Ag and the possibility to screen for those clones of Abs with the desired pattern of specificity. However, generally mAbs have lower affinities to small molecules than pAbs and are more expensive to produce [26].

Recombinant Ab phage display technology, developed during the last decade, has allowed the generation of a variety of Ab fragments mimicking the immune response *in vitro*. In general the whole process include (1) the preparation of Ab encoding libraries (V-genes repertoires) derived by a range of methods (by isolation of mRNA from hybridoma, spleen cells or lymphocyte of immunized or no immunized donors, assembly of gene fragments to create synthetic V-gene repertoires, etc.); (2) cloning of the genes in a bacterial plasmid vector; (3) expression in bacteria (*E.coli*) and then co-infection with helper bacteriophage virus, displaying Ab fragments on its surface as a fusion with normally occurring coat protein; (4) screening for antigen specificity and antigen-driven selection (for reviews see [160,161]). Although the development of these methods has been conducted mainly by therapeutic application, recombinant Ab fragments were used also in environmental analysis [162-165]. Genetic engineering provides an elegant way not only for providing unlimited amounts of biorecognition molecules but also for the alteration of existing properties and the supplementation with additional functions for their application in immunosensors [166,167]. Another very important advantage of these

techniques over the conventional methods for obtaining pAbs and mAbs, is their cost-effective production.

1.3.2. Antibody-based analytical methods

Immunochemical methods are based on the specific (selective) binding of an Ab to an Ag (or analyte). They are bioaffinity methods that can be used in bioanalytical determination (immunoassays) and bioseparation (e.g. immunoaffinity extraction) of a target analyte in a variety of sample matrixes [26,157]. Nowadays, immunoassay methods provide cost-effective, sensitive, and selective analyses for many compounds of environmental and human health concern. Development on immunosensor systems is a great promise for future automation of these immunodetection strategies. Immunoaffinity chromatography methods are also used as efficient sample preparations prior to immunochemical or instrumental detection.

Immunochemical methods are simple, fast, and sensitive, with high throughput, easily learned by nonscientist users and can be very cost-effective, compared to conventional analytical methods which are often time-consuming, labor intensive, expensive, and laboratory oriented. Immunoassays are versatile and can be tailored in various formats to particular purposes, ranging from quantitative laboratory tests to simple "yes/no" screening tests that are field-portable. These advantages can be exploited in monitoring programs where great number of samples needs to be analyzed. The major disadvantage in immunoassays is the tendency toward a positive bias due to matrix effects in real sample measurements (false positives). Thus, as effective screening techniques immunochemical methods are complementary in effect to the standard analytical techniques.

1.3.2.1. Bioanalytical detection methods

1.3.2.1.1. Immunoassays

Immunoassays (IAs) were first applied in clinical settings where their sensitivity and selectivity were used for diagnostic purposes [168]. In the 1970s chemists realized the potential of immunoassays for the environmental monitoring of pollutants [169,170]. Antibody-based analytical detection methods have gained favor in environmental analytical processes beginning in the 1980s, when they were recognized as useful screening techniques for the detection of compounds of environmental regulatory concern [171]. Over the following years a great number of IAs for detection of trace amounts of contaminants, such as pesticides, industrial residues and their degradation products in the environment (water, soil, sediments, etc.) have been developed ([158,172-174]). Nowadays, an important number of IAs for pesticides are commercially available and the US-EPA has validated some of these assays and included 13 of them in the SW-846 methods list [175]. The IA technology have found also wide application in pharmaceutical, food, veterinary and forensic analysis. A more recent application is in the area of human exposure assessment to a variety of industrial chemicals, trace contaminants (such as PAHs, PCBs, PCDDs) and pesticides reviewed in [7,25,26,28,29]. Several IAs have been developed recently for urinary detection of metabolites, such as mercapturates [176-178], phenyl glucuronides [179,180], 3,5,6-trichloro-2-pyridinol [181-184], PAHs [30], etc. Human biomonitoring studies usually require the analysis of large number of samples (e.g., routine urinary screening of workers in a chemical manufacturing plant, or monitoring large populations to determine background levels of xenobiotic metabolites). High sample throughput is a feature of immunoassays that makes them particularly suited to field studies or large-scale monitoring efforts. In addition, Ab cross-reactivity may allow both parent molecule and its metabolite to be registered simultaneously. In this context, EPA encourages the development of immunochemical techniques and devices for human exposure monitoring [26].

Immunoassays are very versatile and they can readily be formatted to suit particular requirements of the intended application [156,185]. They are homogeneous (if the immunoreaction takes place in solution) and heterogeneous (if one of the immunoreagents is immobilized on a solid support (micro plates, tubes, micro spheres, magnetic particles). Heterogeneous assays does not require a separation between the free and bound phases, whereas homogeneous assays require no separation before detection since the Ag-Ab binding modulates the activity of the reporter enzyme or label. Although simpler to perform (quick, no washing steps), homogeneous immunoassays are more hampered by matrix interferences. Heterogeneous formats have found a broader application to many fields and also to different kind of analytes.

In IA procedures the quantification of small molecules is performed under competitive conditions. The general strategy of competitive assays is based on the competition between the free Ag (analyte) and a fixed amount of labeled Ag for a limited amount (low concentration) of Ab. At the end of the reaction the amount of labeled Ag and subsequently the free Ag is determined. The labels used to quantify the immunoreaction can be of different nature. The initially developed IAs (RIA, radioimunoassays) used radioactive reporters [186,187], but due to the generation of radioactive residues and the adverse health and environmental impacts produced, they have been replaced by other types of labelling substances. Nowadays enzymes are the most common used labels in IAs for environmental analysis [188]. The most frequently used enzymes are horseradish peroxidase (HRP), alkaline phosphatase (AP), and glucose oxidase (GO). Enzyme immunoassays (EIA) use the enzyme substrate reaction to produce a chromogen often absorbing in the visible region. The amplifying effect of the enzyme label allows for the development of many sensitive assays using absorbance measurement at the end point. The more common EIA techniques are the competitive homogeneous IA named EMIT (Enzyme Multiplyed Immunoassay Technique) and the heterogeneous EIA, ELISA (***Enzyme Linked Immunosorbent Assay***). ELISAs are the most popular EIAs used in environmental monitoring. The most usual configurations for the analysis of small molecules (direct and indirect ELISA) are shown in **Figure 1.4**. In the direct competitive Ab coating format the equilibrium is established between the Ab bound to the solid phase

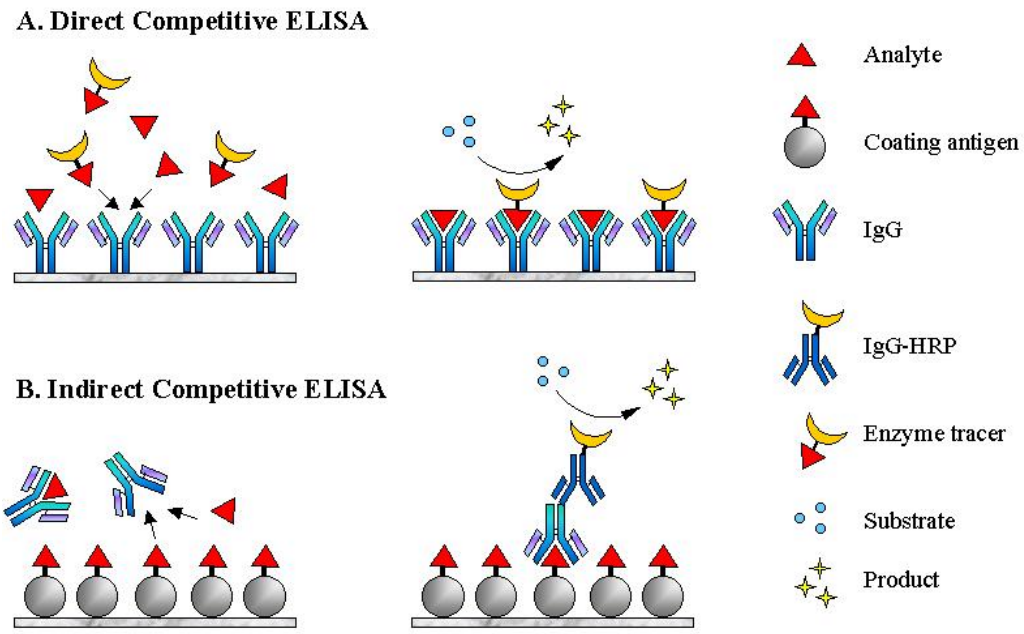


Figure 1.4. Scheme of the most commonly employed heterogeneous IA configurations (ELISA) for the detection of low molecular weight analytes.

(either directly or through orienting agents, such as antibodies generated against immunoglobulins (anti-IgG) or protein A), the analyte and the analyte-enzyme tracer (ET) that are in the solution (see **Figure 1.4.A**). After the main incubation step, the unbound reagents are washed away and the amount of label bound to the solid phase by the Ab is measured. A decrease in the signal is directly proportional to the amount of analyte present in the sample.

Another possibility to perform the direct IA is the Ag coated format, which is based on the competition between the immobilized Ag (or surface derivatized analyte) and the analyte for a fixed small amount of labeled Ab. The indirect competitive ELISA works under the same principle as the coating Ag (CA) format, but the concentration of the analyte is measured in this case indirectly by the quantification of bound Ab with a second labeled Ab (labeled anti-IgG) (see **Figure 1.4.B**).

Fluorescence immunoassays (fluroimmunoassays, FIA) use fluorescent labels, such as fluorescein, rhodamine, cyanines or rare earth quelates (Eu(III), Tb(III)) [189]. Fluorimetry (fluorescence) was introduced in immunological assays mainly because of its superior sensitivity (10-1000-fold higher) compared to that of spectrophotometric methods. The search for single molecule detection [190] has stimulated the development of a variety of FIAs that are gaining popularity. The main limitation of these techniques when applied to biological fluids is the limited sensitivity due to background noise. This has provoked the search for novel labels with longer excitation and emission wavelengths.

The current trends in IAs include an emphasis on non-radioactive labels, more specific reagents, and improved formats for automating or performing IAs [185]. In this context, flow injection methods coupled to immunoassays (FIIA) allows continuous operation and high sample throughput providing rapid results and sensitivity detection [191,192]. Furthermore, IA automation and system integration continue to be some of the most dynamic and innovative areas for research and development in the clinical diagnostic industry [193].

1.3.2.1.2. Immunosensors

Immunosensors are part of the biosensor technology field, which is broad, diverse and multidisciplinary in nature. The term biosensor is defined as an analytical device that consists of a biological component (Ab, enzyme, receptor, DNA, cell, etc.) in intimate contact with a physical transducer that converts the biorecognition process into a measurable signal (electrical or optical) (see **Figure 1.5**). In this sense, immunosensors are affinity-based sensors designed to detect the direct binding of an Ab or an Ag to form an immunocomplex at the transducer surface. Depending on the transducer technology employed, immunosensors can be divided into three principal classes: optical, electrochemical and piezoelectrical (see **Table 1.6**).

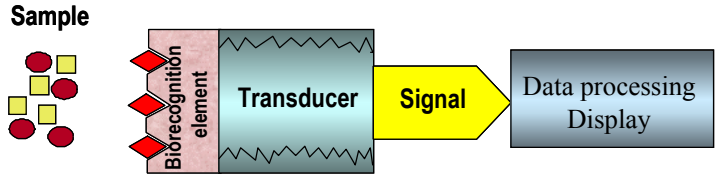


Figure 1.5 Essential components of a biosensor (biorecgnition element, transducer and electronic part involved in data processing and display).

Table 1.6. Transducers and principle of detection in biosensors

Transducer	Method	Principle	Detection [a]
Optical	Surface Plasmon Resonance (SPR)	Changes in refractive index	D
	Total Internal Reflection	Evanescent wave	I
	Photon Correlation Spectroscopy (PCS)	Changes in scattered light	D
Electro chemical	Amperometric	Changes in the oxidation state of the electrochemical substances	I
	Potentiometric	Changes in the surface potential	D, I
	Conductimetric Impedimetric	Changes in the conductivity of the solution	D, I
Piezo electric	Bulk acoustic	Changes in the frequency of a piezoelectric crystal	D
	Surface Acoustic Wave (SAW)		
Thermal	Thermistor devices	Changes in temperature produced as the result of an enzymatic reaction	I

[a] D –direct detection; I – indirect detection.

Furthermore, as they are based on the principles of the solid-phase immunoassays, they can be either direct (where the immunochemical reaction is directly determined by measuring the physical changes induced by the formation of the immune complex) or indirect (where a sensitive detectable label is combined with the Ab or Ag of interest). As environmental contaminants are often small-sized molecules, most of the devices reported perform indirect measurements by using competitive immunoassay configurations and/or labels such as enzymes, fluorescent chemicals, or electrochemically active substances. The sensitivity and specificity of an immunosensor are determined by the same characteristics as in other solid-phase immunoassays, namely, the affinity and specificity of the binding agent, and the background noise of the detection system (transducer).

Challenges still encountered with biosensor development are fabrication, immobilization onto a transducer, effective signal generation, miniaturization and integration.

The majority of biosensor research is currently directed towards clinical applications, but a variety of immunosensors for environmental applications have also been developed in the last years [194-197]. Both types of optical and electrochemical transducers have demonstrated to provide detection limits in the low parts-per-billion to the high parts-per-trillion range. Research on the Ab field is still growing and future perspectives also count on the use of recombinant fragments with desired characteristics, better defined regarding their chemical structure, stability, etc.. As the orientation of the binding molecule (recognition element) after being immobilized onto a transducer would likely to affect the sensitivity it is desirable to equip the recombinant Ab fragments with suitable tags to facilitate their orientation at the sensor surface [166]. However, implementation and commercialization of immunosensor technology is being slow. With further improvement in the fabrication of miniaturized biosensors that are simple, rapid, portable, cost-effective, and with ability to regenerate, it is anticipated that they will provide a powerful tool for environmental and biological monitoring.

1.3.2.2. Bioseparation techniques

Immunochemical techniques are recognized not only as bioanalytical detection methods, but they have found also application for extraction and sample preparation prior to a second analytical method [26,198-200]. ***Immunoaffinity extraction*** (IAE) (or ***immunoaffinity chromatography*** (IAC)) for trace-analysis of low-molecular weight analytes in complex matrices have several advantages over conventional SPE formats: it provides a highly selective extraction based on specific molecular recognition; antibody cross-reactivity allows multiresidue analysis targeting a parent compound and its metabolites or a class of structurally related analytes; IAC allows pre-concentration of analytes from large sample volumes resulting in low detection limits; it minimizes reliance on organic solvents to achieve efficient separation [201-203] and it have great potential for the selective purification especially of polar compounds which are difficult

to isolate by other commonly used supports. A wide range of applications of solid-phase immunoaffinity extraction has been developed, both on-line and off-line, being coupled mostly to HPLC, GC and capillary electrophoresis [200], and to a lesser extent to immunoassays [204-206]. The most common applications are focused on organic pollutants such as pesticides and toxins in environmental samples (water, soil, crops, food) and drugs in biological matrices (plasma, urine, tissue) reviewed in [201,202,207-210]. However, besides its demonstrated versatility and specificity, IAC is still seldom used for human exposure monitoring studies. Isolation of PCDD and PCDF from serum samples using IAC prior to GC-MS has been reported [211-213]. Evaluation of the occupational exposure to PAH has been performed by measuring the urinary excretion of PAH metabolites using IAC-HPLC [214,215].

Although proven to be suitable for sample preparation and cleanup, IAC is still seldom used in routine environmental analysis. This is partly because very effective and relatively cheap solid-phase extraction methods for some contaminants, such as pesticides are already available commercially. In addition, the method requires relatively high amount of Ab and hence it is regarded as uneconomical if Ab is limited. This limitation can be easily overcome by the use of pAbs from large animals (goat, sheep, cattle) or monoclonal Abs. The actual high price of these immunoreagents increases the cost of the IAC technique. In this context recombinant DNA technologies will hopefully decrease the price, facilitating the widespread commercial application of immunoaffinity purification methods.

1.4. Immunochemical techniques for organochlorinated substances

A variety of immunochemical methods have been developed for many organochlorinated substances, such as pesticides (organochlorines, chlorophenoxy acids) and trace contaminants. Some examples for the immunodetection of the most important

organochlorinated contaminants in environmental and biological matrices are presented in **Table 1.7.A** and **B**.

Table 1.7.A. Immunochemical techniques for organochlorinated substances

Compound	Immunochemical technique [c]	Matrix	Reference
Pesticides			
Organochlorines [a]			
DDT	ELISA	buffer	[216]
		water, soil, food	[217]
	CFIA	buffer	[218]
PCP	IA	drinking, ground, surface water	[219]
Triclopyr	ELISA	water	[182,220]
		water, soil	[221]
		urine	[183,184]
	PFIA	tap water	[222]
	Immunosensor	water	[223]
Chlorophenoxy acids [b]			
2,4-D	RIA	buffer	[224]
	ELISA	buffer	[225,226]
		river water, urine	[227]
	EIA	soil	[228]
	IAC	water	[229]
	dipstick IA	water, fruit, urine	[230]
	FIA	buffer	[231-233]
		fruit juice	[234]
	Immunosensor	water	[235-241]
		soil extract	[242]
	Immunosensor	water	[243]
	Immunosensor	buffer	[244]
2,4,5-T	ELISA	apple juice	[245]
	PFIA	buffer	[233]
	Immunosensor	water	[238,240]
Dichlorprop	PFIA	apple	[246]
MCPA	Immunosensor	water	[239]

[a] DDT (1,1,1-(trichloro)-2,2-bis(*p*-chlorophenyl)ethane); PCP (pentachlorophenol); Triclopyr (3,5,6-trichloro-2-pyridinol); [b] 2,4-D (2,4-dichlorophenoxyacetic acid); 2,4,5-T (2,4,5-trichlorophenoxyacetic acid); dichlorprop (2-(2,4-dichlorophenoxy)propanoic acid); MCPA (2-methyl-4-chlorophenoxyacetic acid); [c] ELISA (enzyme linked immunosorbent assay); RIA (radioimmunoasay); EIA (enzyme immunoassay); IAC (immunoaffinity chromatography); FIA (fluoroimmunoassay); PFIA (polarization fluoroimmunoassay); CFIA (chemiluminiscent flow immunoassay).

Table 1.7.B. Immunochemical techniques for organochlorinated substances

Compound	Immunochemical technique [b]	Matrix	Reference
Trace contaminants [a]			
PCBs	ELISA	buffer	[247]
		fish tissue	[248]
		soil	[249]
	IAC	water	[210]
	PFIA	buffer	[250]
	LIA	buffer	[251]
	Immunosensor	river water, soil	[252]
		buffer	[253]
PCDDs	ELISA	buffer	[165,254]
	IAC	milk	[255]
PCDDs/PCDFs	IAC	milk, serum	[256]
		serum	[212,213,257]
2,4-Chlorophenol	ELISA		[258]

[a] PCB (polychlorinated biphenyls); PCDD (polychlorinated dibenzo-*p*-dioxins); PCDF (polychlorinated dibenzofurans). [b] ELISA (enzyme linked immunosorbent assay); LIA (liposome immunoagregation assay); IAC (immunoaffinity chromatography); PFIA (polarization fluoroimmunoassay).

In addition, there are many commercially available immunoassays for environmental analysis of DDT, lindane, 2,4-D, chlorpyrifos and 3,5,6-trichloro-2-pyridinol, PCBs [174]. It is important to note that the US-EPA recognized some of these commercial immunoassays as official methods of detection and they were included in the list of SW-846 methods (see **Table 1.8**).

Table 1.8. Immunoassays methods for organochlorinated compounds accepted by US-EPA

Method	Substance	Matrix
4010	PCP	water, soil
4015	2,4-D	water, soil
4016	2,4,5-T	soil
4020	PCBs	soil, oil
4041	Chlordane	soil
4042	DDT	soil

Based on this overview it can be observed that the only work reported on chlorophenol immunoassays is the development of an ELISA for the detection of 2,4-dichlorophenol [258]. Recently in our group have been developed direct and indirect ELISAs for 2,4,6-TCP [1,259,260]. It was demonstrated that these immunoassays could be applied for the 2,4,6-TCP analysis in drinking water samples. As it was shown in the previous chapters,

2,4,5-TCP could be considered as biomarker of exposure to many organochlorine substances. According to our knowledge there is no existing immunoassay or antibody against this analyte produced. Finally, the immunochemical determination of chlorophenols in biological samples is still not accomplished.

1.5. Objectives

With all these precedents the general goal of this thesis has been to investigate the potential of the immunochemical techniques to assess exposure to organochlorinated compounds. With this aim we have addressed the following objectives:

1. Development of an ELISA for detection and quantification of 2,4,5-TCP.

2. Evaluation and validation of the performance of the immunoassays (ELISAs) for 2,4,5-TCP and 2,4,6-TCP in environmental (water) and biological (milk, human serum and urine) samples.

As a result of these studies it became evident the necessity to develop compatible and efficient sample treatment procedures. For this reason we have set the following additional objective:

3. Development of HTS-SPE-ELISA for urinary detection of 2,4,6-TCP. Validation and application to human biological monitoring for exposure assessment.

As a preliminary study for an automated immunosensor device to analyze 2,4,6-TCP in urine, the following objective was also proposed:

4. Development of QFIA for 2,4,6-TCP based on laser-induced fluorescence detection in microdroplets and its application to urine samples.

2. Development of an ELISA for 2,4,5-trichlorophenol

The most crucial step in the development of an immunochemical technique for low molecular weight environmental pollutants is the hapten design. Immunoassay specificity, selectivity and detectability are mainly determined by the antibody and the chemical structure of the competitor used as CA or ET [158,174,261,262]. Many examples in the literature prove that an appropriate design of the immunizing hapten determines the features of the resulting antibodies. As small organic molecules (Mw < 2000 Da) are not able to elicit an immune response in an animal, it is necessary to transform the analyte to

an immunogenic molecule, which consists of a hapten covalently coupled to a protein. A hapten is a molecule analogous to the target analyte and properly functionalized to allow covalent attachment to a carrier compound. An ideal hapten is one that preserves most of the steric and electronic characteristics of the analyte and mimics the target analyte in chemical structure as closely as possible. It is advisable to avoid modification of the immunogenic groups in the target molecule and/or introducing new ones, as this supposes an alteration of its structural, geometrical and electronic properties, and consequently a reduction of the sites for potential molecular recognition.

The attachment point to the carrier should be placed far from the important sites of the target analyte and separated by a spacer arm to avoid hindrance by the carrier. A 3-6 atom spacer length has often been considered as optimum size [263,264], although not many exhaustive studies have addressed this point. Moreover, IAs have been described when Abs had been raised using haptens with shorter spacer arms [176,265]. Some authors have found a correlation between the length of the spacer arm in the immunogen and the Ab selectivity. Thus, Got *et al.* have demonstrated that a spacer with an average length of 4-6 carbon atoms results in the production of more specific polyclonal Abs [266]. On the other hand, too long spacer arm could result in Ab against the spacer. In addition, the very long chain could fold placing the hapten inside the protein structure. The spacer arm should preferably replace a carbon hydrogen bond instead of a functional group. Ideally, a spacer ram should be a chain of methylenes terminated by a functional group (-COOH, -OH, -SH, $-NH_2$) through which the carrier protein will be attached. The presence of bulky or other functional groups or heteroatoms may lead to the generation of Abs versus the spacer arm and consequently to a poor recognition of the free analyte in the competitive ELISA [267,268].

The introduction of the spacer arm in the molecule may cause deformations of the molecular geometry as well as changes in its electronic distribution. On the other hand, the chemical synthesis of the hapten often is one of the most time-consuming steps of the antibody production process. Therefore, the synthetic effort needed to produce antibodies against an organic molecule should be carefully evaluated in relation to the chances to

obtain a good antibody. Nowadays, it is possible to make computer assisted theoretical and molecular modeling studies in order to predict the suitability of a particular chemical structure as a hapten to raise antibodies against the target analyte [158,259,269-272]. This facilitates the logical comparison of the 3-dimensional structure (3D) and the electrostatic properties of the designed hapten with those of the target analyte. Such an approach has been valuable in the choice of an ideal hapten for synthesis and sensitive immunoassays have been developed using this modeling approach.

2.1. Design of an immunizing hapten to raise antibodies against 2,4,5-TCP. Molecular modeling studies.

Initially, we considered three possible positions in the chemical structure of 2,4,5-TCP where a spacer arm can be introduced leading to three different immunizing haptens presented in **Figure 2.1**. In hapten **A** the spacer arm substitutes the hydrogen atom of the *ortho* position. Although the linker is too close to the phenolic group, it remains free and the three chlorine atoms in this hapten are placed at the same positions as in the target analyte. In hapten **B** the spacer arm is placed through the oxygen atom and the aromatic ring does not suffer any apparent geometric modifications. This maximizes the recognition of the aromatic group and the particular distribution of the chlorine atoms. Finally, hapten **C** respects the phenolic group free with the spacer arm introduced at the *meta* position of the aromatic ring.

As was mentioned, the introduction of a spacer arm into a molecule can cause important geometric deformations. Thus, based on semiempirical models MNDO [273] we optimized the geometries of the three haptens in order to compare them to the geometry of the analyte (2,4,5-TCP). **Figure 2.2** shows these chemical structures at their minimum energetic levels.

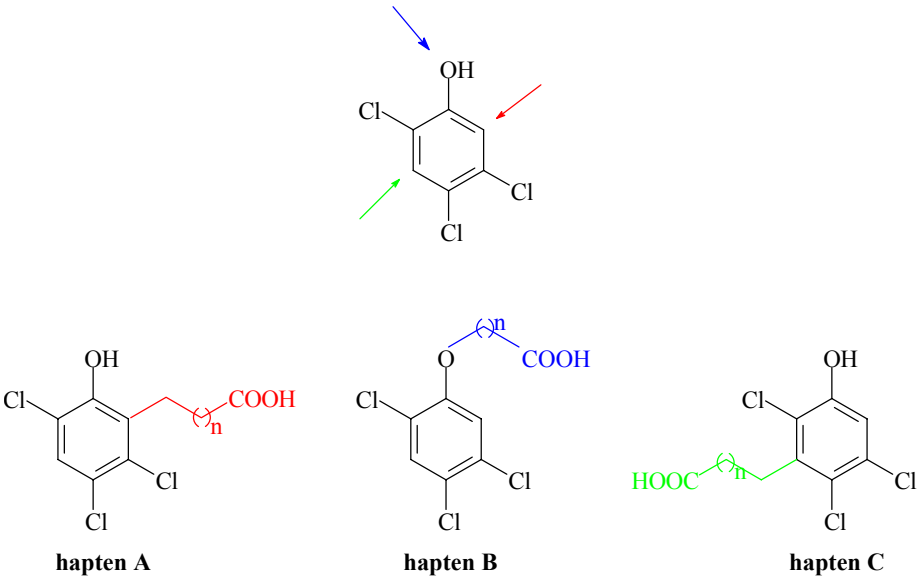


Figure 2.1. Structures of the possible immunizing haptens for the production of antibodies against 2,4,5-TCP

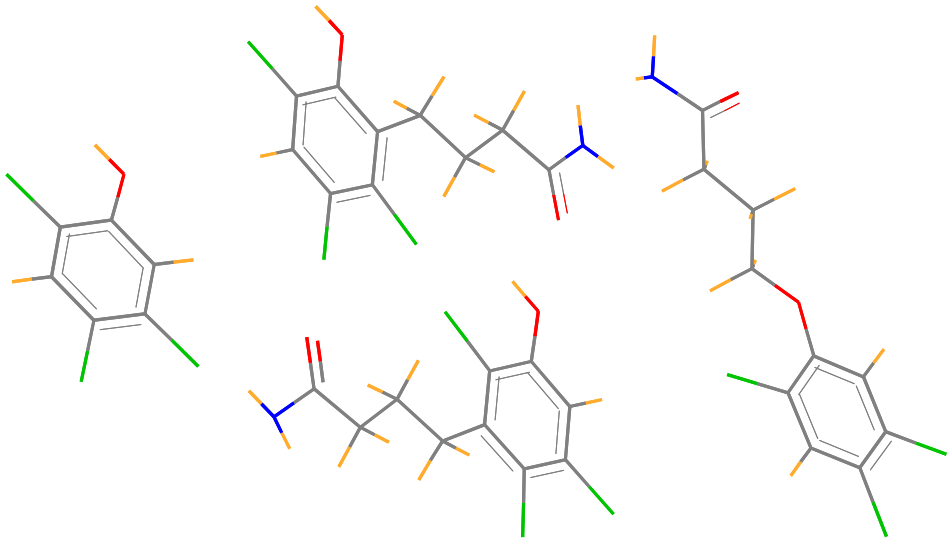


Figure 2.2 Optimized geometries of 2,4,5-TCP and haptens **A**, **B** and **C** according to MNDO models. Calculations have been made using the corresponding amide derivatives to mimic the conjugated haptens. The elements are presented in the following manner: C - gray; H – yellow; O – blue; Cl- green.

As it can be observed the considered haptens' structures do not differ significantly from the geometry of 2,4,5-TCP. The introduction of the spacer arm does not produce significant conformational changes in these molecules. Their planar geometries were preserved as in the target analyte.

Another important factor to be considered in hapten design is the electronic distribution (punctual charge density and total sum of the punctual charges in the molecules). This factor is very important, if we consider that the Ab-Ag recognition is also defined by electrostatic forces, hydrogen bonds or Van der Waals interactions. In the design of immunizing haptens for 2,4,6-TCP [259] the real differences between the electronic distribution of the haptens and those of the target analyte were observed when the acid-base equilibria of the phenolic compounds in aqueous media ($ArOH \rightarrow ArO^- + H^+$) were considered. Both the neutral compounds and the corresponding conjugated bases coexist in an aqueous media in a ratio depending on the pH and their pKa. For example, with a pKa of 7.1, one can assume that both the acid and the conjugated base of 2,4,5-TCP will coexist in a physiological media or in the assay buffer.

Consequently, punctual charges in the aromatic ring for both species (acid and conjugated base) of the haptens **A** - **C** and the target analyte were calculated at their minimum energetic conformations. **Figure 2.3** shows the results obtained for each atom of the ring and the sum of those charges. It can be observed that the punctual charges of the analyte and haptens **A**, **B** and **C** are quite similar, if they considered as organic acids (see **Figure 2.3 A**). Only the balance of the total charge is slightly more positive for hapten B. In contrast, the punctual charges vary significantly if the haptens are considered as conjugated bases (see **Figure 2.3 B**). As phenoxides the charges of the target analyte and haptens A and C follow the same pattern and the total charge of each of them is negative. Since in hapten B the hydroxyl group is blocked and it cannot participate in the acid-base equilibrium, the punctual charge distribution in this hapten is independent on the pH of the media and its total charge remains positive. Therefore, haptens **A** and **C** mimic better the analyte considering their geometry and punctual charges.

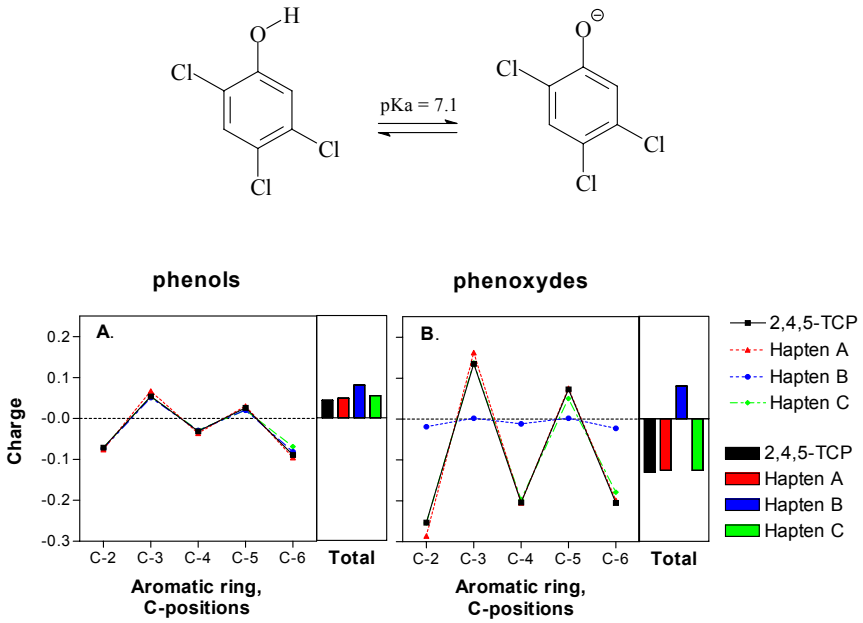


Figure 2.3. Total charge and punctual charges of 2,4,5-TCP and haptens A, B and C as phenols (**A**) and phenoxydes (**B**). The relative amounts of these two forms depend on the corresponding pKa values (see **Table 2.1**). The bars show the total charge. Haptens A and C mimic better the behaviour of the target analyte in both situations. On top it is shown the acid-base equilibrium for 2,4,5-TCP molecule.

It should be noted that the comparisons based on punctual charges are only valid if the phenolic acids have the same tendency to give protons to the media, that means all these compounds have similar pKa values. According to the Gibbs equation on proton transfer reactions, the deprotonation enthalpy (DPE) is directly related to the pKa. Dewar *et al* proved that semiempirical models provide reliable data regarding PA (proton affinities) and DPE values [274]. Using MNDO semiempirical models we could determine the formation enthalpies of 2,4,5-TCP, hapten **A** and hapten **C** as organic acids [H*f* (ArO^-)] and as conjugated bases [H*f*r (ArOH)]. The DPE values of the proton transfer equilibria could be calculated according to the following equations:

$$\Delta H_0 = \Delta Hfp - \Delta Hfr$$

$$\Delta H_0 = [\Delta Hfp\ (H^+) + \Delta Hfp\ (ArO^-)] - \Delta Hfr\ (ArOH) = DPE,$$

where $\Delta H_f r$ is the formation enthalpy of the reagents and $\Delta H_f p$ is the formation enthalpy of the products. According to the literature the formation enthalpy of the H^+ is 367.2 Kcal/mol, [274,275]. **Table 2.1** shows the DPE values for 2,4,5-TCP and haptens **A** and **C** deprotonation equilibrium reactions calculated by MNDO semiempirical models.

Table 2.1. Calculated values for DPE and pK_a[a]

Compound	DPE, kcal/mol	pKa
2,4,5-TCP	354.96	7.10 ± 0.23
Hapten A	359.89	7.38 ± 0.50
Hapten C	355.60	7.14 ± 0.28

[a] The formation enthalpy for H^+ is considred to be 367.2 kcal/mol.

The DPE values clearly indicate the acidic character of these compounds but there are no significant differences between them. This fact was additionally supported by theoretical calculations of the pKa values using suitable software (see **Table 2.1**). Therefore, we can conclude that both haptens **A** and **C** mimic reasonably well the properties of the target analyte. In summary, considering the theoretical studies on geometry, charge distribution and pKa both haptens **A** and **C** could be excellent immunizing haptens for the production of 2,4,5-TCP antibodies.

Finally, the synthetic feasibility is another aspect to be considered. The introduction of an alkylamino group in *ortho* of 2,4,5-TCP, as it appears in hapten **A**, had already been reported by Stokker *et al* for the preparation of a new class of saluretic agents [276]. Hapten **A** (3-(2-hydroxy-3,5,6-trichlorophenyl)propanoic acid) was prepared by R.Galve from our group following a similar procedure [277]. In contrast, the introduction of the spacer arm at *meta* position of the target analyte needed for the preparation of hapten **C** seemed to be more difficult from synthetic point of view. Regioselective nucleophilic substitution at *meta* positions of phenolic substances has been reported by preparing first arene-tricarbonyl-chromium (0) complexes [278] of the corresponding aromatic compound. Another strategy could go beyond using 3(3-hydroxyphenyl)-propenoic acid as starting material (possessing already the spacer arm in *meta*) and introducing the chlorine atoms in the last step [259]. However, the introduction of a chlorine atom on the

less active *meta* position of the aromatic ring could also be tricky. Therefore, we decided to use only hapten **A** as immunizing hapten.

2.2. Antibody production

Hapten **A** was coupled to KLH (keyhole limpet hemocyanine) and BSA (bovine serum albumin) following the mixed anhydride method (MA) and the KLH conjugate was used to raise antibodies in white New-Zealand rabbits (see **Experimental Section**). The obtained antisera were named As53, As54 and As55. Antibodies against hapten **B** were produced in our group within the context of a project aimed to raise antibodies against chlorophenoxy acid herbicides. These antisera were named As56, As57 and As58. With the aim to prove the results of the theoretical studies we decided to evaluate both groups of antiserum for their ability to bind 2,4,5-TCP on a competitive ELISA.

2.3. Competitor haptens

The immunoassays for the analysis of small organic molecules are usually performed under competition. The general strategy for competitive assays is based on the competition between the free analyte (Ag) and the labeled antigen for a limited number of Ab binding sites. The labeled antigen in the direct competitive format is an enzyme tracer (ET) and in the indirect format is a coating antigen (CA) (see **Figure 1.4.**). ETs and CAs are obtained by conjugation of a hapten, called competitor, to an enzyme or proteins.

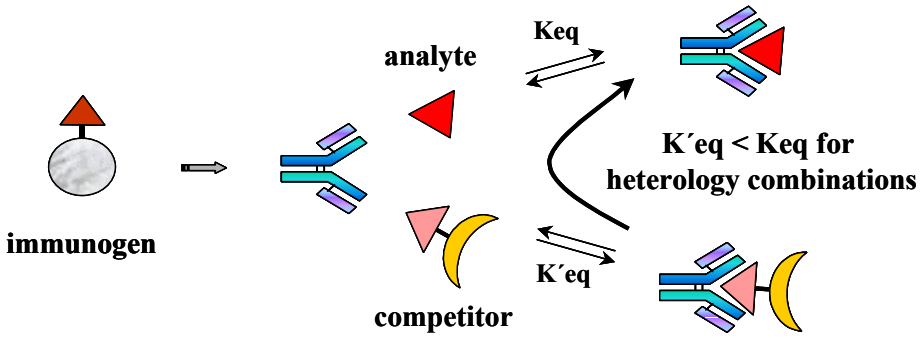


Figure 2.4. Heterology principle

Contrary to the immunizing haptens, requirements regarding similarities between the competitor and the target molecule are not so strict. In general the chemical structure of the immunizing hapten is the main factor influencing IA specificity, while the chemical structure of the competitor mainly defines IA detectability. It has been often reported an increase in the detectability by introducing a certain degree of heterology in the chemical structure of the competitor (so called competitior heterology effect) [181,260,272,279-281].

In this way, the equilibrium constant defining the formation of the immunocomplex competitor-Ab would be lower than the one directing the reaction between the analyte and the Ab (see **Figure 2.4**). Despite the heterology concept, its application is not always general [216,259]. It is suggested that high-affinity Abs may be able to render excellent assays even under homologous conditions while the detectability of IAs based on Abs with low affinity for the analyte may be increased by using heterologous competitors [174].

It is difficult to predict which chemical structure would give the best competitor. Optimal heterologous system is usually accomplished by the screening of several haptens coupled to enzymes or proteins. These haptens may have different degree of heterology depending on the variation of the chemical structure and the length and/or the position of the spacer arm. Thus, it is important to dispose of several competitors with different chemical

structure and to find out experimentally the one who provides the best immunoassay. The chemical structures of the haptens used as competitors in this work are presented in **Figure 2.5**.

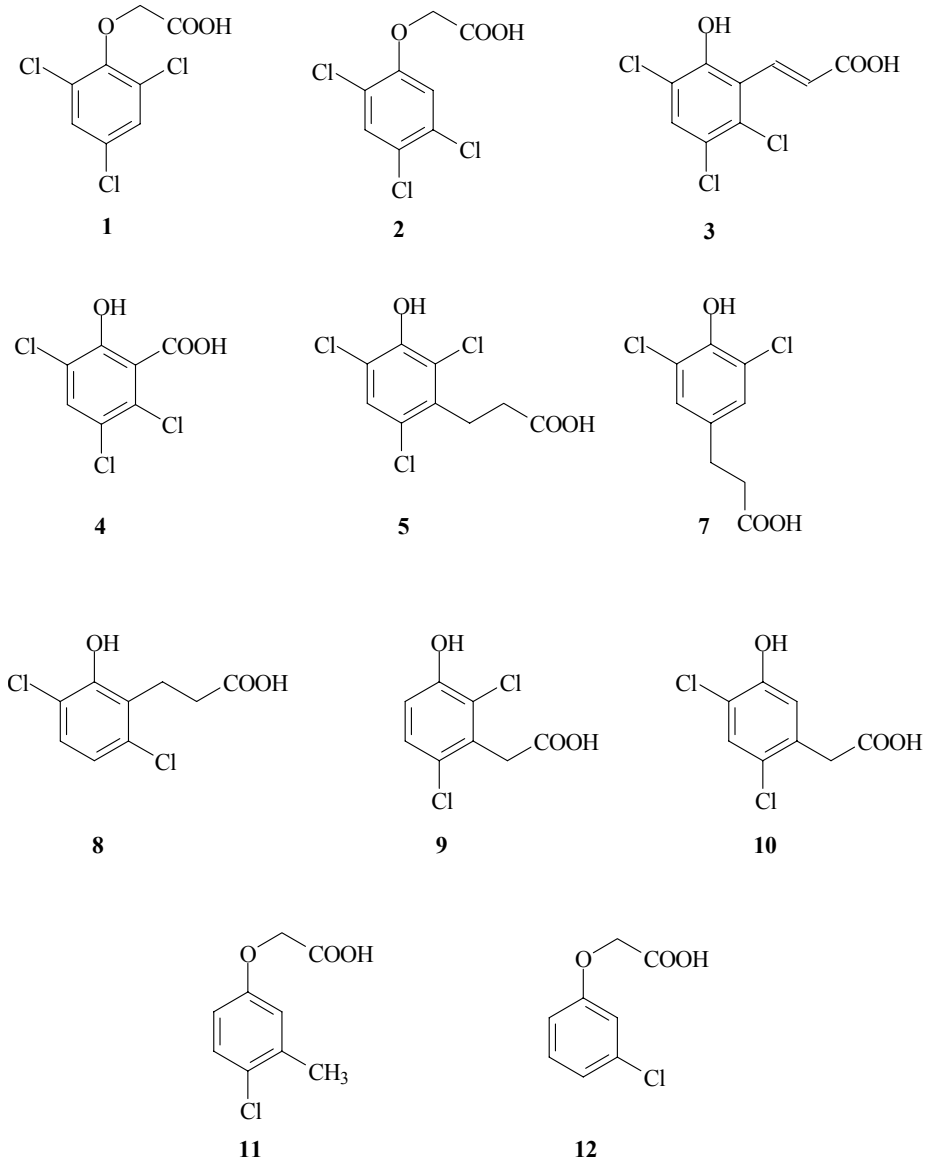


Figure 2.5. Chemical structure of the competitors

Haptens **3**, **4** and **5** keep the phenol group and the three Cl atoms, whereas haptens **1**, **2**, **11** and **12** have the hydroxyl group blocked by the linker. Haptens **7**, **8**, **9** and **10** keep the phenolic group, but posses only two Cl atoms and the spacer arm is introduced in different positions of the aromatic ring. The competitors were prepared by R.Galve from our group [259,260,277].

2.4. Development of a competitive direct ELISA

Initially we decided to develop a direct competitive ELISA format with the Abs immobilized to the plate. As enzyme label we selected horseradish peroxidase (HRP). This enzyme has one or two free lysine groups accessible for bioconjugation [282]. It is advisable to use different conjugation methods for the immunizing hapten and the competitor in order to avoid possible interferences (background noise or Ab recognition) in the assay due to secondary products (sub-products) that may be formed during the immunogen preparation [283,284]. Since the immunizing haptens were prepared by the mixed anhydride (MA) method, we used the active ester (AE) method to attach haptens **A, 1-5** and **7-12** covalently through their carboxylic groups to the lysine residues of the HRP (see **Experimental Section** for general procedure description).

2.4.1. Screening of the antiserum avidity for the ETs (1D and 2D)

On a first set of experiments we selected the ETs recognized by the As using a non-competitive direct ELISA format-1D (one dimension) (see **Figure 2.6**). In this format the plate is coated with the As at a fixed concentration and its interaction with the serially diluted ET is tested (see **Experimental Section**).

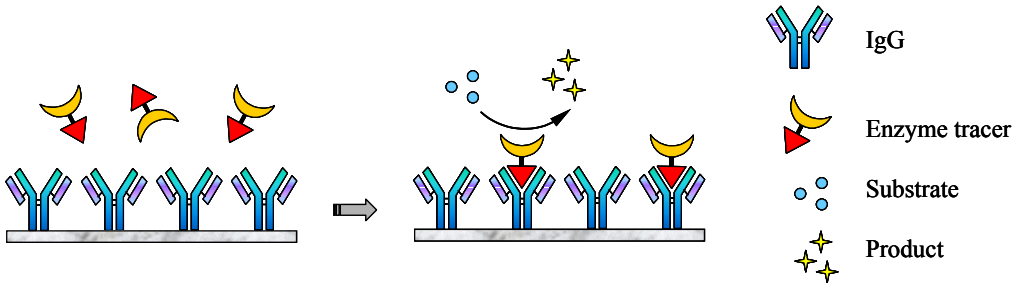


Figure 2.6. Noncompetitive direct ELISA

The A_{max} values obtained for all As/ET combinations are presented in a color scale in **Table 2.2**. Recognition was observed only for the homologous or quasi-homologous ETs. Thus, As53-55 (immunogen **A**-KLH) did only recognize **A**-HRP with an acceptable titer, while **5**-HRP was only recognized by As55. As56-58 (**B**-KLH) did only recognize **2**-HRP that only differs from hapten **B** in the length of the spacer arm.

Table 2.2. Absorbances obtained in noncompetitive direct ELISA 1D experiments for all As/ET combinations.

Immunogen	A-KLH			B-KLH		
As / ET	53	54	55	56	57	58
1						
2						
3						
4						
5						
A						
7						
8						
9						
10						
11						
12						

The plates were coated with As (1/1000 dilution). The absorbance shown corresponds to 1 µg/L ET. 3,3¨,5,5¨-Tetramethylbenzidine was the substrate used for the enzymatic reaction. The absorbance was measured at a wavelength of 450 nm.

Next, the relative avidity of these As versus the recognized ETs was evaluated in a two-dimensional checkerboard titration experiment (2D) (see **Experimental Section**) (see **Figure 2.6**). The binding curves were obtained for seven ET concentrations (see **Figure 2.7**).

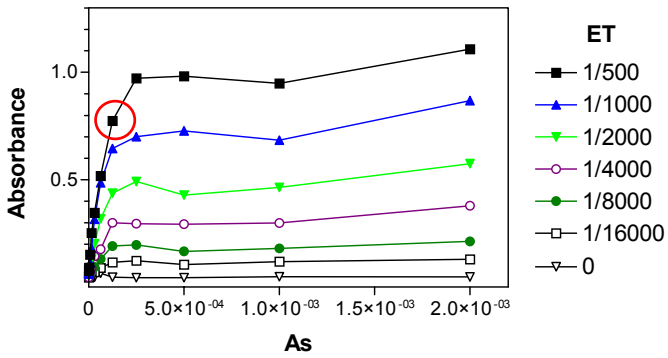


Figure 2.7. 2D experiment for As55/**5**-HRP. The red circle shows the combination of immunoreagents´ concentration chosen for the competitive assay.

From these experiments we selected the appropriate concentrations to perform the competitive assays. A trade-off should be done between concentration high enough to achieve good signal and as lower as possible in order to achieve least LOD. We chose an As concentration giving a 70% of the saturated signal and an ET concentration producing signal between 0.7 and 1 units of absorbance at 450 nm. **Table 2.3** summarizes the selected concentrations of the immunoreagents for all the As/ET combinations and the corresponding A_{max} values.

Table 2.3. Concentration of the immunoreagents selected in non-competitive direct ELISA - 2D.

As / ET	As dilution	ET conc., μg/L	A_{max}
53 / **A**-HRP	1/16000	2	0.86
54 / **A**-HRP	1/16000	2	0.58
55 / **A**-HRP	1/16000	5	0.81
55 / **5**-HRP	1/6000	2	0.80

The As(56-58)/**2**-HRP combinations produced a maximum absorbance of only 0.3 units when the As was diluted 1/500 times and the ET was used at a concentration of 2 μg/mL.

Thus, we focused on the antisera raised against hapten **A** to test their ability to bind 2,4,5-TCP in a competitive assay.

2.4.2. Direct Competitive ELISA

In the direct competitive ELISA (Ab coating format) the analyte and the enzyme tracer in solution compete for the immobilized Abs (see **Figure 1.4.A**). After the main incubation step the unbound reagents are washed and the amount of enzyme-label bound to the solid phase by the Ab is photometrically determined. A decrease in the signal is directly proportional to the amount of the analyte present. Using standard analyte concentrations a dose-response curve can be obtained. It has a sigmoidal shape with linear portion and can be fitted to the four-parameter logistic equation (see **Experimental Section**).

We performed direct competitive immunoassays with the above selected combinations of As/ET using the concentrations presented in **Table 2.3** according to the procedure described in the **Experimental Section**. All tested combinations gave competitive assays, although their detectability was too poor for our purposes. **Table 2.4** shows the parameters of the standard curves for these immunoassays.

Table 2.4. Features of the competitive direct ELISAs

As	ET	A_{max}	A_{min}	Slope	IC_{50}[a]	r^2
53	**A**-HRP	0.81	0	-0.43	18.87	0.989
54	**A**-HRP	0.63	0.06	-0.89	165.48	0.985
55	**A**-HRP	1.27	0.21	-1.03	21.62	0.981
	5-HRP	**0.94**	**0.14**	**-0.97**	**15.84**	**0.971**

[a] IC_{50} values are expressed in µg/L. Shadow boxes show the immunoassay features of the best combination.

The best assay gave a detectability* of 15.84 µg/L in the middle point of the assay. According to the urinary levels recorded for non-occupationally exposed persons (see **Section 1.2.5.3**) an assay with IC_{50} value around 1 µg/L or lower would be desirable.

* We would like to note that in this thesis the term detectability refers to the limit of detection or to the IC_{50} value of the method and the term sensitivity refers to the slope of the calibration curve of the assay.

Therefore, we decided to explore the indirect ELISA format with the aim to find an antiserum/coating antigen combination with a better detectability. Although the indirect format introduces an additional step in the procedure, the absence of the enzyme during the competition made it more promising regarding possible matrix effects from real biological samples [174].

2.5. Development of an indirect ELISA for 2,4,5-trichlorophenol

In the indirect ELISA the coating antigen (CA) is immobilized to the surface of the plate and the concentration of the analyte is measured indirectly by the quantification of the bound specific Ab with a second enzyme-labeled Ab (anti-IgG) (see **Figure 1.4.B**).

Initially we decided to evaluate a battery of 33 CAs obtained by the covalent coupling of 11 competitors (see **Figure 2.5**) to the proteins BSA (bovine serum albumin), OVA (ovalbumin) and CONA (conalbuin). Haptens **1**–**4** and **7**-**12** were conjugated to the three proteins by the AE method and hapten **5** was conjugated by the MA method in a hapten:protein(lysine) molar ratio 2:1. CAs of hapten **A** were not prepared because of limited quantities of hapten **A** available. Unfortunately, the prepared CAs had a limited solubility in 10 mM PBS buffer at 1 mg/mL concentration. This was attributed to their high degree of conjugation (see **Table 2.5**). The hapten molecules in the conjugation reaction displace the free amino groups of the proteins that define their water solubility. The chlorine atoms makes the resulting conjugate more lipophilic (hydrophobic) limiting in this way the protein solubility in buffer.

Due to this lack of solubility, we filtered the working aliquots of 1 mg/mL and evaluated the resulting protein concentration by the Bio-Rad Protein Assay. The Bio-Rad Protein Assay is a binding assay based on the differential color change of a dye in response to

various concentrations of protein. It relies on the Bradford method based on the shift of the absorbance maximum from 465 nm to 595 nm when an acidic solution of Coomassie Brilliant Blue G-250 binds to a protein [285]. In our case we used the microassay procedure with a linear response between 1 μg/mL and 40 μg/mL. As the assay displays protein-to-protein variation we used standard curves for each protein. The analysis demonstrated that the conjugates **3**-BSA, **7**-, **11**- and **12**-CONA were completely insoluble in aqueous buffer and they were not used further. The protein concentrations of the remaining CAs were in the range 120 – 800 μg/mL with a higher solubility found for all CAs prepared by the MA method. The hapten densities of the BSA conjugates were determined by MALDI-TOF-MS (see **Table 2.5**.).

Table 2.5. Hapten densities of the BSA conjugates measured by MALDI-TOF-MS.

Hapten	Hapten density	% conjugation[b]
1	30	85-100
2	25	71-83
3	14	40-47
4	16	46-53
5 [a]	7	20-23
7	25	71-83
8	17	49-57
9	21	60-70
10	26	74-87
11	27	77-90
12	28	80-93

[a] All the conjugates were synthesized by the AE method, except hapten 5-BSA whcih were prepared by the MA method. In both methods the hapten:protein molar ratio employed during the conjugation reaction was 2:1 in terms of lysine residues of the protein. [b] The % conjugation is calculated based on the assumption that the BSA has 30 – 35 free lysine groups.

The analysis of the hapten density demonstrates that the degree of conjugation of the CAs obtained by the AE method was really quite high. This correlates to the statement expressed above that the high degree of conjugation results in less soluble protein conjugates.

2.5.1. Screening of the antiserum avidity for the coating antigens

The recognition of the CAs by the As was evaluated in a non-competitive indirect ELISA realized in 1D configuration (see **Figure 2.8**). The CAs were immobilized on the microtiter plate at a concentration 1 μg/mL and serial dilutions of each As were added.

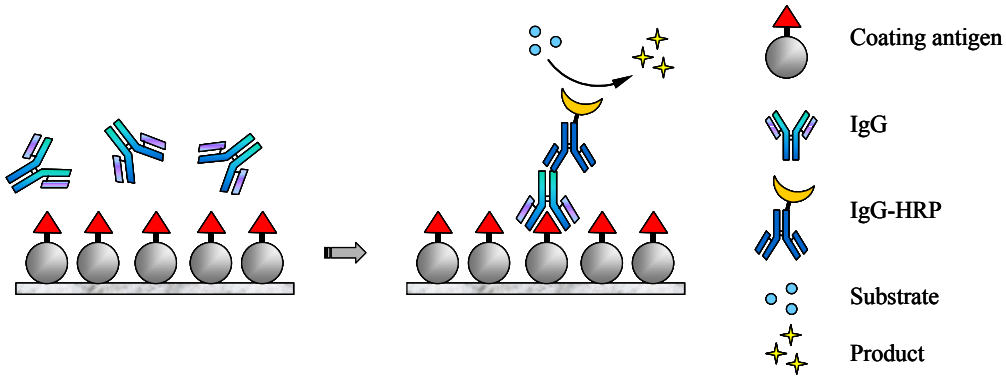


Figure 2.8. Noncompetitive indirect ELISA

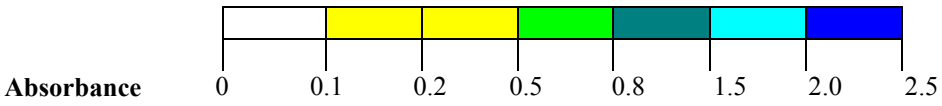
The A_{max} values obtained for all As/CA combinations are presented in a color scale in **Table 2.6**. In general, the CAs were better recognized by the As than the respective ETs. This may be attributed to the fact that the CAs possess more binding centers than the ETs (the number of free lysine groups available for coupling in the proteins is higher than in the HRP). In general, higher recognition was mainly observed for the homologous or quasi-homologous CAs. Thus, As53-55 recognized better haptens **5**, **8**, and **2** and As56-58 recognized in higher degree the haptens with blocked phenolic group (**1**, **2**, **11**, and **12**).

The avidity of As53-58 for the better-recognized protein conjugates was evaluated by two-dimensional checkerboard titration experiments (2D) (see **Experimental Section**). The binding curves for each As/CA combination were obtained (see **Figure 2.9**).

Table 2.6. Absorbances obtained in noncompetitive indirect ELISA 1D experiment for all As/CA combinations.

Immunogen	**A-KLH**			**B-KLH**		
As / CA	**53**	**54**	**55**	**56**	**57**	**58**
1-BSA						
1-CONA						
1-OVA						
2-BSA						
2-CONA						
2-OVA						
3-BSA	nd	nd	nd	nd	nd	nd
3-CONA						
3-OVA						
4-BSA						
4-CONA						
4-OVA						
5-BSA						
5-CONA						
5-OVA						
7-BSA						
7-CONA	nd	nd	nd	nd	nd	nd
7-OVA						
8-BSA						
8-CONA						
8-OVA						
9-BSA						
9-CONA						
9-OVA						
10-BSA						
10-CONA						
10-OVA						
11-BSA						
11-CONA	nd	nd	nd	nd	nd	nd
11-OVA						
12-BSA						
12-CONA	nd	nd	nd	nd	nd	nd
12-OVA						

The plate was coated with CA at 1 μg/L. The absorbances correspond to 1/1000 dilution of the As. All the CAs are prepared by the AE merhod except the CAs of hapten 5.



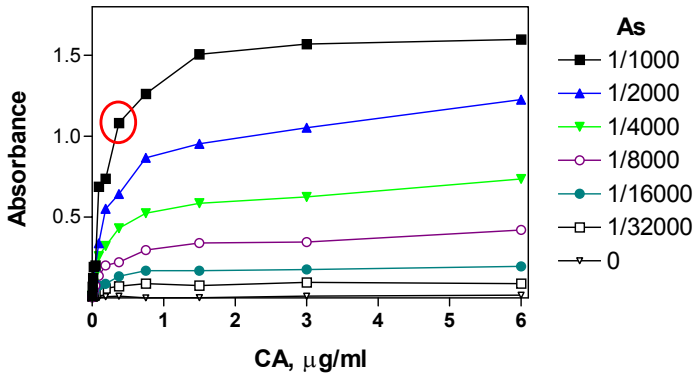


Figure 2.9. Results from the 2D experiment for As53/**7**-OVA noncompetitive assay. The red circle shows the combination of immunoreagents´ concentration chosen for the competitive assay.

The concentrations of the CAs were chosen to produce about 70% of the saturated signal. Antisera dilution factors were selected to rend absorbance values in the range between 0.7 and 1 unit (see **Table 2.7.A** and **B**).

It can be observed that As56-58 have lower avidity than As53-55. This could be explain with the differences in the punctual charges of the competitors haptens and the immunizing hapten B at the assay pH (see **Section 2.1**, **Figure 2.3B**). Thus, most of the competitor haptens had the phenolic group free and according to their pKa values (see **Table 2.9**) an important fraction of their molecules is ionized at pH 7.5. Only haptens **8**, **9** and **10** had a pKa sufficiently high to remain mainly in their protonated forms. The competitors with the hydroxyl group blocked, such as haptens **1**, **2**, **11** and **12** were the best recognized by As56-58. As expected As53-55 showed higher avidity for the haptens with the free phenol group, especially if the chlorine atoms were at the same positions as in the target analyte.

Table 2.7. A. Concentration of the immunoreagents selected in non-competitive indirect ELISA (2D)

As	CA	As dilution	CA conc.	A_{max}
53	1-BSA	1/4000	0.188	0.82
	2-BSA	1/8000	0.188	0.70
	5-BSA	1/4000	0.188	0.96
	8-BSA	1/25000	0.094	0.80
	1-CONA	1/1000	0.375	0.95
	2-CONA	1/2000	1.500	1.05
	5-CONA	1/1000	0.188	0.85
	8-CONA	1/32000	0.094	0.90
	9-CONA	1/1000	0.375	0.83
	10-CONA	1/10000	0.047	0.91
	2-OVA	1/4000	0.750	0.82
	5-OVA	1/4000	0.750	0.74
	7-OVA	1/1000	0.375	1.02
	8-OVA	1/4000	0.375	0.80
	12-OVA	1/1000	1.500	0.49
54	2-BSA	1/8000	0.375	0.90
	5-BSA	1/8000	0.094	1.00
	7-BSA	1/16000	0.023	0.80
	8-BSA	1/32000	0.023	0.92
	10-BSA	1/16000	0.023	0.85
	2-CONA	1/2000	0.750	0.82
	5-CONA	1/4000	0.047	0.90
	8-CONA	1/16000	0.094	0.85
	10-CONA	1/4000	0.047	0.91
	5-OVA	1/4000	0.188	0.69
	7-OVA	1/4000	0.188	0.70
	8-OVA	1/10000	0.375	0.80
	10-OVA	1/4000	0.047	0.59
55	1-BSA	1/1000	0.375	0.93
	2-BSA	1/4000	0.188	0.90
	5-BSA	1/16000	0.047	0.87
	8-BSA	1/16000	0.094	1.02
	10-BSA	1/16000	0.023	0.82
	1-CONA	1/1000	0.750	0.72
	2-CONA	1/2000	0.094	0.70
	5-CONA	1/16000	0.047	0.81
	8-CONA	1/16000	0.094	0.85
	9-CONA	1/2000	0.094	0.72
	10-CONA	1/8000	0.023	0.83
	2-OVA	1/2000	0.188	0.90
	5-OVA	1/4000	0.375	0.80
	8-OVA	1/4000	0.378	0.83
	10-OVA	1/4000	0.094	0.75

[a] All the conjugates were prepared by the AE method except those marked in italics that were prepared by the MA method.

Table 2.7. B. Concentration of the immunoreagents selected in noncompetitive indirect ELISA (2D)

As	CA	As dilution	CA conc.	A_{max}
56	1-BSA	1/1000	0.75	0.80
	2-BSA	1/8000	0.094	1.03
	5-BSA	1/1000	0.375	0.60
	1-CONA	1/1000	0.750	1.00
	2-CONA	1/4000	0.094	0.75
	2-OVA	1/4000	0.187	0.75
	5-OVA	1/1000	1.500	0.65
	8-OVA	1/1000	0.375	0.72
	10-OVA	1/1000	1.500	0.50
57	1-BSA	1/4000	0.375	0.90
	2-BSA	1/4000	0.047	1.00
	5-BSA	1/1000	0.375	0.98
	11-BSA	1/4000	0.047	0.95
	1-CONA	1/4000	0.375	0.94
	2-CONA	1/8000	0.094	0.80
	5-CONA	1/1000	0.187	0.92
	2-OVA	1/4000	0.375	1.00
	11-OVA	1/4000	0.375	1.00
	12-OVA	1/1000	0.750	1.20
58	1-BSA	1/1000	1.500	0.55
	2-BSA	1/4000	0.375	0.80
	2-CONA	1/4000	0.094	0.75
	5-CONA	1/1000	0.094	0.70
	2-OVA	1/2000	0.094	0.92
	11-OVA	1/1000	0.750	0.76
	12-OVA	1/1000	1.500	0.98

[a] All the conjugates were prepared by the AE method except those marked in italics that were prepared by the MA method.

2.5.2. Indirect competitive ELISA: Effect of the hapten heterology

Those As/CA combinations showing reasonable titers were used for screening the ability of 2,4,5-TCP to inhibit the antibody binding to the coated plates (see **Figure 1.4.B**). The concentrations of the immunoreagents (CA, As) used in these assays are presented in **Table 2.7. A** and **B**. In this format it was possible to select some combinations with the As raised against both immunizing haptens. However, in agreement with the results from the molecular modeling studies, only As53-55, raised against hapten **A**, were able to provide competitive assays within the studied concentration interval (0.025 – 100 000 nM 2,4,5-TCP). **Table 2.8** shows the features of all the competitive immunoassays obtained.

Table 2.8. Immunoassay features of the competitive indirect ELISA

As	CA [a]	A_{max}	A_{min}	Slope	IC_{50} [b]	r^2
53	**1-BSA**	**0.61**	**0.03**	**-1.2**	**9.28**	**0.985**
	2-BSA	0.57	0.04	-0.97	11.5	0.979
	5-BSA	**1.02**	**0.08**	**-0.63**	**6.94**	**0.980**
	8-BSA	0.85	0.01	-0.85	15.4	0.984
	1-CONA	1.13	0.09	-1.02	11.79	0.994
	2-CONA	1.78	0.04	-0.95	32.34	0.995
	5-CONA	1.98	0.59	-0.78	30.62	0.990
	8-CONA	1.13	0.03	-0.95	116.80	0.985
	9-CONA	**0.70**	**0.02**	**-0.96**	**5.35**	**0.994**
	10-CONA	**0.86**	**0.01**	**-0.71**	**7.93**	**0.989**
	2-OVA	0.79	0.04	-1.15	14.17	0.979
	5-OVA	**0.68**	**0.03**	**-0.64**	**2.42**	**0.989**
	7-OVA	**0.94**	**0.06**	**-1.2**	**0.83**	**0.990**
	8-OVA	**0.75**	**0.01**	**-0.6**	**6.47**	**0.992**
	12-OVA	**0.57**	**0.13**	**-0.86**	**4.38**	**0.995**
54	2-BSA	1.3	0.04	-0.8	32.18	0.998
	5-BSA	**1.3**	**0.21**	**-0.68**	**8.49**	**0.995**
	7-BSA	**1.05**	**0.01**	**-0.78**	**3.36**	**0.996**
	8-BSA	1.07	0.001	-0.5	6.71	0.959
	10-BSA	0.95	0.01	-0.85	12.14	0.998
	2-CONA	1.03	0.02	-0.87	19.61	0.985
	5-CONA	**1.33**	**0.07**	**-0.84**	**10.04**	**0.990**
	8-CONA	1.24	0.09	-0.9	41.42	0.910
	10-CONA	1.44	0.01	-0.9	43.42	0.983
	5-OVA	**0.56**	**0.03**	**-0.90**	**4.56**	**0.998**
	7-OVA	**0.69**	**0.01**	**-1.0**	**3.05**	**0.990**
	8-OVA	0.54	0.01	-0.7	12.23	0.960
	10-OVA	**0.63**	**0.01**	**-1.0**	**4.87**	**0.990**
55	**1-BSA**	**0.98**	**0.2**	**-0.96**	**4.05**	**0.910**
	2-BSA	**0.82**	**0.02**	**-1.2**	**8.66**	**0.990**
	5-BSA	0.75	0.01	-0.9	16.35	0.990
	8-BSA	0.99	0.01	-0.8	12.06	0.970
	10-BSA	0.86	0.01	-0.97	28.24	0.998
	1-CONA	0.987	0.32	-1.4	13.5	0.900
	2-CONA	0.75	0.04	-1.1	15.82	0.970
	5-CONA	0.9	0.02	-0.7	21.66	0.996
	8-CONA	1.4	0.19	-0.6	31.81	0.989
	9-CONA	0.79	0.02	-1.0	61.78	0.970
	10-CONA	1.11	0.01	-0.76	78.3	0.990
	2-OVA	**1.01**	**0.04**	**-1.3**	**9.64**	**0.940**
	5-OVA	0.99	0.02	-0.9	21.22	0.940
	8-OVA	**0.73**	**0.04**	**-1.2**	**8.53**	**0.970**
	10-OVA	0.90	0.03	-1.0	20.53	0.970

[a] All the conjugates were prepared by the AE method instead of those marked in italics that were prepared by the MA method. No competition was observed for As56, 57, 58.

[b] IC_{50} values are expressed in µg/L. The combinations with IC_{50} < 10 µg/L are presented in bold and the combinations with IC_{50} < 5 µg/L are in blue. Shadow boxes show the immunoassay features of the best combinations used for further optimization.

It has been suggested that there is a correlation between immunoassay detectability and the degree of competitor heterology regarding punctual charge distribution and structural geometry [260]. In this work we tried to provide objective data about hapten heterologies and their correlation with the IC_{50} values of the immunoassays obtained. Thus, all haptens **1**-**12** and the target analyte were optimized to find their minimum potential energies using a semi-empiric PM3 model. The ratio phenol:phenoxide was calculated for each hapten according to their pKa values and used to determine the ultimate punctual charges of the molecule (see **Table 2.9**).

Table 2.9. Relative similarities between the target analyte and the haptens regarding punctual charge distribution

Hapten	pKa [a]	RO^- (%) [b]	RMSE[c], pH=7.5	Hapten density [d]
1	-	-	0.436	30
2	-	-	0.425	25
3	6.59	89.05	0.081	14
4	5.51	98.99	0.113	16
5	6.63	88.11	0.120	7
7	7.37	57.43	0.157	25
8	7.82	32.37	0.224	17
9	7.99	24.45	0.320	21
10	7.99	24.45	0.249	26
11	-	-	0.411	27
12	-	-	0.421	28
A	7.38	56.86	0.093	10
2,4,5-TCP	7.1	71.5		-

[a] pKa values of the haptens are calculated in their amide form. [b] Percentage of the phenoxide species when the pH is 7.5. [c] The similarity between the haptens and the analyte has been expressed as the root mean square error (RMSE) of the differences between the punctual charges of the corresponding atoms of both molecules (hapten *vs* analyte) at pH=7.5. The punctual charge on each atom was calculated considering the percentage of the neutral molecule and their anion form at pH=7.5 according to their pKa values. [d] Hapten densities of the BSA conjugates are measured by MALDI-TOF-MS. All the conjugates are synthesized by the AE method, except hapten 5 conjugate which are prepared by the MA method. In both methods the hapten:protein molar ratio employed during the conjugation reaction is 2:1 in terms of lysine residues of the protein.

The heterology, as the difference between the charge distribution of each hapten and the analyte, has been expressed as the root mean square error (RMSE) of the addition of the errors for each equivalent position of the aromatic rings (competitor *vs* target analyte). Thus, the following heterology order according to the electronic distribution on these molecules can be defined: hapten **1** > hapten **2** ≥ hapten **12** > hapten **11** >> hapten **9** >

hapten **10** > hapten **8 >** hapten **7** > hapten **5** > hapten **4** > hapten **A** > hapten **3**. Surprisingly, the homology of hapten **3** to the analyte was greater than those of hapten **A**, used as immunizing hapten. This fact is strongly related to the pKa values of these substances. While hapten **A** has approximately only half of the molecules ionized at pH 7.5, hapten **3** and 2,4,5-TCP have 89 % and 71%, respectively, in the ionized form. Based on these results, the use of hapten **3** as immunizing hapten could have been considered. However, it must be noted that using hapten **3** as immunizing hapten would had the risk of introducing additional undesirable epitopes for antibody recognition.

According to these results we can observe that the best competitive assays were afforded by competitors with a high homology with the target analyte (see **Table 2.8**). Thus, haptens **5** and **7** provided the most sensitive assays except for the As55. Unfortunately, we did not have sufficient hapten **A** to prepare coating antigens by the AE method to prove if this was also true for this competitor. Furthermore, the CAs of haptens **3** and **4** were not tested on competitive assays due to their low recognition observed in 1D experiments (see **Table 2.7**). In the case of hapten **3** this may be attributed to the limited solubility observed in aqueous buffer and the poor recognition of hapten **4** is probably due to the short spacer arm of this hapten (see **Figure 2.5**). However, in addition to the hapten homologies, other factors may affect the availability of the competitor (as part of the protein conjugate) for the antibody interaction. Thus, the hapten density or the protein nature of the CA, are additional aspects to be considered. Similarly, the hydrophobic or hydrophilic nature of the compound may also determine its tendency to be hidden or not inside the tertiary structure of the protein.

2.5.3. Indirect competitive ELISA: Effect of the hapten density

Immunoassays As53/**5**-OVA and As53/**7**-OVA were selected for further optimization and evaluation because of their detectability, good signal-to-noise ratio, acceptable slope and reproducibility (see **Table 2.8**.). Initially our aim was to improve immunoassay detectability. It has been reported that the degree of hapten density of the competitors can

affect the immunoassay detectability [259,286,287]. The carrier protein OVA has 20 lysine residues, most of which are available for hapten coupling [155]. The CAs from the first battery were prepared at hapten:protein molar ratio 2:1 resulting in high degree of conjugation of the BSA conjugates as was determined by MALDI-TOF-MS (see **Table 2.9**). Therefore, we decided to prepare a new battery of **5**- and **7**- coating antigens with lower degree of conjugation simply by varying the initial molar ratio of both species in the conjugation procedure using the MA and AE method. We synthesized **5**-BSA, **5**-OVA, **7**-BSA and **7**-OVA by the AE method at four different hapten:protein ratios: (1:1), (1:2.5), (1:5), (1:10) and **5**-BSA and **5**-OVA by the MA method at three different hapten:protein ratios: (1:1), (1:2.5), (1:5). We did not prepare CAs with (1:10) ratio by the MA method, because according to our experience the MA method usually gives lower yields.

Although the hapten:lysine molar ratio in this second battery was lower, we encountered again solubility problems with the CAs. The protein concentrations had to be determined again by Bradford assay after filtration. This test revealed that **5**-BSA (1:1) (AE) was completely insoluble in 10 mM PBS. As previously observed, the CAs prepared by the MA method showed a higher solubility in aqueous buffers than the AE ones. In general, the OVA conjugates were more soluble probably due to its high number of the –COOH groups. The resulting degree of conjugation for the BSA and OVA conjugates was determined by MALDI-TOF-MS (see **Table 2.10**.).

The avidities for the new CAs were tested only with As53 using by 2D experiments. As expected higher concentrations of the less conjugated CAs were required to give appropriate absorbances using similar As dilutions [226,287]. Using the most suitable immunoreagent concentrations we obtained competitive assays for each combination. Their parameters are presented in **Table 2.10.** It can be observed that, as reported by other members of our group [1], the MA method afforded lower conjugation yields. For example, the 5-BSA conjugate prepared at molar ratio 1:2.5 by the MA method had a hapten density of 5, while the same conjugate prepared by the AE method had a hapten density of 13. Moreover, for conjugates with the same hapten:protein molar ratio, those

prepared by the MA method gave assays with better detectability (compare the IC_{50} values of **5**-BSA/-OVA (MA) with those of **5**-BSA/-OVA (AE)). These results demonstrate that it is not always necessary to use a heterologous coupling procedure to obtain a suitable immunoassay.

Table 2.10. Effect of the hapten density of the competitor on the immunoassay features.

CA	Hapten: protein [a]	δ [b]	% conjug. [c]	A_{max}	A_{min}	Slope	IC_{50}	r^2
5-BSA (MA)	2:1	7.5	21-25	1.02	0.08	-0.63	6.94	0.980
	1:1	9	26-30	1.04	0.01	-0.46	1.30	0.980
	1:2.5	5	14-17	0.92	0.01	-0.52	1.94	0.987
	1:5	3	9-10	0.92	0.02	-0.45	1.56	0.989
5-OVA (MA)	2:1	n.d.[e]		0.68	0.03	-0.64	2.42	0.989
	1:1	6	30	0.82	0.01	-0.56	1.76	0.990
	1:2.5	3	15	0.87	0.02	-0.54	1.92	0.990
	1:5	3	15	0.61	0.01	-0.5	0.45	0.990
5-BSA (AE)	1:1[d]	n.d.[e]		-	-	-	-	-
	1:2.5	13	37-43	1.13	0.01	-0.48	15.52	0.981
	1:5	8	23-27	0.8	0.01	-0.64	11.1	0.988
	1:10	5	14-17	1.1	0.01	-0.56	9.13	0.989
5-OVA (AE)	1:1	20	100	1.19	0.01	-0.65	16.81	0.997
	1:2.5	12	60	1.28	0.02	-0.72	9.70	0.980
	1:5	6	30	1.24	0.01	-0.60	10.30	0.985
	1:10	3	15	0.79	0.01	-0.68	4.80	0.988
7-BSA (AE)	2:1	25	72-84	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]
	1:1	22	63-73	0.37	0.03	-1.69	0.24	0.964
	1:2.5	13	37-44	0.59	0.01	-0.98	0.28	0.985
	1:5	7	20-23	0.3	0.01	-0.71	0.1	0.986
	1:10	4	11-13	0.1	0.01	-1.19	0.06	0.919
7-OVA (AE)	2:1	n.d.[e]		0.94	0.06	-1.20	0.9	0.990
	1:1	12	60	0.77	0.03	-1.24	0.57	0.990
	1:2.5	**10**	**50**	**0.72**	**0.01**	**-1.13**	**0.29**	**0.990**
	1:5	3	15	0.65	0.02	-1.00	0.21	0.990
	1:10	3	15	0.74	0.02	-1.20	0.23	0.990

In all cases the antiserum used was As 53. Shadow boxes show the immunoassay features of the immunoassay used for further optimization. [a] Hapten:protein molar ratio used for the conjugation reaction. The molar ratio of the protein has been calculated in terms of the lysine residues available. [b] Hapten densities (δ) are expressed as mol of hapten per mol of protein, and the data reported has been measured on the corresponding BSA or OVA conjugate respectively by MALDI-TOF-MS. [c] The % conjugation is calculated based on the assumption that the BSA has 30 – 35 free lysine groups and OVA has 20 free groups. [d] 5-BSA (AE) (1:1) was not soluble. [e] nd, not determined.

The hapten density and the ratio A_{max}/IC_{50} as a function of the hapten:protein molar ratio used in the conjugation of the CAs are presented in **Figure 2.10.** As it can be seen a

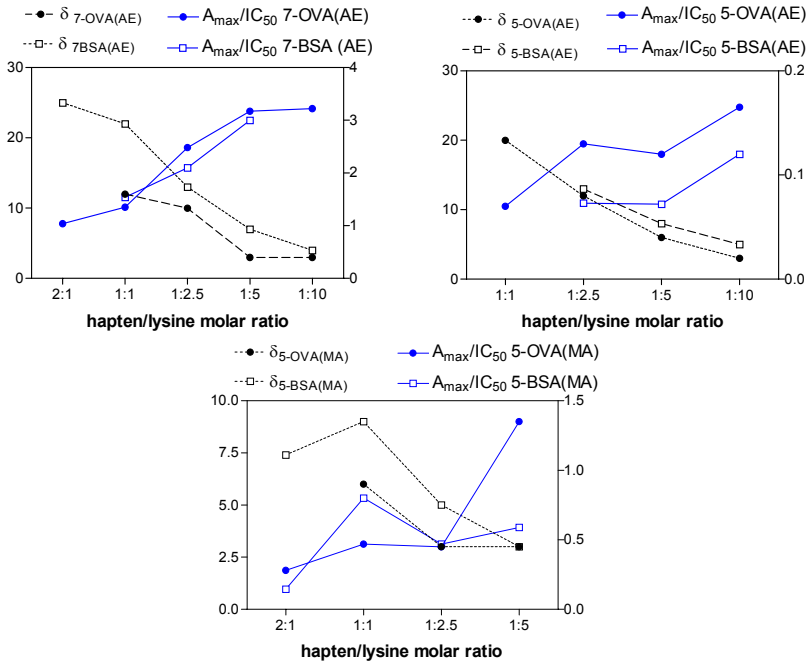


Figure 2.10. Hapten density determined by MALDI-TOF-MS and A_{max}/IC_{50} as a function of the hapten/lysine molar ratio used in the conjugation of the CAs

decrease in the substitution degree of the free lysine groups in the proteins corresponds to a decrease in the hapten density of the CA resulting in a improvement of the immunoassay detectability (increases the ratio A_{max}/IC_{50}). This is observed for all the combinations studied. The same effect has been observed on competitive indirect immunoassays for 2,4-dichlorophenoxyacetic acid [226], sulfamerazine [286], chlorpyrifos [287] and 2,4,6-TCP [259]. This is consistent with the statement that the least hapten centers are available in the coating antigen, the least amount of analyte in solution is needed to compete for the limited number of antibody binding sites.

In general, at the lowest conjugation ratio the OVA conjugates performed better than the BSA conjugates. Finally, hapten **7** afforded the assays with the lowest IC_{50} values and the OVA conjugate presented the highest A_{max}/IC_{50} ratio. Therefore, the coating antigen **7**-OVA (AE) with molar ratio (1:2.5) was selected for further studies. Lower molar ratios

did not increase significantly the immunoassay detectability and required higher concentrations of immunoreagents to obtain the same maximum absorbance in the assay.

2.5.4. Optimization and evaluation of the indirect competitive ELISA As53/7-OVA

It is well known that immunoassay performance may be affected by many physico-chemical features of the media and by a variety of experimental conditions. Here we focus our studies on the evaluation of the immunoassay behavior under several conditions, such as immunoreaction time, detergent, pH, ionic strength, etc.

2.5.4.1. Effect of the preincubation time

It has been reported that the detectability of an immunoassay can be improved by preincubation of the analyte with the As prior to the competition step [272,288]. In our case no significant effect on the immunoassay detectability was observed after an overnight incubation of the As with the analyte at 4ºC (IC_{50} 0.29 µg/L versus 0.31 µg/L)

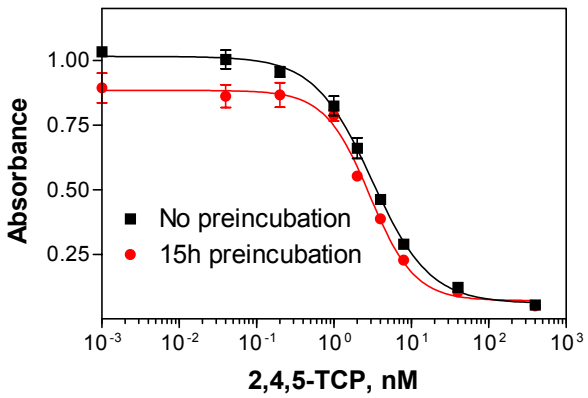


Figure 2.11. Effect of the preincubation of the As and the analyte before the competitive step. The preincubation took place overnight at 4°C. The data correspond to the average of two replicates.

(see **Figure 2.11**). The same effect was observed for ELISAs developed in our group for other phenolic compounds, such as 4-nitrophenol [289] and 2,4,6-TCP [259,277].

2.5.4.2. Effect of the length of the competition step

The influence of the length of the competition step was also studied. As shown in **Figure 2.12** the best detectability (the lowest IC_{50}) were obtained with shorter incubation periods. A reduction of the duration of the competitive step from 2 h to 10 min led to a decrease in the IC_{50} value of three times, but at the same time a decrease of the maximum assay signal (A_{max}) was also produced. Therefore, a competitive incubation period of 30 min was chosen as a compromise because of the greater A_{max} /IC_{50} ratio encountered (see **Figure 2.12**).

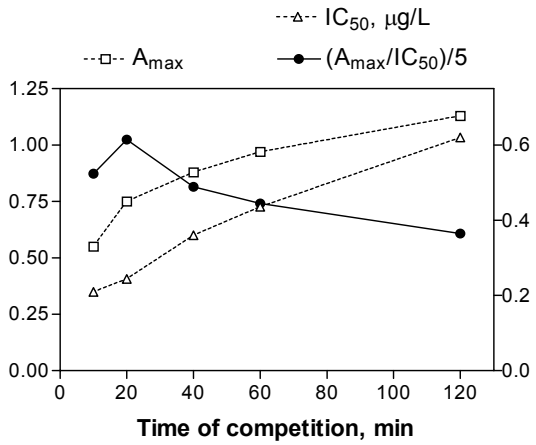


Figure 2.12. Variation of the immunoassay parameters (IC_{50} and A_{max}) as a function of the length of the competitive step. The analyte, 2,4,5-TCP and the antibody were incubated for different periods of time (between 10 and 120 min) in the antigen-coated plates. The results reported are extracted from the four-parameter equation used to fit the standard curves. Each standard curve was run in duplicate.

2.5.4.3. Effect of the detergent Tween 20

Tween 20 is a nonionic surfactant commonly used in immunoassay protocols to reduce non-specific interactions. For most pesticide immunoassays its usual concentration is 0.05%. It was demonstrated in several works that lower concentrations of Tween 20

improve the immunoassay detectability for nonpolar small organic analytes such as endosulfan [290] and chlorpyrifos [287]. In contrast, highly polar analytes, such as chlorophenols [260,277], nitrophenols [289,291] and 3,5,6-tricloro-2-pyridinol [182] are less influenced by the presence of detergents in the immunoreaction media because of their inability to establish non-hydrophobic interactions with the detergent. Our results are in agreement with the above statement. **Figure 2.13** shows that Tween 20 concentrations lower than 0.05% have no effect on the immunoassay detectability for the polar 2,4,5-TCP. Concentrations higher than 0.5% significantly decreased the detectability reaching IC_{50} values close to 6 μg/L without affecting A_{max}. Thus, subsequent experiments were performed at 0.025% Tween 20 final concentration

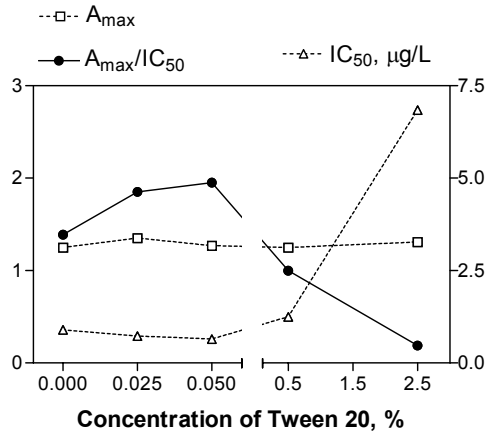


Figure 2.13. Effect of the concentration of Tween 20 on the indirect competitive ELISA. Standard curves and the As were prepared in PBS with different concentrations of Tween.

(preparing the curve in PBS and adding the As diluted in 0.05% PBST).

2.5.4.4. Effect of the pH

The effect of the pH on the immunoassay performance is presented on **Figure 2.14.** The parameters of the 2,4,5-TCP standard curves are quite stable in media with pH values between 6.6 and 10.5. At pH lower than 5.5 the assay is totally inhibited. This behavior is similar to the one reported by our group for other immunoassays for phenolic compounds

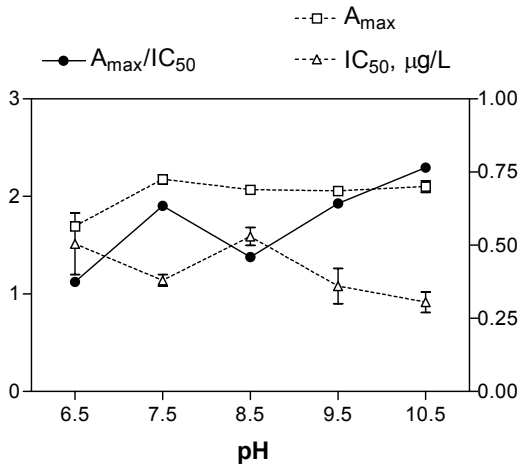


Figure 2.14. Effect of the pH on the 2,4,5-TCP immunoassay. Several standard curves were prepared using PBS 10 mM at different pH values and added to the antigen-coated plates. The As dilutions were also prepared with the same buffers and Tween 20 was added to each of them at 0.05%. Data correspond to the average effect observed for each pH value, on two assays run in separate microtiter plates.

[259,260,289,291]. The greater tolerance to the basic conditions (2,4,5-TCP pKa=7.1: pH> pKa) has been interpreted as a larger participation of the phenolate form in the formation of the analyte-antibody complex. At pH 7.5 about 72% of the 2,4,5-TCP molecules exist as phenolate forms, while only 55% of the competitor hapten **7** are ionized, according to its pKa value (pKa=7.4). This difference may be one of the reasons favoring the competition of the target analyte *versus* the competitor.

2.5.4.5. Effect of the ionic strength

The effect of the ionic strength on the immunoassay performance was evaluated in media with conductivity values ranging from 0 to 80 mS/cm (0 to 50 mM PBS) (**Figure 2.15**). It was observed that in the absence of salts the assay was almost inhibited due to a high increase of the assay noise (A_{min}/A_{max} = 0.7). This behavior confirms the above-mentioned implication of the ionic species in the stabilization of the antigen-antibody complex. A marked increase of the detectability (decrease in IC_{50}) was produced from 10 to 20 mS/cm and the IC_{50} remains almost constant from 30 to 80 mS/cm. The A_{max} slightly diminishes from 1 to 0.5 units within this range, but its values allow a usable assay. The greatest variability of the immunoassay performance was observed in media

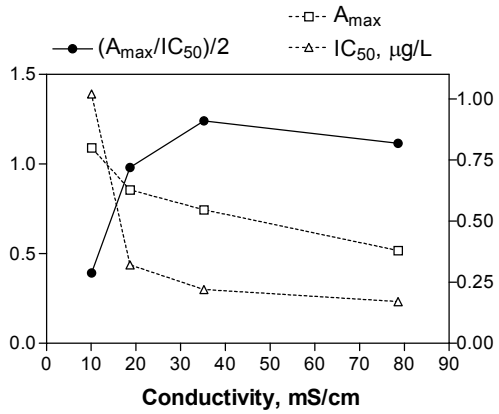


Figure 2.15. Variation of the immunoassay features with the ionic strength of the media (expressed in mS/cm). Different concentrations of PBS (0, 5, 10, 20, 50 mM) were used to prepare standards and As solutions. Each standard curve was run in duplicate in the same microtiter plate. The data presented are extracted from the four-parameter equation used to fit the standard curve**.**

ranging from 10 to 20 mS/cm. Therefore it was considered necessary to place the assay conditions under ionic strength regions not as much affected by this parameter. The initial 10 mM PBS assay buffer (conductivity near 15 mS/cm) was not the most appropriate. In contrast a 20 mM PBS buffer with conductivity values between 20 and 30 mS/cm placed the assay under conditions where small variations of the conductivity of the media would not produce important variations on the immunoassay parameters.

2.5.4.6. Summary of the immunoassay features

As a result of the immunoassay evaluation described above we can conclude that some parameters allowed the improvement of the ELISA detectabilty. While the time of preincubation and competition do not affect significantly the imunoassay, we have established the conditions of best immunoassay performance regarding Tween 20 concentration, pH and conductivity. The factors determining the best immunoassay performance are summarized in **Table 2.11** and the corresponding standard curve is presented in **Figure 2.16**. Under these conditions we have studied the reproducibility of the immunoassay As53/**7**-OVA running nine calibration curves in two-well replicates in three different days. The parameters of the standard curves are summarized in **Table**

2.11. The IC_{50} value is 0.23 µg/L (1.165 nM), the dynamic range is between 0.093 and 0.725 µg/L (0.471-3.671 nM) and the limit of detection is 0.053 µg/L (0.268 nM).

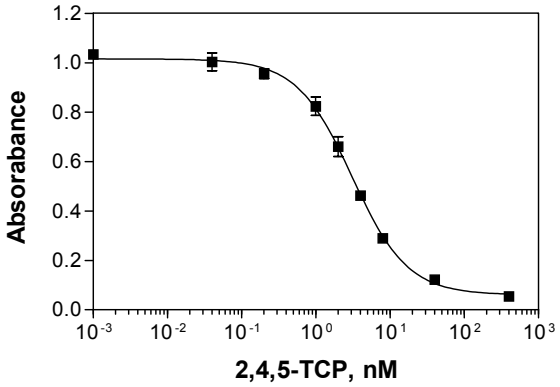


Figure 2.16. Standard curve for the As53/**7**-OVA (1:2.5) immunoassay. The corresponding characteristics are presented in **Table 2.11**.

Table 2.11. Optimum conditions and features of the 2,4,5-TCP immunoassay (As53/**7**-OVA)

Condition	Values	Parameter	Values [a]
Preincubation time	0 min	$\mathbf{A_{max}}$	0.838±0.020
Competition time	30 min	$\mathbf{A_{min}}$	0.031±0.009
pH	7.5	**IC_{50}, µg/L**	0.231±0.011
Ionic strength	20 mS/cm (20 mM PBS)	**Dynamic range** [b]	0.093±0.004 to 0.725±0.106
Tween 20	0.025%	**Slope**	1.399±0.070
		LOD [c]**, µg/L**	0.053±0.004
		$\mathbf{r^2}$	0.995±0.001

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. The data presented correspond to the average of nine calibration curves run in three different days. Each curve was build using two-well replicates. [b] The dynamic range is defined by the concentrations corresponding to 20 and 80% of the assay response at zero dose. [c] The limit of detection (LOD)is the analyte concentration corresponding to 90% of the assay response at zero dose.

2.5.4.7. Specificity study

Immunoassay specificity was evaluated by testing 27 structurally related compounds as competitors in the ELISA. We have selected a variety of chlorophenols, bromophenols

and phenol. Standard curves for all the selected compounds were run in the range 0.04 and 10 000 nM and compared to the 2,4,5-TCP curve. The cross-reactivity values were calculated according to the following formula:

$$CR\% = \frac{IC_{50}(2,4,5-TCP)}{IC_{50}(phenolic\_compound)} \times 100$$

The results regarding the IC_{50} for each analyte and the crossreactivity (%) values demonstrate that the immunoassay is quite specific (see **Table 2.12**).

Table 2.12. Interference caused by structurally related chemicals, expressed by their IC_{50} and the percentage of cross-reactivity [a].

# [b]	Phenolic Compounds	IC_{50} (nM)	% CR	Brominated analogues	IC_{50} (nM)	% CR
5	**PCP**	101.3	1.5	**PBP**	361.9	0.42
4	**2,3,4,6-TtCP**	8	18.9			
	2,3,4,5-TtCP	33	4.6			
	2,3,5,6-TtCP	304	0.5			
3	**2,4,5-TCP**	1.52	100			
	2,4,6-TCP	8.9	17	**2,4,6-TBP**	5.5	27.7
	2,3,4-TCP	168.9	0.9	**2,4,6-TIP**	6.3	24
	2,3,5-TCP	1520	0.1	**2,4,6-TFP**	>10000	<0.01
	2,3,6-TCP	380	0.4			
	2,4,5-TCA	524.5	0.29			
	2,4,6-TCA	5066,7	0.03			
2	**2,4-DCP**	43.4	3.5	**2,4-DBP**	14.5	10.5
	2,5-DCP	168.9	0.9	**2,5-DBP**	8	15
	2,6-DCP	337.8	0.45	**2,6-DBP**	108.6	1.4
	3,4-DCP	>10000	<0.01	**2-B-4-CP**	14.9	10.2
1	**2-CP**	2533.3	0.06			
	3-CP	>10000	<0.01			
	4-CP	>10000	<0.01	**4-BP**	475	0.32
0	**phenol**	>10000	<0.01			

[a] Cross-reactivity is expressed as % of the IC_{50} of 2,4,6-TCP/IC_{50} phenolic compound;
[b] Number of halogens. B, bromo; C, is chloro; DCP, dichlorophenol; TCP,trichlorophenol; TtCP, tetrachlorophenol;, PCP, pentachlorophenol; BP, bromophenol; DBP, dibromophenol; TBP, tribromophenol; PBP, pentabromophenol; TIP, triiodophenol; TFP, trifluorophenol; TCA, trichloroanisol.

It can be observed that the degree of recognition was directly related to the presence of two chlorine atoms (one at *ortho* and another at *para* position) and one hydrogen atom in *meta* position as in the target analyte (see **Figure 2.17**). For example, 2,3,4,6-tetrachlorophenol (TtCP) possessing these three atoms on one of its moieties was the most recognized compound with 19 % cross-reactivity value, while 2,3,4,5-TtCP lacking the hydrogen atom in *meta* was less recognized (4.6%) and 2,3,5,6- TtCP lacking the chlorine atom at *para* position and possessing two chlorine atoms at *meta* positions cross-reacted only 0.5%. Similarly, among the trichlorophenols 2,4,6-TCP is the most recognized (17%) possessing both *orto* and *para* chlorine atoms and a *meta* hydrogen, in the same range as 2,3,4,6-TtCP. The lack of the hydrogen in *meta* (2,3,4-TCP) or of the chlorine atom in *para* (2,3,5-TCP and 2,3,6-TCP) decreases significantly the recognition. Analytes with only two, one or none chlorine atoms had a negligible (≤3.5%) or void interference in the assay. It also worth noting that blocking the hydroxy group as an ether reduced drastically the recognition. Thus, 2,4,5-trichloroanisol was only recognized at 0.29%, although it possesses the three chlorine atoms in the same position as the target analyte. This lack of recognition could be attributed to the fact that the phenolate species are the forms participating mostly in the stabilization of the immunocomplex. As was demonstrated by theoretical models, blocking the hydroxyl group produced significant electronic differences with the target analyte. Similarly to ELISAs for 2,4,6-TCP developed in our group [259,260,277], brominated phenols were also highly recognized in this assay. Thus, 2,4-dibromophenol (DBP) and 2,5-DBP cross-reacted 10.5% and 15%, respectively, while their homologous chlorinated analytes 2,4-DCP and 2,5-DCP were only recognized in 3.5% and 0.5%, respectively. Similarly, 2,4,6-tribromophenol (TBP) was recognized 27.7% while 2,4,6-TCP interfered only 17%. Unfortunately, at the time of writing this thesis we have not been able to test 2,4,5-TBP, but we would expect recognition greater than 100%.

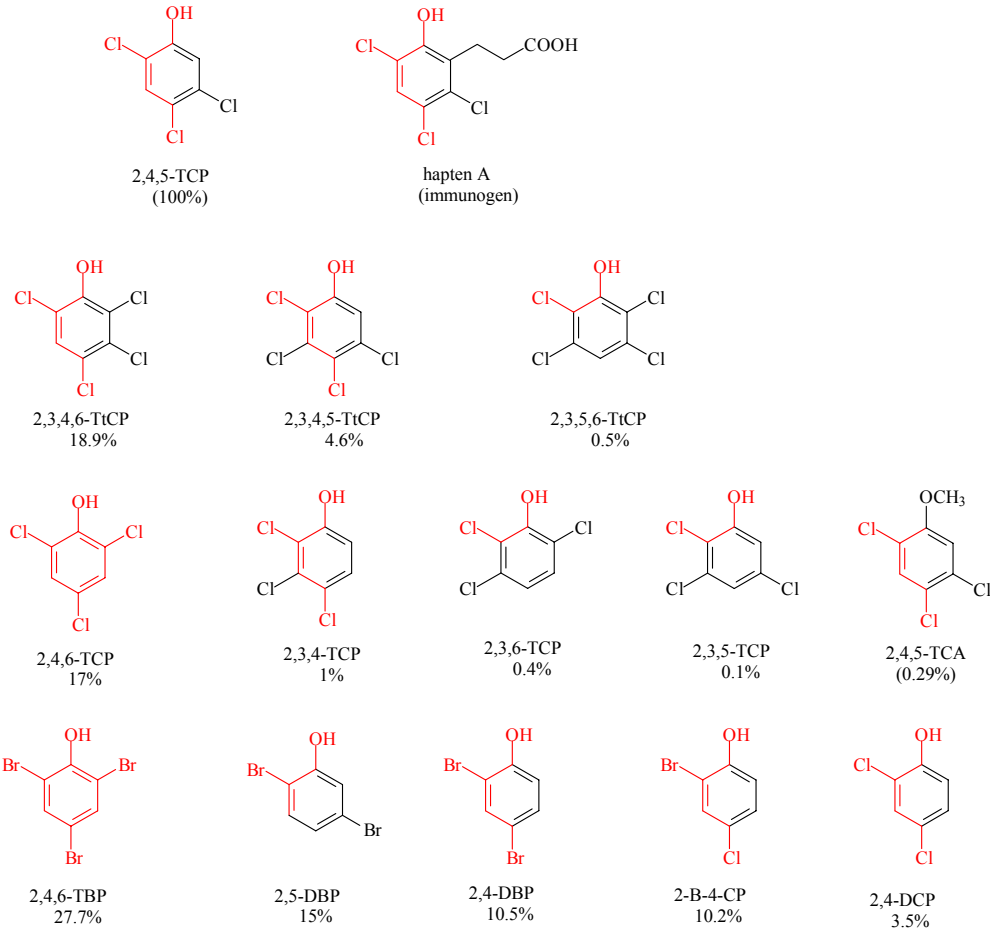


Figure 2.17. Chemical structures of the analyte, the immunogen used for As 53 production and the crossreactants. The fragments of the structures colored in red are possibly recognized by the As. From left to the right the cross-reactivity is decreasing.

2.5.4.8. Precision

Once we set the optimum performance conditions of the immunoassay (see **Table 2.11**), we studied its precision. The within assay precision was evaluated by the analyses of spiked PBS samples in triplicate inside a plate and the precision between different assays was calculated based on the analyses of the same samples in different days.

Table 2.13. Precision of 2,4,5-TCP immunoassay

	Spiked concentration, μg/L		
	0.1	**0.3**	**1.5**
N[a]	9	9	9
Mean	0.094 ± 0.018	0.285 ± 0.031	1.453 ± 0.174
% CV (within assay) [b]	15	5.1	7.9
% CV (between assay)	20	10.9	12

[a] Samples were prepared in PBS and analyzed in three-well replicates in three consecutive days.
[b] The % CV is the average of the % CV of all measurements.

The coefficients of variation obtained are below 12% except in the case when the measurement takes place at the limits of the working range of the assay.

2.5.4.9. Accuracy

Similarly, the accuracy of the assay was tested by recovery experiments. Eight PBS samples with increasing 2,4,5-TCP concentrations varying from 0.1 to 50 μg/L were analysed by the ELISA and the measured values were compared to the spiked concentrations (see **Figure 2.18**). The correlation was very good (r^2 = 0.999) and the slope of the regression curve was close to 1 (slope =1.07) that is an indication of the assay accuracy.

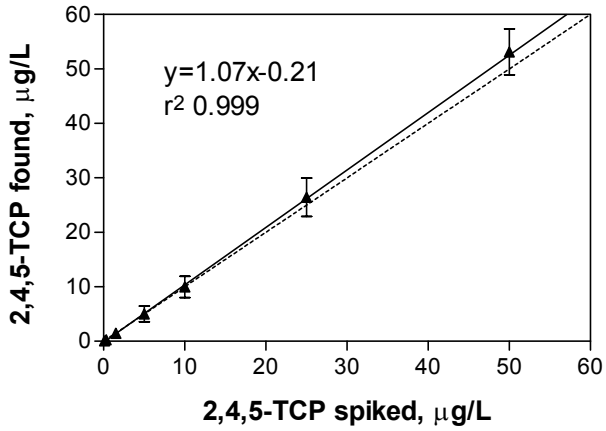


Figure 2.18. Correlation between the spiked and the measured concentration values. Samples were prepared by spiking 20 mM PBS with different concentrations of 2,4,5-TCP. The data shown correspond to the average and the standard deviation of the results obtained from the immunoassay run in three different days. The dotted line corresponds to a perfect correlation (slope = 1).

3. Evaluation of the effect of environmental and biological matrices on the immunochemical determination of 2,4,5-TCP and 2,4,6-TCP

The final objective of any immunoassay is to obtain correct results when measuring a particular analyte in a real sample. However, the IA performance may be affected by interferences from the sample matrix [25,174,292]. The matrix effect represents the deviation from the parameters of the standard curve in buffer caused by the sample properties or its components (except the analyte). Interferences on IAs are categorized into specific and non-specific. All of them may affect Ab-Ag interaction and/or the

enzyme activity if enzyme labels are used. Specific interferences (cross-reactants) refer to those kind of sample components which are recognized by the Ab. Non-specific interferences involve other components that bear no structural resemblance to the analyte and physicochemical parameters, such as pH, ionic strength, chaotropic agents, organic compounds, etc.

As the interfering substances in many cases are completely unknown, the matrix effects of real samples should be evaluated when environmental and biological monitoring is performed by an immunochemical method. There are several approaches to asses matrix effects. If a "blank" (zero analyte concentration) sample matrix is available, standard curve run in the matrix of interest is compared to the standard curve in buffer. If there is no matrix effect, no differences should be observed between the parameters of both curves. Another possibility is to measure fortified samples and compare the expected and measured recovery values. In an IA with interferences, recovery suffers deviations (it should be around 100%) and differences between observed and measured values usually decrease with dilutions. If an appropriate "blank" sample is not available, a sample can be used, preferably one in which the analyte is present in very low levels. The method of standard additions is then recommended for evaluating the effect of the matrix [293]. Briefly, in this method the sample is split and one split is fortified with a known concentration of analyte. Both are measured by ELISA and the difference between them should be the level of the fortification. If it is not, a matrix effect may be assumed. Another way to verify if there is a matrix effect is to analyze the sample at several dilutions. A plot of the absorbance obtained from each dilution should be parallel to the slope of the calibration curve in the absence of matrix effects.

Once the presence of a matrix effect is confirmed, the next step is to find out the best way to eliminate it. Matrix effects in IAs can be overcome by sample dilution, purification or changes in the assay format. Sample dilution is the easiest way to overcome negative matrix effects, but it limits the assay detectability. Another alternative is to run the standard curve in the matrix of interest, so that the influence on quantification would be normalized. Finally, adding some additives to the working buffer could mimic the matrix

of interest and in this way the buffer calibration curve will be placed close to the matrix one. Nevertheless, it should be noted that interferences may vary with reagent batches and matrix sources and thus must be checked frequently by a combination of running appropriate blanks and controls, and confirming positive samples by an alternate analytical method. This is crucial when ELISA is used for monitoring samples of unknown origin when corresponding blanks are not available.

In general, when ELISA is applied to environmental and/or biological monitoring the objective is to find out the easiest and less time-consuming sample treatment resulting in a correct analyte determination. In this chapter we describe the evaluation of the effect of environmental (water) and biological (milk, serum and urine) matrices on the performance of the two immunoassays for trichlophenols (2,4,5- and 2,4,6-TCP ELISA) developed in our group. As mentioned in the **Introduction** an indirect ELISA for 2,4,6-TCP (As43/**8**-BSA(1:5)) had been developed before by R.Galve [277]. The As was raised against *3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid)* (hapten **5**) (see **Figure 2.5**) covalently coupled by the MA method to KLH. The indirect ELISA uses a heterologous coating antigen prepared by conjugation of *3-(2-hydroxy-3,6-dichlorophenyl)propanoic acid* (hapten **8**) to BSA using the AE method. The optimum hapten density for the coating antigen was found to be 3 mols hapten/mol of protein. The assay parameters and the optimum conditions of its performance are summarized in **Table 3.1.**

Table 3.1. Optimum conditions and features of the 2,4,6-TCP immunoassay (As43/**8**-BSA)

Condition	Values	Parameter	Values [a]
Preincubation time	0 min	$\mathbf{A_{max}}$	0.796±0.174
Competition time	30 min	$\mathbf{A_{min}}$	0.018±0.021
pH	7.5	$\mathbf{IC_{50}}$**, µg/L**	1.132±0.361
Ionic strength	15 mS/cm (10 mM PBS)	**Dynamic range**	0.288±0.045 to 3.117±0.679
Tween 20	0.05%	**Slope**	1.22±0.23
As43	1/4000	**LOD, µg/L**	0.175±0.027
8-BSA	0.625 µg/mL	$\mathbf{r^2}$	0.991±0.006

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. The data presented correspond to the average of 34 calibration curves run in different plates in a 3-months period. Each curve was built using two-well replicates.

The assay shows a limit of detection of 0.175±0.027 μg/L and it is performed in about 1.5 h. (see **Experimental Section** for detailed procedure). Regarding the effect of the physicochemical properties of the media a tolerance to basic conditions was observed. The assay performs well between pH 7.5 and 9.5 and it is inhibited at pH lower than 6. The immunoassay detectability do not change significantly when the ionic strength of the media is in the range of 12 - 25 mS/cm. The ELISA for 2,4,6-TCP is quite specific but some cross-reactivity with other chlorinated phenols such as 2,3,4,6-TtCP (21%), 2,4,5-TCP (12%) and 2,3,5-TCP (15%) is observed. Brominated phenols (BP) are even more recognized than the corresponding chlorinated analogues (e.g. 2,4,6-TBP, 710%; 2,4-DBP, 119 %).

The development of the indirect ELISA for 2,4,5-TCP (As53/**7**-OVA(1:2.5)) have been described in the previous chapter (see **Section 2.5.4** for the influence of several physicochemical parameters, see **Table 2.12** for the immunoassay features, and **Table 2.13** for cross-reactivity data).

In this chapter we report the evaluation of the 2,4,5- and 2,4,6-TCP ELISAs for environmental and biological monitoring programs. As environmental samples we have evaluated water samples from different sources. In this case the objective was to prove if these ELISA methods would be able to reach a limit of detection close to the values set by the EU as maximum concentration of pesticides in drinking water (0.1 μg/L for individual compounds and 0.5 μg/L for total).

For biomonitoring purposes we have considered suitable to evaluate ELISA performance in several body fluids, such as milk, serum and urine. In contrast to the much more lipophilic organochlorinated compounds (such as PCP, HCH, HCB, DDT, DDE, PCBs) that are widely detected in milk and serum (or plasma) [94, Wrbitzky, 1995 #2535,124,127,294-296], there are very few examples on the detection of trichlorophenols in these matrices [64,134,135]. Based on the reference values established for PCP in serum (12 μg/L) and for several organochlorinated pesticides in human milk (for all of them the reference values are lower than 1 μg/g fat) [20], we could assume that

the limit of detection to be accomplished for TCP in these samples should be similar. In addition, the evaluation of the ELISA in serum samples could be useful not only for biomonitoring but also for toxicological studies. Completely different is the case of the excretion of trichlorophenols in urine that has been covered by several authors [75,121,123,124] (see **Introduction** for more information). Attending to the information found in the literature our purpose was to evaluate the interferences caused by this matrix, keeping in mind that a LOD near 1 μg/L would be desirable in order to measure levels of exposure of the general population.

3.1. Performance of the 2,4,5- and 2,4,6-TCP ELISAs in water samples

As it was mentioned in the **Introduction** chlorophenols have been identified as usual contaminants of surface waters [39], industrial wastewater [32] and drinking water [52]. According to Directive 76/464/CEE and US-EPA method 8041, 2,4,5-TCP and 2,4,6-TCP are included in the lists of the most important phenolic contaminants to be controlled in waters.

With the aim to evaluate the effect of the matrix we selected two types of water samples, a well water and drinking water from water-supply mains. The pH and the conductivity of both water samples are presented in **Table 3.2.** The conductivities of these samples were lower than the buffer conductivity.

Table 3.2. Properties of the water samples studied

Water	pH	Conductivity, mS/cm
well	6	2.31
water supply	7.3	0.52
2x PBS [a]	7.4	30

[a] 2xPBS is 20mM PBS buffer.

As mentioned in **Chapter 2**, the 2,4,5-TCP ELISA is inhibited in the absence of any salts. Therefore, the water samples were buffered by adding 10% (v/v) of 100 mM PBS.

In this way their pH and conductivity was adjusted to the buffer values. A white precipitate was formed when adjusting pH of the well water sample and it was removed by centrifugation. **Figure 3.1.A.** shows the standard curves for 2,4,5-TCP obtained for both water samples after this small treatment. It can be observed that their behavior is equal to the standard curve prepared in the assay buffer indicating the absence of undesirable matrix effects. Similarly, buffering of natural water samples has been demonstrated to be efficient to overcome the interferences on the ELISA for 2,4,6-TCP (see **Figure 3.1.B**) [1]. Only for the case of the well water sample this treatment showed to be not completely efficient.

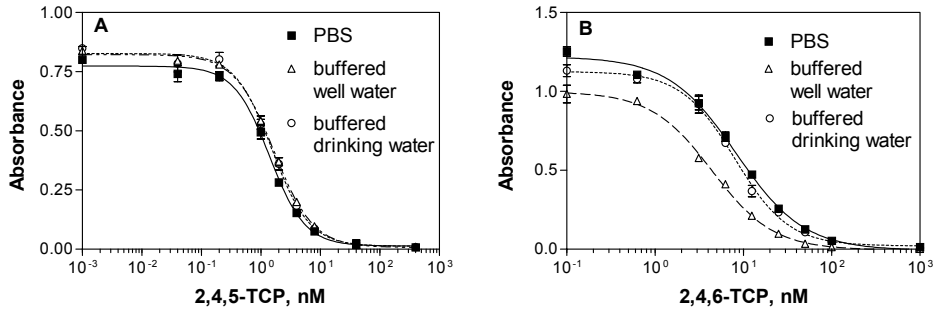


Figure 3.1. Results from the studies of the matrix effect produced by well and drinking water on the ELISAs for 2,4,5-TCP (**A**) and 2,4,6-TCP (**B**). Samples were buffered by adding 10% (v/v) of 100 mM PBS and used directly in the immunoassay. (**B**) is reprinted from [1]

In order to prove the reliability of the method, drinking water was spiked with 2,4,5-TCP at several concentration levels. Spiked samples were conditioned as already described and analyzed by ELISA without further treatment. Results from the analysis are shown in **Table 3.3**. The comparison between the amount of 2,4,5-TCP measured by ELISA and the amount added indicates a good agreement, with recovery values in the range between 92% and 105%. The average intra-assay CV was 6.7%, ranging from 2.2% to 12.7%.

Table 3.3. Accuracy of the analysis of 2,4,5-TCP in drinking water samples by the immunoassay (As53/7-OVA)

Spiked [a], μg/L	Measured (mean ± SD) [b], μg/L	Recovery, %
0.2	0.184 ± 0.023	92
0.4	0.372 ± 0.008	93
1	1.053 ± 0.027	105.3
5	5.190 ± 0.490	103.8

[a] Spiked drinking water samples were conditioned for pH and ionic strength by adding 10% (v/v) of 100 mM PBS and analysed without further treatment. Concentrations were calculated from the PBS standard curve. [b] Data correspondto three determinations performed in the same ELISA plate.

The accuracy of the ELISA for the determination of 2,4,6-TCP in water samples was evaluated by eight spiked samples. Each sample was split in two parts, one of which to be analyzed by ELISA and the other one, by a GC-ECD method. Samples were directly measured by ELISA, while for GC-ECD determination the samples were extracted in toluene in the presence of an internal standard, then derivatized and finally injected in the chromatograph (see and **Annex I**). The results obtained are presented in **Figure 3.2**. It can be observed that ELISA (slope=0.89) shows better accuracy than the GC-ECD method (slope= 0.7). This can be explained by the fact that ELISA measurements take place directly in the aqueous samples without special treatment. For instrumental analysis an extraction step is necessary and in our study we have used a standard curve built on

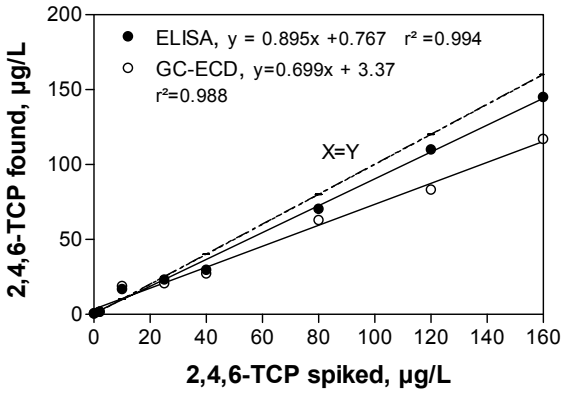


Figure 3.2. Accuracy of the 2,4,6-TCP ELISA method compared to the GC-ECD method. Spiked samples were prepared in PBS and splitted in two parts to be analyzed by both methods. Samples were directly measured for ELISA, while for GC-ECD samples were extracted in toluene in the presence of an internal standard, derivatized and then injected in the chromatograph. ELISA shows better accuracy than the GC-ECD method. Correlation between both techniques agrees to the following equation y=1.26x–4.27 and has a regression coefficient of r^2=0.993

2,4,6-TCP standards in toluene (not coefficient of extraction efficiency is applied). Correlation between both techniques agrees to the following equation y=1.26x–4.27 and has a regression coefficient of r^2=0.993.

In conclusion, the results from the water matrix effect studies demonstrate the suitability of both immunoassays for a direct and precise determination of 2,4,5-TCP and 2,4,6-TCP in water samples. The detectability of the IA methods for 2,4,5-TCP and 2,4,6-TCP in water are summarized in **Table 3.4.**

Table 3.4. Detectability features of the TCP ELISAs in drinking water samples (μg/L)

Parameters	2,4,5-TCP	2,4,6-TCP
LOD	0.07 ± 0.01	0.18 ± 0.03
IC_{50}	0.32 ± 0.02	1.13 ± 0.36
Dynamic range	0.13 ± 0.01 - 1± 0.15	0.29 ± 0.05 – 3.12± 0.68

The reported parameters correspond to the standard curves run in buffered water.

The detection limits of both ELISAs are below the LOD of the standard chromatographic methods established by EPA for environmental monitoring of trichlorophenols (0.64 μg/L 2,4,6-TCP)[144,145]. Finally, water contamination with trichlorophenols can be evaluated according to the EU legislation (Directive 80/778/CCE) on maximum levels of contaminants in drinking water samples.

3.2. Performance of the 2,4,5-TCP ELISA in milk samples

Organochlorine compounds (insecticides, pesticides, PCBs) are lipid-soluble and tend to accumulate in the food chain and store in tissues and lipid-rich organs. Milk secretion is the most important route of excretion of those type of compounds in women. The presence of organochlorines in human breast milk has been reported from various countries [94,294,297-299], since analysis of breast milk supply information on the exposure of different populations to organochlorine contaminants. In addition, several

studies demonstrate the transfer of dioxins/furans to milk of cows grazing near a municipal solid waste incinerator or following ingestion and exposure to pentachlorophenol-treated wood [87,88,300].

In order to evaluate the performance of the immunoassay for 2,4,5-TCP in milk we used skimmed milk (see **Table 3.5** for its properties).

Table 3.5. Properties of skimmed milk

pH	6.8
Conductivity	5.25 mS/cm
proteins	31 g/L
fat	≤ 5 g/L
Ca	1.2 g/L

Several standard curves were prepared in milk diluted in 2x PBS (1/5, 1/20, 1/100). Although under these conditions the pH and the conductivity values of the milk samples were closed to those of the buffer, the signal was completely inhibited. This suggested that the milk interfered with the Ab-Ag binding reaction. According to the literature [301] matrix effects in milk are likely produced by the fat and the protein content of the sample. In addition, the analyte properties can also be crucial for the good performance of the assay in milk. Thus, it has been reported that there were substantial fluctuations in the absorbance values at low analyte levels due to the difficulty in spiking raw milk (a two-phase fat and aqueous system) with a highly hydrophobic analyte, such as sulfamerazine [286]. On the other hand, immunoassays in milk have been described for the highly polar diflubenzuron [302], paraquat [303], aldrin and dieldrin [304], and atrazine [305].

With the aim to eliminate the observed interferences we decided to remove proteins by alkaline (0.2M KOH) precipitation of the milk sample but unfortunately no improvement was observed (see **Figure 3.3.A)**.

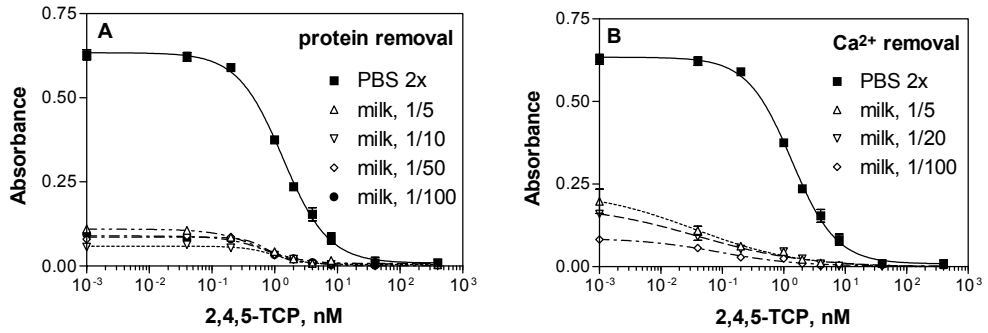


Figure 3.3. Matrix effects on the 2,4,5-TCP ELISA caused by milk after different sample treatments and subsequent dilutions with 2xPBS buffer. **A.** Protein precipitation was produced by NaOH; **B.** Ca^{2+}ions were precipitated by Na_2SO_4;. Detailed procedures are described in **Experimental Section**.

Another source of matrix effect could be the high content of calcium ions in milk (for example the 2,4,6-TCP ELISA is inhibited by Ca^{2+} ions, see **Section 3.4.3**). Some authors have reported the removal of Ca^{2+} ions by precipitation with Na_2SO_4 [302] or with oxalic acid [306]. We treated the milk samples with Na_2SO_4 and after centrifugation of the $CaSO_4$ precipitate the supernatant was diluted in PBS buffer. The resulting curves are presented in **Figure 3.3.B** and show that no improvement in immunoassay performance was accomplished. These results suggested that for the analysis 2,4,5-TCP in milk it would be necessary to develop more complex clean-up procedures such as filtration, fat removal, 2,4,5-TCP extraction, etc. Therefore, we decided to go forward evaluating other biological samples as potential matrices for biomonitoring of trichlorophenols as indicators of exposure (see **Introduction**).

3.3. Performance of the 2,4,5- and 2,4,6-TCP ELISAs in human serum

As it was mentioned in the **Introduction** human exposure assessment can be performed by analysis of trichlorophenols in blood serum [64,134] and plasma [135]. In addition,

toxicological studies on organochlorine contaminants require the determination of their metabolites (chlorophenols) in animal tissues (blood, liver, etc.) [78].

3.3.1. ELISA for 2,4,5-TCP

With the aim to determine trichlorophenols in serum by ELISA, we approached the evaluation of the immunoassay performance in a commercially available pooled human serum sample (pH=7.8; conductivity: 11 mS/cm). Initially, we tested the effect of the matrix just diluting the serum in the assay buffer. The pH and salinity of serum dilutions were the same as the buffer values. As it is shown in **Figure 3.4**, the serum did not have a significant effect on the A_{max} of the assay, although the non-specific adsorption (A_{min}) increased. The serum standard curves were parallel to the one prepared in PBS and shifted towards higher concentration region, diminishing in this way the assay detectability. Thus, the standard curve prepared in 20 times diluted serum produced an IC_{50} value of 25 μg/L. This behavior was attributed to the high concentration of albumins in human serum (range 3500-5500 mg/mL for adults in central Europe) [307]. It is well known that albumins have the ability to bind many substances and play an important role as carrier proteins. The analyte, present in the samples or in the standards, could bind the serum albumins preventing in this way the reaction with the antibody. As a consequence, the real analyte concentration would be underestimated if the buffer standard curve is used for quantification (serum curves are shifted to the right). Therefore, we focused on the evaluation of sample treatment procedures to reduce the protein content of the serum samples.

Protein removal is perhaps the crudest clean-up procedure applied in biofluid analysis and it can be realized by filtration (dialysis) or protein precipitation (by heat, acids, alkalis, organic solvents) [308]. Our aim was to find the best procedure to eliminate proteins in terms of immunoassay compatibility and analyte recoveries. ***Ultrafiltration*** of the serum using nitrocellulose microfilters (10 kDa cut-off, Millipore) improved significantly (3 times) the immunoassay detectability (see **Figure 3.4.B**), but the reproducibility was not

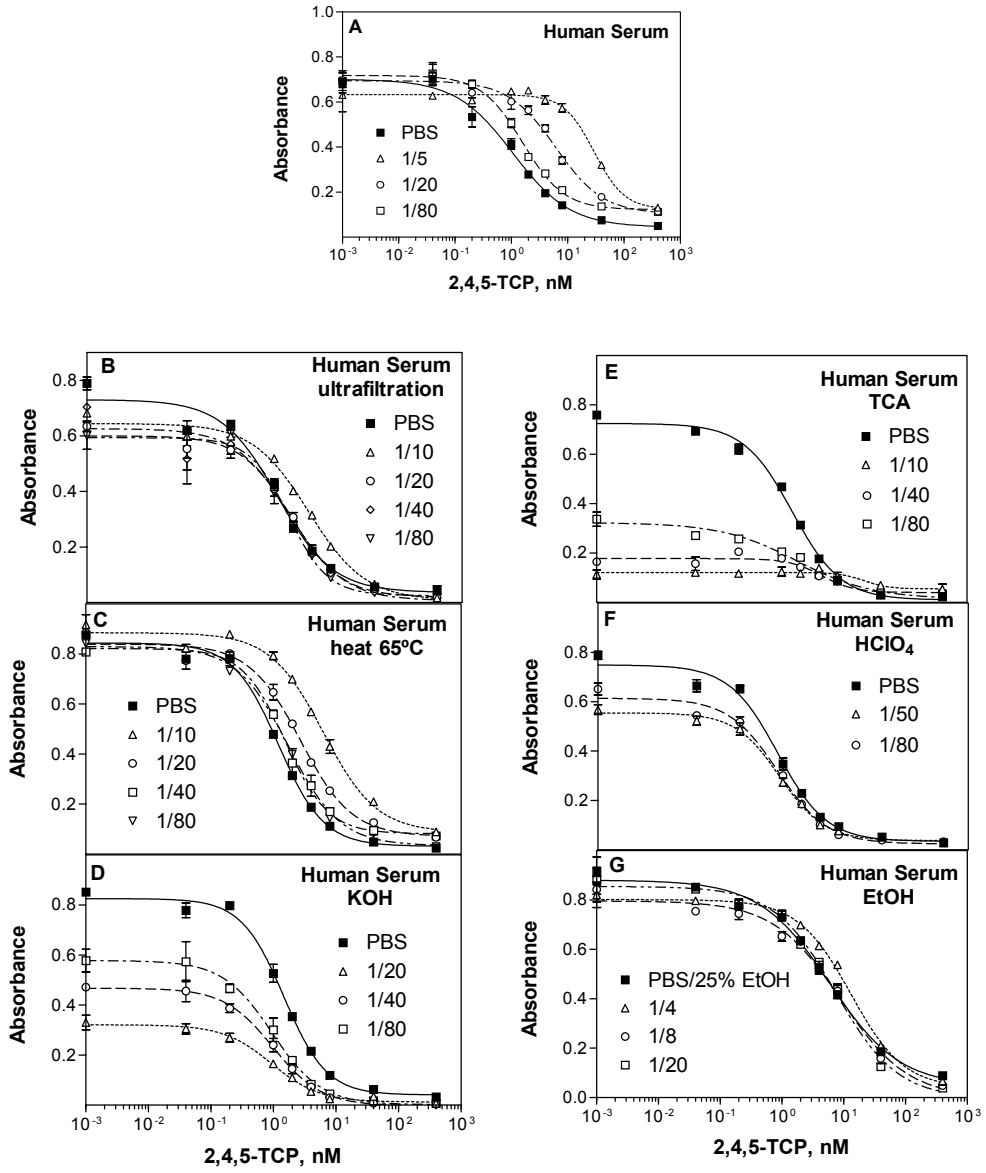


Figure 3.4. Matrix effects on the 2,4,5-TCP ELISA caused by the human serum after several sample treatments and subsequent dilutions with PBS buffer. **A,** Serum without any treatment; **B**. Ultrafiltration**;** Protein precipitation was produced by: **C,** heating the serum at 65ºC; **D,** KOH; **E**, trichloroacetic acid; **F;** perchloric acid; **G**, absolute ethanol. Detailed protein removal procedures are described in Experimental section.

very good and the recovery from spiked and filtered serum samples was low probably due to analyte adsorption on the microfilters. Similar problems were encountered after protein denaturation by ***heat***. Partial protein removal by heating the serum at 65°C during 30 min reduced the matrix effect on the ELISA (compare **Figure 3.4.C** with **3.4.A**). However, the recoveries ranged from 20 % to 55 % when 1/20 PBS serum dilution was used and the quantification was performed with a standard curve run in PBS (see **Table 3.6**). Complete protein removal was achieved by heating up to 100ºC for 15 min. In this case 1/10 PBS serum dilution was enough to eliminate completely the matrix interferences (data not shown), but the recoveries were lower than 34% (see **Table 3.6**).

Table 3.6. Accuracy of 2,4,5-TCP analysis in human serum by the immunoassay.

Protein removal	Spiked, μg/L	Measured (mean ± SD) [e], μg/L	Recovery, %
Heat 65ºC [a]	6	1.206 ± 0.704	20
	12	2.604 ± 0.660	21.7
	20	11.06 ± 2.762	55.3
Heat 100ºC [b]	5	1.691 ± 0.105	33.8
	12	3.602 ± 0.410	30
	20	5.720 ± 0.425	28.6
$HClO_4$ [c]	12	11.364 ± 0.477	94.7
	20	13.360 ± 0.922	66.8
	40	13.040 ± 2.060	32.6
EtOH [d]	3.2	3.080 ± 0.600 [f]	95.50
	8	7.844 ± 0.512 [f]	98.05
	32	24.440 ± 0.784 [f]	76.57

[a] Spiked serum samples were deproteinized by heating at 65ºC for 30 min, diluted 20 times with PBS. Further dilutions were performed using 1/20 PBS diluted serum. The concentrations were evaluated from a standard curve run in PBS. [b] Spiked serum samples were deproteinized by heating at 100ºC for 15 min and diluted 10 times with PBS. Further dilutions were performed in 1/10 PBS diluted serum. The concentrations were evaluated from a standard curve run PBS.
[c] Spiked serum samples were deproteinized by 6% perchloric acid and the supernatant diluted 80 times with PBS. The concentrations were evaluated from a standard curve run in PBS.
[d] Spiked serum samples are precipitated by absolute ethanol (1:1, v/v) and the supernatant diluted with PBS to final 25% EtOH and 1/8 serum concentration. The concentrations were evaluated from a standard curve run in 25% EtOH/PBS. [e] Data obtained from three determinations performed in the same ELISA plate, except [f] that was obtained from two different samples precipitated with ethanol and each samples was analyzed in triplicates on the same ELISA plate.

Protein precipitants, such as acids and bases, commonly used in preparing biological samples prior to analysis, were also tested. We observed that ***alkaline denaturation*** with 0.2M KOH [309] removed most of the proteins in the serum, but the serum supernatant had to be diluted more than 80 times to avoid matrix effect. This was probably caused by

the high increase of the ionic strength of this sample (see **Figure 3.4.D**). When ***trichloroacetic acid*** was used as protein precipitating agent [310], the ELISA was even more inhibited in spite that the pH and the conductivity had been previously adjusted (see **Figure 3.4.E**). Deproteinisation using 3% (w/w) ***perchloric acid*** [311] performed slightly better, although it was still needed to dilute the serum supernatant at least 80 times to obtain an acceptable standard curve close to the one obtained with the assay buffer (see **Figure 3.4.F**; the pH and the conductivity had also been adjusted to the buffer values for all dilutions). Additionally, the recovery values of this sample treatment were low, especially at high 2,4,5-TCP concentration levels (40 μg/L - 32.6%, see **Table 3.6**). A co-precipitation of the trichlorophenol with the serum proteins may occur due to its polar and lipophilic properties.

Finally, we approached deproteinization of the serum by organic solvents. We chose ethanol because of its ability to precipitate more than 99% of proteins without changing the pH of the sample [308]. Additionally, hydro-alcoholic extracts are often compatible with the immunoassay performance. In general immunoassays tolerate a concentration at about 10% of alcohols [177,180,265,312,313]. The effect of the EtOH content on the immunoassay for 2,4,5-TCP is presented in **Figure 3.5**.

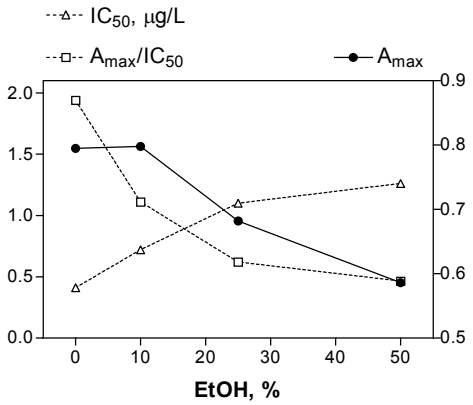


Figure 3.5. Variation of the 2,4,5-TCP immunoassay features as a function of the EtOH concentration in PBS buffer. Different concentrations of EtOH/PBS (0, 10%, 25%, 50%) were used to prepare standards and As solutions. Each standard curve was run in duplicate in the same microtiter plate. The data presented are extracted from the four-parameter equation used to fit the standard curve.

A clear decrease in maximumum signal and detectability (IC_{50}) is observed when increasing amounts of the solvent are added to the assay buffer, although reasonable values of A_{max} and IC_{50} can be accomplished up-to 25% EtOH. A 25% EtOH content produces an assay with IC_{50} value 2-3 times higher than the IC_{50} of the buffer assay keeping similar A_{max}. Even at 50% EtOH content the immunoassay is not inhibited.

Taking in consideration the 2,4,5-TCP levels found in the general population and the reference values accepted in some countries (see **Section 1.2.5.3**), we considered that a LOD between 0.1 and 0.2 μg/L could still be good enough for biological monitoring studies. Therefore, human serum was treated with absolute ethanol to a 50% concentration, centrifuged and the supernatant diluted 2 times with PBS. This solution represents 1/4 serum dilution and it contains a 25% EtOH. Further serum dilutions were performed using 25% EtOH/PBS to maintain the organic solvent concentration. The serum standard curves compared to the 25% EtOH/PBS curve are presented in **Figure 3.4.G**. It can be observed that, under these conditions a 1/4 dilution eliminates the interferences caused by the serum matrix. Additionally, this sample treatment improves significantly immunoassay performance when compared to the 1/5 diluted serum assayed without any treatment (see **Figure 3.4.A**).

A 1/8 diluted deproteinized serum was chosen to test immunoassay accuracy. Three spiked and one non-spiked serum samples were prepared in duplicates for each concentration value, treated to remove proteins as described before and measured with the ELISA using the standard curve run in 25% EtOH/PBS. As it is shown in **Table 3.6**, the percentage of the coefficient of variation is quite low and the recovery values ranged from 76% to 98%. From these results it can be concluded that this procedure is appropriate to analyze 2,4,5-TCP in serum samples. A LOD of about 0.8 μg/L can be accomplished in serum using this procedure (see **Table 3.7** at the end of this section). This value can be improved if further dilutions of the serum supernatant are performed in PBS reducing thus the ethanol content until a 12.5% (LOD= 0.05 μg/L x 8 = 0.5 μg/L).

3.3.2. ELISA for 2,4,6-TCP

Next, we evaluated the serum matrix effect on the immunoassay for 2,4,6-TCP. As can be seen at **Figure 3.6.A** the serum matrix effect is quite similar to that observed for the 2,4,5-TCP ELISA: at least 80-fold serum dilution would be necessary for a correct 2,4,6-TCP quantification in serum samples.

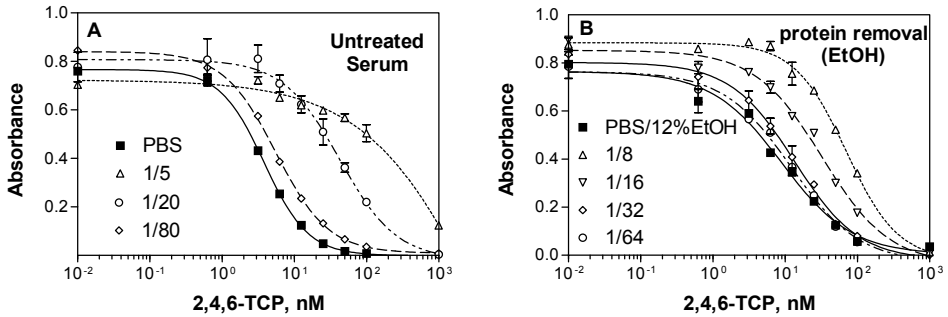


Figure 3.6. Matrix effects on the 2,4,6-TCP ELISA caused by the human serum. **A,** Serum without any treatment.; **B**, Protein precipitation was produced by absolute ethanol. Detailed protein removal procedure described in Experimental section.

Therefore, we have decided to apply the most effective serum treatment procedure established above, the protein precipitation by EtOH. First, it was necessary to evaluate the effect of EtOH content on the performance of 2,4,6-TCP ELISA (see **Figure 3.7**). The increase in the percentage of EtOH affected negatively the immunoassay parameters limiting its detectabilty. Ethanol concentration values higher than 15% clearly modified the assay parameters and at 56% EtOH the absorbance values of the standard curve fitted very poorly to a sigmoidal curve. It should be noted that the 2,4,5-TCP ELISA tolerates ethanol concentrations even at 50% EtOH (see **Figure 3.5**). Therefore, a lower EtOH concentration in the serum sample after the protein removal had to be used to avoid its negative effect on the 2,4,6-TCP ELISA. Thus, we followed the same protein precipitation procedure (50% EtOH) but then the sample was diluted with PBS buffer until 12% EtOH.

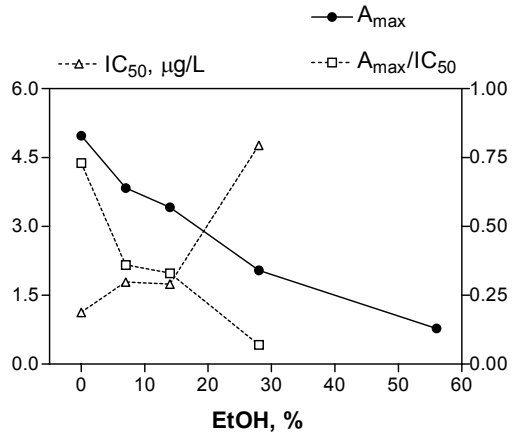


Figure 3.7. Variation of the 2,4,6-TCP immunoassay features as a function of EtOH concentration in the PBS buffer. Different concentrations of EtOH/PBS in the range 0-50% were used to prepare standards and As solutions. Each standard curve was run in duplicate in the same microtiter plate. The data presented are extracted from the four-parameter equation used to fit the standard curve.

The effect of the serum on the immunoassay after this treatment is presented in **Figure 3.6.B.** Surprisingly, this immunoassay showed higher matrix effect than the 2,4,5-TCP ELISA and a higher serum dilution (1/32) was needed to accomplish the same behavior as that of the 12%EtOH/PBS buffer (compare to **Figure 3.4.G**). Therefore, the observed matrix effect results in a higher LOD for 2,4,6-TCP in serum samples (4.6 µg/L) (see **Table 3.7**). It is possible that a further optimization of the ELISA protocol applied to serum extracts or an introduction of a clean-up procedure would allow an improvement in method detectability.

Table 3.7. Detectability features of the TCP ELISAs in serum samples (µg/L).

Parameters	2,4,5-TCP [a]	2,4,6-TCP [b]
LOD	0.80 ± 0.16	4.576 ± 0.68
IC_{50}	6.46 ± 0.25	85.12 ± 2.55
Dynamic range	1.2 ± 0.14 - 40 ± 6.96	15.04± 2.73 - 288.8 ± 40.46

[a] The reported values are obtained multiplying the parameters of the 1/8 serum standard curve by 8. Serum is precipitated by ethanol. [b] The reported values are obtained multiplying the parameters of the 1/32 serum standard curve by 32. Serum is precipitated by ethanol.

In conclusion, the detection concentrations accomplished with both ELISAs following the optimized procedure of serum treatment (EtOH precipitation) are much lower than the

established reference value for PCP in serum (12 μg/L) and we believe that this would allow the biomonitoring of trichlorophenols in the general population.

3.4. Performance of the 2,4,5- and 2,4,6-TCP ELISAs in human urine

As was mentioned in the **Introduction**, trichlorophenols are considered to have significant toxicological effects and potential carcinogenicity to humans. Chlorophenols are mainly excreted in urine as such or as sulfate or glucuronide conjugates, the amount of conjugation depending on the particular chlorophenol and its concentration in urine [64]. Urinary excretion of chlorophenols can be produced also as a consequence of the exposure to other chlorinated substances, such as phenoxyacid herbicides, hexachlorocyclohexanes and chlorobenzenes [74,122,314]. Similarly, there have been many attempts to correlate the trichlorophenol levels in urine with a potential exposure to dioxin [35,36,107-110].

3.4.1. Characterization of urine samples

Urine samples are very complex matrices that may interfere with immunoassays. Urine physicochemical properties from various individuals are quite different. The pH values usually vary from 5 to 7 and the ionic strength may range between 5 to 30 mS/cm. Individual differences in urine samples are related to particular habits and distinct physiological conditions. Creatinine content (an indicator of renal clearance) is usually employed for correction of urine dilution. This parameter may vary significantly from one to another urine sample (from 0.3 to 4 g/L, average value 1g/L). The variation in the creatinine concentration is caused by differences in the sweating rate, the fluid intake and the renal efficacy.

Taking into consideration this variability we decided to proceed with the evaluation of the trichlorophenol immunoassays performance using two types of representative urine samples. Sample A was collected over two days from a healthy male volunteer and then mixed to have a large and homogeneous urine sample. Sample B was a pooled human urine from different individuals purchased by Bio-Rad Laboratories, Irvine, USA. The most important characteristics (pH, conductivity and creatinine) of both samples are shown in **Table 3.8**.

Table.3.8. Properties of the urine samples used in this studies

Properties	**Sample A** [a]	**Sample B** [b]
pH	6.2	6.6
Conductivity, mS/cm	25	13.6
Creatinine, g/L	1.8	0.8

[a] Sample A is collected over two days from a healthy male volunteer. [b] Sample B is pooled human urine from different individuals. [c] Creatinine concentration was determined by the Jaffé method (see **Experimental Section**)

As it was mentioned in the **Introduction**, exposure to organochlorinated compounds is quite common within the general population through the contaminated environment or edible products. Thus, there was a risk of presence of chlorophenols in these urine samples, if we wanted to use them as "blank" samples for matrix effect studies. Therefore, on a first instance, both urine samples were characterized by GC-MS to establish the presence of the most important halophenolic compounds potentially interfering with the ELISAs. According to the specificity studies (see **Table 2.12**. and [277]) the most important specific interferents are 2,4,6-TCP (2,4,5-TCP respectively) 2,3,4,6-tetrachlorophenol (2,3,4,6-TtCP), 2,4,6-tribromophenol (2,4,6-TBP) and 2,4-dibromophenol (2,4-DBP). Thus, both urine samples were hydrolyzed to release potential chlorophenols from their corresponding glucuronides and sulfate conjugates. The hydrolysis was performed with concentrated sulfuric acid according to the procedure described in the **Experimental Section**. Next, samples from both urines, hydrolyzed and non-hydrolyzed were extracted with toluene and derivatized with *N, O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) prior to GC-MS analysis (see **Annex 1**). **Figure 3.8** shows the selected ion chromatograms of urine sample A after acid hydrolysis compared to the mixture of standards selected according to cross-reactivity ELISA

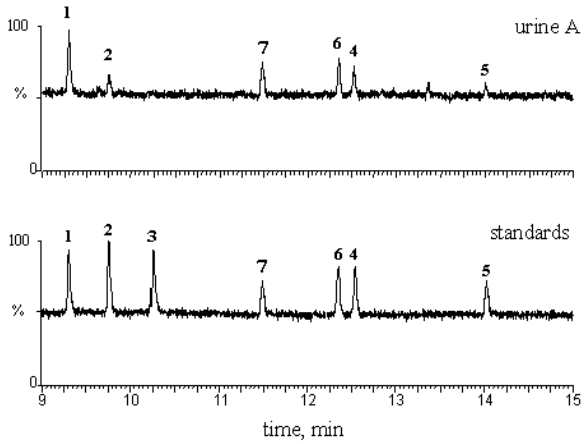


Figure 3.8. Gas chromatograms obtained for the standards and the hydrolyzed urine sample A using MS detection in the SIM mode following the ions of the corresponding halophenolic compounds interfering the assay. Peaks: **1** - 2,4,6-TCP (m/z 253, 255, 268), **2** - 2,4,5-TCP (m/z 253, 255, 268), **3**-2,4-DBP (m/z 307, 309, 324), **4** - 2,3,4,6-TtCP (m/z 287, 289, 304), **5** - 2,4,6-TBP (m/z 387, 389, 402), **6** - 2,3,5,6-TtCP (m/z 287, 289, 304), **7** – ISt (m/z 203, 259, 261). For details see **Annex I**.

studies. The characteristic ions for each chlorophenol were monitored in the corresponding window. In addition to the retention time, the ratio of the ions selected and the fragmentation pattern were used for identification. The qualitative analysis revealed the presence of halophenols in both urine samples. The appearance of chlorophenols is in agreement with the observation made by some authors [74,122] regarding the exposure of the general population through the contaminated environment or edible products. It was more surprising the presence of bromophenols, although some sources for this contamination could be the use of organobrominated compounds as flame retardant agents [315,316] and also peoples´ diet, since bromophenols have been identified in prawns, ocean fish, benthic organisms and other seafood [317,318].

Table 3.9.A shows the urinary concentrations of free and conjugated halophenols determined for sample A and B. In general, the levels found are lower than the mean concentrations reported for the general population: 0.61 μg/L 2,4,6-TCP [123] and 2 μg/L 2,4,6-TCP [121]. It should be noted that the 2,4,6-TCP concentration is higher in the hydrolyzed urine. The degree of conjugation is about 75% for urine A and 50% for urine B. These conjugation levels are in agreement with [64].

Table 3.9.A. Concentration levels (µg/L) of chlorophenols found in urinnes A and B by GC-MS.

Phenols	Urine A		Urine B	
	hydrolyzed	non-hydrolyzed	hydrolyzed	non-hydrolyzed
2,4,6-TCP	0.24	0.06	0.24	0.12
2,4,5-TCP	0.06	nd	nd	nd
2,4-DBP	nd [a]	0.1	nd	0.12
2,3,4,6-TtCP	0.2	nd	0.12	nd
2,4,6-TBP	0.06	nd	0.12	nd

The GC-MS results are calculated assuming that complete acid hydrolysis and extraction took place for all halophenols. The extraction ratio used was urine:toluene=100:1. [a] nd, not detected

In order to estimate the interference of these halophenols in our ELISAs we calculated their equivalents in terms of 2,4,6-TCP and 2,4,5-TCP immunoreactivity (IR) equivalents according to the cross-reactivity data obtained for both immunoassays. The results are shown in **Tables 3.9.B** and **3.9.C** for the 2,4,6- and 2,4,5-TCP ELISA, respectively.

Table 3.9.B. Urinary chlorophenol levels expressed as 2,4,6-TCP-IR equivalents

Phenols	Cross reactivity	Urine A		Urine B	
		hydrolyzed	non-hydrolyzed	hydrolyzed	non-hydrolyzed
2,4,6-TCP	**1**	0.24	0.06	0.24	0.12
2,4,5-TCP	**0.12**	0.0072	nd	nd	nd
2,4-DBP	**1.19**	nd	0.1	nd	0.12
2,3,4,6-TtCP	**0.206**	0.04	nd	0.025	nd
2,4,6-TBP	**7.1**	0.43	nd	0.852	nd
Total 2,4,6-TCP-IR equiv. [a]		**0.72**	**0.16**	**1.12**	**0.24**

[a] Total TCP-IR equivalents are calculated as the sum of the cross-reactivity of each compound multiplied by its concentration (µg/L). The LOD of 2,4,6-TCP ELISA is 0.175 µg/L

Table 3.9.C. Urinary chlorophenol levels expressed as 2,4,5-TCP-IR equivalents

Phenols	Cross reactivity	Urine A		Urine B	
		hydrolyzed	non-hydrolyzed	hydrolyzed	non-hydrolyzed
2,4,6-TCP	**0.17**	0.041	0.010	0.041	0.0204
2,4,5-TCP	**1**	0.06	nd	nd	nd
2,4-DBP	**0.10**	nd	0.011	nd	0.013
2,3,4,6-TtCP	**0.19**	0.038	nd	0.023	nd
2,4,6-TBP	**0.28**	0.017	nd	0.033	nd
Total 2,4,5-TCP-IR equiv. [a]		**0.155**	**0.021**	**0.097**	**0.033**

[a] Total TCP-IR equivalents are calculated as the sum of the cross-reactivity of each compound multiplied by its concentration (µg/L). The LOD of 2,4,5-TCP ELISA is 0.053 µg/L.

For the case of non-hydrolyzed urines, in both samples the amount of specific interferences expressed as equivalents of 2,4,6- or 2,4,5-TCP-IR is below the LOD of the ELISAs (0.18 and 0.05 μg/L, respectively). Only the 2,4,6-TCP-IR equivalents for sample B are slightly above this value. However, since the halophenol content of these samples is sufficiently low, we can consider both non-hydrolyzed urines as "blank samples", free of chlorophenols.

In contrast, for the hydrolyzed urine sample A the specific interferences were equal to 0.7 and 0.15 μg/L of 2,4,6- and 2,4,5-TCP-IR equivalents, respectively. For the hydrolyzed urine sample A the detected halophenols corresponds to 1.12 μg/L 2,4,6-TCP-IR equivalents and to 0.09 μg/L 2,4,5-TCP-IR equivalents. These concentration values are too high and they exceed the LOD of the respective ELISAs. Therefore, these levels had to be taken into account in the evaluation of the matrix effect and a criterion such as the method of the "standard additions" mentioned at the beginning of this chapter should be applied.

3.4.2. Evaluation of the matrix effect produced by non-hydrolyzed urine

Initially, we tested the effect of non-hydrolyzed urine sample B on the performance of 2,4,5- and 2,4,6-TCP ELISAs. Once the urine sample was defrosted, its pH was adjusted to the standard buffer conditions (pH=7.5) and the solid materials observed were removed by centrifugation. Subsequently the urine was serially diluted in PBS and the pH and the conductivity adjusted to maintain them close to the PBS buffer values to exclude possible matrix effects due to these physicochemical parameters. The PBS diluted urine solutions were used to prepare standard curves and run them in the ELISA to compare their parallelism to the buffer standard curve. The sigmoidal curves obtained are shown on **Figure 3.9.A**. The 2,4,5-TCP ELISA was very tolerant to the urine matrix of sample B. Only a small decrease in the absorbance was observed at the highest urine concentration

used (1/5 PBS diluted) but this small effect was completely eliminated after a 1/20 PBS dilution.

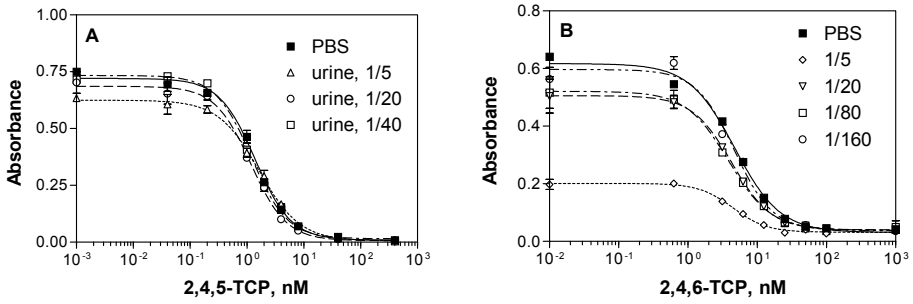


Figure 3.9. Interferences caused by non-hydrolyzed sample B on the ELISAs: **A**. 2,4,5-TCP and **B**. 2,4,6-TCP. Urine sample B was serially diluted with PBS buffer and the pH and conductivity were adjusted.

From practical point of view, we could considered that this urine sample could be analyzed with this assay just by diluting the matrix 5-times with PBS and employing this matrix as sample diluent. Under these conditions a LOD of 0.26 µg/L and an IC_{50} of 1.28 µg/L 2,4,5-TCP in urine could be defined. Considering the reference value set for this analyte in Germany (4.5 µg/L) based on the urinary levels of the general population [81], it can be predicted that the method could be useful for the determination of 2,4,5-TCP in the urine of occupationally non-exposed persons without any sample treatment.

To prove this fact, human urine sample B was spiked at several concentration levels. The spiked samples were diluted 5 times in PBS buffer and measured by the ELISA using a standard curve prepared PBS. To ensure appropriate quantification within the linear range of the standard curve further dilutions of the spiked urine samples were performed in a 1/5 PBS diluted blank urine to maintain constant the effect of the matrix. The recoveries observed are close to 100% and the %CV is between 7.2% and 12.7% (see **Table 3.10**). These results demonstrate the accuracy of this ELISA method to measure 2,4,5-TCP in urine samples.

Table 3.10. Accuracy of the 2,4,5-TCP ELISA in human urine.

Spiked [a], μg/L	Measured (mean ± SD) [b], μg/L	Recovery, %
0.75	0.815 ± 0.049	108.7
1.5	1.545 ± 0.129	103
4	4.0566 ± 0.556	101.4
10	9.64 ± 0.875	96.4

[a] spiked urine samples (sample B) were diluted 5 times with PBS. Higher dilutions (for higher spiked levels) were performed in urine 1/5. The concentrations were evaluated from a standard curve run in PBS. [b] data obtained from three determinations performed in the same ELISA plate.

Similarly to the serum matrix effect (see **Section 3.3**), the urine matrix interfered much more with the ELISA for the detection of 2,4,6-TCP. Although the pH and the conductivity were adjusted to place the urine within the working conditions of the immunoassay, a significant matrix effect was observed (see **Figure 3.9.B**). About 100 fold dilution of the urine sample B was needed to accomplish the same behavior of the immunoassay as in buffer. Since the 2,4,6-TCP-IR equivalents of sample B determined by GC-MS are below or close to the LOD of the immunoassay (see **Table 3.9B**), the non-hydrolyzed urine sample B could be considered as "blank" matrix. Therefore, the strong matrix effect observed was attributed to unidentified non-specific interferences. Therefore, the direct ELISA analysis of non-hydrolyzed urine samples followed by 100-fold buffer dilution results in a high LOD (about 17.5 μg/L 2,4,6-TCP). Under these conditions the assay would be suitable only for the determination of free 2,4,6-TCP in the urine of occupationally exposed persons of particular industrial sectors with a high risk of exposure. In **Table 3.11** are summarized the detection concnetrations of 2,4,5- and 2,4,6-TCP in non-hydrolyzed urine.

Table 3.11. Detectability features of the TCP ELISAs in non-hydrolyzed urine samples (μg/L).

Parameters	2,4,5-TCP [a]	2,4,6-TCP [b]
LOD	0.26 ± 0.024	17.5 ± 2.7
IC_{50}	1.28 ± 0.064	113.2 ± 36.1
Dynamic range	0.52 ± 0.048 - 5.3 ± 0.854	28.8 ± 4.5 to 311.7 ± 67.9

[a] The reported values are obtained by multiplying the parameters of the 1/5 diluted urine standard curve by 5. Urine is diluted in PBS. [b] The reported values are obtained multiplying the parameters of the PBS standard curve by 100.

As it was mentioned above, urine samples from different individuals can vary widely in composition. Thus, we have decided to test the performance of the 2,4,5- and 2,4,6-TCP immunoassays with another urine (sample A). Surprisingly, a significant matrix effect of the non-hydrolyzed urine sample A was observed on the 2,4,5-TCP ELISA requiring a 100 fold dilution of the sample to eliminate the interferences. In the case of 2,4,6-TCP ELISA the matrix effect observed was again higher than for the 2,4,5-TCP immunoassay (see **Table 3.12**).

Table 3.12. Summary of the matrix effect of non-hydrolyzed and hydrolyzed urine samples on the 2,4,5-TCP and 2,4,6-TCP ELISAs.

Treatment	Urine sample	ELISA	
		2,4,5-TCP	**2,4,6-TCP**
Non-hydrolyzed	**A**	1/100	> 1/100
	B (pooled)	1/5	1/100
Acid hydrolysis	**A**	n.d.	> 1/128
	B (pooled)	> 1/100	1/200
Enzymatic hydrolysis	**A**	n.d.	> 1/200
	B (pooled)	n.d.	> 1/200

Matrix effect is expressed as the dilution factor needed to eliminate the interferences.

The different behavior of sample A and B prompted us to check the effect of several non-hydrolyzed urine samples with different creatinine content taken from different individuals (see **Figure 3.10**). The pH and the conductivity of these urine samples were adjusted to the assay buffer values. The results shown in **Figure 3.10** clearly demonstrate that the effect of urine samples from different origins is not universal. As a general rule we have observed that there is a relation between the creatinine value and the intensity of the matrix effect produced. Urine samples with creatinine values higher than 2 g/L inhibited completely both assays. Different matrix effects on immunoassays by urines from different individuals have been also reported by other authors [177,179,319].

In conclusion, both ELISAs could be applied directly to the analysis of non-hydrolyzed urine samples when the LOD required for biomonitoring is around 18 ug/L for 2,4,6-TCP and 0.26 ug/L for 2,4,5-TCP (see **Table 3.11**). However, since one of our purposes was to establish the real level of exposure of the general population and to identify sources of exposure in certain occupational sectors, we needed much better limit of detection for

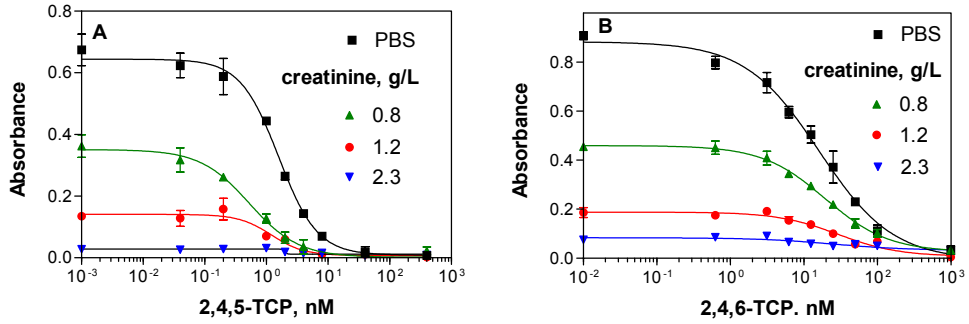


Figure 3.10. Matrix effect of non-hydrolyzed urine samples from different individuals on the ELISAs for 2,4,5-TCP (**A**) and 2,4,6-TCP (**B**). Standard curves corresponds to non-diluted urine samples with different creatinine content (0.8, 1.2 and 2.3 g/L).

2,4,6-TCP analyses. This limited detectability as well as the strong variation of the urine matrix effect with the sample origin (from different individuals) point for the necessity to introduce a sample treatment prior to ELISA analyses.

3.4.3. Evaluation of the matrix effect produced by hydrolyzed urine

Since trichlorophenols in urine are mainly conjugated [64], we decided to evaluate the immunoassay for hydrolyzed urine. Most of the methods reported involve acid hydrolisys using HCl [64,320] or H_2SO_4 [117,121,123]) and enzymatic hydrolysis [64,120]. Firstly, we have performed the deconjugation step under acidic conditions [117]. After acid hydrolysis by H_2SO_4 the pH of urines A and B were adjusted to 7.5 with NaOH and compared to the one run in the assay buffer. The hydrolyzed urines interfere with both ELISAs to seriously hinder their sensitivity and dynamic range. More than 100-fold dilution was needed to reach the buffer behavior (see ***acid hydrolysis*** in **Table 3.11)**.

The strong assay inhibition observed for hydrolyzed urine could be attributed to a possible ionic strength effect of the media, because acid hydrolysis followed by pH adjustment resulted in very high conductivity (300 mS/cm) while both immunoassays perform well until 30 mS/cm (see **Chapter 2**). We tried to eliminate the excess of sulfate

ions introduced in the hydrolysis step precipitating them either as $BaSO_4$ or $CaSO_4$. For this purpose, the hydrolyzed urine sample A was split in two parts, one of them was treated with $BaCl_2.2H_2O$ and the other one with $Ca(OH)_2$. The precipitated $BaSO_4$ and $CaSO_4$ were removed by centrifugation. The pH of the supernatant was adjusted with NaOH to 7.5 resulting in a conductivity of 137 mS/cm. After 10-fold dilution of the sample with water the buffer conductivity was reached. However, the matrix effect remained very similar to the urine sample prior to its treatment with $BaCl_2.2H_2O$ (data not shown). The immunoassay was totally inhibited in the case of $Ca(OH)_2$ – treatment.

It is expected that, in contrast to the acid hydrolysis, the enzymatic hydrolysis would yield a cleaner matrix (25 mS/cm, pH, less hydrolyzed interfering compounds) because it is performed under milder conditions and it is more specific. Based on this assumption we studied the matrix effect of both urine samples after enzymatic hydrolysis on the 2,4,6-TCP ELISA [64]. Surprisingly, the matrix effect was even higher: about 200-fold dilution was needed to eliminate the interferences (see ***enzymatic hydrolysis*** in **Table 3.11**). This effect could be attributed to the presence of the enzyme glucuronidase in the solution that could suppress the antibody – antigen binding. The high levels of cross-reactants found in the hydrolyzed urine (e.g. 0.72 μg/L 2,4,6-TCP-IR equivalents for sample A and 1.12 μg/L for sample B, see **Table 3.9.B** and **3.9.C**) could result in a small inhibition but does not explain the strong effect produced by the hydrolyzed urines on the 2,4,5- and 2,4,6-TCP ELISAs. Therefore, the matix effect observed was due to non-specific interferences.

At this point we considered the idea of using a "representative" urine sample as "assay buffer" and diluent with the hope that this method would allow the direct analysis of TCP in urine samples. To accomplish this aim it was necessary to change the immunoassay conditions on such a way that the assay would not be inhibited.

3.4.4. Development of 2,4,6-TCP ELISA in urine as assay media

We decided to use sample B as a standard urine matrix because it is a pooled sample collected from different individuals and was commercially available in high volumes. On a first step we performed an indirect non-competitive ELISA (2D experiment) directly in the urine with the pH and the conductivity adjusted and with the necessary percentage of Tween 20 (pH=7.5, 14 mS/cm, 0.05% Tween 20). The objective was to choose the appropriate concentration of the immunoreagents needed for the competitive assay. A signal of A_{max} at about one unit was given with 1/1000 dilution of the As43 and with 1.5 µg/mL of the coating antigen 8-BSA. These concentrations were higher than those used when PBS was the assay buffer (see **Table 3.1**). On a second step we performed competitive assays with the selected conditions using again urine sample B as an assay buffer. The parameters of the immunoassay are shown in **Table 3.13**. The detectability obtained was a little bit lower than that of the ELISA performed in PBS but inside the acceptable values. The rest of the parameters such as slope and background noise were also good.

Table 3.13. Features of the 2,4,6-TCP immunoassay performed in urine sample B

Condition	Values	Parameter	Values [a]
Preincubation time	0 min	**A_{max}**	1.05
Competition time	30 min	**A_{min}**	0.06
pH	7.5	**IC_{50}, µg/L**	2.48
Ionic strength	14 mS/cm	**Dynamic range**	0.8 - 10
Tween 20	0.05%	**Slope**	1.1
As43	1/1000	**LOD, µg/L**	0.3
8-BSA	1.5 µg/mL	**r^2**	0.997

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve run in two-well replicates.

The following step was to compare the 2,4,6-TCP ELISA performance in the standard urine (sample B) to its performance in other urine samples. Three urine samples from different individuals (see their properties in **Table 3.14**) were diluted 2-fold in the standard urine sample B (used as buffer) and their pH and conductivity were adjusted.

The physicochemical parameters of the urines prior and after this treatment are shown in **Table 3.14**.

Table 3.14. Properties of the urine samples studied.

Urine samples	Creatinine, g/L	Untreated urine		Treated urine [a]	
		pH	Conductivity, mS/cm	pH	Conductivity, mS/cm
St.urine B	**0.8**	**6.6**	**13.6**	**7.5**	**14.4**
1	0.3	8	5.4	7.6	16
2	1.2	5.2	26	7.3	26.7
3	2.8	5.8	19.2	7.5	17.9

[a] After 2-fold dilution in "standard urine B" and adjustment of the pH and the conductivity.

We selected urines with different creatinine values: low (0.3 g/L), medium (1.2 g/L, similar to the "standard urine B") and high (2.8 g/L). After the pH and conductivity adjustment all urines, except sample 2, had values close to those of the "standard urine B". Sample 2 differed only in the conductivity value that was much higher than the others, but still inside the working interval of the 2,4,6-TCP ELISA (see **Chapter 3**).

To assess the effect of these individual urine matrices on the 2,4,6-TCP ELISA we prepared again standard curves and compared them to the curve run in urine B. The standard curves obtained are presented in **Figure 3.11**. As it can be observed the "diluted" urine sample (0.3 g/L creatinine) gives an immunoassay with higher signal than in the standard urine matrix (0.8 g/L creatinine) and the samples with higher creatinine

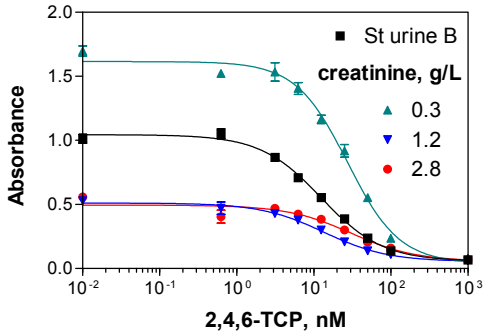


Figure 3.11. Matrix effect on the ELISA for 2,4,6-TCP of non-hydrolyzed urine samples from different individuals with different creatinine content using urine sample B as standard matrix.

content have less signal. The decrease in the signal produced by the urine sample 2, could be attributed to its higher conductivity value, since the creatinine concentration was similar to that of urine B, used as standard.

These studies demonstrate that, although the concentration of the immunoreagents had been adapted to the urine media, urine samples from different individuals still cause important variations in the immunoassay response. In conclusion, the approach of using a "standard" urine matrix could not be applied to the analysis of urine samples of different origin.

3.5. Summary

In this chapter we have evaluated the performance of 2,4,5- and 2,4,6-TCP ELISAs in environmental (water) and biological matrices (milk, serum and urine).

TCP analysis in drinking water by the ELISAs require only buffering of the samples. The recovery of spiked drinking water samples and the validation of the immunoassays by GC-ECD demonstrated excellent accuracy of the ELISA method. The assessed LOD of 0.07 μg/L 2,4,5-TCP and 0.18 μg/L 2,4,6-TCP in water allow the evaluation of drinking water contamination with chlorophenols according to the requirements of the EU on the maximum admissible levels.

Unfortunately, strong signal inhibition was observed for the 2,4,5- and 2,4,6-TCP ELISAs when applied directly to skimmed milk samples. Precipitation of milk proteins and calcium ions did not improve the immunoassay performance. Further studies on milk clean-up procedures, such as filtration, fat removal, or analyte extraction are needed.

The effect of non-treated human serum on the ELISAs consisted in a parallel shift of the serum standard curves toward higher concentrations with respect to the buffer standard curve. Serum interference on the IAs was eliminated by protein removal. Several

procedures were tested (ultra filtration; heat, acid, alkaline and organic solvent precipitation). The most effective serum treatment regarding matrix effect and analyte recovery is the precipitation with absolute ethanol (1:1, v/v) and further dilution of the serum with PBS: 8-fold in the case of 2,4,5-TCP ELISA and 32-fold for the 2,4,6-TCP ELISA. It should be noted that the immunoassay for 2,4,5-TCP tolerates better the serum matrix interferences. Therefore, the LOD in serum samples are 0.8 μg/L 2,4,5-TCP and 4.6 μg/L 2,4,6-TCP. Considering the reference value set for PCP in serum (12 μg/L) [20], we hope that these LOD values could be applied to the biomonitoring of the general population and to toxicological studies.

The urine samples mainly used in the matrix effect studies were initially characterized by GC-MS for the presence of cross-reacting chlorinated substances. Non-hydrolyzed urines could be considered as "blank" samples but hydrolyzed samples had TCP-IR levels higher than the LOD of the IAs. The observed matrix effect of the urine (signal inhibition) can be eliminated in a certain extent after corresponding dilution of the sample with PBS and pH adjustment. In this way, free (non-conjugated) TCPs can be determined in non-hydrolyzed urine with LOD of 0.26 μg/L 2,4,5-TCP and 17.5 μg/L 2,4,5-TCP. Although these level allow the urinary determination of 2,4,5-TCP in the general population, the higher LOD for 2,4,6-TCP limits the application of the 2,4,6-TCP ELISA only to the occupational exposure assessment in industrial sectors with high levels of contamination. Hydrolyzed (acid and enzymatically) urines produced strong matrix effect on the 2,4,5- and 2,4,6-TCP ELISAs that could not be attributed to the presence of cross-reactants in the samples. Furthermore, it was observed that urine samples from different origin have different matrix effect on the IAs related to the creatinine value of the sample. Finally, it was explored another alternative to overcome urine matrix effects, e.g. the 2,4,6-TCP ELISA was developed in a "standard pooled urine sample". However, our studies shows that urien samples from different individuals cause significant variations in the IA response. Therefore, the results obtained with urine samples up to now suggest the introduction of a sample clean-up step prior to ELISA measurements.

4. Development of SPE (C_{18} and IAC)-ELISA method for urinary detection of chlorophenols

Solid-phase extraction (SPE) is continuously growing in importance, and is currently a routine sample preparation technique employed in numerous environmental and bioanalytical applications, such as determination of trace amounts of pesticides and other organic contaminants in water, drugs and endogeneous compounds in biological samples [321,322]. SPE, introduced in the early 1970s, avoids and/or minimizes the disadvantages of the liquid-liquid extraction (LLE). Compared to LLE, SPE offers faster and easier manipulation, and higher concentration factors; it requires much smaller amounts of organic solvents. SPE techniques offer large choice of sorbents and the possibility of introducing new formats, such as the 96-well SPE plates and the microfibers for solid-

phase microextraction. Furthermore, solid-phase eluent can be selected to be compatible with the analytical system to be used.

The general SPE procedure consists in four steps (see **Figure 4.1**): conditioning (activation of the sorbent by passing through an appropriate solvent), retention (during sample application the analytes are retained by the sorbent), removal of interfering compounds (washing off interferences less attracted to the sorbent), and elution (desorption) of the anlyte(s) leaving interferences with greater attraction bound to the sorbent. The different mechanisms of retention and elution are due to intermolecular forces between the analyte, the active site on the surface of the adsorbent and the liquid phase or matrix. The hydrophobic, polar and ionogenic properties of both the solute and the sorbent play a crucial role for effective SPE. In general, non-selective SPE is based on

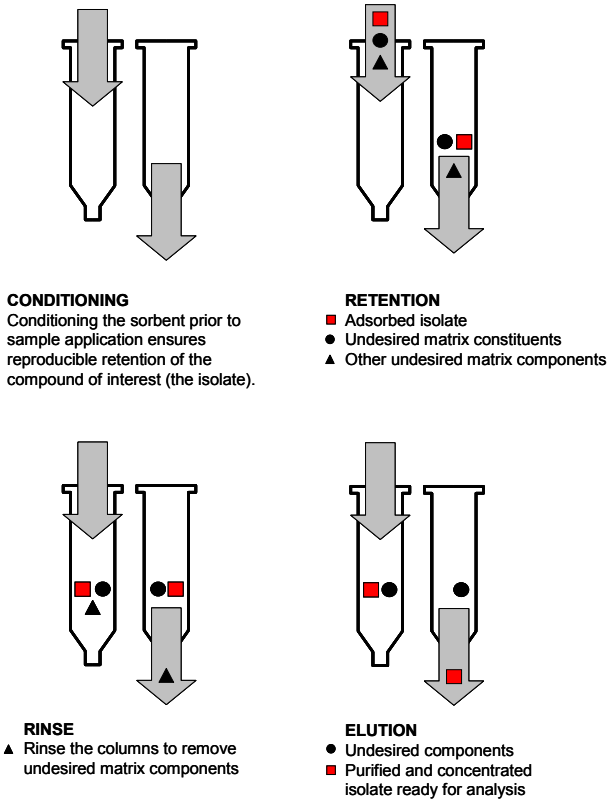


Figure 4.1. General principle of a SPE procedure

Van der Wals hydrophobic (reversed-phase bonded silica sorbents, graphitized carbon black, polymeric sorbents) or electrostatic interactions (ion exchange). Sometimes it is possible to separate the analytes into two or more groups by judicious choice of the solid phase and the eluting conditions and/or a better sample conditioning such as pH or ionic strength [323,324]. However, analyte retention is based on hydrophobic interactions and their selectivity is often too low for trace analysis in many complex environmental, food and biological samples. Co-extraction of analytes and matrix interferences generally occurs, and this can even become a major problem when analytes of interest are at trace level and interferences at high concentrations. Additional clean-up procedures are required, but, then the sample pre-treatment involves several steps and consequently the risk of loss or contamination increases and the reliability of the results is reduced. Thus, the goal is to achieve extraction and clean up in a single step. That can be achieved by using highly selective SPE sorbents based upon molecular recognition, such as immunosorbents (immunoaffinity extraction sorbents) [201-203,325,326] and molecularly imprinted polymers (MIP) [327-329]. The first approach involves antigen-antibody interactions. Antibodies are covalently bonded onto an appropriate sorbent to form the so-called immunosorbent (IS). Single analytes can be targeted but thanks to the cross reactivity of the antibodies, immunoextraction sorbents have been also designed to target group of structurally related analytes. Due to antibody specificity, the problem of the co-extraction of matrix interferences is circumvented. The high selectivity provided by immunoextraction has led to attempt to synthesize antibody mimics in recent years, such as MIPs. This second approach involves the preparation of polymers with specific recognition sites for certain molecules. Like immunosorbents, the recognition is due to shape and a complex of hydrogen, hydrophobic and electrostatic interactions. However, they have the advantages to be prepared more rapidly and easily, using well-defined methods, and to be stable at high temperature, in a large pH range and in organic solvents. At the moment the application of MIP-based SPE to biosamples is still limited and further research focusing on the analytical performance is warranted [329].

In the previous chapter we demonstrated that the direct analysis of 2,4,5- and 2,4,6-TCP in body fluids with the corresponding ELISAs was troublesome at the desired limits of

detection (at about 1 μg/L in urine). The observed significant interferences prevented the analysis unless the matrix was diluted appropriately with the assay buffer diminishing in this way the method detectability. On the other hand, it was found that the undesirable urine matrix effect depends on each individual. Therefore, the need to develop a clean-up procedure to improve the detectability of the immunoassays and to avoid individual variability was considered crucial to provide a robust and reliable analytical method for biomonitoring studies.

In this chapter we present the evaluation and the development of clean-up procedures for the urine samples prior to ELISA detection. According to our aim the clean-up step should fulfill the following requirements: i) to extract efficiently the target analyte from the urine matrix; ii) to rend extracts compatible with the ELISA method; iii) to be easily adaptable to the 96-well format of the ELISA microplates in order to avoid the loss of analysis efficiency; iv) to ensure the desired limits of detection. These studies have been carried out with the 2,4,6-TCP ELISA, although we believe that the results obtained could be exploited to other immunoassays aimed to be used for biomonitoring purposes.

4.1. Evaluation of a C_{18} reversed-phase SPE as urinary clean-up method prior to ELISA analysis of 2,4,6-TCP

Most of the nonselective SPE methods developed for organic pollutants, such as phenols and acidic herbicides, from aqueous samples utilizes reversed-phase (bonded silica, polymeric resins, graphitized carbons) and ion-exchange sorbents (for recent reviews see [148,330]. SPE with ion exchange particles has been used to extract ionic analytes or analytes that can be converted to ionic form by adjusting the sample pH [330]. As phenols are acidic compounds, sorbents with quaternary ammonium sites work as anion exchangers and are used to retain phenols in water samples adjusted to basic pH. Thus, an anion exchanger with quaternary ammonium groups was applied to extract 2,4,6-TCP

from non-hydrolyzed urine in order to diminish its interference in the ELISA [1]. Urine sample was loaded to the cartridge at pH 8 where the TCP is at its anionic form. An acidic solution (citrate buffer, pH= 3.5) was used to elute phenols from the anion-exchange column converting them back to their molecular form. However, this urine clean-up was not sufficient to eliminate completely the matrix effect on the ELISA and at least 10 fold urine dilution was needed to reach buffer behavior.

Reversed-phase SPE is used to isolate relatively nonpolar analytes from a polar sample (water, urine). Octadecylsilane silica (ODS or C_{18}) is by far the most widely used non-polar reversed-phase sorbent. It has been successfully applied for trace enrichment of chlorophenols from non-hydrolyzed and hydrolyzed human urine [120,121]. Octylsilane (C_8), cyclohexyl, phenyl and cyano are other silica-based sorbents that have also been used for SPE of phenols in water samples [148]. With the aim to eliminate efficiently urine matrix components interfering with the 2,4,6-TCP ELISA we approached the development of reversed-phase (C_{18}) SPE procedure. C_{18}-SPE methods have been successfully applied for isolating small organic molecules from human urine prior to ELISA measurements [176-178,313]. Most of these protocols use methanol as eluting solvent, because it is usually tolerated by the immunoassays until a certain percentage. Thus, we evaluated the effect of the methanol on the 2,4,6-TCP ELISA (see **Figure 4.2**).

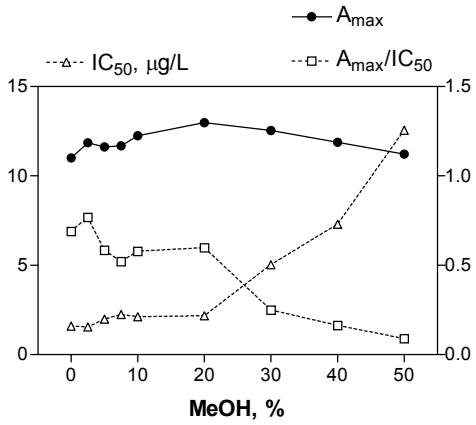


Figure 4.2. Effect of methanol on the ELISA for 2,4,6-TCP. Different concentrations of MeOH (0-50%) in the assay buffer PBS were used to prepare standard curve and As solutions. The data presented are extracted from the four-parameter equation used to fit the standard curves.

Increasing MeOH concentration in the assay buffer did not affect the signal A_{max}, but results in an increase of the IC_{50} values. Nevertheless, the immunoassay features were not seriously affected until 20% MeOH concentration.

In many SPE procedures of organic acids the acetic acid (AcOH) is used at low concentrations (0.1%) in the eluting buffer to improve their interactions with the hydrophobic solid-phase. Thus, it was also evaluated if this organic modifier could interfere in the ELISA. The results demonstrated that the presence of very low concentration of AcOH (0.01%) in the assay buffer in fact increases the detectability of the 2,4,6-TCP ELISA [1]. We have also proved that the presence of both organic solvents (MeOH and AcOH) in the PBS buffer does not affect significantly the immunoassay parameters. For example, an assay buffer containing 8.5% MeOH and 0.01% AcOH was completely tolerated by the 2,4,6-TCP ELISA without significant modification in the immunoassay features (A_{max}= 0.55, IC_{50}= 1.25 μg/L, LOD= 0.15 μg/L, dynamic range 0.3 – 4 μg/L, slope= -1.1, r^2= 0.996).

After these preliminary experiments we approached the evaluation of the extraction efficiency of the C_{18}-SPE of 2,4,6-TCP from urine samples. We also investigated the efficiency of this procedure as a clean-up step necessary to eliminate ELISA matrix interferences.

4.1.1. Recovery studies

For our studies we have selected commercial Sep-Pak®Plus Cartridge (Waters S.A.) with C_{18} as sorbent. Preliminary experiments performed by R. Galve in our laboratory demonstrated that it was necessary to introduce a washing step with a high content of MeOH to eliminate an important fraction of the ELISA interferences and that the retained 2,4,6-TCP starts eluting at about 60% MeOH in the mobile phase [1]. However, this fact was only proved evaluating the effect of the matrix on the ELISA and performing very

few recovery studies. In addition, the possible presence of specific interferences in the urine sample used was not considered.

Therefore, we focused our studies on the determination of the recovery of the purification process by two methods: ELISA and GC-ECD. Initially, we evaluated the C_{18}-SPE procedure using PBS standard of 40 μg/L 2,4,6-TCP. To ensure a complete elimination of the urine interferences we used a procedure that consisted in the following steps: conditioning of the C_{18} cartridge with MeOH followed by 0.1% aqueous AcOH; loading of 10 mL sample; washing with 50% MeOH/H_2O, and finally elution of the retained 2,4,6-TCP with 65% MeOH/0.1% AcOH/H_2O solution. Each eluted fraction (1 mL) was measured by ELISA after a 10-fold dilution with PBS buffer and was quantified using a standard curve run in 6.5% MeOH/0.01% AcOH/PBS buffer. As can be seen from the results presented in **Table 4.1.A** the 2,4,6-TCP eluted in the first three fractions (F1, F2 and F3) corresponding to a recovery of 103%. No 2,4,6-TCP was detected in the washing fractions demonstrating that all the analyte has been retained during loading.

Table 4.1.A. Recovery of C_{18}-SPE of 2,4,6-TCP from PBS.

Elution	**65% MeOH**	
Sample loaded	**PBS**	
Fraction	**ELISA**	**GC-ECD**
F1	23.3	23.5
F2	15.2	11.8
F3	2.7	3.2
F4	1.7	n.d.
F5	0.5	n.d.
Total, μg/L	41.2	38.5
Recovery, %	103	96.3

10 mL PBS standard of 40 μg/L 2,4,6-TCP is loaded. Elution is performed with 65% MeOH/0.1% AcOH/water solution. Each fraction is of 1 mL. The 2,4,6-TCP concentration (μg/L) in the eluted fractions is determined by ELISA and GC-ECD. The recovery is based on the concentration in the shadowed fractions and is calculated *vs* the spiked amount.

The eluted fractions were also measured by GC-ECD after a toluene extraction and sylilation of 2,4,6-TCP (see **Experimental Section**). A good agreement between ELISA and GC-ECD values was obtained (see **Table 4.1.A**). The cartridge was effectively regenerated by washing with absolute MeOH (the control blanks were free of analyte).

With these results we moved towards the application of the same protocol to urine samples.

As was mentioned in the previous chapter, urine may contain a variety of unknown components that could be retained by the C_{18} reversed-phase, because this SPE is not specific. It was important to evaluate the possible co-elution of interferences when the C_{18}-clean-up was applied to real urine samples. We decided to use urine A as a model urine matrix in C_{18}-SPE studies because its content in total 2,4,6-TCP-IR equivalents was lower than in sample B (see **Table 3.9B**), and its behavior could be considered closer to a "blank matrix" regarding specific interferences. Thus, we applied the above clean-up procedure to a non-spiked and to a spiked (40 μg/L 2,4,6-TCP) non-hydrolyzed sample. The concentration of each eluted fraction after 10-fold dilution was determined by the 2,4,6-TCP ELISA run in 6.5% MeOH/0.1% AcOH/PBS in PBS. **Figure 4.3A** shows the recovery values calculated *vs* the spiked amount. The non-spiked urine produced a positive response in the ELISA that could be attributed to specific and/or to non-specific interferences. However, the ELISA responses in spiked urine sample were not equivalent after multiple dilutions, suggesting the presence of non-specific interferences. In addition, the total amount of 2,4,6-TCP-IR equivalents extracted in the 5 mL (F1+F2+F3+F4+F5) from non-spiked urine corresponds to 18.1 μg, which is much more than the expected amount present in 10 mL non-hydrolyzed urine sample A (1.6 μg, see **Table 3.9B**). Therefore, the clean-up performed had not been sufficient to remove the urinary interferences in spite of having used a washing step with a 50% MeOH.

Taking into consideration the elution of non-specific interferences, we evaluated the real recovery of 2,4,6-TCP as a difference between the response of the spiked and non-spiked urine (see **Table 4.1.B**). The recovery of 111% corresponding to the first three fractions (3 mL) eluted with 65%MeOH was similar to the recovery obtained loading PBS standards (103%). In spite of the co-elution of non-specific interferences, we expected to be able to measure urine samples by applying a higher dilution factor to the eluted fractions. When 3 fractions are collected the final dilution of the urine is 3 times (10 mL loaded, 3 mL collected, 30 mL used for ELISA determination).

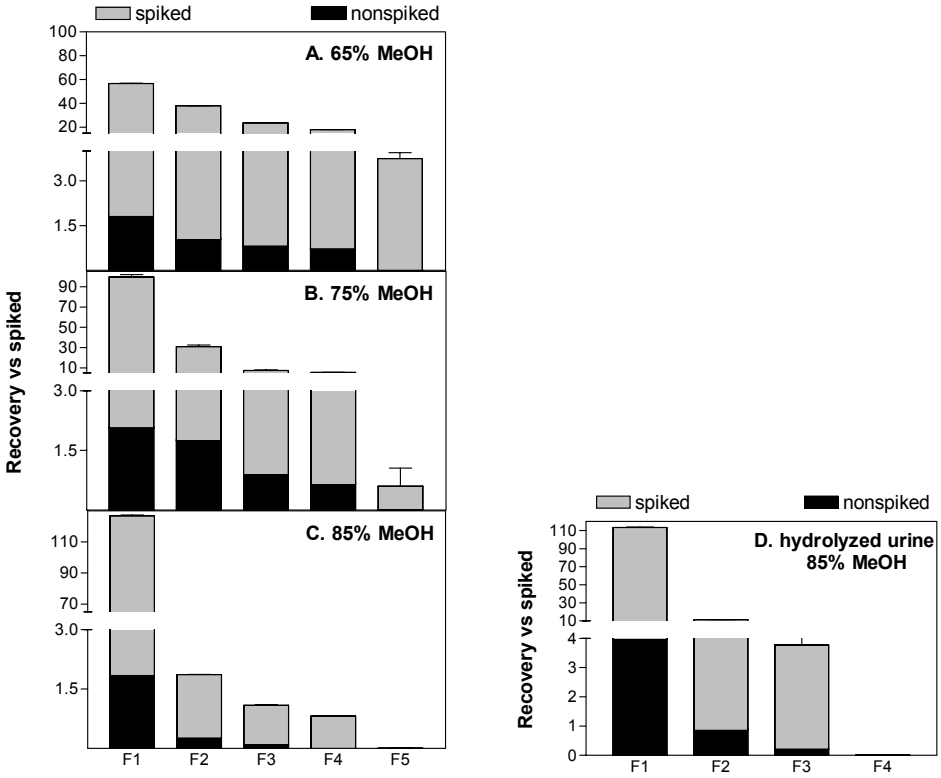


Figure 4.3. Elution profile of C_{18}-SPE clean-up of urine sample A (spike and non-spiked) with different MeOH concentration in the elution solvent. Non-hydrolyzed urine: (**A**) 65% MeOH, (**B**) 75% MeOH, (**C**) 85% MeOH; Hydrolyzed urine: (**D**) 85% MeOH.The concentration in each eluted fraction was determined by the 2,4,6-TCP ELISA and the recovery *vs* spiked amount was calculated. A washing step using 50%EtOH was applied before eluting the

Table 4.1.B. Recovery of 2,4,6-TCP in the C_{18}-SPE urinary purification.

Elution	**65% MeOH**	**75% MeOH**	**85% MeOH**		
Sample loaded	**NH [a] urine**	**NH [a] urine**	**NH [a] urine**	**AH [b] urine**	
Fraction	**ELISA**	**ELISA**	**ELISA**	**ELISA**	**GC-ECD**
F1	21.2	38.2	49.3	42.2	33
F2	14.4	11.0	0.5	3.8	n.d.
F3	8.8	2.2	0.3	1.3	n.d.
F4	6.6	1.7	0.3	n.d.	n.d.
F5	1.5	0.2	< 0.15	n.d.	n.d.
Total, μg/L	44.4	49.2	49.3	42.2	33
Recovery, %	111	123	123.3	105.5	82.5

Urine sample A (non-spiked and spiked with 40 μg/L 2,4,6-TCP) was loaded. Elution is performed with different concentrations of MeOH/0.1% AcOH/water solution. Each collected fraction is of 1 mL. The concentration of 2,4,6-TCP (μg/L) in the eluted fractions is determined by ELISA and GC-ECD and corresponds to the difference between spiked and non-spiked sample. The recovery is based on the concentration in the shadowed fractions and is calculated *vs* the spiked amount.
[a] NH urine is non-hydrolyzed urine; [b] AH urine is acid hydrolyzed urine.

A reduction of the elution volume could allow increasing of the dilution factor applied to eliminate non-desired matrix effect before ELISA measurements. Moreover, it could be possible that varying the elution conditions the non-specific interferences would elute differently. All these considerations encouraged us to optimize the elution conditions directly with the urine sample. Thus, non-spiked and spiked (40 μg/L 2,4,6-TCP) urine samples were loaded to the C_{18} cartridge and eluted with different MeOH concentration (65%, 75%, 85%) in the elution buffer. The concentration of 2,4,6-TCP in the eluted fractions was measured by ELISA standard curves run in 6.5% MeOH/0.01% AcOH/PBS, 7.5% MeOH/0.01% AcOH/PBS and 8.5% MeOH/0.01% AcOH/PBS buffer, respectively (see **Table 4.1.B**). The overestimation in the recovery (110-123%) will be explained in **Section 4.1.2**. by the matrix effect of the urine extract to the immunoassay (see **Figure 4.4**).

The effect of the MeOH concentration in the elution buffer on the elution profile of 2,4,6-TCP for the non-hydrolyzed urine is presented in **Figure 4.3 A, B, C**. Increasing MeOH concentration resulted in a reduction of the volume needed for a complete 2,4,6-TCP elution. More than 90% of the spiked amount eluted in 3 mL using 65% MeOH and in 1 mL when 85% MeOH was employed. The same was valid for the elution of the interferences from non-spiked urine sample. It should be noted that the contribution of the non-specific adsorption (calculated as the ratio of the ELISA response for non-spiked *vs* spiked urine) in each fraction increases with the number of the eluted fraction (see **Table 4.2**).

Table 4.2. Immunoreactivity (expressed in %) measured in the ELISA due to non-specific interferences present in the urine extracts after C_{18}-SPE.

Urine	MeOH %	F1	F2	F3	F4
Non-hydrolyzed	**65%**	3.37	2.91	3.71	4.35
	75%	2.15	6.06	14.23	13.99
	85%	1.5	19.05	12.8	nd
Hydrolyzed	**85%**	3.65	8.57	7.09	nd

The percentage is calculated for each fraction according to the following formula: [2,4,6-TCP-IR equiv (non-spiked urine)/ 2,4,6-TCP-IR equiv (spiked urine)]*100%.

For example, using 75% MeOH elution of non-hydrolyzed urine the percentage of the non-specific interferences to the total immunoreactivity measured for spiked sample was 2.15% for fraction F1 and 13.99% for F4. That means that increasing the elution volume collected (lower MeOH elution concentration) would result in an overestimation due to higher non-specific adsorption. If we consider the volume needed to extract the total spiked amount of 2,4,6-TCP (3 mL at 65% MeOH, 2 mL at 75% MeOH and 1 mL at 85%MeOH), it can be observed that at 85% MeOH elution the contribution of the nonspecific adsorption is lowest, and it is only 1.5% (compare shadow boxes in **Table 4.2**). Thus, we chose 85% MeOH elution for further studies.

Thus, the C_{18} SPE procedure using 85% MeOH for analyte elution was applied as a clean-up of acid hydrolyzed (spiked and non-spiked) urine sample A. The recovery *vs* the spiked amount of 2,4,6-TCP for each fraction is presented in **Figure 4.3.D.** Similarly to the non-hydrolyzed urine (see **Figure 4.3.C**), 2,4,6-TCP eluted mainly in the first fraction together with most of the non-specific interferents (non-spiked urine). The presence of non-specific interferents can be confirmed by the fact that the amount of 2,4,6-TCP-IR equivalents detected in non-spiked acid hydrolyzed urine was twice (20.8 μg) the amount of the cross-reactants evaluated by GC-MS in the same sample (7.2 μg). Furthermore, it should be noted that the contribution of the non-specific adsorption in the eluted fraction F1 for the acid hydrolyzed urine (3.65%) was higher than those for the non-hydrolyzed urine (1.5%) (see **Table 4.2**). This was expected, since the hydrolyzed sample is a less clean matrix than the non-hydrolyzed one. Finally, if the 2,4,6-TCP concentration measured in spiked acid hydrolyzed urine is corrected with the IR measured in non-spiked urine, good recovery can be observed (see **Table 4.1.B**).

4.1.2. Matrix effect of C_{18}-SPE purified urine on the 2,4,6-TCP ELISA

In order to evaluate the efficiency of the C_{18}-SPE clean-up method we tested the matrix effect of the urine extract (85% MeOH elution) on the immunoassay. According to the established procedure (10 mL urine loaded, 1 mL extract collected and diluted 10 times

with PBS) the C_{18}-SPE does not pre-concentrate the sample. Thus, non-hydrolyzed and acid hydrolyzed non-spiked samples (urine A) were loaded on the C_{18}- cartridge and the eluted fractions (F1, 1 mL) were diluted with PBS to the initial sample volume (10 mL). Further dilutions were performed with 8.5% MeOH/0.01% AcOH/PBS in order to maintain constant solvent concentration. Standard ELISA curves were run in the diluted extracts and compared to the curve run in 8.5% MeOH/0.01% AcOH/PBS. **Figure 4.4.** shows that an important improvement had been achieved by introducing this clean-up method, because now urine analysis can be accomplished just by a 25-fold dilution with PBS. Previously, we demonstrated that both non-hydrolyzed and hydrolyzed urine samples had to be diluted 100 and 128 times, respectively (see **Table 3.11**). Therefore, after a C_{18} clean-up step the LOD accomplished for the analysis of 2,4,6-TCP in both hydrolyzed and non-hydrolyzed urine is of 3.75 µg/L 2,4,6-TCP ($LOD_{MeOH/AcOH/PBS}$ x 25 = 0.15 x 25= 3.75 µg/L*)*. This means that the SPE clean up allows 5-6 times lower detection limit than the direct analysis of 2,4,6-TCP in urine without any purification.

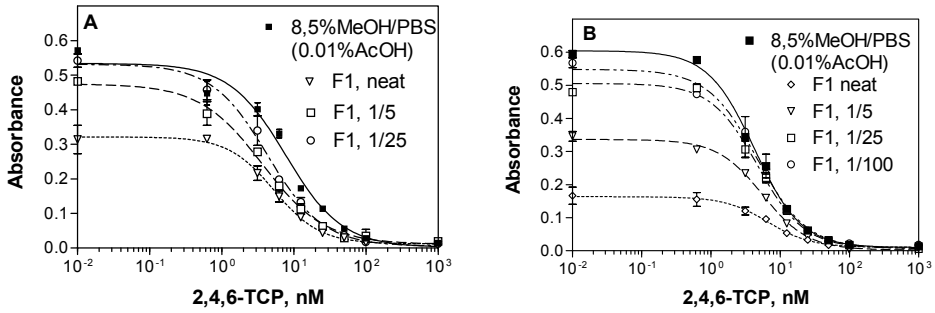


Figure 4.4. Matrix effect of urine sample A after C_{18}-SPE clean-up on the 2,4,6-TCP ELISA: (**A**) non-hydrolyzed, (**B**) acid hydrolyzed. 10 mL urine was loaded to the C_{18}-cartridge, washed by 50% MeOH, and eluted by 85% MeOH. The eluted fraction F1 was reconstituted to the original volume with PBS, so its final MeOH concentration was 8.5%. Further dilutions (more than 5 times) were performed with 8.5% MeOH/0.01% AcOH/PBS in order to maintain constant the organic solvent content.

We can assume that the matrix effect observed for non-hydrolyzed urine is due to the presence of non-specific interferences in the eluted extract (the 2,4,6-TCP IR equiv. in non-hydrolyzed urine sample A (0.16 µg/L) are close to the $LOD_{MeOH/AcOH/PBS}$ of the ELISA = 0.15 µg/L) (see **Section 3.4.1**). However, the slightly higher matrix effect

produced by the hydrolyzed urine (see **Figure 4.4.B**) could be attributed not only to non-specific interferences, but also to the specific interferences present in this sample. As mentioned, these substances would produce an immunoreactivity equivalent of 0.72 µg/L 2,4,6-TCP that is above the LOD of the immunoassay.

Finally, the efficiency of the SPE procedure can be observed in **Figure 4.5** that presents the GC-ECD chromatograms obtained for non-purified urine and for urine purified by

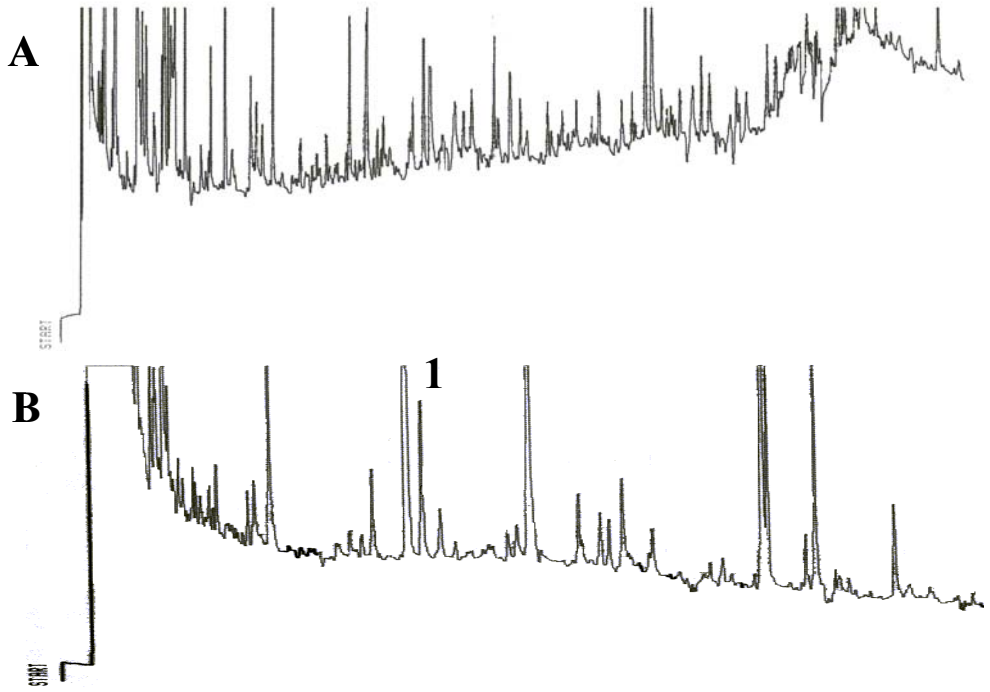


Figure 4.5. GC-ECD chromatograms obtained for: **A**. Non-purified urine: 100 mL of acid hydrolyzed urine A were extracted with 1 mL of toluene. The obtained extract was 10-fold diluted with toluene; **B**. after C_{18}-SPE: 10 mL of acid hydrolyzed urine A spiked with 1 µg/L 2,4,6-TCP were used. The collected elution fraction (85% MeOH) was diluted 10-fold with PBS and extracted with 1 mL of toluene. The concentration of 2,4,6-TCP in the toluene extract corresponds to 10 µg/L; In both cases the toluene extracts were derivatized with BSTFA and the overall sample concentration is 10 times (urine: toluene=10:1). Peaks: (1) 2,4,6-TCP.

C_{18}-SPE. Although, an important part of the matrix components are eliminated by the clean up step, there is still a presence of non-specific interferences in the C_{18}-extract.

In conclusion, the matrix effect studies showed that the introduction of a C_{18}-SPE procedure is an effective way to eliminate an important part of the interferences produced on the ELISA by the urine sample A. Good recovery and accurate quantification could be achieved after appropriate (at least 25 fold) dilution of the urine extract prior to ELISA determination of 2,4,6-TCP. The procedure (C_{18}-SPE-ELISA) described in this chapter would allow the determination of free chlorophenols in non-hydrolyzed urine and total (free and conjugated) chlorophenols in hydrolyzed urine samples with a LOD at about 4 μg/L. This LOD is low enough for occupational exposure assessment, if we consider that the general population can have background levels of 2,4,6-TCP up to this value (reference value set for Germany is 4.5 μg/L [81]). However, it would be important to find more effective urine treatment procedure to allow trichlorophenol quantification at lower levels in order to perform risk assessment of the non-occupationally exposed population and to confirm the reference values set by Wrbitzky *et al* [81]. One possible approach to improve the LOD of the method could be to reduce even more the matrix effect still observed in the C_{18} purified urine. The idea to extract selectively the analyte from the urine making use of the antibodies already obtained was a challenge. As mentioned in the **Introduction** selective SPE methods have proved to be useful tool for urine sample preparation prior to chromatographic analysis [214,331-336]. In our group P.Bou had successfully applied immunosorbents to purify and pre-concentrate the antifouling agent Irgarol 1051 from sea water samples prior to ELISA analysis [206]. In spite of the higher complexity of the urine we focused on this sample treatment strategy as a good approach to accomplish our purposes.

4.2. Evaluation of an IAC-SPE procedure as urinary clean-up method prior to ELISA analysis of 2,4,6-TCP

The first step in the development of an IAC-SPE method is the preparation of the immunosorbent. It is prepared by covalently immobilizing the Ab on the surface of a rigid or semirigid ***support***. The selected support should be chemically and biologically inert, easily activated, mechanically stable, uniform in particle size, and its pore size should be large enough to allow access to the Ab and the Ag [337]. It should be hydrophilic in order to avoid any non-specific interactions, and be pressure resistant for direct use in on-line techniques. Two types of affinity supports have been shown to be appropriate for making ISs (for review see [200-202,207]. The first one includes traditional supports used in IAC, such as carbohydrate-related materials (i.e. agarose and cellulose) and synthetic organic supports (e.g., acrylamide polymers, copolymers or derivatives, polymethacrylate derivatives and polyethersulfone matrixes). Many of these supports are commercially available under various trademarks. As they cannot be operated at high flow-rates because of their limited stability (high pressure generates compacting and fouling), they are called low-performance supports. The second category of appropriate sorbents is pressure resistant, so-called high-performance supports. They include derivatized silica, glass beads, and hydrophilic organic polymers (vinyl and polystyrene based). These materials are attractive for use with standard HPLC equipments.

Appropriate ***coupling chemistry*** to attach the Ab is essential for any affinity separation. Ideally the immobilization conditions should keep the biospecific activity of Abs. The activation and coupling chemistry procedures determine the efficiency of the immobilization procedure. A variety of supports are commercially available from different suppliers (Pharmacia, Biorad, Pierce, etc.). Usually, activation of the support occurs through its functional groups (i.e. hydroxyl groups: agarose, silica; carboxy groups: alkaline hydrolysis of polyacrylamide, etc.), which are appropriately derivatized to introduce a spacer arm with the desired chemistry. The existing coupling methods can be mainly grouped in two categories: random and oriented Ab immobilization. The

easiest and most frequently used is the first route, random immobilization. In this case the covalent attachment of the Abs is realized by reacting of their free amino groups (lysine residues are distributed throughout the Ab molecule) with supports that are activated with agents such as carbonyldiimidazole, cyanogens bromide or N-hydroxysuccinimide (NHS), or with supports that have been treated to produce reactive epoxide or aldehyde groups on their surface. Oriented immobilization procedures that keep free the F_{ab} sites for antigen recognition, use several strategies, such as binding of Abs via their carbohydrate moieties located on the F_c region (heavy chain) to amine or hydrazide containing supports, covalent coupling with iodoacetyl- or maleidimide-activated supports through the thiol groups of antibody fragments, or non-covalent binding of the F_c region of the antibodies to streptavidine or protein A- or G-based sorbents.

The solid-phase extraction properties of the immunosorbents are bonding density, capacity, recovery, breakthrough volume, elution conditions, IS regeneration, flow-rate, and non-specific interactions. The ***bonding density*** is defined by the number of antibodies linked to the surface of the sorbent and it determines the antigen-binding capacity. It depends on the specific surface area of the sorbent accessible for the immobilization of the Abs. The reasonable minimal pore size is in the order of 2-5 times antibody diameter for immunoextraction of small molecules. The second factor defining binding density is the degree of purification of the Ab solution. Non-purified antisera is used in many cases, but reduced bonding density can be expected due to co-immobilization of other proteins. Better Ab density will occur using the purified IgG fraction obtained by ammonium sulfate precipitation or using protein A (G) affinity column. Although it has been suggested that the use of hapten specific affinity chromatography isolated fraction of the polyclonal Abs increases IS capacity, it is not often used, because exists the risk of an incomplete recovery of the highest affinity fraction of the antisera. Using monoclonal Abs, these drawbacks are circumvented. Furthermore, Ab fragments (F_{ab} or F_v) can also be used. The theoretical coupling capacity of the support is usually provided by the commercial supplier, but the amount of immobilized Ab should be nevertheless measured. The determination of the amount of Abs immobilized onto a matrix consists in

measuring the Ab concentration of the bonding solution by spectrophotometry UV at 280 nm.

The ***capacity*** of the IAC column indicates how much analyte can be retained by the IS. It strongly depends on the support, the Ab immobilization procedure employed, the total number of immobilized active Abs (specific for the target analyte) (the availability of the Ab binding sites). Moreover, the random orientation and steric hindrances may prevent the Ag from access to Ab binding sites. But, for a standard immobilization procedure , the theoretical binding capacity can be estimated. If, for example in the case of polyclonal Abs, with the hypothesis that (i) 10% of a purified IgG fraction is considered specific, (ii) the Ab activity is fully retained and (iii) active site accessibility is complete, then taking into account the coupling density, the molecular weight of the Ab (150 kDa) and two binding sites per Ab, it is possible to estimate the theoretical capacity of the IS. Usually the binding capacity is determined experimentally by loading the column with varying amount of the antigen. In general, the experimental capacity is lower than the theoretical one [201]. On the other hand, due to Ab cross-reactivity, structurally related analytes present in a sample will compete for the same Ab binding sites, modifying the behavior of the IS. In this case the real IS capacity should be evaluated using a mixture of these structural analogs [338].

The immunoextraction procedure consists of four steps: sample loading, washing, elution, and regeneration. (see **Figure 4.6**). During the ***loading*** step, the binding reaction between the immobilized Abs and the analyte from the sample takes place. For effective Ab-Ag recognition (interaction) it is recommended the applied sample to have properties close to the physiological conditions (i.e. neutral pH application buffer with low-to-moderate ionic strength). The ***flow-rates*** govern the speed of the Ab-Ag reaction. The binding reaction is less efficient with faster flow-rates. Flow rates between 0.4 and 4.0 mL/min are common. In general, it is best to determine the optimum flow-rate for each separation.

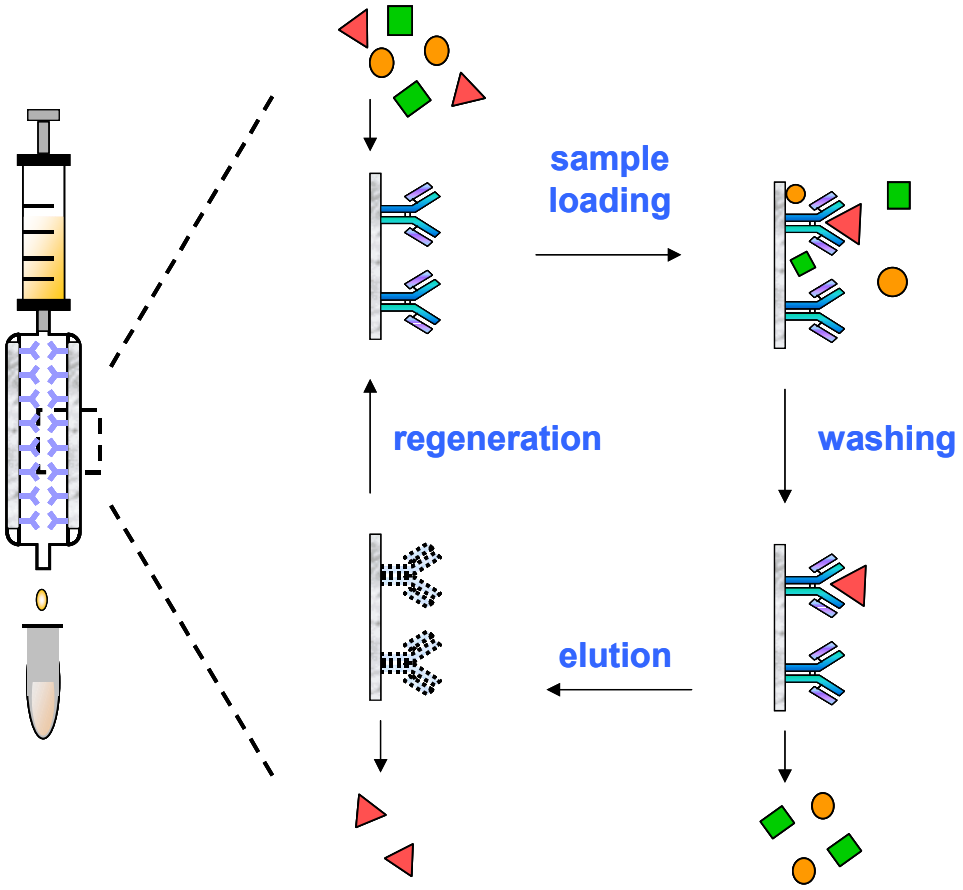


Figure 4.6. IAC-SPE procedure.

Another important aspect related to the loading step is the ***breakthrough volume***, i.e. the maximum sample volume that can be percolated without loss in recovery. In general, immunoaffinity extraction (IAE) is used to extract compounds at trace levels. So, the greater the breakthrough volume and the capacity are, the better the limit of detection is. Sample volume is different depending on the field of application. Biological samples are limited to the low-mL order for blood and plasma samples and up to a maximum of 50 mL for urine samples. Therefore, the breakthrough volume is not a basic parameter for biological applications, but it is important in environmental analysis. Two parameters can affect the recovery, which are the IS capacity and/or affinity of Abs towards compounds. Insufficient retention induces a low breakthrough volume and an incomplete recovery.

The function of the ***washing*** stage is to remove unwanted material from the matrix and nonspecifically bound compounds (see **Figure 4.6**). The washing step is characterized by the composition of the buffer and the number of column volumes necessary to remove nonspecifically bound substances. If their equilibrium adsorption association constant is lower than the equilibrium adsorption association constant of the Ab-Ag binding (K_a), the buffer used for column loading is adequate for the washing procedure. If not, harsher conditions should be applied to remove the unwanted non-specifically bound impurities avoiding joint elution of the specifically retained analyte.

Some of the most important parameters in IAE are the ***elution*** conditions. During the sample percolation, the Ab-Ag complex is formed by electrostatic forces, secondary hydrogen bonds and Van der Waals forces. To desorb the analyte, this complex has to be disrupted, i.e. the strategy of elution is based on creating a local environment that reduces the association constant K_a. The choice of elution buffer is important in analytical applications of IAC, since it is usually desirable to elute the analyte as quickly as possible while avoiding any irreversible damage to the immobilized antibody support. Common eluents used in IAC are acidic or basic buffers, high ionic strength solutions, chaotropic agents, detergents, organic solvents and displacer agents [200,201]. In the literature large molecules are traditionally desorbed using aqueous solutions, whereas desorption of low molecular weight organic molecules from the IS is better-achieved using organic solvents altering the polarity of the system. Unfortunately, a universal system that ensures an efficient desorption of the analytes does not exist. Every IS should be checked for the best elution conditions.

The last step in IAC-SPE is the column ***regeneration***. Regeneration is the re-equilibration of the column at the conditions required for loading in a new purification cycle. This entails, at the very least, removing the elution buffer from the column. Immunosorbent reusability depends on the concentration and conformation of the immobilized Abs, on the chemical and mechanical stability of the support used and the elution conditions. The key parameter to follow the evolution of an IS is its capacity.

4.2.1. Immunosorbent preparation. Determination of the coupling efficiency and the theoretical capacity of the column

As immunosorbent for 2,4,6-TCP urine extractions we used polyclonal As45 [259]. We have isolated the IgG fraction from As45 by ammonium sulfate precipitation in order to remove serum albumins. As solid support for IAC-SPE we chose a commercially available NHS-activated Sepharose™ HiTrap column (1 mL bed) consisting of pre-packed highly cross-linked agarose. This material is resistant to high pressure gradients and due to its hydrophilic properties non-specific interactions are avoided. The IgG was immobilized to the Sepharose gel by covalent coupling of their free amino groups with the NHS-activated solid phase leading to random antibody orientation. Throughout this thesis three IAC-Ab45 columns were prepared following manufacturer's instructions. Briefly, a solution of the purified As45 in carbonate buffer (pH=8.3) was injected onto the HiTrap column at a constant flow rate. The remaining active sites were deactivated by alternate and repetitive injections of ethanolamine and acetate buffers. The coupling efficiency of the HiTrap column was estimated as the difference between the total amount of IgG initially loaded onto the column and the amount of IgG found in the eluted buffer after the coupling took place. The IgG concentration in both solutions was determined by UV measurement at λ=280 nm. Previously, the NHS co-eluting with the antisera solution after coupling was removed using a HiTrap™ desalting column prepacked with Sephadex®G-25 Superfine. A coupling efficiency of 94.5 ± 0.98% (N=3) was achieved. This corresponds to a maximum ***theoretical binding capacity*** of approximately 1.24 ± 0.12 μg (6.27 ± 6.14 nmol) (N=3) 2,4,6-TCP, based on the amount of IgG coupled (9.4 mg) and the assumptions that bivalent binding takes place; 10% of the polyclonal IgG are specific [339] and 50% of the immobilized IgG are not accessible due to steric hindrance, or due to a random antibody orientation.

4.2.2. Characterization of the immunosorbent. Optimization of an IAC-SPE procedure using PBS standards of 2,4,6-TCP

4.2.2.1. Evaluation of the experimental column binding capacity and establishment of the desorption conditions

The aim of the elution is to have efficient desorption with a high recovery in a volume as small as possible, without denaturation of the Abs. Efficient desorption of the retained analyte from the immunosorbent requires that the total volume of eluent needed should be kept small to avoid extensive dilution of the analyte in the final extract. Our aim was to find out best elution conditions regarding effective IAC-SPE, but also regarding compatibility of the eluent with the ELISA method. Initially we approached the elution with an aqueous acidic buffer in order to obtain a final aqueous extract that could be measured by ELISA.

A. Elution with an acidic buffer: glycine/HCl, pH=3

One of the most often employed strategies for analyte elution from immunosorbents is lowering the pH below 2.5 with a glycine-HCl buffer. Since the Sepharose gel stability was limited below pH=3, we decided to test the elution of the analyte with 0.05 M glycine-HCl at pH=3. Another important point was to place the physicochemical properties (pH, conductivity) of the eluted fractions within the immunoassay conditions. As the 2,4,6-TCP ELISA performs better at neutral and basic conditions, it was necessary to neutralize the eluted fractions. By adding 0.2N aq NaOH until pH=8.6 a buffered media could be obtained (glycine-NaOH, pH=8.6-10.6). Both acidic and basic glycine buffers have a very low conductivity (3.5 mS/cm) but it could be corrected by the addition of NaCl until the necessary ionic strength of the IA media (15 mS/cm). Therefore, we established a protocol where to each milliliter of the eluted fractions a solution of 87 μL of 0.2N aq NaOH (18M NaCl) was added to obtain a buffer system containing 0.05M glycine-NaOH (18M NaCl) (pH=8.6 and conductivity of 15 mS/cm).

Before moving forward it was necessary to determine how the ELISA behaved in this buffer system. **Figure 4.7** shows the standard curves obtained with the PBS and the glycine-NaOH(NaCl) buffer. There is slight displacement of the curve to higher concentration range resulting in the following immunoassay parameters: A_{max}= 0.77, IC_{50}= 2.47 µg/L, LOD= 0.5 µg/L, dynamic range 0.7 – 10 µg/L, slope= -1.13, r^2= 0.997. The assay detectability was still good enough under these new conditions.

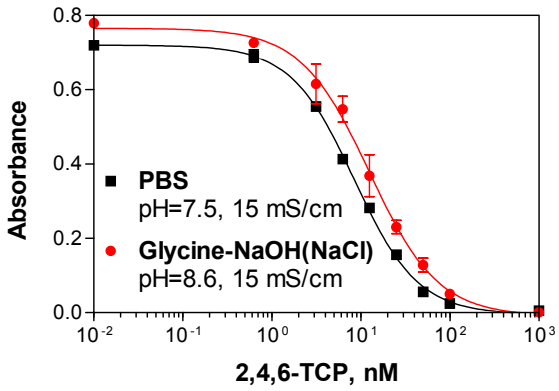


Figure 4.7. Effect of the 0.05M glycine-NaOH (NaCl) buffer (pH=8.6, 15 mS/cm) on the 2,4,6-TCP ELISA.

The next step was to evaluate the accuracy of this protocol. Thus, 2,4,6-TCP standards were prepared in 0.05M glycine-HCl, pH=3 and after adding the corresponding amount of NaOH and NaCl, they were measured on the ELISA and their concentration was determined from a standard curve run in glycine-NaOH(NaCl) (pH=8.6, 15 mS/cm). **Table 4.3** shows the measured concentration values compared to the initial spiked amounts. A good agreement can be observed between these values indicating the accuracy and the variability of the procedure.

Table 4.3. Accuracy of the analysis of 2,4,6-TCP in glycine solutions

Spiked, µg/L	**Measured (mean ± SD), µg/L**
1	0.78 ± 0.13
1.25	1.13 ± 0.15
2.5	2.4 ± 0.25
5	4.95 ± 0.35
10	10.57 ± 0.57

Samples prepared in 0.05M glycine-HCl (pH=3) were treated with 0.2N aq NaOH/18M NaCl until pH=8.6 and conductivity of 15 mS/cm before ELISA measurements.

Therefore, we loaded the IAC column with 5 mL of 2,4,6-TCP PBS solutions at three different concentration levels corresponding to 97% (1.2 μg), 40% (0.5 μg) and 4% (0.05 μg) of the theoretical immunosorbent capacity. After washing the column with 5 mL PBS, we eluted the analyte using 10 mL 0.05M glycine-HCl buffer (pH=3). As it is shown in **Figure 4.8** 10 mL were sufficient to remove the trapped analyte completely from the immunoextraction column. The recovery was greater than 80% for the three samples loaded.

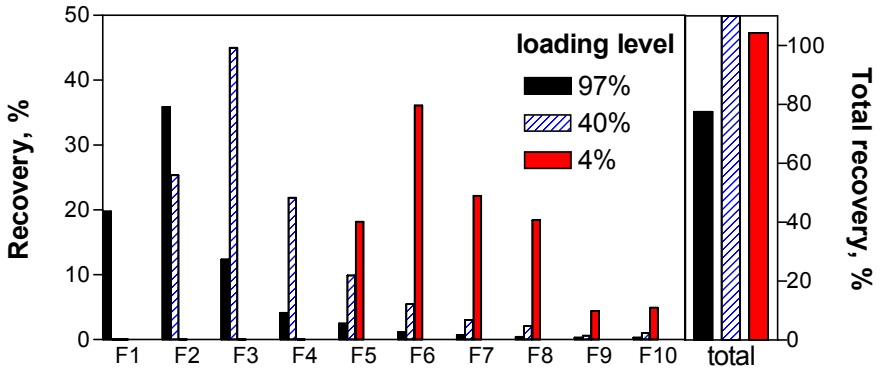


Figure 4.8. Elution profile of 2,4,6-TCP with glycine/HCl buffer (pH=3). The IAC was loaded with 5 mL of PBS standards of 2,4,6-TCP at three different loading levels, washed with PBS, and eluted with glycine/HCl, pH=3. Each elution fraction is of 1 mL. After adjustment of pH and conductivity the fractions were measured by ELISA standard curve run in glycine-NaOH/NaCl (pH=8.6, 15 mS/cm). Recovery for each elution fraction is calculated *vs* spiked amount. The total recovery represents the sum of the recovery in all elution fractions.

However, the elution profile was strongly dependent on the loading level, i.e. the lower the loading level, more volume had to be percolated through the column to disrupt Ab-Ag binding. Thus, it can be observed that at a loading level of 97% the analyte starts to elute from the first fraction but at 40% it starts to elute later (in fraction 2) and at 4% loading it appears in fraction 5. Moreover, 2,4,6-TCP elutes slowly being distributed within a different number of fractions, also depending on the loading level. Higher analyte loadings produce narrower elution bands and vice versa. These results indicate that 0.05M glycine-HCl buffer (pH=3) cannot effectively dissociate the Ag-Ab complex. For practical reasons the elution of the analyte must take place in the same way for all loading

levels. Therefore, we decided to explore other more efficient elution systems such as organic solvents frequently described in the immunoaffinity purification of organic molecules from complex samples.

B. Elution with an organic solvent: Ethanol

Elution has been in many cases achieved by increasing the content of an organic modifier in the solution. Pure methanol, methanol-water [331,336,340], ethanol-water [206,208,340], acetone-water [341], and acetonitrile-water [215] mixtures have been used successfully for the desorption of low molecular weight compounds from an immunosorbent. As mentioned before, in previous experiments performed in our group ethanol-water mixture was proven to be useful to desorb Irgarol 1051 from the same type of immunosorbent [206]. Thus, we decided to use this elution system.

As it was described in **Chapter 3** (see **Figure 3.7**), the 2,4,6-TCP ELISA tolerates EtOH concentration in the PBS up to 10-15%. Thus, a simple dilution of the final ethanol extract would be compatible with the subsequent ELISA analysis. In order to define the ethanol content under which the desorption takes place we applied a step elution to an IAC column loaded with 0.05 μg 2,4,6-TCP (5 μg/L, 10 mL PBS, 4% column loading). We selected this amount because it was close to the reference value of 2,4,6-TCP in urine. As can be seen from **Figure 4.9** the elution started at about 30% EtOH and about 16% of the loaded analyte eluted in 3 mL (F11, F12, F13). Subsequent elution with 50% EtOH gave too broad profile (four fractions of 1 mL were needed to collect 66% of the amount loaded). In addition, this EtOH concentration was not enough to desorb all the analyte bounded to the immunosorbent. Finally, at 70% EtOH the remaining 16% of the loaded 2,4,6-TCP eluted. Therefore, we decided to use 70%EtOH/water as elution solvent in further studies.

As before the optimum volume of 70% EtOH elution solvent was determined at three loading levels for both of the IAC columns prepared (see **Figure.4.10**). At high loading levels (97%, 1.2 μg 2,4,6-TCP) complete elution was achieved in just one mL fraction. At

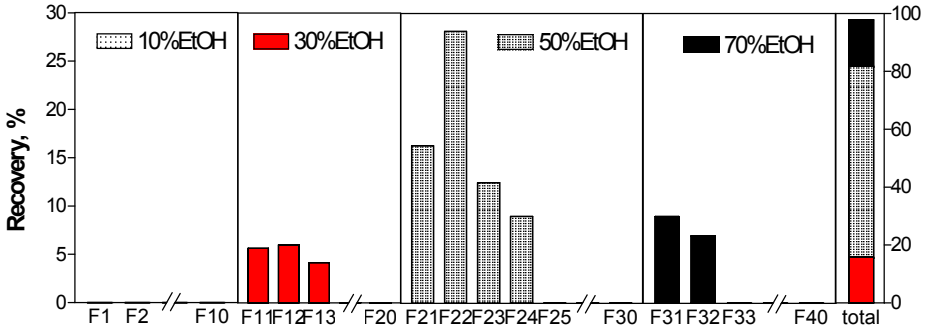


Figure 4.9. Step elution of 2,4,6-TCP with different EtOH concentration in the elution buffer. The IAC is loaded with 0.05 µg 2,4,6-TCP (5 µg/L, 10 mL PBS, 4% loading level). Each elution fraction is of 1 mL. Elution was performed with 10 mL 10% EtOH/water, followed by 10 mL 30% EtOH/water, 10 mL 50% EtOH/water, and finally by 10 mL 70% EtOH/water.

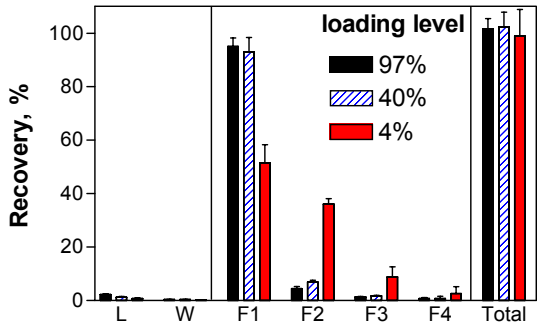


Figure 4.10. Elution profile of IAC-SPE of 2,4,6-TCP with 70% EtOH. PBS standards 10 mL (0.05, 0.5 and 1.2 µg 2,4,6-TCP) were loaded to the IAC column, washed with PBS and eluted with 70% EtOH. Eluted fractions (F) were diluted 10 fold with PBS and analyzed by ELISA using standard curve run in 7% EtOH/PBS buffer. The 10 mL collected fraction after loading (L) corresponds to the amount which is not retained by the immunosorbent. Loading (L) and washing fractions (W) (10 mL) were measured directly by ELISA using standard curve run in PBS buffer. Recoveries (%) are calculated *vs* spiked amount. Data presented correspond to the average of two different IAC columns.

lower loading level (4%, 0.05 µg 2,4,6-TCP) the elution volume needed for 100% recovery was 3 mL. This difference was probably due to the fact that mainly the higher affinity antibodies are participating at low loading levels. An important aspect of this result is that now the elution started in the first fraction (F1) for all loading levels indicating the higher efficiency of this system in dissociating the Ag-Ab complex.

As it was expected to detect low urine levels in real samples from the non-exposed population, we decided to collect 3 mL elution volume for further experiments. In addition, we should note that 2,4,6-TCP was not detected in the volume collected after loading of the standard neither in the collected washing fractions (see **Figure 4.10**). This indicated that the chlorophenol was retained completely by the immunosorbent. Even at the highest loading levels studied (97%) that correspond to the theoretical capacity of the column, the analyte was completely recovered in the elution fractions. Therefore, it can be concluded that in PBS the ***binding capacity*** of the IAC column is 1.2 μg or greater (note that an arbitrary correction factor of 50% of sterically hindered antibody molecules had been applied in the calculation of the theoretical capacity).

The IAC column ***breakthrough volume*** was determined by loading 0.05 μg 2,4,6-TCP in PBS samples with different volumes (5, 10, 20 and 50 mL). No dependence on the sample volume was found. The recoveries were still over 93% in all experiments and the analyte eluted in the first 3 mL. This result indicates that a high sensitivity could be achieved because large sample volumes could be loaded to the column and then collected in just 3 mL fraction.

4.2.2.2. Specificity of the IAC column

As it was previously described, halogenated phenols structurally related to 2,4,6-TCP are usually present in urine samples. Therefore, it was necessary to evaluate the selectivity of the IAC-SPE procedure established above. The specificity studies were carried out by GC-ECD analysis of the eluted fractions after toluene extraction and derivatization of the chlorophenols with BSTFA.

The specificity of the IAC column was evaluated towards 15 halophenols. Initially, PBS standard solutions of each compound were loaded individually at a 20% loading level. Each column was washed with PBS and the halophenol was eluted with 3 mL 70% EtOH. Then the eluted fractions were diluted 10 times with PBS until 7% EtOH and extracted with toluene for GC-ECD analysis. Previously we have proven that the recoveries of

toluene extraction of di-, three-, and tetra-chlorophenols from 7% EtOH water solutions were higher than 80% (see **Annex 1**). The results of the experiments performed with individual analyte loading and PBS column washing are presented in **Table 4.4**.

Table 4.4.. Specificity of the IAC column expressed in percentage recovery for each compound.

	IAC (As45)				ELISA (As43)		
Loading	**PBS**			**Urine** [c]	**Direct** [d]	**Indirect**	
	Individual [a]	**Mixture** [b]		**Mixture** [b]			
Washing / **Compound**	**PBS**	**PBS**	**20%EtOH**	**20%EtOH**		**A** [e]	**B** [f]
PCP	81	100	25	20	10	4	<0.05
2,3,4,6-TtCP	98	103	81	83	86	89	21
2,3,4,5-TtCP	104	101	< 10	n.d.	n.d.	n.d.	n.d.
2,3,5,6-TtCP	91	116	< 10	n.d.	3	<0.4	<0.05
2,4,6-TCP	**100**	**95**	**80**	**78**	**100**	**100**	**100**
2,4,5-TCP	107	103	< 10 [g]	n.d.	41	49	12
2,3,4-TCP	83	82	< 10 [g]	n.d.	0.5	<0.4	n.d.
2,3,5-TCP	94	105	< 10 [g]	n.d.	12	<0.4	13
2,3,6-TCP	97	98	< 10 [g]	n.d.	<0.001	3	n.d.
2,4,6-TBP	79	86	68	71	1550	391	710
2,5-DCP	< 12 [g]	n.d.	n.d.	n.d.	0.7	<0.4	n.d.
2,6-DCP	< 12 [g]	n.d.	n.d.	n.d.	2.3	<0.4	<0.05
3,4-DCP	< 12 [g]	n.d.	n.d.	n.d.	<0.001	<0.4	n.d.
2,4-DBP	93	103	17	n.d.	136	n.d.	119
2,6-DBP	37	n.d.	n.d.	n.d.	5	n.d.	<0.05
2,4,6-TCA	16	n.d.	n.d.	n.d.	<0.001	n.d.	n.d.

PBS solutions of the halogenated compounds were loaded to the column and eluted by 70%EtOH. After 10-fold water dilution they were extracted with toluene, derivatized with BSTFA and their concentration was determined by GC-ECD analysis. The recovery was calculated as the ratio of the peak area of the compound in the eluted fraction to the peak area corresponding to the loaded standard extracted with toluene. Rows in gray indicate the compounds that are strongly retained by the IAC column and are also the main cross-reactants in the ELISAs. n.d.- not determined;
[a] The loading level for each compound is 20%; [b] The total loading level for the mixture is 20%; the individual loading level is 1.7%; [c] 10 mL acid hydrolyzed urine spiked with a mixture of the halophenols is loaded; [d] Immunoassay As43/5-HRP. Data reported is from [259]. [e] Immunoassay As43/2-BSA. Data reported is from [260]. [f] Immunoassay As43/8-BSA. Data reported is reprint from [277]. [g] The limit of quantification in these experiments correspond to a recovery of 12% (DCPs) and 10% (TCPs).

The specificity of the IAC column is lower than those of the ELISA in both direct and indirect formats. Thus, all penta-, tetra- and tri-chlorophenols were retained in more than 80%. The dichlorophenols were not detected in the elution fractions. Regarding bromophenols, 2,4,6-TBP and 2,4-DBP were completely retained, as expected from the

cross-reactivity data of the ELISA. In contrast, 2,6-DBP was only retained in a 37%. It must be noted that this analyte was not recognized in the ELISA, in spite of the greater recognition of the brominated analogues due to the lack of the halogenated atom at *para* position.

It is well known that the Ab affinity for a given analyte is not the same when it is present individually in the solution and when it is in presence of other structurally related compounds [332]. In immunoassays individual analyte cross-reactivity is a function of the concentration of the cross-reactant present [342]. In IAC it is also demonstrated that there is such type of threshold concentration of the cross-reactant above which there is an effect on the analyte recovery [343]. The capacity measured for each analyte using a mixture of compounds strongly depends on the competition for the Ab binding sites, i.e. on the number of compounds and on their concentrations [338]. Since chlorophenols appear in urine as a mixture and in low levels, we determined IAC recoveries loading a mixture of halophenols, corresponding all together to 20% total column loading (each compound - 1.7% loading) and washing the column with PBS. No difference respect to the individual loading was observed under these conditions. All compounds were retained by the IAC column in higher than 80% (see **Table 4.4**). It is important to note that the recovery of the analyte 2,4,6-TCP (95%) was not affected by the presence of other similar compounds in the loaded mixture. The results clearly indicate a high affinity of the immunosorbent (Ab45) for all these halophenols.

It has been reported that a step desorption varying the MeOH concentration in the elution buffer can be a means for assessing the affinity order of analytes within a group (weakly bound compounds are recovered with lower % MeOH and the strongest bound analytes are recovered with 70% MeOH) [344]. Moreover, potential non-specific interferences present in the urine could be washed out more efficiently by introducing a washing step with certain EtOH content. Before we had demonstrated that the elution of the analyte did not start until at least 30% EtOH in the elution buffer. Thus, when we decided to introduce a washing step with 20% EtOH, a higher selectivity was observed (see **Table 4.4**). Only 2,4,6-TCP, 2,4,6-TBP, 2,3,4,6-TtCP, PCP and 2,4-DBP remained retained by

the IS. The organic modifier in the washing buffer disrupted the interactions between the Ab and the most weakly bounded analytes. The chlorophenols retained now followed the same recognition pattern as in the ELISA (see rows in gray, **Table 4.4**). The presence of hydroxyl group and three chlorine atoms at *ortho* and *para* positions in the aromatic ring of the halogenated compound was a necessary condition to remain bound to the IS (see **Figure 4.11**).

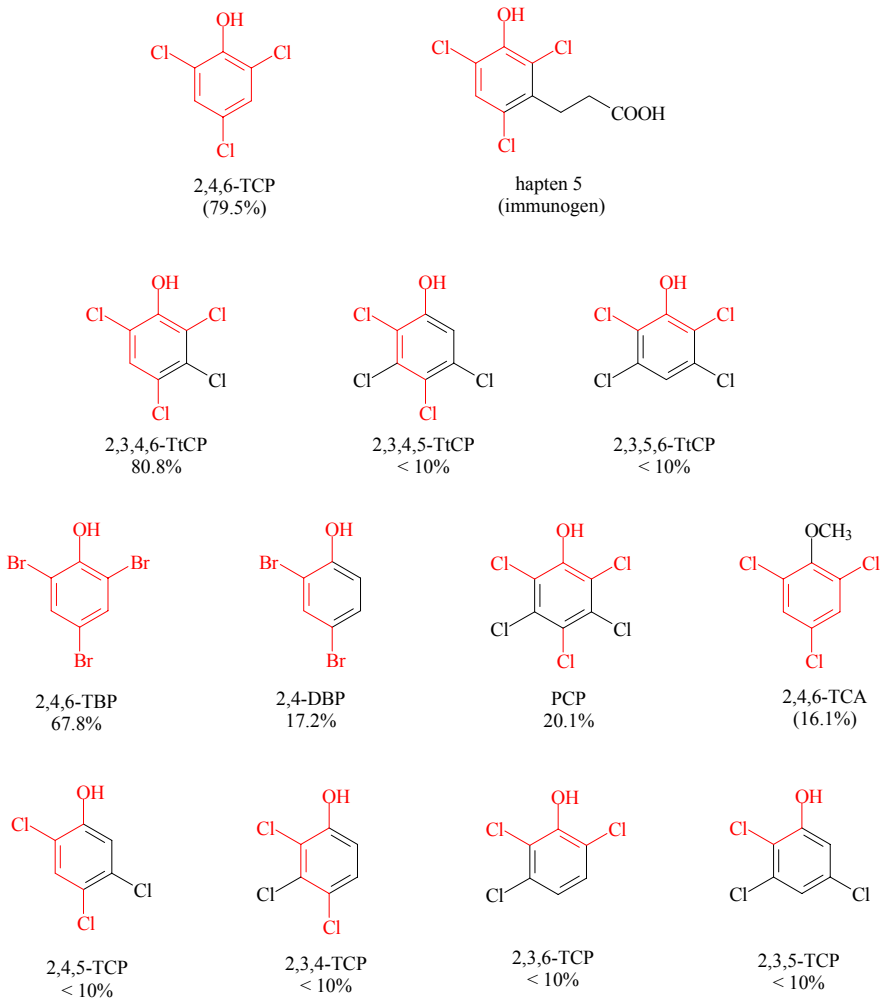


Figure 4.11. Chemical structures of the analyte, the immunogen used for Ab45 production and the cross-reactants tested on IAC column. The fragments colored in red are the ones mostly recognized by the Ab and determine analyte retention onto the immunosorbent. The recovery (%) presented corresponds to 20% EtOH washing of the IAC column loaded with a PBS mixture of the halogenated phenols (1.7% individual loading level, 20% total loading).

Although some authors have pointed that the cross-reactivity observed in a classical immunoassay does not necessarily has to mimic that seen in immunoaffinity extraction format using the same Ab [202], in our case the specificity of the IS (Ab45) was similar to the cross-reactivity of Ab43 shown in the three different ELISA formats developed in our group (see **Table 4.4**). Ab43 demonstrated higher affinity towards the same compounds. Although the Ab used for the preparation of the IAC column was obtained from another rabbit (Ab45), both antibodies (Ab43 and 45) were raised against the same immunogen (hapten **5**-KLH). These observations demonstrate the importance of the structure of the immunogen for the Ab selectivity.

Finally, the IAC specificity was also investigated loading the column with acid hydrolyzed urine sample (loading in a mixture, 20% EtOH washing). The behavior observed indicated that the urine matrix did not affect the selectivity of the Ab-Ag recognition. The halogenated compounds were retained by the immunosorbent in a similar manner as when they were loaded in PBS sample (see **Table 4.4**).

In summary, the IS prepared can trap several analytes from the same structural group when PBS washing is applied. Thus, this procedure can be used as a class-selective IAC-SPE of chlorophenols from urine samples that later can be analyzed by chromatographic (GC, HPLC) or immunochemical methods. A more selective extraction can be performed by washing with 20% EtOH buffer without any loss of recovery in the analyte of interest. If ELISA is used as a detection method after both types of IAC-SPE procedures, only the halogenated phenols with significant immunoassay cross-reactivity would be detected. However, if chromatographic methods were employed, the choice of IAC-SPE protocol (PBS or EtOH washing) would allow performing a class-selective or more specific analysis. Finally, it is important to note that the problem with the specific interference effect on the 2,4,6-TCP ELISA discussed in the previous chapter will persist after IAC-SPE of urine, because ELISA cross-reactants are also extracted by the immunosorbent. This will be considered when matrix effect studies with urine samples will be performed.

4.2.2.3. Immunosorbent regeneration and stability

When water-organic modifier mixture is used for elution, the presence of non-polar solvents reduces the hydrophobic binding component of the Ab-Ag interaction. However, it also affects the stability of the hydrophobic bonds that maintain the Ab tertiary structure resulting in the release of the antigen. These harsh eluting conditions can irreversibly denature Abs, but as small volumes are required, contact times can be minimized. In our studies, the regeneration of the IS was performed by passing 10 bed volumes of PBS through the column. Between uses the column was stored at 4°C in PBS in the presence of NaN_3.

To study the IS regeneration and stability, 2,4,6-TCP standards prepared in PBS were used regularly as quality controls of the IS binding capacity within several applications of urine (non-hydrolyzed/hydrolyzed) samples. **Figure 4.12**. shows the long-term stability of the IAC column monitored by loading it with 2,4,6-TCP in PBS at three concentration levels corresponding to 97%, 40% and 4% of the maximum capacity (1.2, 0.5 and 0.05 μg). In the first 10-20 analyses a 50% decrease of the maximum capacity followed by stabilization is observed. This phenomenon is often observed [331,336] and can be explained by irreversible denaturation and ligand leakage during the early runs. This capacity decrease will not affect the recovery of 2,4,6-TCP from real urine samples

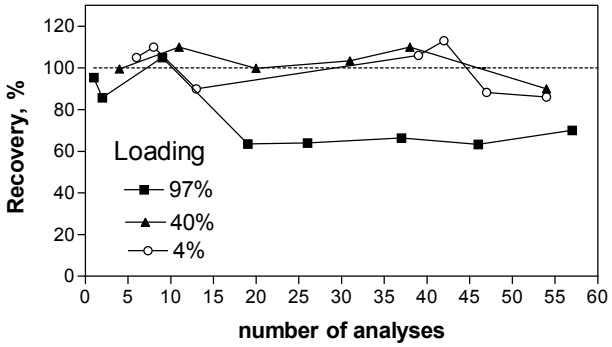


Figure 4.12. Performance of the IAC as a function of the number of analyses. For each control determination the IAC column was loaded with a standard PBS solution. The 2,4,6-TCP concentration in the eluted fractions were determined by ELISA.

because the actual concentrations present in most of the population are placed between 0 - 4% (non-exposed) and 40% (exposed). As it is demonstrated in **Figure 4.12** at medium (40%) and low (4%) loading levels recoveries remain constant of around 90 - 100% up to 50 analyses. Thus, the IAC capacity and recoveries were not affected adversely and the column can be efficiently regenerated and used for many cycles of urinary chlorophenol extraction.

4.2.3. Evaluation of the IAC-SPE procedure with urine samples

4.2.3.1. Matrix effect studies

Non-specific interactions can occur between the IS support, the Ab proteins, the analyte and the sample matrix. Most commonly hydrophobic and ionic forces are responsible for undesirable adsorption, which results in a decrease of the selectivity and the LOD. Due to their hydrophilic character agarose-based ISs have been shown to minimize the interactions generated by the solid support.

With the aim to evaluate the efficiency of the IAC- clean up we have tested the matrix effects of the IAC-purified urine samples A and B (non-hydrolyzed and hydrolyzed) on the 2,4,6-TCP ELISA. With this purposes we loaded 30 mL urine into the IAC column, washed it with PBS, collected the elution fractions (3 mL 70% EtOH) and then diluted them 10 times with PBS (neat). Further dilutions used for the ELISA were performed in 7% EtOH/PBS in order to keep the solvent concentration constant. In this way the IAC-SPE procedure consists just in a clean up step without concentration (30 mL urine loaded – 30 mL recovered for ELISA analysis). The standard curves compared to the one run in 7% EtOH/PBS are presented in **Figure 4.13 A, B, C,** and **D**. Although a small inhibition of the A_{max} is observed for all the cases studied (non-hydrolyzed, acid and enzimatically hydrolyzed urine A; alkaline hydrolyzed urine B), the significant matrix effect previously

observed for the non-purified urine (see **Table 3.12**) and for the C_{18}-SPE extracted urine (see **Figure 4.4**) has been eliminated.

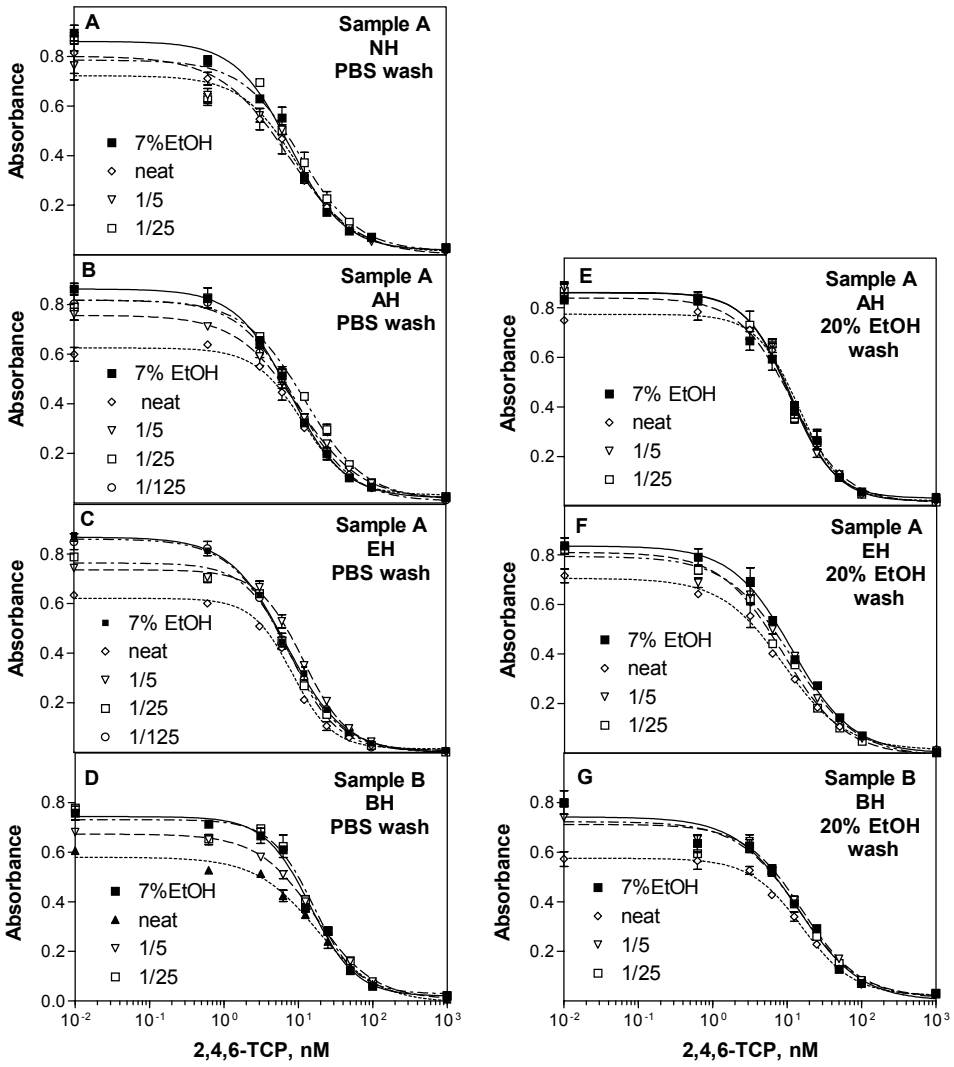


Figure 4.13. Matrix effect on the 2,4,6-TCP ELISA of urine sample A (**A**, **B**, **C**, **E**, **F**) and urine samle B (**D**, **G**) after IAC-SPE clean-up in different concentrations. (**A**) non-hydrolyzed (NH), (**B, E**) acid hydrolyzed (AH), (**C, F**) enzimatically hydrolyzed (EH), (**D, G**) base hydrolyzed (BH). 30 mL of urine sample was loaded to the IAC column, washed with PBS (**A**, **B**, **C**) or with 20% EtOH (**E**, **F**) and eluted with 70% EtOH. The collected 3 mL elution fractions were diluted 10 times with PBS. Further dilutions were preformed with 7% EtOH/PBS. The IAC-SPE for sample B (**D**, **G**) was realized with mini-columns using volumes proportional to the single IAC-SPE procedure (see **Chapter 5**).

At the light of these results we thought on impoving the clean-up of the matrix by increasing the strenght of the washing buffer. Thus, the co-extraction of interfering analytes (specific or non-specific) could be reduced or removed by applying a washing solution made of water with a small percentage of organic solvent. Based on our previous studies on the specificity of the IAC column (**Table 4.4**) we decided to apply a 20% EtOH washing step to the hydrolyzed urine samples in order to improve clean-up and to achieve higher selectivity. Following the above described procedure we evaluated the matrix effect on the ELISA after washing the IAC with 20% EtOH (see **Figure 4.13**. **E**, **F**, **G**). The inhibition of the A_{max} seems to be smaller than in the case of PBS washing. It is important to remind that EtOH washing of the IAC column provide higher selectivity of the clean up step and especially one of the main ELISA cross-reactants (2,4,5-TCP) is not co-eluted under these conditions (**Table 4.4**).

In an attempt to explain the origin of the small reduction of the A_{max} still observed on the ELISA, we considered the presence of chlorophenols in urine samples A and B (**Table 3.9**) co-eluting with the analyte 2,4,6-TCP from the IAC column (**Table 4.4**). Thus, we compared the 2,4,6-TCP-IR equivalent values obtained by the IAC-ELISA method and those determined by GC-MS analysis of samples A and B after direct toluene extraction (see non-purified urine TCP-IR equiv. GC-MS **Table 4.5**). The results are presented in **Table 4.5**. We evaluated the 2,4,6-TCP-IR equivalents for the urines using the standard curves prepared in 7% EtOH/PBS (ELISA in **Table 4.5**). On the other hand, we corrected the concentration of each CP determined by GC-MS considering its IAC recovery, and the ELISA cross-reactivity (TCP-IR equiv. $GC\text{-}MS_{corrected}$ in **Table 4.5**). Under PBS washing conditions all endogeneously present CPs (cross-reactants) are IAC-extracted (see **Table 4.4)**. For 30 mL urine sample loaded at low loading level (4%) and PBS washing 85-100% IAC recovery can be applied for NH sample and 55% IAC recovery - for AH urine (according to the results presented in **Figure 4.15**). The IAC recovery considered for AH urine sample at 20%EtOH washing is 65% (see **Figure 4.16.B**).

Table 4.5. The 2,4,6-TCP-IR equivalents according to GC-MS, ELISA cross-reactivity and IAC specificty are compared to the 2,4,6-TCP-IR determined by the 2,4,6-TCP ELISA after IAC-SPE of urine sample A and B.

Urine sample		Non-purified urine	IAC purified urine			
			PBS wash		20%EtOH wash	
		TCP-IR equiv. GC-MS	TCP-IR equiv. $GC\text{-}MS_{corrected}$ [d]	ELISA	TCP-IR equiv. $GC\text{-}MS_{corected}$ [d]	ELISA
A	NH	0.16	0.14- 0.16 [e]	0.3 [b]	n.d.	n.d.
	AH	0.72	0.4 [f]	0.66	0.46 [g]	0.4
	EH	n.d. [a]	n.d. [a]	0.58	n.d. [a]	0.43
B	AH	1.12	0.62 [f]	1.1 [c]	0.73 [g]	0.9 [c]

[a] TCP-IR for EH urine are not determined, but if complete hydrolysis is assumed they should be equal to the TCP-IR equivalents determined for AH urine by GC-MS. [b] This value is close to the LOD of the ELISA $_{7\%EtOH/PBS}$ = 0.2 µg/L. [c] Alkaline hydrolysis

[d] The values for TCP-IR equiv. are estimated as follows: (CP concentration in urine according GC-MS, µg/L – **Table 3.9A**) x (IAC recovery corresponding to each CP) x (ELISA cross-reactivity – **Table 3.9B**). This is calculated for each CP and the values are summarized to obtain the total TCP-IR. [e] 85-100% IAC recovery is considered for all CPs according to **Figure 4.15** (30 mL NH urine sample loaded, 4% loading level, PBS wash). [f] 55% IAC recovery is considered for all CPs according to **Figure 4.15.B.** (30 mL AH urine sample loaded, 4% loading level, PBS wash). [f] 65% IAC recovery is considered for all CPs according to **Figure 4.16.B.** (30 mL AH urine sample loaded, 4% loading level, 20%EtOH wash)

The TCP-IR values measured by ELISA correlate quite well to the values expected according to GC-MS (see **Table 4.5**). In general, the ELISA values are slightly higher or equal to the GC-MS estimated values. The overestimation is observed especially in the case of PBS washing. It is possible that other ELISA cross-reactants are present in the IAC urinary extracts (not considered by us in the GC-MS urine characterization), such as 2,3,5-TCP, which has 12% ELISA cross-reactivity and it is retained by the IAC column under PBS washing conditions (see **Table 4.4**). Therefore, we can conclude that the IAC purified urine samples have no matrix effects on the 2,4,6-TCP ELISA. The observed "matrix effect" is only specific and it is due to the low levels of chlorophenols present in the samples. In addition, it can be observed that there is no significant difference in the concentrations determined by IAC-ELISA of acid and enzymatically hydrolyzed urine sample A. This is another confirmation for the "specific matrix effect" observed, if efficient hydrolysis in both cases is assumed.

Finally, we have confirmed our statement by GC-ECD and GC-MS analysis of the IAC-extracted urine sample A (30 mL, AH, PBS wash). The presence of 2,4,6-TCP, 2,4,5-TCP, 2,3,4,6-TtCP, 2,3,5,6-TtCP and 2,4,6-TBP was observed and their concentrations corresponds to 0.56 μg/L 2,4,6-TCP-IR equiv. which agrees with the ELISA measured value of 0.66 μg/L. Furthermore, a comparison of the GC-ECD chromatograms for the IAC-extracted urine and the C_{18}-SPE extracted urine demonstrates the efficiency and specificity of the IAC-SPE (see **Figure 4.14**). While urine matrix components are co-eluting under C_{18}-SPE producing a matrix effect on the ELISA (**Figure.4.4**), with IAC-SPE only the specific compounds are eluted (**Figure.4.13**).

4.2.3.2. Recovery studies. Effect of loading level, volume loaded and type of urine hydrolysis.

A. Non-hydrolyzed urine

Once we demonstrated that there was no matrix effect of the IAC- extracted urine on the 2,4,6-TCP ELISA, we approached IAC-recovery studies. The evaluation of the IAC-SPE procedure was performed using urine sample A characterized by GC-MS in **Chapter 3**. Different volume of non-hydrolyzed urine (pH=7.5, 25 mS/cm) and spiked at two concentration levels corresponding to 4% and 40% of the maximum column capacity were passed through the IAC column. The unbound material was washed with PBS buffer. The eluted 2,4,6-TCP was determined by ELISA after a 10-fold dilution with PBS and using a standard curve run in a 7%EtOH/PBS buffer. The recoveries were calculated with respect to the spiked amount and substracting the TCP-IR equivalents present as background level in sample A.

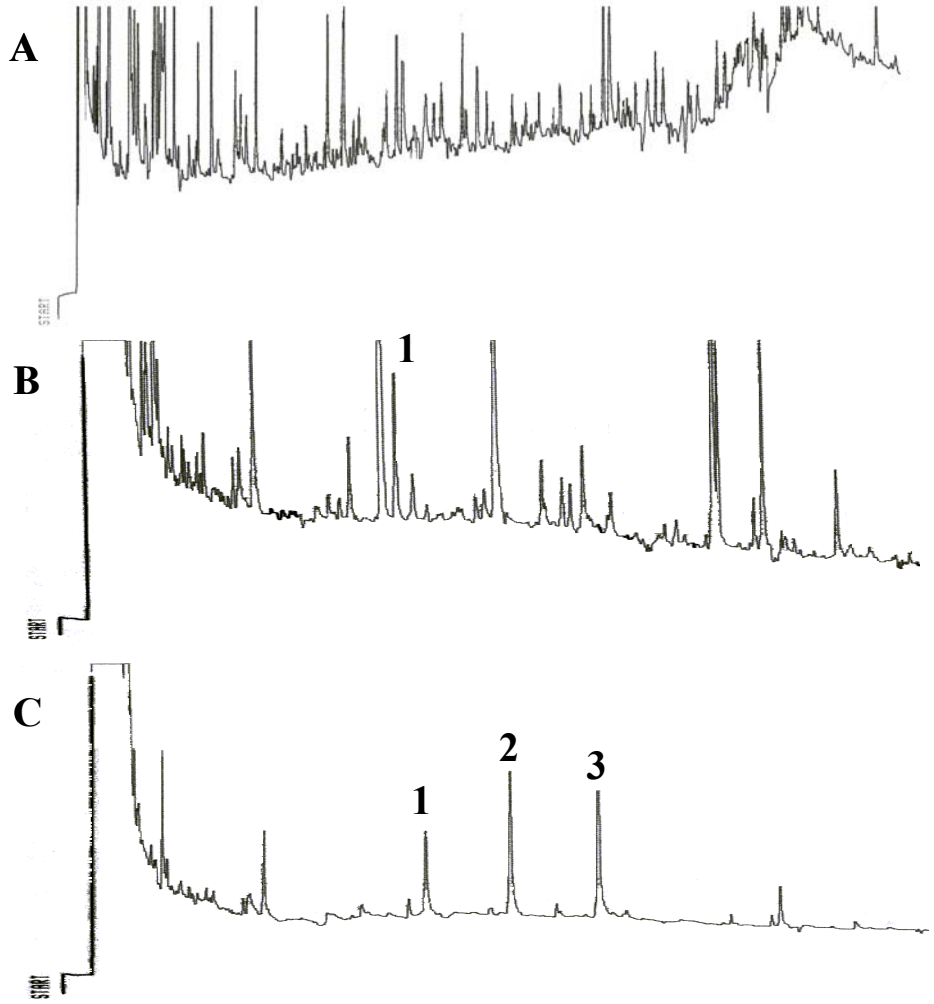


Figure 4.14. GC-ECD chromatograms obtained for: **A**. Non-purified urine: 100 mL of acid hydrolyzed urine A were extracted with 1 mL of toluene. The obtained extract was 10-fold diluted with toluene; **B**. after C_{18}-SPE: 10 mL of acid hydrolyzed urine A spiked with 1 μg/L 2,4,6-TCP were used. The collected elution fraction (85% MeOH) was diluted 10-fold with PBS and extracted with 1 mL of toluene. The concentration of 2,4,6-TCP in the toluene extract corresponds to 10 μg/L; **C**. after IAC-SPE: 10 mL of acid hydrolyzed urine A spiked with a mixture of 2,4,6-TCP, 2,3,4,6-TtCP and 2,4,6-TBP (0.25 μg/L each) were used. The collected fraction of 3 mL after the IAC-SPE procedure (20% EtOH washing) was diluted 10-fold with PBS and extracted with 1 mL of toluene. Peaks: (1) 2,4,6-TCP, (2) 2,3,4,6-TtCP and (3) 2,4,6-TBP The concentration of each chlorophenol in the toluene extract corresponds to 7.5 μg/L; All toluene extracts were derivatized with BSTFA, In the three cases the overall sample concentration is 10 times (urine: toluene=10:1). Peaks: **1** – 2,4,6-TCP, **2** – 2,3,4,6-TtCP, **3** - 2,4,6-TBP.

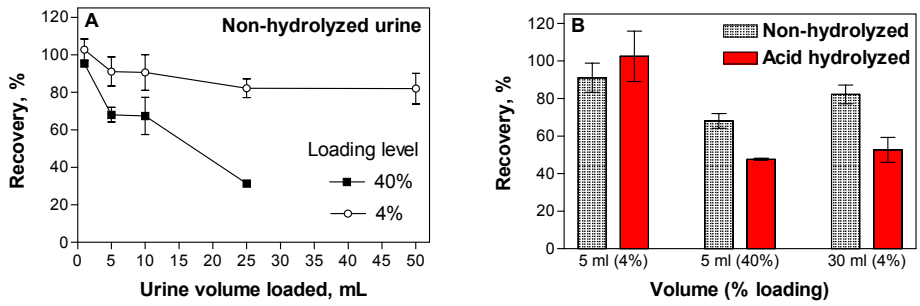


Figure 4.15. Effect of sample volume and loading level on the recovery of the IAC-SPE procedure (PBS washing and 70% EtOH elution) applied to urine sample A. (**A**) non-hydrolyzed urine; (**B**) acid hydrolyzed urine. 100% loading level corresponds to 1.2 μg 2,4,6-TCP. The 2,4,6-TCP concentrations in the purified urine are measured by ELISA. Recovery is calculated with respect to the spiked amount substracting the background level of TCP-IR equiv. in urine sample A. The standard deviation corresponds to three column replicates.

As can be seen from **Figure 4.15A**, there are two main factors affecting the recovery from non-hydrolyzed urine: the volume loaded and the spiked concentration. In general, the recovery is higher at lower loading levels. An explanation could be found considering that at low loading levels only the antibodies with high affinity participate in the binding of the analyte, while at high loads the analyte is also retained by antibodies with low affinity, and the binding forces of those can be more affected by other components of the urine matrix affecting thus the recovery. Similar to the IAC of spiked PBS samples, where no breakthrough effects were observed until 50 mL loaded volume, the 2,4,6-TCP recovery of non-hydrolyzed urine spiked with 50 ng 2,4,6-TCP (4% lading level) is higher than 80% for all the volumes tested (from 1 mL to 50 mL). In contrast, at 40% loading level (500 ng 2,4,6-TCP) breakthrough effects become evident upon sample volume increases. Such type of breakthrough volume is defined at about 5 mL, where the recovery drops to 70% and further at 25 mL, where it becomes only 30%.

Since the immunoreaction taking place during sample loading may be interfered by unknown urine components, this effect could be minimized if urine sample would be loaded diluted. Furthermore, urine conductivity (25 mS/cm) is slightly higher than usual

buffer conditions that favour Ab-Ag interactions (15 mS/cm). Thus, spiked urine samples (500 ng 2,4,6-TCP, 5mL) were loaded as such and after 2- and 10-fold water dilution. The recoveries obtained were 67.7%, 67.8% and 68.8%, respectively. As no improvement was achieved, we decided to apply non-hydrolyzed urine to the IAC in 2-fold dilution in further experiments.

B. Hydrolyzed urine

We have applied the same IAC-SPE procedure (PBS wash) to acid hydrolyzed (AH) urine A. After acid hydrolysis the urine was adjusted to pH 7.5, the precipitated salts were removed by centrifugation and the supernatant was spiked at 4% and 40% loading levels as before. As the conductivity of the solution remained very high (100 mS/cm), we diluted the urine sample 3 times with water prior to loading the column. The recoveries obtained are compared to those of the non-hydrolyzed urine in **Figure 4.15.B**. At 4% loading level the breakthrough volume for the AH urine was smaller than those for the NH urine: while the recovery for NH urine was higher than 80% when up to 50 mL were loaded, the recovery for AH urine was near 50% when 30 mL were loaded. At medium loading levels (40%) the recovery reached this value with just 5 mL AH urine sample. In addition, no effect of urine dilution prior to IAC was observed. Therefore, IAC column capacity for acid hydrolyzed urine is lower than for non-hydrolyzed one.

As was previously demonstrated, 20% EtOH washing of the IAC column resulted in an IA extraction with higher specificity. We also observed that clearer (colorless) extracts were obtained from acid hydrolyzed urine after ethanol washing. Thus, we decided to test the efficiency of the IAC-SPE of AH spiked urine with 20% EtOH washing. The effect of the volume and the sample concentration on the recovery is presented in **Figure 4.16**. The results are quite similar to the recoveries obtained with PBS washing (see **Figure 4.15 B**). The results also revealed that 10 mL of urine with a 4% loading level could be purified without loss of analyte (recovery higher than 80%). In an attempt to determine the maximum capacity of the column when 10 mL of AH urine were loaded, we also tested 2,4,6-TCP loading corresponding to 8% of the theoretical capacity. We observed that the

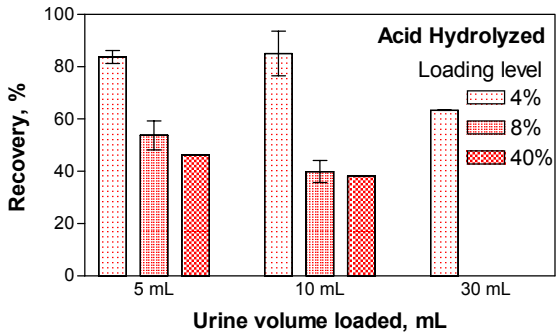


Figure 4.16. Effect of sample volume and loading level on the recovery of the IAC-SPE (20% EtOH washing and 70% EtOH elution) for acid hydrolyzed urine A. The 2,4,6-TCP concentration in the purified urine was measured by ELISA. Recovery was calculated with respect to the spiked amount resting the background level of TCP-IR in urine sample A. The standard deviation corresponds to two column replicates.

recovery dropped below 50% under these conditions. The recovery was below 60% even when 5mL of AH urine spiked with 8% loading level was loaded. Therefore, it can be considered that for the AH urine the maximum capacity is near 50 ng and the breakthrough volume is around 10 mL. This means that levels up to 5 μg/L 2,4,6-TCP (10 mL, 50 ng 2,4,6-TCP) in acid hydrolyzed urines can be determined quantitatively by the IAC-ELISA method.

The TCP-IR equivalent urinary concentration in general population [75,124] and occupationally exposed persons [36,117] could exceed 5 μg/L. In an attempt to achieve higher recoveries we decided to study the IAC-SPE of enzimatically hydrolyzed (EH) urine. Although we expected more efficient immunoaffinity extraction from this cleaner matrix, the recovery obtained was a little bit higher, but the improvement was not significant. Since enzymatic hydrolysis would increase the cost of routine urine analysis, we approached alkaline (base) hydrolysis (BH). Thus, 10 mL of NH, AH and BH urine samples were spiked at different concentrations of 2,4,6-TCP and purified by IAC-SPE (20% EtOH washing). **Figure 4.17** shows the effect of the loading level on the recoveries obtained for these three types of urine. Excellent recoveries were obtained for all type of urines until a 4% loading level. Furthermore, the IAC column can extract efficiently even 200 ng 2,4,6-TCP (16% loading) from NH and BH urine samples. In contrast, the IAC

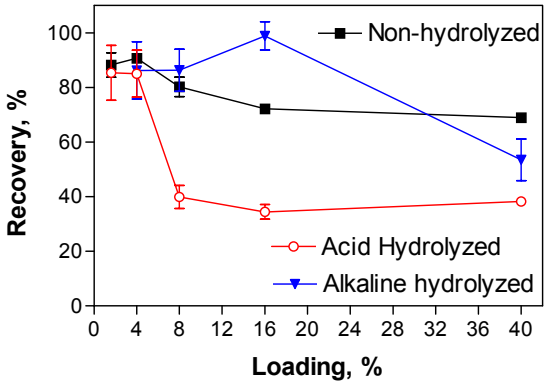


Figure 4.17. Effect of the loading level on the recovery of IAC-SPE (20% EtOH washing and 70% EtOH elution) for non-hydrolyzed and hydrolyzed (acid, alkaline) urine sample A. Sample volume loaded is 10 mL. 2,4,6-TCP concentration in purified urine is measured by ELISA. Recovery is calculated with respect to the spiked amount resting the background level of TCP-IR in urine sample A. The standard deviation corresponds to two column replicates.

capacity for AH urine remained limited to 50 ng 2,4,6-TCP (4% loading). These results suggested that alkaline hydrolysis seems to be more appropriate than the acid hydrolysis of the urine for the IAC-SPE protocol.

Finally, each type of hydrolysis (alkaline and acid) was evaluated regarding possible loss of the analyte. Spiked urine samples (60 ng and 200 ng 2,4,6-TCP) were hydrolyzed by both methods. Simultaneously, non-spiked samples were treated in the same way and spiked with the same concentrations after the hydrolysis took place. All the samples were IAC purified and their concentrations were determined by ELISA. The data presented in **Table 4.6** demonstrates that both hydrolysis methods have similar recoveries.

Table 4.6. Hydrolysis evaluation.

Loading, %	**Recovery ± SD (%)**	
	Acid hydrolysis	**Alkaline hydrolysis**
5	81.2±4.7	71.2±11.6
16	76.9±5.3	75.3±5.7

Urine samples (10 mL) spiked at two concentrations (60 ng and 200 ng 2,4,6-TCP corresponding to loading levels 5% and 16%) were hydrolyzed by KOH and H_2SO_4. Simultaneously, non-spiked samples were treated in the same way and spiked after hydrolysis. All the samples were IAC purified and their concentrations were determined by ELISA. The recoveries are calculated as (spiked+hydrolyzed)/hydrolyzed+spiked)]*100. Data are the result of three replicates.

However, since the alkaline hydrolysis allows quantitative immunoaffinity extraction at higher loading levels (see **Figure 4.17**), the use of this type of hydrolysis of urine samples is more convenient for the detection of 2,4,6-TCP in urine of occupationally exposed persons.

In conclusion, we established the following IAC-SPE procedure for clean up of urine samples (see **Figure 4.18**): 10 mL hydrolyzed or non-hydrolyzed urine sample is loaded to the IAC column, then the column is washed with 5 mL 20% EtOH; the analyte is eluted by 3ml 70%EtOH; the IAC column is regenerated by 10 mL PBS. The eluted urine extract is 10-fold diluted with PBS and the concentration of 2,4,6-TCP is measured on the ELISA. In this procedure urine is diluted 3 times (10 mL urine is finally converted in 30 mL purified urine). The overall LOD of the IAC-ELISA method is determined by the LOD of the ELISA run in 7% EtOH/PBS (0.2 µg/L 2,4,6-TCP), by the 3-fold urine dilution in the IAC procedure, by the IAC-recovery (see **Figure 4.17**) and by the recovery in the hydrolysis step if total (free + conjugated) chlorophenol concentration is quantified

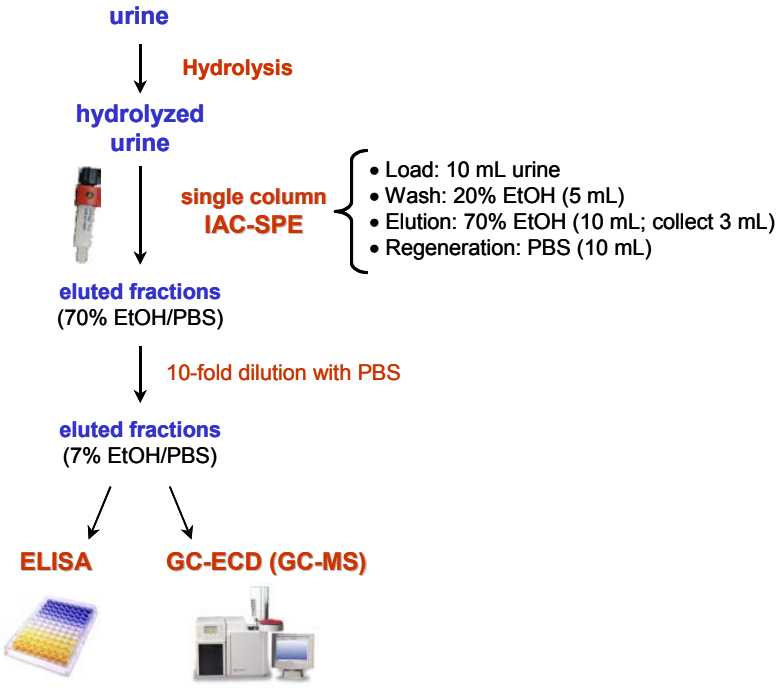


Figure 4.18. IAC-SPE procedure. 2,4,6-TCP concentration in purified urine can be determined by ELISA and/or gas-chromatography (GC-ECD/MS).

(see **Table 4.6**). The range of the method (low LOD and maximum detectable concentration) where quantitative analysis (overall recovery > 70%) can be performed is summarized in **Table 4.7**.

Table 4.7. Range of quantitative detection for the IAC-SPE-ELISA method for different types of urine samples

Urine sample	LOD [a], μg/L	MDC [b], μg/L
Non-hydrolyzed	0.66	50
Acid hydrolyzed	0.83	5
Alkaline hydrolyzed	0.99	20<MDC<50

The range correspond to the protocol where 10 mL urine sample is loaded to the HiTrap column, washed with 20% EtOH, and eluted in 3 mL 70% EtOH. [a] The $LOD_{7\%EtOH/PBS}$=0.2 μg/L is corrected with the corresponding IAC and hydrolysis recoveries. [b] MDC (maximum detectable concentration with recovery >70%)

Free 2,4,6-TCP can be determined with a LOD of 0.66 μg/L 2,4,6-TCP-IR equivalents in 10 mL non-hydrolyzed urine (if 90% IAC recovery is considered). Non-hydrolyzed urine can be extracted efficiently (recovery > 70%) until 50 μg/L (500 ng in 10 mL). For hydrolyzed urine the LOD is about 0.83 μg/L for acid and 1 μg/L for alkaline one (if 10 mL sample volume is loaded and recoveries from **Table 4.6** and **Figure 4.17** are considered). If acid hydrolysis is used, 2,4,6-TCP can be extracted efficiently in the range between 0.83 and 5 μg/L urinary concentration. In the case of alkaline hydrolysis quantitative analysis can be performed in the range 1 - 20 μg/L 2,4,6-TCP urinary concentration. As for higher concentrations the recovery is about 60%, those samples giving concentration values higher than 20 μg/L will have to be repeated after dilution of the urine with PBS.

The chlorophenols extracted from urine samples following the established IAC-SPE protocol can be analyzed also by GC-ECD (GC-MS) after toluene extraction and derivatization (see **Annex 1**). Thus, the concentration of the halogenated phenols mainly extracted by the IAC column can be determined. The LOD of the IAC-SPE-GC method depends on the hydrolysis recovery, the IAC recovery, the pre-concentration factor used in the toluene extraction and on the instrumental LOD. For example, if we consider the recoveries related to the IAC-SPE procedure, the instrumental LOD in the GC-ECD for

2,4,6-TCP (0.5 µg/L) and a pre-concentration factor of 5 in the toluene extraction, then the overall LOD of the IAC-SPE-GC-ECD would be 0.11 µg/L, 0.14 µg/L and 0.17 µg/L 2,4,6-TCP for NH, AH and BH urine respectively.

4.2.3.3. Validation of the IAC-SPE-ELISA for 2,4,6-TCP analysis in urine by GC-ECD

For a validation study the protocol described above was applied to the analysis of spiked urine samples (see **Figure 4.18**). 10 mL of NH and AH urine sample A was spiked at four levels 2, 5, 10 and 20 µg/L 2,4,6-TCP (corresponding to 1.6%, 4%, 8% and 16% IAC column loading). After IAC selective extraction the eluted fractions were diluted 10 times with PBS buffer and split in two parts: one part (0.5 mL) was measured directly by ELISA and the other part (29.5 mL) was extracted with toluene and after derivatization the chlorophenol concentration was determined by GC-ECD (see **Annex 1**). The data presented in **Figure 4.19.** clearly demonstrate the potential of the method developed. The linear regression analysis of hydrolyzed and nonhydrolyzed urine samples by both methods showed a very good correlation. Correlation between both techniques agrees to the following equation y=0.878x+1.19 with a regression coefficient $r^2 = 0.990$ for NH and to y=0.91x+0.56 with a regression coefficient $r^2 = 0.977$ for AH urine.

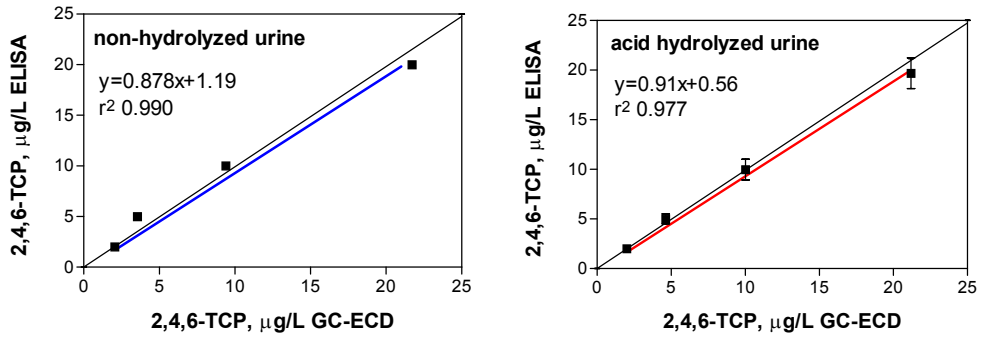


Figure 4.19. Validation of IAC-ELISA by GC-ECD. Urine samples (non-hydrolyzed and acid hydrolyzed) were spiked with 2,4,6-TCP and IAC purified. The eluted fractions were determined both by ELISA and by GC-ECD. For GC-ECD calibration toluene extracts of 2,4,6-TCP PBS standards were used. For high concentrations (10 and 20 µg/L 2,4,6-TCP) an IAC-recovery coefficient was applied according to **Figure 4.16**.

4.2.3.4. IAC-SPE application to urine samples from different individuals with not known exposure to 2,4,6-TCP

In the course of the IAC-SPE method development we used urine sample A collected from one individual. Finally, we decided to apply the optimized procedure to urine samples from different individuals with not known exposure to 2,4,6-TCP. Previously, we had shown that when urines from different individuals with different creatinine content affected on a different manner ELISA performance. In this case, we used seven urine samples (C, D, E, F, G, H, I) with the pH in the range from 5.7 to 6.5 and conductivity between 7.1 and 24.3 mS/cm. The creatinine content was determined according to the protocol described in the **Experimental Section**. As it is shown in **Table 4.8** the creatinine concentration covers a broad range (0.2 –2.3 g/L), thus the urine samples can be considered as representative in this validation study.

Table 4.8. Recoveries obtained for urines from non-occupationally exposed persons.

Urine sample	Creatinine [a], g/L	Recovery [b], %
C	0.2	109.25
D	0.3	115.5
E	0.8	116
F	1	89.25
G	1.3	81.75
H	1.5	85
I	2.3	80.25

10 mL acid hydrolyzed urine samples spiked with 5 μg/L 2,4,6-TCP (4% lading level) were purified by IAC-SPE (20%EtOH washing, 70%EtOH elution) and analyzed by ELISA.
[a] The creatinine concentration was determined according to the procedure described in the Experimental Section. [b] The recovery is calculated *vs* the spiked amount

The samples were hydrolyzed with H_2SO_4 and after IAC-SPE they were measured by ELISA. In this case it was possible to use AH since it was not expected to find any sample with concentration values higher than 5 μg/L. Thus, as expected in none of them 2,4,6-TCP was detected (LOD = 0.83 μg/L, see **Table 4.7**). Then 10 mL of each acid hydrolyzed urine sample were spiked with 50 ng 2,4,6-TCP (4% loading level) and analyzed by IAC-ELISA method. The recoveries obtained were higher than 80% (see

Table 4.8). These results demonstrate the versatility of the IAC-ELISA method developed and its applicability to urine samples of different origin.

In conclusion, in this chapter a standard protocol for an IAC-SPE-ELISA was established (see **Figure 4.18**). The LOD of the method as well as the maximum detectable concentration defined by the IAC column capacity and by the type of hydrolysis used (see **Table 4.7**) allow the quantitative determination of free and conjugated 2,4,6-TCP in the general population and in persons with occupational exposure to organochlorinated compounds**.** Every 10 loadings a quality control of the IAC-SPE procedure has to be made using PBS standards of 2,4,6-TCP. The IAC-SPE-ELISA method was validated with GC-ECD and is applicable to urine samples from different individuals.

5. Development of HTS-IAC-ELISA method for urinary detection of chlorophenols

The need for fast and efficient sample preparation methods in bioanalytical analysis is growing rapidly as it is often the bottleneck step avoiding speeding up the overall analysis process [345]. In the last years there is an increasing trend in developing parallel sample processing approaches. Although the 96-well format has been utilized for many years for immunoassays and in vitro binding assays, its use for SPE is relatively new. SPE devices in a 96-well plate format were introduced in 1996 and they enjoyed widespread application and rapid acceptance in biotechnology and pharmaceutical laboratories where high-throughput screening (HTS) is seeking. The traditional bulky SPE cartridges have

been replaced by small-volume SPE cartridges with a packed bed lower than 50-100 ng and by membrane-extraction discs. The 96-well SPE formats have been designed to fit automated plate handling system and to reduce the void volume as well the sorbent bed masses in order to have the minimum desorption volume. In addition to these advantages (fast sample preparation, parallel processing of 96 samples at once, automation, and volume reduction) the simultaneous sample processing overcomes the problems with analyte/biological sample stability found in serial processing methods.

Sample preparation in the 96-well format is mainly used in conjunction with liquid chromatography- tandem mass spectrometry (LC-MS-MS) [346] or coupled to optical detection [347]. The commercialisation of 96-channel robotic liquid handling workstations [348] as well as the wide selection of 96-well SPE sorbents [349] afford the rapid development and automation of SPE methods to eliminate traditional time-consuming and labor-intensive sample preparation steps for environmental water [350], plasma [351-353] and urine samples [354,355].

All these applications of 96-SPE formats are based on non-selective SPE sorbents (C_2, C_8, C_{18}, ion-exchange, etc.). However, the trends in SPE-research are not only oriented towards reduction of the SPE format and the automation for a high throughput, but also towards the development of more selective extraction procedures, such as those using immunoextraction sorbents [322]. There are no many examples in the literature regarding immunoaffinity extraction performed in 96-well format. A high-throughput mass spectrometric immunoassay system for the analysis of proteins directly from plasma has been reported recently [356]. In this work a 96-well format robotic workstation has been used to prepare antibody-derivatized affinity pipette tips for subsequent use in the extraction of specific proteins from plasma and deposition onto 96-well format MALDI-TOF-MS targets. The approach represents a rapid (~ 100 samples/2h, reagent preparation-to-data) and accurate means of characterizing specific proteins present in large numbers of individuals for proteomic and clinical/diagnostic purposes.

Another rapid screening technique in 96-well format is the Enzyme-Linked Immunofiltration Assay (ELIFA) [357,358]. Although immunofiltration implies similarities to immunoaffinity extraction, it is not used as a clean up procedure and it can be regarded as an own immunoassay format. The immunofiltration methodology is based on the use of membranes with immobilized Abs, which are subject to a usual competitive immunoassay with colorimetric detection. It has been applied for semi-quantitative and quantitative determination of several compounds in environmental, food and clinical samples [359,360].

In the previous chapter we have described the development of an IAC-SPE-ELISA method for urinary determination of 2,4,6-TCP. Although the ELISA analysis allows the simultaneously quantification of 96 samples, the overall method was limited by the tedious, manually performed immunoaffinity urine extraction in single column format. To meet the ever-increasing demands for higher throughput in biological monitoring (clinical analysis), it is crucial to streamline all aspects of the process, from sample treatment to analyte measurement. Therefore, we have approached the development of HTS-IAC-SPE (96-column format), which would allow the direct quantification of the 96 urine extracts by subsequent ELISA. We have chosen the Versaplate 96-Well SPE system (Varian, Palo Alto, CA) because it permits the cartridges to be easily snapped into a separate 96-well base plate [361,362]. Furthermore, the system is available with separate empty columns, which can be packed with the desired immunosorbent.

Transferring methods from standard SPE cartridges to 96-well format is often nontrivial. Differences in packing and sorbent bed dimensions, in pressures and flow-rates can create significant differences in method performance. Therefore, a re-optimization procedure should be undertaken. Furthermore, the HTS-IAC-SPE method development should include inter- (intra-) day precision and recovery evaluation of matrix samples at different concentration levels.

5.1. Immunosorbent preparation

As solid support for HTS-IAC-SPE we have used NHS-activated Sepharose 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden). It is a bead-formed highly cross-linked 4% agarose matrix, quite similar to the phase of the commercial HiTrap column used in the previous chapter, but with approximately two times higher substitution level (16-23 μmol NHS/mL drained gel). The IgG fraction of As45 was covalently immobilized to the Sepharose gel (see **Experimental Section**) keeping the ligand density (ratio Ab mg/NHS μmol) the same as in the single column coupling. Throughout this thesis the Ab coupling was performed at different scale (using 1 mL, 5 mL, 12 mL and 24 mL Sepharose suspension) with a coupling efficiency at about 97% in all cases. Using the prepared immunosorbent we have packed 96 mini-IAC columns with 0.2 mL drained gel bed (see **Experimental Section** and **Figure 5.1**). This corresponds to maximum theoretical binding capacity for each mini-column (0.2 mL bed) of approximately 1 μg (5.1 nmol) 2,4,6-TCP, based on the amount of IgG coupled (9.7 mg) and the assumptions that bivalent binding takes place, 10% of the polyclonal IgG are specific and 100% of the immobilized IgG are accessible. If we assume a 50% steric hindrance or wrong Ab orientation, the theoretical binding capacity would be: 0.5 μg (2.22 nmol) 2,4,6-TCP.

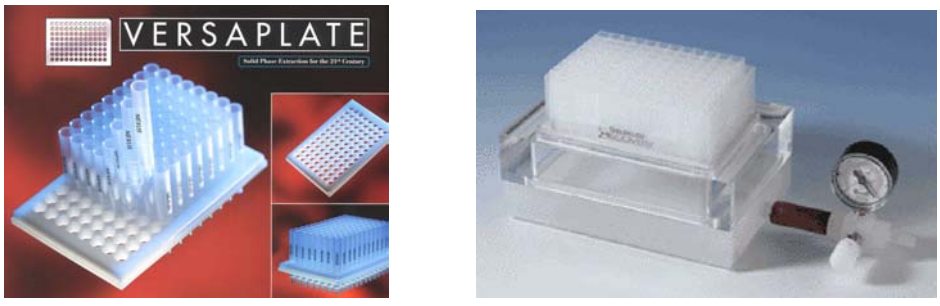


Figure 5.1. VersaPlate 96-SPE System

5.2. Characterization of mini-IAC columns. Optimization of HTS-IAC-SPE procedure with PBS standards of 2,4,6-TCP

The prepared mini-IAC columns were assembled in a 96-format SPE vacuum manifold (VersaPlate System, Varian) (see **Experimental Section** and **Figure 5.1**). The detailed general HTS-IAC-SPE procedure is described in details in the **Experimental Section**. All steps of the IAC cycle were performed using a multichannel pipette and controlling the applied vacuum to maintain constant flow rate in the range of 1-2 mL/min.

5.2.1. Evaluation of the binding capacity of mini-IAC columns

Taking in consideration the similarities between the HiTrap (1 mL) and the mini-IAC columns (0.2 mL) we performed an initial evaluation with PBS standards of 2,4,6-TCP with the aim to adapt the IAC-SPE procedure established above to a smaller scale. Based on our experience with the IAC-SPE development we used initially PBS for washing and 70% EtOH for elution. We investigated the elution profile for four different loading levels corresponding to 150% (0.75 μg), 100% (0.5 μg), 20% (0.1 μg) and 2% (0.01 μg) of the theoretical immunosorbent capacity. The sample volume loaded in these experiments was 6 mL. The concentration of 2,4,6-TCP was determined by ELISA in the loading fraction collected after passing the mini-column (L), in the washing fractions (W) and in the the elution fractions (F). Each collected fraction was 0.2 mL (1 bed volume). The recoveries obtained are presented in **Figure 5.2**. When the column is loaded with 750 ng 2,4,6-TCP, 20% of this amount is not retained by the IS and it is detected in the fraction collected after loading (L); about 12% is washed with PBS buffer (W) and the remaining amount (69.7%) is eluted resulting in a total recovery of 101%. A small amount of 2,4,6-TCP was also detected in L and W fractions when the mini-columns were loaded with 500 ng. However, the recoveries in the elution fractions were still high (90%). At a loading concentration lower than 0.5 μg, the analyte is completely retained by the immunosorbent: 2,4,6-TCP is not detected in the volume collected after loading of

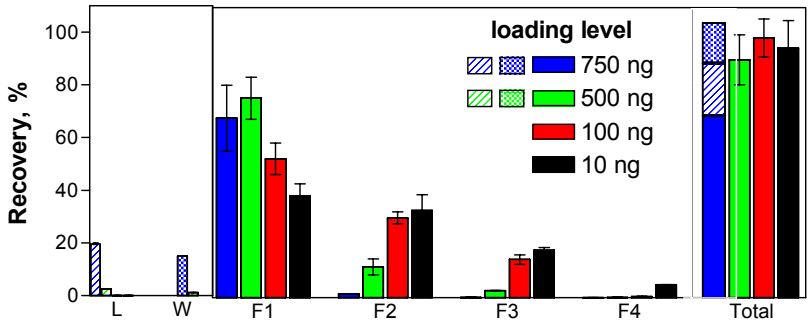


Figure 5.2. Elution profile of 2,4,6-TCP IAC-SPE by mini-IAC columns (VersaPlate System). PBS standards 6 mL (750 ng, 500 ng, 100 ng, 10 ng 2,4,6-TCP) were loaded to the columns, washed with PBS and elute with 70%EtOH. Eluted fractions of 0.2 mL were collected. Each was diluted with PBS buffer and measured on the ELISA using standard curve run in 7%EtOH/PBS. The 6 mL collected fraction after loading (L) and washing fractions (W) (6 mL) were determined directly by ELISA standard curve run in PBS. Recovery is calculated *vs* spiked amount of 2,4,6-TCP. Data correspond to the average of three mini-IAC columns processed simultaneously.

the standard (L) neither in the collected washing fractions (W) and the eluted amount corresponds to a complete recovery (around 95%). Therefore, the experimentally determined maximum binding capacity corresponds to the theoretically evaluated value of 0.5 μg 2,4,6-TCP.

Similarly to the HiTrap column, the elution profile of the mini-IAC columns is influenced by the loading level (compare **Figure 5.2** to **Figure 4.10**) and a volume, three times the column volume (3 x 0.2 mL = 0.6 mL) was also needed to ensure complete recovery of the trapped analyte from the immunoextraction column. Therefore, we decided to collect 0.6 mL of the elution fractions in further experiments.

As the mini-IAC columns were packed manually, it was important to evaluate the variation in the capacity of different columns. We designed an experiment where different columns placed in different positions of the rack holder were loaded with different amounts of 2,4,6-TCP. In **Table 5.1** are presented the recoveries obtained for HTS-IAC-SPE of 2,4,6-TCP from PBS standards of different concentration.

Table 5.1. Recovery obtained for HTS-IAC-SPE of 2,4,6-TCP from PBS standards (PBS washing, 70%EtOH elution in 0.6 mL).

Loading level, %	2,4,6-TCP, ng	N [a]	Recovery ± SD, %
100%	**500**	10	101.05 ± 4.74
20	**100**	3	84.81 ± 13.25
2.4	**12**	16	97.75 ± 16.84
0.72	**3.6**	16	95.77 ± 26.04

[a] N is the number of mini-IAC columns used

It should be noted that at the maximum capacity the columns were very reproducible (%CV = 4.69, N=10). The highest CV of 27.19% (N=16) was observed for the lowest loading level (0.72%). In general, we can conclude that, in spite of the manual packing, the reproducibility within columns was very good.

5.2.2. Effect of EtOH washing for different loading levels

In single column IAC-SPE we determined that 2,4,6-TCP starts to elute at 30% EtOH, when a 4% loading level was applied (see **Figure 4.9**). This allowed washing the HiTrap column with 20% EtOH to achieve higher specificity. The smaller scale of HTS-IAC (smaller maximum capacity of the mini-IAC columns and smaller sample volume to be loaded, 6 mL) required a more rigorous evaluation of the effect of EtOH in the washing step. Taking advantage of the fact that in HTS-IAC simultaneous processing of many columns is possible, we decided to define more precisely the ethanol content under which the desorption starts for several loading levels (100%, 40%, 10%). Three 2,4,6-TCP standards (0.5 μg, 0.2 μg and 0.05 μg 2,4,6-TCP in 6 mL PBS each) were loaded in triplicate. We applied a step elution increasing the EtOH concentration from 10% to 70% and collecting 0.6 mL for each EtOH concentration. All columns were processed simultaneously using the VersaPlate System. The recoveries calculated based on the ELISA determination of the 2,4,6-TCP eluted in each fraction are presented in **Figure 5.3**. The higher the loading level, the lower the EtOH concentration needed to start the elution. For example, at 100% loading level 10% of the loaded amount is already eluted with 10% EtOH, whereas at 10% loading, 30% EtOH is needed to start the elution. This could be explained by the heterogeneity in the affinity of the polyclonal Abs of the

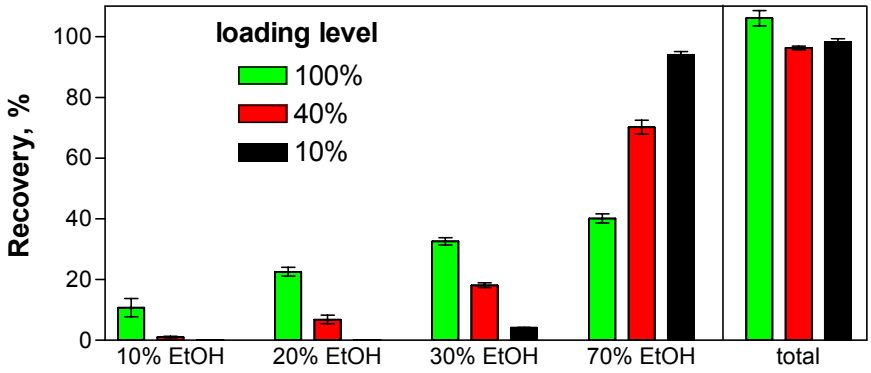


Figure 5.3. Step elution of 2,4,6-TCP with different EtOH concentration in the elution buffer in HTS-IAC-SPE (VersaPlate System). PBS standards 6 mL (500 ng, 200 ng, 50 ng 2,4,6-TCP) were loaded to the mini-IAC columns. The column was washed first with 0.6 mL 10%EtOH, then with 0.6 mL 20% EtOH, followed by 0.6 mL 30% EtOH and finally eluted with 0.6 mL 70%EtOH. Each collected fraction (0.6 mL) was diluted with PBS buffer and measured on the ELISA using standard curve run in 1%, 2%, 3% and 7%EtOH/PBS respectively. Recovery is calculated *vs* spiked amount of 2,4,6-TCP. Data correspond to the average of three mini-IAC columns processed simultaneously.

immunosorbent. At high loading levels all the Abs (low and high affinity) are participating in the immunoextraction and at low EtOH concentration part of the interactions (the weak ones) are disrupted. At low loading levels only high affinity Abs trap the analyte from the sample, thus higher EtOH concentration is necessary to disrupt Ab-Ag interaction.

From practical point of view, the washing step could be performed with 20% EtOH without loss of analyte until 10% loading level (50 ng, 6 mL, 8 μg/L). This result agrees with the observation in single column IAC-SPE, where for 4% loading level the elution starts at 30% EtOH. At 40% loading level (200 ng, 6 mL, 32 μg/L) 6.83% of the loaded analyte can be washed with 20% EtOH. However, 32 μg/L is a quite high concentration only possible when monitoring groups of population with very high risk of exposure. Therefore, we decided to use a 20% EtOH as washing buffer in the HTS-IAC-SPE procedure.

5.2.3. Column stability

In the previous chapter we have shown that the 70% EtOH elution does not affect the capacity of the HiTrap column and it can be regenerated and used for up to 55 cycles without loss of stability (see **Figure 4.12**). Thus, we expected that the mini-IAC columns prepared with the same immunosorbent, would have similar stability. As we did with the HiTrap column, we included regularly cycles where quality controls (QC) at different loading levels were applied to the mini-columns. In **Figure 5.4.** is presented the long-term stability of three mini-IAC columns monitored by loading them with three levels of 2,4,6-TCP in PBS (0.5, 0.1, 0.05 and 0.01 μg) corresponding to 100%, 20%, 10% and 2% loading levels.

As it is shown in the figure, quantitative recovery (in the range of 80-100%) was

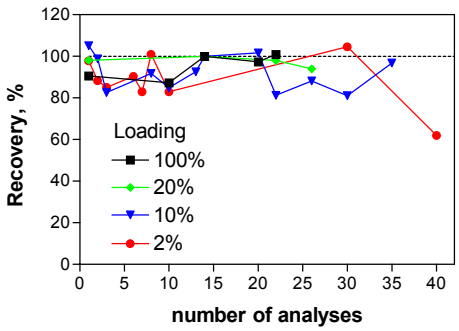


Figure 5.4. Capacity of mini-IAC columns as a function of the number of analyses. PBS standards 6 mL (500 ng, 100 ng, 50 ng, 10 ng 2,4,6-TCP) were loaded to the mini-IAC columns and eluted with 70%EtOH. Recovery is calculated *vs* spiked amount of 2,4,6-TCP. Data correspond to the average of three mini-IAC columns processed simultaneously.

maintained for up to 35 analyses for all levels. Therefore, the IAC capacity was not affected adversely and the columns can be efficiently regenerated and used for many cycles of urinary chlorophenol extraction. However, in HTS-IAC-SPE washing and elution procedures are performed until the columns get dried (in order to avoid error in the collected volume), which can have negative effect on the immunosorbent stability. In addition, the back pressure formed is not equal for all the columns, because different resistance is created by the manually put frits (different packing). The applied pressure to

all the mini-columns in the 96-rack is not homogeneous. Some columns get more dried under elution. All these can create problems with column stability. We have observed that the recovery of 6% from the 96 mini-IAC columns prepared (at 2.4% lading level) became lower than 40% after 30 cycles. This drawback of the HTS-IAC format can be improved with better packing of the columns or using more resistant sorbent.

5.3. Application of HTS-IAC-SPE to urine samples

In the previous chapter we have demonstrated that the concentration and the volume of the urine sample and also whether it is hydrolyzed or not affect the extraction recovery of 2,4,6-TCP in the IAC-SPE. No breakthrough effects until 50 mL were observed for non-hydrolyzed urine at low loading levels corresponding to the concentration usually found in the general population. In contrast, a breakthough volume of 30 mL was observed for acid hydrolyzed urine (see **Section 4.2.3.2**). As in biological monitoring studies it is important to determine the total chlorophenol concentration, here we have focused our studies only on hydrolyzed urine. Considering the effect of the type of the hydrolysis on the single IAC-SPE procedure (see **Figure 4.17**), here we decided to apply only alkaline hydrolyzed urine. Because of the smaller scale of HTS-IAC-SPE set up it was important to define the effect of the sample volume and the 2,4,6-TCP concentration on the recovery. The evaluation of the HTS-IAC-SPE was realized using the pooled urine sample B. We have already discussed the matrix effect of the alkaline purified urine by mini-IAC columns on the 2,4,6-TCP ELISA (see **Figure 4.13 D, G**).

5.3.1. Recovery studies. Effect of loading level, breakthrough volume

Alkaline hydrolyzed urine B was spiked with three different amounts of 2,4,6-TCP (200 ng, 75 ng, 20 ng) corresponding to 40%, 15% and 4% loading level. Each amount was loaded in different volumes (6 mL, 12 mL and 18 mL) to the mini-IAC columns. The immunoaffinity extraction was performed washing with 20% EtOH and eluting with 0.6 mL 70% EtOH. The 2,4,6-TCP concentrations were determined by ELISA. The recovery was calculated considering the background level of 2,4,6-TCP-IR equiv. in the hydrolyzed urine sample B according to GC-MS (0.24 μg/L) and is presented in **Figure 5.5**. It can be observed that the breakthrough volume is placed between 12 and 18 mL for the three loading levels tested. If 12 mL sample was used, quantitative recovery (>80%) could be achieved for concentrations lower or near 6.25 μg/L (75 ng, 15% loading level). When the loading level reached 40% of the maximum capacity (200 ng) using the same volume, the recovery already dropped to 70%. On the other hand, if 6 mL sample was used, efficient extraction without loss of analyte could be achieved for concentrations until 33 μg/L 2,4,6-TCP (200 ng, 40% loading level).

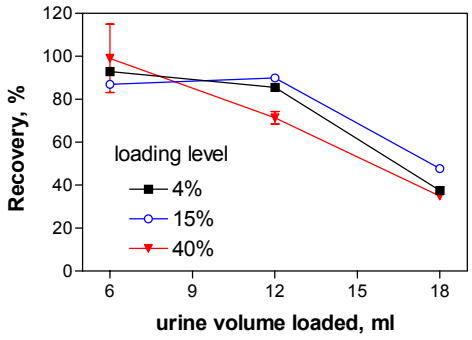


Figure 5.5. Effect of sample volume and loading level on the recovery of mini-IAC columns forming part of HTS-IAC-SPE. Alkaline hydrolyzed urine sample B was spiked with different amount of 2,4,6-TCP (200 ng, 75 ng and 20 ng) and loaded at different volumes, washed with 20% EtOH and eluted with 0.6 mL 70% EtOH. The concentration was determined by ELISA. Recovery is calculated *vs* spiked amount of 2,4,6-TCP resting the background level of TCP-IR in urine sample B. Data correspond to the average of three mini-IAC columns processed simultaneously.

Lower sample volume means more rapid extraction. Thus, we selected 6 mL as appropriate volume to be loaded, since it ensures the quantitative recovery of the analyte even at concentrations present in exposed persons. Extraction in 0.6 mL and further 10-fold dilution with PBS buffer will result in no pre-concentration neither dilution of the initial analyte concentration. Therefore, the overall LOD of the HTS-IAC-ELISA/GC analysis will be determined mainly by the LOD of the method used for analysis. In the case of ELISA (7% EtOH/PBS) the LOD would be around 0.2 μg/L 2,4,6-TCP-IR equivalents considering a 100% recovery in the purification process. If GC method was used each extracted chlorophenol would be detected with the corresponding LOD (see **Annex II**). **Figure 5.6** shows the scheme of the finally established procedure for the analysis of 2,4,6-TCP in urine samples. About 12 mL of urine are sufficient to extract chlorophenols in duplicate and to analyze them by ELISA (in triplicate) and to compare the result with GC-MS or GC-ECD. After ELISA measurements the remaining 7% EtOH/PBS solution can be extracted with toluene and derivatized.

Urine samples with negative ELISA response will indicate a level of exposure lower than the LOD (about 0.2 μg/L). Urine samples giving values between 0.2 and 30 μg/L could in principle be accurately quantified. Finally, urine samples with concentration values near or higher than 30 μg/L, will have to be repeated with a lower load (e.g. 3 mL urine) for a quantitative recovery and accurate analysis.

5.3.2. Validation of HTS-IAC-ELISA method

5.3.2.1. Precision and accuracy of the urine analysis by HTS-IAC-ELISA

The objective of the experiments presented here was to determine the precision and the accuracy of the whole procedure (HTS-IAC-ELISA) taking into account column variability (each column could behave differently), position of the columns in the 96-column rack (flow-rate could vary depending on the position in the rack) and day-to-day

variability. With this purpose the following analyses were performed in three separate days according to the HTS-IAC-ELISA procedure described in **Figure 5.6**.

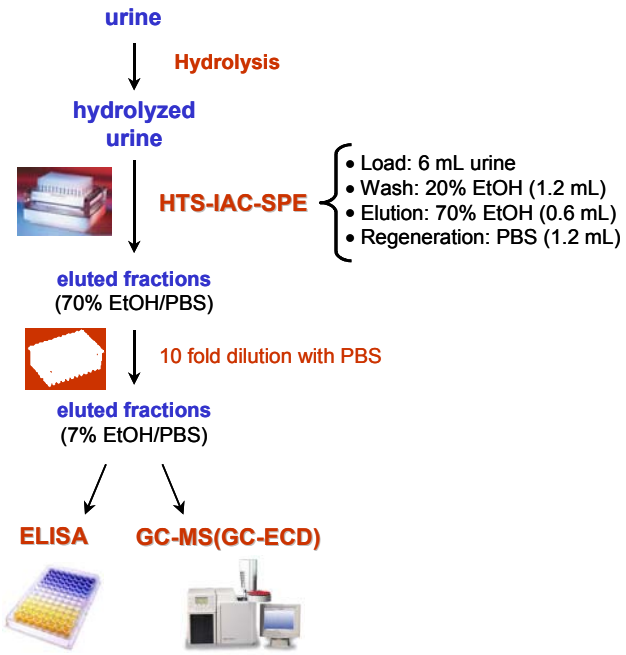


Figure 5.6. Procedure established for HTS-IAC-SPE (VersaPlate System) of 2,4,6-TCP from urine samples. The 2,4,6-TCP concentration in the extracted urine can be determined by ELISA or by gas chromatography technique (GC-MS or GC-ECD).

One experiment consisted of the simultaneous analysis of 3 sets of samples. Each set was composed of non-spiked urine samples and quality controls (QCs) of defined 2,4,6-TCP concentrations in PBS buffer (*b*QCs) and in urine (*u*QCs). The *u*QCs were prepared in pooled urine B after its alkaline hydrolysis in big batch (200 mL). Both *b*QCs and *u*QCs were prepared at three 2,4,6-TCP-concentration levels: low (LQC - 0.7 µg/L), medium (MQC - 2 µg/L) and high (HQC - 8 µg/L) that correspond to IAC-loading levels 0.84%, 2.4% and 9.6% respectively. Loading of the 96-column rack was performed according to the design shown in **Figure 5.7**. Thus, urine QCs (*u*LQC, *u*MQC, *u*HQC), buffer QCs (*b*LQC, *b*MQC, *b*HQC) and non-spiked urine samples were loaded in 24, 3 and 15 replicates, respectively. Some of the non-spiked urine samples were spiked in triplicate after the HTS-IAC-SPE procedure. All extracted samples were measured on ELISA using

standard curve run in 7%EtOH/PBS. This analysis was repeated in three consecutive days varying the sample distribution through the 96-IAC plate. The precision and accuracy data for the buffer and urine QC samples are summarized in **Table 5.2.**

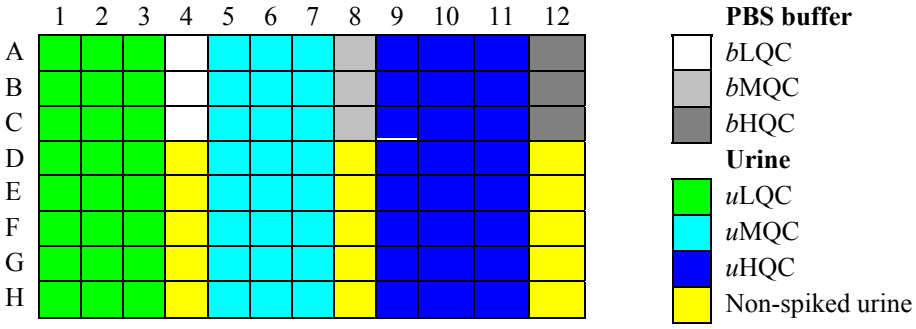


Figure 5.7. Experimental scheme for precision and accuracy study of HTS-IAC-SPE urine clean-up. Quality controls at three concentration levels were used: LQC - 0.7 µg/L, MQC - 2 µg/L and HQC - 8 µg/L 2,4,6-TCP. Buffer quality controls (*b*LQC, *b*MQC, *b*HQC) were prepared in PBS buffer. Urine quality controls (*u*LQC, *u*MQC, *u*HQC) were prepared in alkaline hydrolyzed urine sample B.

Table 5.2. Precision and accuracy of the 2,4,6-TCP analysis by HTS-IAC-SPE urine clean-up followed by ELISA.

	urine QCs				buffer QCs			
Spiked concentration, µg/L	**N** [d]	**0.7**	**2**	**8**	**N** [d]	**0.7**	**2**	**8**
Mean	**72**	0.61	1.55	7.06	**9**	0.65	1.74	7.62
Mean recovery [a]**, %**	**72**	87.5	77.4	88.3	**9**	92.7	87.2	95.3
Interday precision (% CV)[b]	**24**	22.9	20.6	17.4	**3**	17.1	19.2	13.7
Intraday precision (% CV)[c]	**72**	11.3	6.2	10.4	**9**	5.4	9.35	12.5

[a] The recovery for urine QCs is calculated as [(pre-extracted spiked urine)/(post-extracted spiked urine)]*100; The recovery buffer QCs is calculated as (measured/nominal value)*100. [b] The % CV is the average of the % CVs for each concentration for each day (within one 96-SPE procedure). [c] The % CV corresponds to the recovery obtained for each concentration for three days (between three 96-SPEs). [d] N is the number of mini-IAC columns used for each concentration.

The recovery (accuracy) for the buffer QCs was determined as the ratio of the measured *vs* the nominal value. The recovery for urine QCs was assessed by comparing the concentration of 2,4,6-TCP extracted from spiked urine samples (pre-extraction spike) to the non-spiked urine samples processed identically and spiked with the corresponding concentration after their processing (post-extracted spike). The results show that the method is consistent and reliable. The accuracy (recoveries >80%) allows quantitative

determination of 2,4,6-TCP in urine samples by HTS-IAC-SPE coupled to ELISA. Both inter- and intra-assay precision (% CV) are lower than 20% except in the case when the measurements takes place at the ELISA limit of detection.

No significant difference between urine concentration obtained using the HTS-IAC-SPE method and the previous traditional single column IAC-SPE method was observed. Thus, the TCP-IR level of urine sample B was 0.9 μg/L determined by the single IAC column – ELISA protocol (see **Table 4.5**) and 0.81±0.13 μg/L (N=6) evaluated by HTS-IAC-ELISA (the HTS-IAC was performed in three different days in 4 replicate each day).

5.3.2.2. Analysis of urine samples from different individuals. Validation ELISA/GC-MS of the HTS-IAC-SPE procedure.

About 117 urine samples from different individuals that belong to various occupational groups were used to validate the HTS-IAC-SPE-ELISA procedure by comparing the 2,4,6-TCP-IR equivalents measured by this method with the concentration of immunoreactive chlorophenols found when measuring the same samples by GC-MS. We selected MS detection in order to identify and confirm the presence of halogenated phenols in the urines that are the main ELISA cross-reactants.

The urine samples from different individuals were analyzed following the procedure described in **Figure 5.6**. The samples were hydrolyzed with KOH and processed following the HTS-IAC-SPE protocol. The eluted fractions (70% EtOH) were diluted 10 times with PBS and split in two parts. About 500 μL were sufficient for the ELISA analysis in triplicate and the rest of the sample was extracted with toluene and derivatized with BSTFA to identify and quantify the cross-reactants (2,4,5-TCP, 2,4-DBP, 2,3,4,6-TtCP and 2,4,6-TBP) by GC-MS (see **Annex 1**).

The correlation between the values determined by ELISA (2,4,6-TCP-IR equivalents) and the concentration of 2,4,6-TCP determined by GC-MS indicates a significant

overestimation by the ELISA method (see **Figure 5.8.A**). This result is not surprising, because the immunoassay response corresponds not only to the concentration of 2,4,6-TCP, but also to the concentration of the cross-reactants present in the sample. If we consider the concentration of each cross-reactant determined by GC-MS analysis and the cross-reactivity of the 2,4,6-TCP ELISA (see **Table 3.9.B**), assuming similar IAC-SPE extraction recovery, we can calculate the 2,4,6-TCP-IR equivalents that will contribute to the ELISA response. Thus, the GC-MS 2,4,6-TCP-IR equivalents were obtained as the sum of the % CR multiplied by the cross-reactant concentration determined by GC-MS for each cross-reactant in the sample. The correlation between the 2,4,6-TCP-IR equivalents determined by ELISA and by GC-MS (ELISA TCP-IR and GC-MS TCP-IR) is shown in **Figure 5.8.B** and it indicates an excellent correlation between both techniques following the equation y=1.14x-0.21 with a regression coefficient r^2=0.912. From these experiments we can conclude that the HTS-IAC-ELISA procedure developed in this thesis can be an excellent tool to assess risk of exposure analyzing urinary biomarkers. In this case, the HTS-IAC-ELISA method is addressed to the efficiently screening of chlorophenols in urine as potential biomarkers of exposure to certain organochlorine compounds or to dioxins. The usefulness of 2,4,6-TCP as biomarker has not been demonstrated yet, although as mentioned in the introduction many research groups have reported the appearance of this urinary metabolite in certain occupationally exposed groups or in the general population due to the contact with residues with herbicides or preservatives metabolized to chlorophenols.

In **Table 5.3** are summarized the main features of the HTS-IAC-ELISA method compared to the single column IAC-SPE-ELISA method. The significant increase in sample throughput, the decrease of urine sample volume required for the assays and the absence of dilution in the clean-up step, are the main advantages of the HTS-IAC-SPE. The method developed allows the processing at about 100 samples/day with overall LOD of 0.3 μg/L 2,4,6-TCP-IR equivalents. About 6 mL of urine are sufficient to provide enough replicates for ELISA, even performed in different days and to compare the result in the case of positive result, by a reference method such as GC-MS. The selective extraction results in no false positive results.

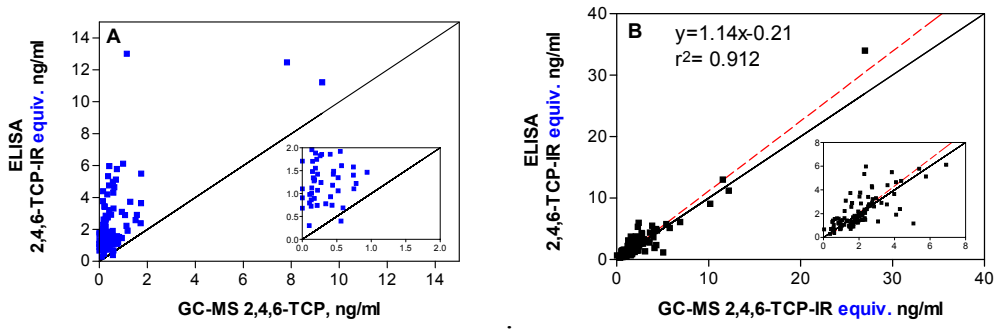


Figure 5.8. Validation HTS-IAC-SPE-ELISA/HTS-IAC-SPE-GC-MS. Urine samples from different individuals (N=117) were hydrolyzed by KOH and their 2,4,6-TCP concentration was determined by HTS-IAC-SPE coupled to ELISA. Cross-reactants in purified urines were identified and quantified by GC-MS. **A**. Correlation between 2,4,6-TCP-IR equivalents determeined by ELISA and 2,4,6-TCP concentration determined by GC-MS. **B**. Correlation between 2,4,6-TCP-IR equivalents determined by ELISA and by GC-MS. The GC-MS TCP-IR are defined as ∑(%CR x cross-reactant concentration determined by GC-MS).

Table 5.3. Features of the urinary analysis of 2,4,6-TCP by single column IAC-ELISA and HTS-IAC-ELISA

Parameters	single IAC-ELISA	HTS-IAC-ELISA
sample volume	10 mL	6 mL
clean-up	3-fold dilution	no dilution
speed of IAC clean-up	96 samples / **8 days**	96 samples/ **1 h**
speed of total analysis	96 samples / **9 days**	96 samples/ **1 day**
LOD [a]**, µg/L**	0.99	0.3
LOQ [b]**, µg/L**	1.4	0.55
MDC [c]**, µg/L**	20<MDC<50	30
% CV [d]	5.1% - 18.4% for 5 - 30 µg/L	17.4% – 22.9% for 0.7 – 8 µg/L
Number of false positives	0	0
Number of false negatives	0	0

[a] The limit of detection is evaluated according to the LOD of the ELISA ($LOD_{7\%EtOH/PBS}$=0.2 µg/L, 90% of the signal at zero analyte concentration) and the corresponding IAC an hydrolysis recoveries. [b] The limit of quantification is evaluated according to the LOQ of the ELISA ($LOQ_{7\%EtOH/PBS}$=0.37 µg/L, 80% of the signal at zero analyte concentration) and the corresponding IAC an hydrolysis recoveries. [c] MDC (maximum detectable concentration with recovery >70%)
[d] The coefficient of variation (% CV) corresponds to the interday analysis.

The availability of efficient and reliable biomonitoring tools such as HTS-IAC-ELISA may help to collect sufficient toxicological and exposure data to assist on the establishment of biomarkers and also to assess risks derived from exposure.

5.3.4. Preliminary case-study biomonitoring of chlorophenols in different population groups

We applied the HTS-IAC-ELISA method to the biological monitoring of three population groups living in two different towns of Cataluña, Spain. The people under study belong to different employment sectors: a petrochemical industry (town A, N=43 samples collected in 2001), services (town B, N=41 samples collected in 2002) and general population living in the vicinity of a waste incinerator (town B, N=33 samples collected in 1999). No additional information on the health status and particular habits of these individuals was available for us at the time when this study was performed.

The urines were treated following the procedure described in **Figure 5.6**. Each urine sample was analyzed by ELISA and by GC-MS after chlorophenol extraction in duplicate by HTS-IAC-SPE. PBS standards of 2,4,6-TCP (2 and 5 μg/L) were used as quality controls to test the HTS-IAC-SPE procedure. The recovery for the control samples was 89.77 ± 12.86%. The ELISA LOD (90% inhibition) was 0.2 μg/L (0.16 ug/g creat) and the LOQ (80% inhibition) was 0.6 μg/L. According to our procedure the urinary LOD of the GC-MS method (see **Annex 1**) was at about 0.1 μg/L (0.08 ug/g creat) for 2,4,6-TCP and 2,4,5-TCP and 0.2 μg/L (0.16 ug/g creat) for 2,4-DBP, 2,3,4,6-TtCP and 2,4,6-TBP.

To get accurate data on the chlorophenol excretion by the general population it was one of our concerns to avoid contaminations of the biological specimen. As phenolic compounds were not detected in the blanks, we assume that chlorophenol excretion of the examined population is really caused by environmental uptake of these substances themselves or halogenated compounds transformed to phenolic substances in the human body.

The primary analytical variable to be considered in biomonitoing is the percentage of specimens with levels greater than or equal to the limit of detection of the method used. We observed that according to ELISA determination 92% of both petrochemical and services workers groups and practically all urine samples from the general population living in the surroundings of a waste incinerator had detectable concentrations of 2,4,6-TCP-IR equivalents. Moreover, the GC-MS analysis of the urine purified by HTS-IAC-SPE confirmed this fact and revealed the presence of chloro- and bromo-phenols in the three groups. The frequency of detection (positive sample *vs* total number of samples) for the extracted halogenated phenols is present in **Table 5.4**.

Table 5.4. Frequency of detection (%) of all the analytes determined by HTS-IAC-SPE-GC-MS for the three groups examined.

Compound	Petrochemical workers	Services workers	General population close to incinerator
2,4,6-TCP	100	89	86
2,4,5-TCP	57	57	52
2,3,4,6-TtCP	79	95	93
2,4-DBP	60	76	79
2,4,6-TBP	81	100	100

The frequency of detection corresponds to the number of positive samples (with concentration higher than the LOD) with respect the total number of samples analyzed for each group. LOD for TCPs is 0.1 μg/L and for 2,3,4,6-TtCP, 2,4-DBP and 2,4,6-TBP it is 0.2 μg/L.

It is striking that all the analytes were detected in more than 50% of the population, likely indicating frequent exposure of the general public to the parent compounds or their derivatives. Among the chlorophenols 2,4,6-TCP and 2,3,4,6-TtCP are most widely distributed (>80%). 2,4,5-TCP was found in 50-57% of the population. There is no significant difference between the three groups studied. For comparison, the frequency of detection of trichlorophenols in USA adults in 1995) was 20% for 2,4,5-TCP (LOD 1 μg/L) and 9.5% for 2,4,6-TCP (LOD 2 μg/L) [75]. It is remarkable that our study led to similar results regarding 2,4,6-TCP: we found that 19% of the petrochemical workers, 2.7% of the office workers and 6.9% of the incinerator vicinity group had concentrations higher than 1 μg/L 2,4,6-TCP (for the three groups the average is 10% 2,4,6-TCP higher than 1 μg/L). However, 2,4,5-TCP was less distributed in our populations: about 3% of

the individuals from the three groups had levels higher than 1 μg/L. A higher frequency of detection was observed for the general population living in Germany: 54% for 2,4,5-TCP (LOD = 0.8 μg/L) and 37% for 2,4,6-TCP (LOD = 1.2 μg/L) [121]. However, it must be noted that the most important companies producing chlorophenols were located there (e.g. BASF) and the frequency of detection may be influenced by many other factors, such as geographical region, nutrition habits, etc.

It must be pointed out that all chlorophenols found in the urine samples are environmental contaminants and may be introduced in the human body through the contact with the sources of exposure or through edible products. As was mentioned in the **Introduction** 2,4,5-TCP and 2,4,6-TCP are also reported to be metabolites of HCB, lindane, pentachloronitrobenzene, PCP, 1,3,5-trichlorobenzene, pentachlorobenzene, 1,2,4-trichlorobenzene which are ubiquitous contaminants in the environment. TtCP detected in the urine samples are supposed to result from the same sources as TCP. Particularly, for the general population living in town B urinary PCP and TtCPs (2,3,4,6- and 2,3,5,6-) were found in all samples in a study performed in 1986 [128]. In the same study urinary TCPs were not detected, but HCB and PCP were present in all serum samples tested at levels higher than those reported in other countries (e.g. England) reflecting that HCB was one of the main pollutants in town B. Unfortunately, we have not find more recent studies regarding organochlorine exposure in this area. Furthermore, the levels of PCDD/Fs in blood and adipose tissue [91,363,364] and human milk [94] of the general population of town A have been found to be similar or higher than the levels reported for other populations (Madrid, Germany, Sweden, USA, Canada). High intakes of seafood and smoking habits should also be considered.

It was surprising the presence of bromophenols in the urine samples studied. These pollutants have not been subject of serious risk assessment studies until now, but recently they have been defined as emerging environmental contaminants. Tribromophenol is being used as flame retardant in plastics and wood products. It is detected in river waters [365], in sewage sludge from wastewater treatment plants [366], in food [367], etc.. Bromophenols are considered degradation products and metabolites of brominated flame

retardants (polybrominated diphenyl ethers, polybrominated biphenyls, etc.) and are widely distributed in the environment (for recent reviews see [315,368,369]). Furthermore, one should consider that 1600 naturally occurring organobromine compounds are known today [370].

The results of the biological monitoring of chlorophenols evaluated by ELISA are presented in **Figure 5.9,** which shows the relative frequency distribution for the three groups studied, and in **Table 5.5** are listed the corresponding statistic parameters. The concentrations in urine are given in μg/g creatinine to correct the results for urine dilution. The variation in the creatinine concentration may be caused by differences in the sweating rate, the fluid intake or the health status of the individuals. The urinary creatinine content of the analyzed samples ranged from 0.3 to 3.0 mg/mL urine. The mean urinary creatinine concentration of all the subjects was 1.24 mg/mL urine.

Table 5.5. Urinary levels of 2,4,6-TCP-IR equivalents (mean concentration, range, concentrations at selected population percentiles) for the three groups.

Population group	N	Mean ± SD	Min	5%	25%	Median	75%	95%	Max
Service workers	41	1.33±0.78	0.42	0.61	0.85	1.13	1.44	2.67	4.69
Petrochemical workers	43	1.90±1.68	0.18	0.65	0.92	1.33	2.02	5.68	8.06
Living in vicinity of incinerator	33	3.86±4.36	0.59	0.78	1.54	2.19	3.93	14.47	22.69

The concentrations are determined by HTS-IAC-ELISA and expressed in μg/g creatinine. The detection limit is 0.17 μg/g creatinine based on the LOD of the ELISA (0.2 μg/L) and the mean urinary creatinine concentration of all the subjects (1.24 mg/mL urine).

The relative frequency distribution for the three groups demonstrates that most of the population has levels around 1 μg/L. These are 75% of the services workers, 50% of the petrochemical workers and only 25% of the population living close to the incinerator. However, 30% of the last group have levels of 2 μg/L. Furthermore, the median and 95% percentiles values increase following the order: services < petrochemical < incinerator. The highest detected level (23 μg/L 2,4,6-TCP-IR equivalents) also belongs to the third group.

The results presented in **Figure 5.9** and **Table 5.5** clearly demonstrate that the 2,4,6-TCP-IR equivalent levels in the urine of the population living in the surroundings of the incinerator are highest among the three groups. It is difficult to interpret these results, because we have no information about the real risk of exposure of these groups of workers to organochlorine compounds and, as mentioned above, differences on their health status and particular habits must also be taken into consideration. It is well known that workers in a refinery are mainly exposed to benzene, toluene, pentane, and hexane [371,372]. If no occupational exposure of petrochemical workers is assumed and they are considered exposed only to environmental contamination as the general population like service workers from town B, their higher TCP-IR levels could be attributed to regional differences in the contamination levels. It is important to note that town A is situated in a region of chemical industry.

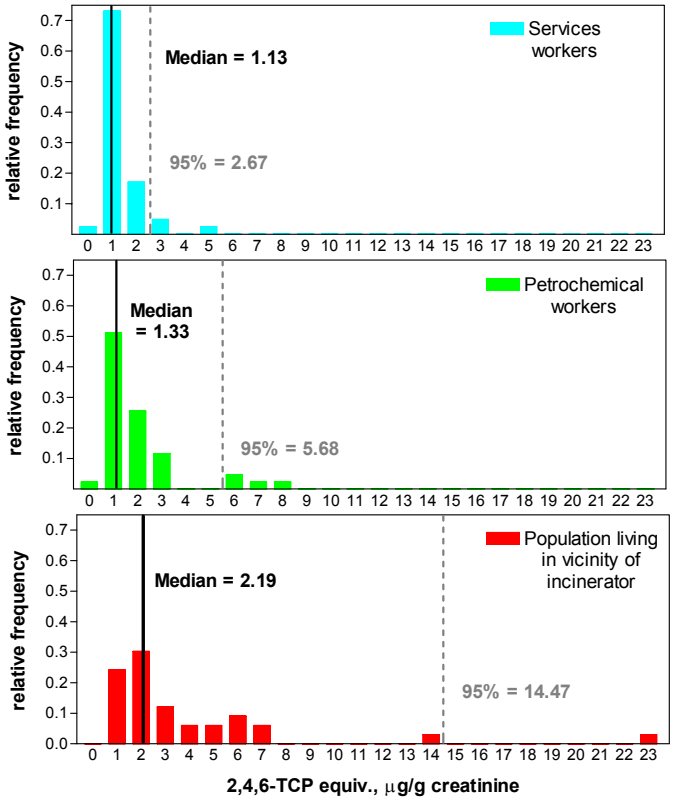


Figure 4.28. Relative frequency distribution of 2,4,6-TCP-IR equivalents determined by HTS-IAC-ELISA urine analysis for petrochemical workers, services workers and general population leaving close to waste incinerator. ——— Median (50-percentile); ------ 95-percentile.

On the other hand, this study clearly indicates that the general population living in the vicinity of a waste incinerator has levels higher than the population living in the same town and working in the service sector. It is well documented that chlorophenols, hexachlorobenzne, polyaromatic hydrocarbons and dioxins are potentially emitted from incineration plants [373,374], although the amount of internal exposure and of potentially resulting health effects for the workers of the plant and the general population is a matter of controversy [35,102,118,123,375]. In general, the excretion of chlorophenols could be regarded as an indirect indicator of current exposure to dioxins [36], although to our knowledge it is not reported any rigorous epidemiological study where this fact can be proved.

It is worth noting that the general population living close to the incinerator in our study was also subject to biomonitoring of PCDD/PCDF and PCB levels in blood as a part of evaluation of the effect of the incinerator performance on the public health [376-378]. These studies revealed that for the period of 4 years of incinerator functioning (1995-1999) for the residents living close to the incinerator the blood levels of PCDD/PCDF increased with 40-48% and the PCB levels remain constant. However, given the low dioxin stack emissions from the plant, the fact that the blood dioxin levels were not found to depend on the residence distance to the incinerator, and that similar dioxin levels were found in a control population living in an area with not known source of industrial dioxin contamination, the authors concluded that it seemed unlikely that the increase in dioxin blood levels resulted from the incinerator's emissions. The authors attributed the observed levels to the consumption of contaminated food or other sources [379]. However, it is important to note that the increasing trend in PCDD/PCDF levels was surprising, considering that the general tendency has been a decrease in levels over the past few years in other developed countries [92].

As was mentioned above, all urine samples were analyzed with GC-MS in order to confirm the presence of halogenated phenols by an independent technique. The urinary concentrations of 2,4,6-TCP, 2,4,5-TCP, 2,3,4,6-TtCP, 2,4-DBP and 2,4,6-TBP (mean,

minimum and maximum, 5th, 25th, 50th (median), 75th, 95th – percentiles in μg/g creatinine) for the different groups are reported in **Table 5.6**.

Table 5.6. Urinary levels of all the analytes (mean concentration, range, concentrations at selected population percentiles) for the three groups.

Analyte / group	N	Mean	Min	5%	25%	Median	75%	95%	Max
2,4,6-TCP									
Services	37	0.18	<d.l.	<d.l.	0.11	0.13	0.17	0.58	1.24
Petrochemical	41	0.74	<d.l.	0.1	0.26	0.48	0.78	2.36	4.89
Vicinity of incinerator	29	0.43	<d.l.	<d.l.	0.17	0.26	0.49	1.28	2.33
2,4,5-TCP									
Services	37	0.08	<d.l.	<d.l.	<d.l.	0.08	0.11	0.32	0.52
Petrochemical	41	0.27	<d.l.	<d.l.	<d.l.	0.15	0.42	0.83	1.77
Vicinity of incinerator	29	0.52	<d.l.	<d.l.	<d.l.	0.08	0.24	0.72	11.04
2,3,4,6-TCP									
Services	37	0.25	<d.l.	<d.l.	0.18	0.21	0.29	0.63	0.67
Petrochemical	41	0.33	<d.l.	<d.l.	<d.l.	0.22	0.33	0.97	2.2
Vicinity of incinerator	29	0.31	<d.l.	<d.l.	<d.l.	0.19	0.3	1.11	1.33
2,4-DBP									
Services	37	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	0.1	0.27	0.4
Petrochemical	41	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	0.18	0.28
Vicinity of incinerator	29	0.18	<d.l.	<d.l.	<d.l.	0.13	0.16	0.72	0.93
2,4,6-TBP									
Services	37	0.22	<d.l.	<d.l.	0.15	0.19	0.25	0.48	0.52
Petrochemical	22	0.16	<d.l.	<d.l.	<d.l.	0.15	0.21	0.37	0.4
Vicinity of incinerator	29	0.42	<d.l.	<d.l.	0.17	0.24	0.48	1.44	2.0

The concentrations are determined by HTS-IAC-GC/MS and expressed in μg/g creatinine. The detection limit is 0.08 μg/g creatinine for TCPs and 0.16 μg/g creatinine for DBP, TtCP and TBP based on the LOD of the GC-MS and the mean urinary creatinine concentration of all the subjects (1.24 mg/mL urine).

For better comparison of the level of each analyte in the different groups we present the 5th, 50th (median), and 95th percentile concentrations at **Figure 5.10.** The average excretion of 2,4,6-TCP in urine is highest for petrochemical workers (0.48 μg/g), followed by the general population living in vicinity of the incinerator (0.26 μg/g). The services workers excreted the lowest level of 2,4,6-TCP in urine (0.13 μg/g). The highest levels of 2,4,5-TCP are observed again for petrochemical workers (0.15 μg/g) according to the median concentrations, although the incinerator group has the highest maximum

concentration of 2,4,5-TCP (11 µg/g). It is important to note that the mean concentrations for 2,4,6-TCP (0.74 µg/g) and 2,4,5-TCP (0.27 µg/g) determined by us for the petrochemical workers are very similar to the values recently reported by Schumacher *et al* for some persons living in the same town (0.6 µg/g and 0.4 µg/g for 2,4,6-TCP and 2,4,5-TCP, respectively) [118]. These mean levels are referred to the administrative workers from a waste incinerator situated in town A. If we consider them as representative for the general population of this area, we can conclude that the

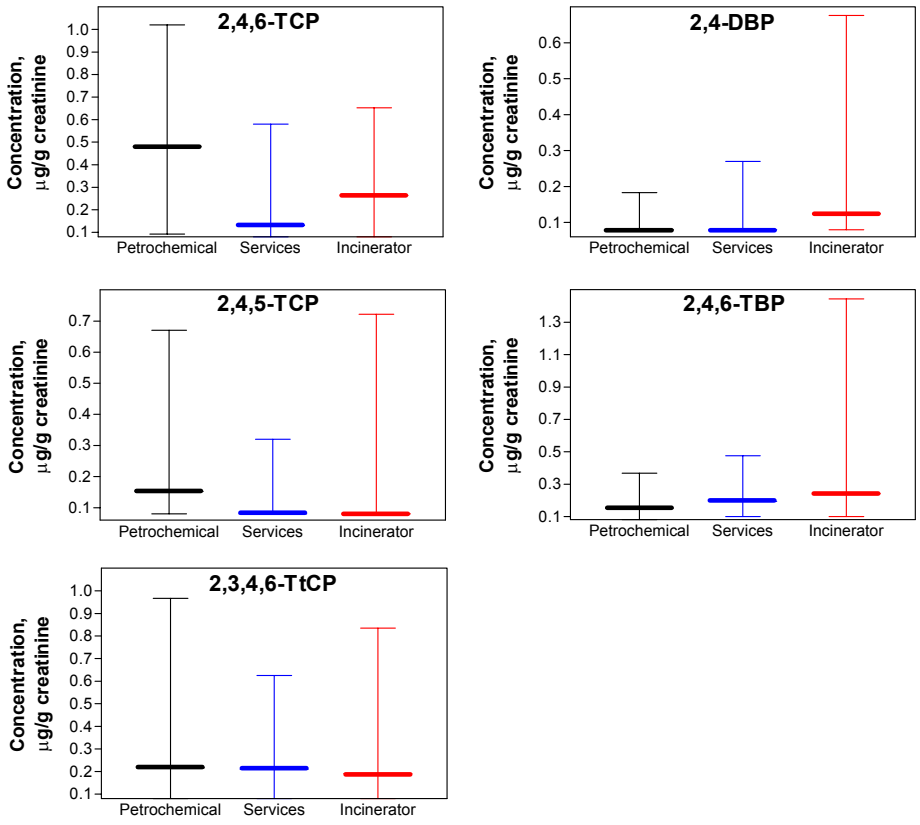


Figure 5.10. Description of the concentrations of chloro- and bromo- phenol in the urine of petrochemical workers, services workers and general population living in vicinity of waste incinerator. The top of each bar is the 95th percentile, the bold bar in the middle is the median, and the lower bar is the 5th percentile. The concentrations are determined by HTS-IAC-GC/MS and expressed in µg/g creatinine. The detection limit is 0.08 µg/g creatinine for TCPs and 0.16 µg/g creatinine for DBP, TtCP and TBP based on the LOD of the GC-MS and the mean urinary creatinine concentration of all the subjects (1.24 mg/mL urine).

petrochemical workers subject of our study have urinary TCP levels similar to those found for non-occupationally exposed persons and therefore, they do not suffer any occupational exposure to organochlorine compounds.

The excretion of 2,3,4,6-TtCP seems to be similar for the three groups stuided. It is very important to note that the urinary levels (median, 75^{th}, 95^{th}, maximum) of brominated phenols (2,4-DBP and 2,4,6-TBP) are highest for the incinerator vicinity population followed by the services workers and finally by the petrochemical workers. These results suggest that the general population living in the surroundings of the incinerator suffers higher exposure to these compounds, although the source is uncertain. There are only limited amount of data on emissions of brominated compounds from incinerators and most of the data in the literature were generated in laboratory experiments [380]. On the other hand, it is possible that the lowest bromophenol levels in the petrochemical workers is due to the fact that they live in another city (town A), where may be the contamination of the drinking water and food with this emerging pollutants is lower.

In addition, these high levels of bromophenols in the urine of the incinerator vicinity group explains the results from the urinary TCP-IR evaluation by the ELISA method, which indicated that this group is subject to highest exposure (see **Figure 5.9**). Although the 2,4,6-TCP levels in the incinerator group are lower than the petrochemical group according to GC-MS analysis, due to the high cross-reactivity of these brominated compounds in the 2,4,6-TCP ELISA (700% for 2,4,6-TBP and 119% for 2,4-DBP), the chlorophenol effect is compensated and the total TCP-IR exposure results highest for the incinerator group. In fact, this incinerator is a modern facility where the residues have to be minimized.

Finally, compared to other studies on the general population the concentrations in the present study are either lower or of the same magnitude. For example, the highest values determined by us for 2,4,5-TCP in the urine of the petrochemical workers (median = 0.15 μg/g (0.12 μg/L), 95^{th} =0.83 μg/g (0.67 μg/L)) are lower than the reported values for German children (median = 0.25 μg/L, 95^{th} = 1.1 μg/L) [123] and German adults (median

= 0.85 μg/L, 95th = 2.7 μg/L) [124]. Similarly, the urinary concentrations of 2,4,6-TCP for the petrochemical workers from our study (median = 0.48 μg/g (0.39 μg/L), 95th =2.36 μg/g (1.93 μg/L)) are lower or in the same order as the general population in Germany: median = 0.6 μg/L, 95th = 2.4 μg/L [124] and children (median = 0.6 μg/L, 95th = 1.7 μg/L) [123]. In general, the trichlorophenol levels detected in the catalan population studied do not exceed the established reference values for 2,4,5-TCP and 2,4,6-TCP in urine in Germany (5.0 μg/g creatinine) [36,81] and in USA (3.3 μg/g creatinine for 2,4,6-TCP and 3.0 μg/g creatinine for 2,4,5-TCP [75]. The only exception is the maximum concentration of 2,4,5-TCP (11 μg/g creatinine) detected in one individual of the group of population living in the surroundings of a waste incinerator.

Our aim was to validate the developed HTS-IAC-ELISA method and to compare the population's contamination levels from different regions of Cataluña. Another aim was to evaluate the reference values for trichlorophenols in the urine of the general population. Such values allow an estimation of the extent of background contamination. The reported values will serve as a basis against which to compare concentrations in subjects who may have been occupationally exposed to halogenated phenols or toxic compounds that metabolize to them.

Although the results shown here draw a tendency on the potential risk or level of exposure of these population groups, we can not make any definitive conclusion because much more information and criteria should be taken into consideration. However, these results and the comparison between ELISA and GC-MS methods prove the potential usefulness of the procedure described and developed in this thesis.

6. Quenching Fluorescence Immunoassay for Chlorophenols based on Laser-Induced Fluorescence Detection in Microdroplets

Fluorescent detection methods have led to major improvements in bioanalytical applications because of their extraordinary sensitivity and selectivity [381]. One of the most exciting aspects of fluorescence technologies is their ability to support decreasing sample sizes down to the single-molecule detection level [190,382-384], which in turn provides the opportunity for miniaturization and high throughput screening. Fluorescence immunoassays (fluoroimmunoassays, FIAs) employ a fluorescent signal for analyte detection. Homogeneous FIAs are separation-free, making automation easy and

permitting simple adaptation to existing instrumentation. A variety of homogeneous FIA systems have been developed based on detection schemes such as fluorescence polarization, fluorescence energy transfer, time-resolved fluorescence, fluorescence quenching or enhancement (see recent reviews [185,189,385]). Fluoroimmunoassays have become a common clinical chemistry procedure for the analysis of a wide range of analytes, such as drugs, hormones, and proteins. They have also found application in environmental analysis for the determination of pesticide contaminants in water [222,232,234,250,386-388] and their metabolites in urine sample [389,390]. Direct quenching FIA (QFIA) are assays in which the antibody-binding reaction significantly decreases the fluorescence signal of the labeled antigen due to changes in the microenvironment around the fluorophore. The direct quenching approach has proven suitable only for haptens of small size, because the antibody binding site lies sufficiently close to the label to influence its signal. Competitive direct QFIA based on the fluorescence quenching of a labeled hapten upon binding its antibody (Ab) have been developed for the detection of gentamycin [391] and cortisol [392] in serum, atrazine [393], 2,4-dichlorophenoxyacetic acid [231] and propazine [394] in nonpolar organic media, and pyrethroid metabolites in urine [390].

More recently, fluoroimmunoassays have been combined with laser-induced fluorescence (LIF) technology to make use of sensitive optical microscope and charge-coupled device (CCD) systems [395-399]. Hence, using modern lasers and CCD cameras it is possible to construct compact and highly sensitive detection systems. Laser excitation allows significant reduction of the illuminated sample-volume, minimizing the background interferences resulting from Raman and Rayleigh scattering, and impurity fluorescence. This results in an improved fluorescence-to-background signal-to-noise ratio, making detection at single molecule levels feasible [382,400,401]. LIF detection of single fluorescent molecules in solution has been performed in flowing streams, in sheath flow cuvettes, and in capillaries [402,403]. One of the main drawbacks of these flowing techniques is that the optically defined probe volume is smaller than the flow cell itself, so that many of the molecules may not be probed by the laser. As a result of these

limitations, research has been focussed on alternative detection schemes, including levitated [400,404-407] and falling [408-411] microdroplets.

The microdroplet approach to single molecule detection in liquids is unique in several aspects. The probe volume in this case is defined by the droplet instead of by the laser beam, which means that all fluorophore molecules can be interrogated. Small detection volumes afforded by micrometer-sized droplets minimize background fluorescence. Contact of the sample to container walls is avoided, eliminating scattering from walls and possible binding of reagents to the wall. In addition, spontaneous emission in spherical microdroplets is significantly enhanced due to cavity resonances that increase optical emissions significantly.

In the previous chapters we have shown that the 2,4,6-TCP ELISA requires sample pre-treatment to allow urine analysis with sufficient detectability for biomonitoring occupationally exposed persons. In this chapter, we describe the improvement in a TCP assay by developing a LIF-microdroplet-QFIA. This novel biodetection system combines the selectivity of immunoassays and the sensitivity of laser-induced fluorescence detection in microdroplets. It has been evaluated for water and urine analysis. This work was realized in collaboration with the Department of Mechanical and Aeronautical Engineering and the Department of Entomology of University of California, Davis.

6.1. Synthesis of the fluorescent tracers

The feasibility of using a homologous (immunizing hapten is the same as the competitor hapten) direct enzyme immunoassay to determine 2,4,6-TCP has already been demonstrated in our group [259]. Thus, the same approach was initially planned to develop a fluoroimmunoassay by coupling hapten **5** (*3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid*) to a fluorescent label. For maximum sensitivity, the fluorophore should have a high molar extinction coefficient, high quantum yield and large Stokes shift. Fluorescent dyes from the fluorescein family (e.g. fluorescein, rhodamine,

Texas Red, etc.) have relatively high molar extinction coefficients (60 000-100 000 M^{-1} cm^{-1}) and fluorescence quantum yields greater than 85% [412]. In particular, fluorescein has an effective excitation wavelength range of about 488-495 nm, closely matching the emission of an Ar laser (488 nm). Frequently, fluoresceinamine is used for labeling haptens containing a -COOH group, employing dicyclohexyl carbodiimide (DCC) [222,390,413] or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [232] as an activating agent to form the amide bond. *N*-substituted carbodimides react with carboxylic acids to create a highly reactive *O*-acylisourea intermediate, which then react with the primary amine to form an amide bond with release of the isourea by-products according to the general reaction mechanism shown in **Figure 6.1**. A potential undesirable effect of carbodiimide coupling reactions is the spontaneous rearrangement of the *O*-acylisourea intermediates to an inactive *N*-acylurea.

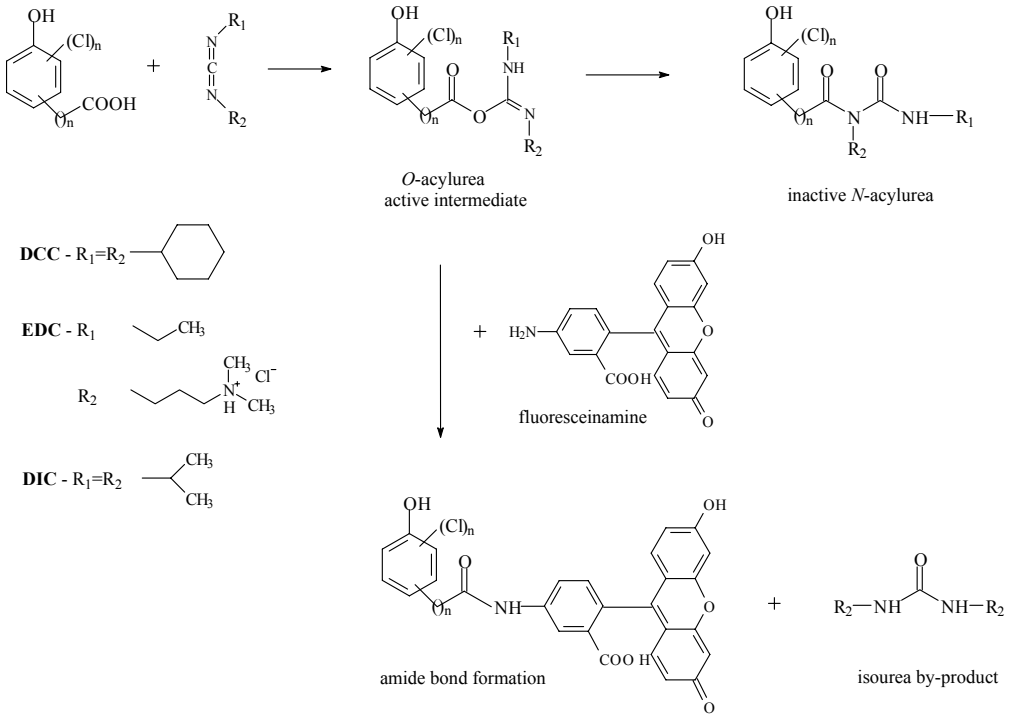


Figure 6.1. General reaction mechanism of carbodiimide-mediated coupling of carboxylic acids and amine nuclephile.

Initially we tried to find out the best reaction conditions (type of carbodiimide, solvent, reagents concentration, etc.) using as model hapten 3-hydroxyphenylacetic acid. The coupling reaction with fluoresceniamine (FNH_2) in anh. THF was studied using DCC and EDC. An excess of carbodiimide with respect to the hapten was used to avoid the formation of anhydride products. Anhydrous solvents and Ar atmosphere were used to avoid competing hydrolysis of the activated ester intermediate, forming an isourea. The reaction conditions and yields calculated with respect to the FNH_2 are presented in **Table 6.1**.

Table 6.1. Conjugation of 3-hydroxyphenylacetic acid (HO-PhCOOH) to FNH_2 in THF.

Reagents	Molar ratio	Time, T°	HOPh-F yield [a], %
HO-PhCOOH : **DCC** : FNH_2	1:1.25:1	20h, RT	20
HO-PhCOOH : **EDC** : FNH_2	1:1.25:1	20h, RT	15
	1:2:1	3h, 30°C	15

[a] The yield is calculated based on the ratio of the signals of the fluorescein conjugate of the 3-hydroxyphenylacetic acid (HOPh-F) and of the FNH_2 in the aromatic region of the ^{1}H-NMR spectra of the reaction mixture.

The coupling reaction was followed by TLC and ^{1}H-NMR. The characteristic signals dd at δ 6.96 and d at δ 7.05 corresponding to the hydrogen at the closest positions to the NH_2 group in the aromatic ring of the fluoresceinamine molecule are shifted to δ 7.78 and δ 8.28, respectively, when the amide bond is formed. In addition, the signal (s, 2H, -CH_2COO-) at δ 3.4 in the 3-hydroxyphenylacetic acid shifts to δ 3.57 (s, 2H, -CH_2CONH-) under coupling. In all the cases the reaction yield was very low (<20%).

As the dicyclohexylisourea formed when DCC was used could not be easily removed (insoluble in ethylacetate, ether, toluene, hexane), we continued with the EDC. We studied the reactivity of the –COOH and NH_2- groups when EDC was employed using phenylacetic and 3-hydroxyphenylacetic acid as model haptens and 2,6-diethylaniline instead of the flruorescein (see **Table 6.2**). Although 50% yield was achieved in the reaction between phenylacetic acid and 2,6-diethylaniline, the presence of the OH-group in the aromatic ring when 3-hydroxyphenylacetic acid was reacted produced a decrease of the yield to 30%.

Table 6.2. EDC coupling in THF. The reagent concentration was: -COOH:ED**C**:FNH_2=1:1.25:1 and the reactions were performed at RT.

-COOH	-NH_2	Time	yield [a], %
Phenylacetic acid	2,6-diethylaniline	4h	50 [a]
3-Hydroxyphenylacetic acid	2,6-diethylaniline	4h	30 [a]
Phenylacetic acid	F-NH_2	20h	30 [b]
3-Hydroxyphenylacetic acid	F-NH_2	20h	15 [b]

[a] The yield is calculated based on the ratio between the signal (s, 2H, -CH_2CONH-) of the conjugate and the signal (s, 2H, -CH_2COO-) in the respective acid according to the ^{1}H-NMR spectra of the reaction mixture. [b] The yield is calculated based on the ratio of the signals of the fluorescein conjugate and of the FNH_2 in the aromatic region of the ^{1}H-NMR spectra of the reaction mixture.

In addition, the amino group of the fluoresceinamine resulted less reactive than the amino group of the 2,6-diethylaniline. A possible explanation could be the limited solubility of fluoresceinamine in the solvent used (THF). To prove this fact we performed the reaction in presence of a co-solvent to increase fluoresceinamine solubility. We also decided to use diisopropyl carbodiimide (DIC) instead of EDC because is more soluble in organic solvents and allows the elimination of the non-reacted DIC and the diisopropylurea by-product formed by evaporation facilitating subsequent purification steps of the product. However, aprotic organic solvents may favor the formation of the corresponding *N*-acylurea derivative. Therefore, it was very important to determine precisely the appropriate co-solvent and its concentration. **Table 6.3** presents the results obtained with different co-solvents and concentrations.

Table 6.3. DIC coupling of 3-hydroxyphenylacetic acid (HO-PhCOOH) and FNH_2

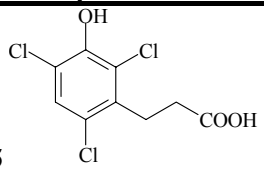
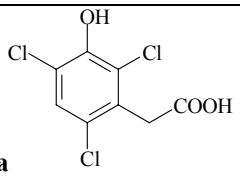
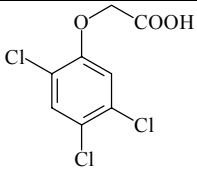
Solvent	Time	HOPh-F yield [a], %	HOPhCOOH non-reacted	N-acylurea
100%DMF	20h	0	0	100
10%DMF/THF	4h	53	10	37
3%DMF/THF	4h	57	0	43
3%DMSO/THF	4h	30	30	30
5%ACN/THF	4h	30	5	65

[a] The yield is calculated based on the ratio of the signals of the conjugate vs the FNH_2 in the aromatic region of the ^{1}H-NMR spectra of the reaction mixture.
The reagent concentration was: -COOH:DI**C**:FNH_2=1:1.5:1

Since highest yields were achieved at 3% and 10% DMF, we decided to use DMF as co-solvent at low concentrations (3-10%).

It has been demonstrated that in polarization fluorescence immunoassays higher sensitivity was achieved using short bridge length based tracers [233,414,415]. Therefore, we approached the synthesis of three different fluorescent tracers using DIC in THF containing 6% of DMF (see **Table 6.4**). The homologous hapten **5** (*3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid*) was coupled to fluoresceinamine with a reaction yield of 50%.

Table 6.4. DIC coupling of haptens and FNH_2.

Hapten	Solvent	Yield [a], %
 hapten **5**	6%DMF/THF	50
 hapten **5a**	6%DMF/THF	10
 hapten **2**	10%DMF/THF	0

The reagent concentration was: -COOH:DI**C**:FNH_2=1:1.5:1 and the reactions were performed for 20h at RT. [a] The yield is calculated based on the ratio of the signals of the conjugate vs the FNH_2 in the aromatic region of the ^{1}H-NMR spectra of the reaction mixture.

The formation of the conjugate was confirmed by the presence of the following signals in the ^{1}H-NMR spectra: δ: 2.68 (t, J = 8.4 Hz, 2H, -CH_2CONH-), 3.32 (t, J = 8.4 Hz, 2H, PhCH_2-), 7.38 (s, 1H_{Ar} meta, hapten 5), 7.85 (dd, J_1 = 8.4 Hz, J_2 = 1.9 Hz, 1H, FNH_2), 8.3 (d, J = 1.8 Hz, 1H, FNH_2). The product was purified by TLC and the overall yield after purification was 37.7%. Following the same procedure we have tried to synthesize the fluorescein conjugate of the hapten **5a** with a shorter spacer arm *(3-hydroxy-2,4,6-trichlorophenylacetic acid*), but the reaction yield *vs* FNH_2 was only 10%. This could be attributed to some type of stearic hindrance of the chlorine atoms from the aromatic ring situated very close to the –COOH group. Furthermore, the synthesis of hapten **2** (*2,4,5-trichlorophenoxyacetic acid*)-fluorescein was also unsuccessful.

6.2. Optical characterization of hapten 5-fluorescein (TCP-F)

The fluorescence properties of the synthesized tracer hapten 5-fluorescein (TCP-F) were initially tested in a standard spectrofluorometer where the solution is subject to excitation/emission in a quartz cuvette of 1 mL. Maximum excitation was observed at ~490 nm (see **Figure 6.2.A**) and maximum emission was obtained at ~515 nm (see **Figure 6.2.B**). Increasing the TCP-F concentration resulted in an increased intensity of the emitted fluorescence. At high concentrations (μM) dimmers are formed and the maximum emission at ~515 nm is auto-quenched.

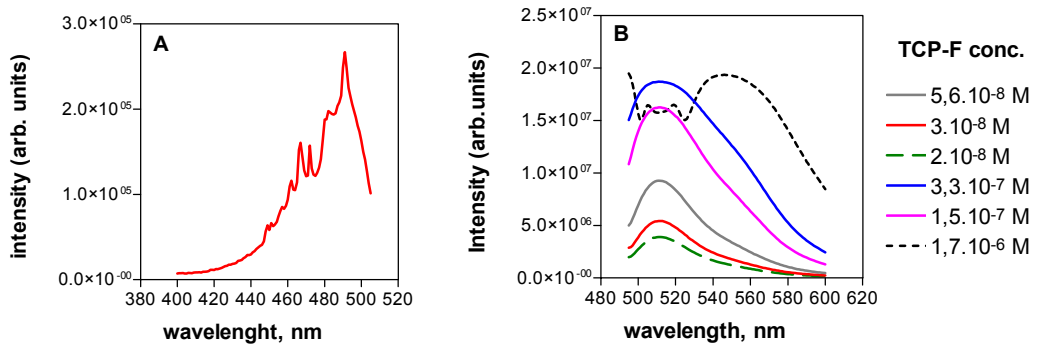


Figure 6.2. Excitation and emission spectra of the fluorescein tracer TCP-F recorded in standard spectrofluorometer. **A**. Excitation spectrum is obtained for 10^{-8}M TCP-F in PBS (pH=7.5) buffer at emission wavelength of 520 nm. **B**. Emission spectrum is obtained for several concentrations of TCP-F in PBS (pH=7.5) buffer at excitation wavelength of 490 nm.

The fluorescence intensity of the fluorescein conjugate is very dependent on pH of the solution, showing enhanced intensity in basic conditions (see **Figure 6.3**). For each pH a linear relationship was observed between the maximum fluorescence intensity and the TCP-F concentration. Since the usual immunoassay media is the PBS buffer (pH=7.5) and suitable fluorescence intensity was observed at this condition, we have decided to perform our following studies only at pH=7.5.

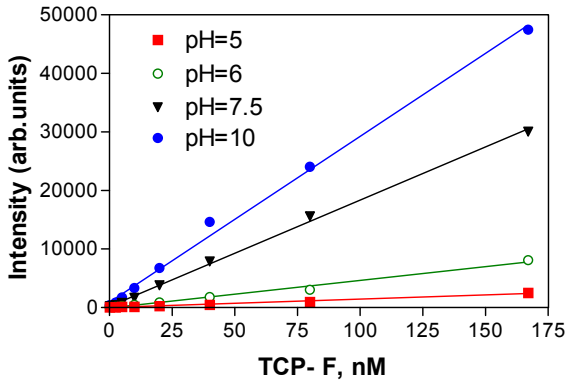
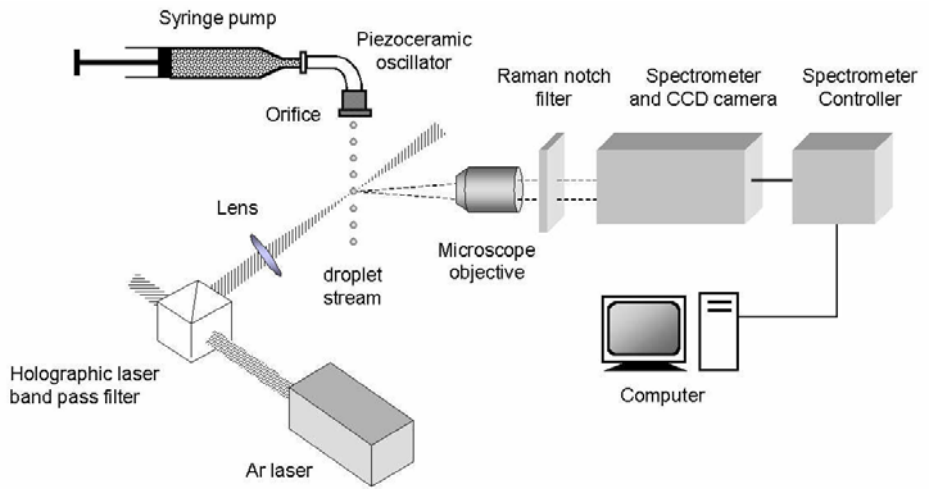


Figure 6.3. pH effect on the fluorescence intensity of the fluorescein tracer TCP-F. The fluorescence intensity was measured in a spectrofluorometer (λexc=490 nm) for several concentrations of TCP-F at different pH.

6.3. LIF detection of TCP-F in microdroplets

Laser induced fluorescence in microdroplets was measured using the instrumental set-up presented in **Figure 6.4**. The microdroplets were generated by the vibrating orifice aerosol generator and illuminated by the laser beam (λ = 488 nm). The fluorescence was collected by a microscope objective lens and focused onto the entrance slit of the imaging spectrometer. Emission spectra were recorded with a thermoelectrically-cooled camera with a CCD detector. Microdroplet size and shape have very important effects on fluorescence detection. For a fixed orifice diameter these parameters are determined by the piezoelectric frequency of the droplet generator and the liquid flow rate. In our experiments a frequency of 40 kHz was used with a liquid flow rate of 0.25 mL/min (4.16 x 10^{-3} cm^3/s). According to a simple relationship between these parameters, the diameter of the generated droplet was 58.4 μm and its volume was 104 pL. With a shutter exposure time of 1 second, 40000 droplets passed the laser beam and, hence, the detected volume was 4.2 μL.



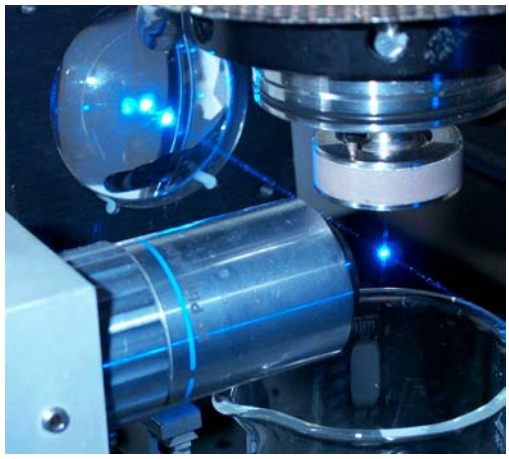


Figure 6.4. Scheme of the instrumental set-up. Microdroplets are generated by a vibrating orifice aerosol generator (orifice diameter = 10 μm) consisted of syringe pump and piezoceramic oscillator. Droplets are illuminated by a laser beam (continuous Ar ion laser, λ = 488 nm) that is focused to a laser beam waist of ~200 μm at the trajectory of the droplet stream. The beam diameter is chosen to be larger than the droplet diameter to ensure that all fluorophores in the sample are illuminated. A holographic laser band pass filter eliminates undesirable plasma lines from the laser source and transmits only the laser line at 488 nm. The fluorescence is collected by a microscope objective lens (N.A. of 0.55) and focused onto the entrance slit of the imaging spectrometer. Spectra are recorded with a thermoelectrically-cooled camera with a 512 x 512 pixel charge coupled device (CCD) detector. A 488 nm holographic Raman notch filter placed in front of the slit of the spectrometer blocks elastically scattered laser radiation.

Figure 6.5.A compares the fluorescence emission spectra obtained from droplets of the TCP-F conjugate and from droplets of the dye molecule fluoresceinamine (FNH_2) used for labeling. As expected, the maximum emission for both molecules is at ~520 nm (close to the 488 nm laser excitation). The insert present the microdroplets image recorded with the CCD camera. Furthermore, a linear dependence of the maximum fluorescence intensity on the TCP-F concentration was observed for both laser powers used in this study (see **Figure 6.5.B**). The regression coefficients are 0.996 and 0.998 for laser power 60 mW and 120 mW, respectively. Transit times were of the order of 100 μs, leading to a very short interaction time between excitation source and fluorophore, consequently avoiding photobleaching. The absolute detection limit of the fluorescein conjugate in the microdroplets is around 5×10^{-10} M (0.5 nM) corresponding to 5.2×10^{-20} mol TCP-F per microdroplet, while in a cuvette (2 mL) or in a microwell (200 μL) the absolute detection limit was 2×10^{-12} mol and 2×10^{-13} mol, respectively, which demonstrates one of advantages of the microdroplet approach allowing detection close to single molecule levels. A concentration of TCP-F of 10 nM was chosen for further studies as a compromise between the goal of maximizing the signal-to-background ratio (to facilitate observation of the antibody quenching), and the recognition of the fact that low TCP-F concentrations would provide lower detection limits in the competitive assays.

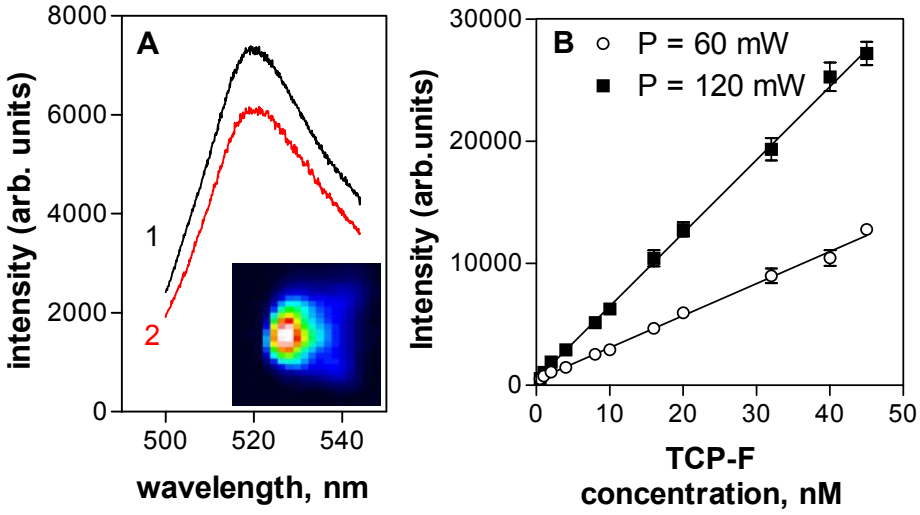


Figure 6.5. A. Fluorescence emission spectra detected in microdroplets of 20 nM FNH_2 (1) and 20 nM TCP-F (2). The laser power is 60 mW, the exposure time is 1 sec. The insert represents the microdroplets image obtained with the CCD camera. **B**. Fluorescence intensity *vs* TCP-F concentration. The measurements were made in PBS buffer (pH=7.5), the exposure time was 1 second and the laser power (P) was set at 60 and 120 mW. The data shown correspond to the average and the standard deviation of three measurements.

6.4. LIF-microdroplet-QFIA

6.4.1. Quenching effect on TCP-F fluorescence upon Ab binding

It is well known that an immunochemical reaction between a fluorescent-labeled antigen and its specific antibody can cause a decrease in the fluorescence of the labeled antigen, whereas the free fraction retains its full signal [189]. The effect of the antibody binding reaction on the fluorescent properties of the TCP-F conjugate was tested on a noncompetitive LIF-microdroplet-QFIA for three different Abs specific for 2,4,6-TCP (Ab43, 44, 45). **Figure 6.6** shows the quenching effect observed after 20 min of immunoreaction time between TCP-F (10 nM) and the three Abs (10 μg/mL). The decreased fluorescence intensity is presented as a ratio between the fluorescence intensity of TCP-F in presence of Ab (I_{Ab}) and the fluorescence intensity of TCP-F in the absence of Ab (I_{TCP-F}).

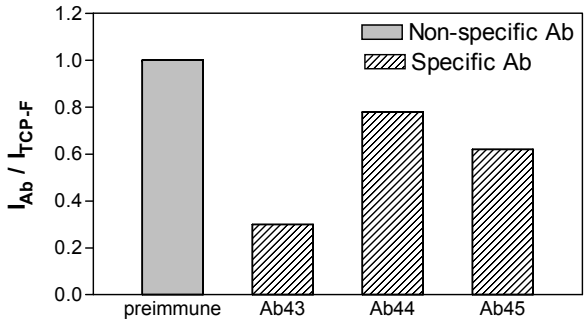


Figure 6.6. Quenching effect on the fluorescence of the TCP-F conjugate upon Ab binding. The TCP-F and the Ab concentration were 10 nM and 10 μg/ml, respectively. Measurements were made after 20 min of immunoreaction. The decreased fluorescence intensity is presented as a ratio between the fluorescence intensity of TCP-F in presence of Ab (I_{Ab}) and the fluorescence intensity of TCP-F in the absence of Ab (I_{TCP-F}). The preimmune serum is used as control of non-specific binding.

A pre-immune serum, used as a negative control, did not show fluorescence quenching, demonstrating the specificity of the observed effect using Ab43-45. The inhibition of the fluorescence signal was highest for Ab43, followed by Ab45, and is lowest for Ab44. A similar correlation (Ab43 > Ab45 > Ab44) was found regarding the Ab affinity for hapten 5-HRP on a direct ELISA developed in our group [259]. Higher Ab affinity means stronger binding, resulting in larger changes in the microenvironment of the fluorescent label, which is expressed in a stronger quenching effect [416]. Hence, the Ab with the strongest quenching effect (Ab43) was chosen for further study.

The kinetics and the fluorescence emission spectra of the TCP-F fluorescence quenching in microdroplets using different concentrations of Ab43 was investigated in order to ascertain the time needed for maximum quenching and to determine the most suitable Ab concentration for further competitive assays. Several mixtures of TCP-F/Ab43 were prepared in PBS with constant TCP-F concentration (10 nM) and Ab43 concentration in the range 0.6 - 15 μg/mL. The fluorescence emission from microdroplets of each solution was measured every 5 minutes. It was observed that at high Ab concentrations (15 μg/mL), a steady-state level was reached in about 15 minutes, while with lower Ab concentrations more time (30-40 min) was needed to reach equilibrium (see **Figure 6.7 A**). Thus, an incubation time of 45 min was selected to ensure complete immunoreaction between Ab43 and TCP-F. The emission spectra and the Ab titration curve for 10 nM TCP-F obtained after 45 min incubation time are presented in **Figure 6.7. B** and **C**. It should be noted that complete quenching of the signal was not accomplished even at very high Ab concentrations. Thus, in a situation where all binding sites of the antibody are saturated with TCP-F (i.e., Ab concentration 10 μg/mL) a maximum 70% quenching of the TCP-F fluorescence signal was accomplished (see **Figure 6.7.C**). This is in agreement with other direct quenching fluoroimmunoassays with small haptens where the fluorescence of the tracer is only partially quenched upon Ab binding [390,392,416]. In fact, complete quenching (100%) has only been reported on biotin-avidin systems characterized by even a stronger binding than the Ag-Ab; it has been attributed to the very tight association that brings avidin right to the surface of the biotin-fluorophore conjugate [416]. It can be expected that a hapten with a shorter spacer arm in the fluorescent tracer would give rise to a more efficient quenching due to closer proximity of

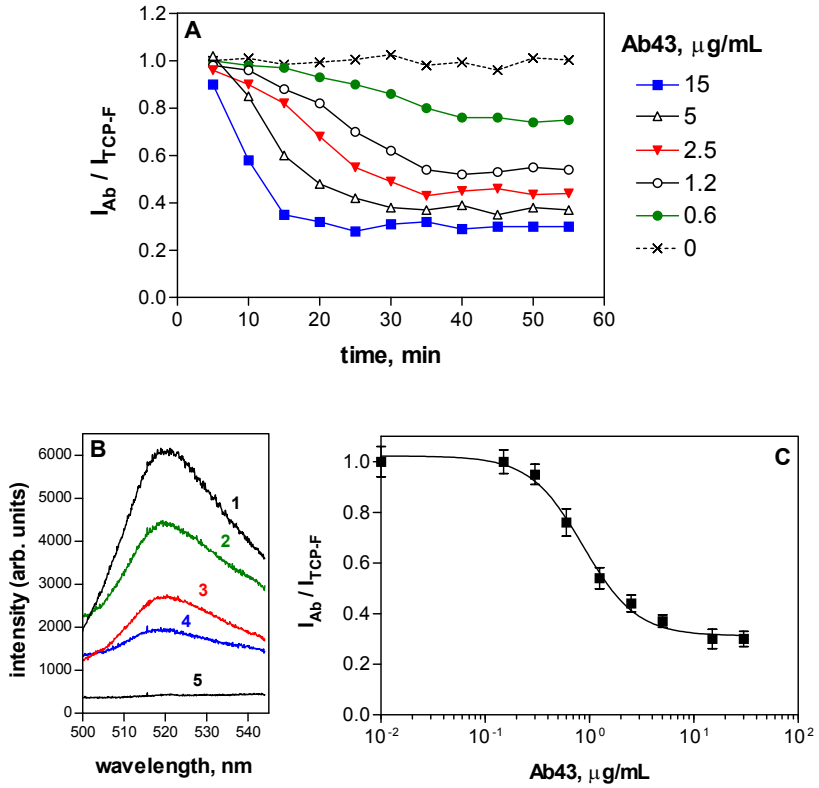


Figure 6.7. A. Kinetics of 10 nM AF fluorescence quenching by different concentrations of Ab43 in 10 mM PBS buffer at room temperature. **B**. Fluorescence emission spectra of 10 nM TCP-F (1) and a mixture of 10 nM TCP-F and Ab43 at varying concentrations: 0.6 μg/mL (2), 2.5 μg/mL (3), 15 μg/mL (4); 15 μg/mL Ab43 in the absence of TCP-F (5). **C.** Antibody titration curve for 10 nM AF at increasing Ab43 concentration. The immunoreagents were incubated 45 min at room temperature. The decrease in the fluorescence intensity is presented as a ratio between the fluorescence intensity of TCP-F in presence of Ab (I_{Ab}) and the fluorescence intensity of TCP-F in the absence of Ab (I_{TCP-F}). All data shown correspond to the average of three measurements.

the fluorophore to the Ab in the Ab/TCP-F complex. Unfortunately, as already described our efforts to synthesize the respective fluorescein tracer using 3-(3-hydroxy-2,4,6-trichlorophenyl) acetic acid have failed. For the competitive QFIA, an Ab concentration of 2.5 μg/mL was selected, producing a signal around 80% of the complete saturation (80% of the maximum quenching). We must also mention that the antibody solutions themselves did not show a significant fluorescence background (see **Figure 6.7. B**).

6.4.2. Competitive LIF-microdroplet-QFIA

Using the above selected concentrations of the immunoreagents we have performed a homogeneous competitive QFIA with LIF detection in microdroplets. The standard curve obtained for PBS buffer is presented in **Figure 6.8.** The TCP-F tracer competes with the analyte (2,4,6-TCP) for a limited number of binding sites. At low analyte concentrations, the TCP-F conjugate is preferentially bound to the Ab and the fluorescence of the label is quenched. Higher analyte concentrations lead to a decreased occupation of the Ab binding sites by the TCP-F conjugate and an increase of the free TCP-F (homogeneous assay) in the solution, resulting in an increase of the fluorescence signal. The parameters of the standard curve performed in 10 mM PBS (pH=7.5) are: IC_{50} = 2.3 nM (0.45 μg/L); the dynamic range is between 0.54 and 11.5 nM (0.11 to 2.27 μg/L); the LOD is 0.2 nM (0.04 μg/L), slope = -0.9, r^2=0.976.

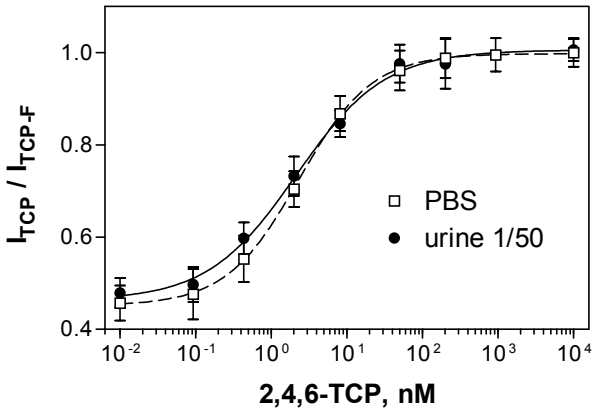


Figure 6.8. Calibration curves of the LIF-microdroplet-QFIA for 2,4,6-TCP in 10 mM PBS buffer and in 50-fold diluted urine. The concentration of TCP-F and the Ab43 was 10 nM and 2.5 μg/mL, respectively. The immunoreagents and the analyte were allowed to compete for 45 min at room temperature. The fluorescence intensity is presented as a ratio between the fluorescence intensity of TCP-F in presence of Ab and analyte (I_{TCP}) and the maximum fluorescence intensity of TCP-F in the absence of Ab (I_{TCP-F}). The data shown correspond to the average of three measurements.

The LOD of an immunoassay is often determined by the relative affinity of the Ab for the analyte and the competing hapten-label conjugate. However, it also depends on other parameters, such as the label detection system. We have compared the main features of the QFIA performed in 96-well plate, the LIF-microdroplet-QFIA, and a conventional microplate ELISA [259] (see **Table 6.5**). It should be noted that the three immunoassays (ELISA and QFIAs) were performed under similar conditions: the immureagents used were hapten **5** and Ab43; the reaction media was PBS buffer (pH=7.5); the competition step took place for about 30-40 min. As it can be observed in **Table 6.5**, both microplate immunoassays (heterogeneous ELISA and homogeneous QFIA) have very similar LOD and IC_{50} values in spite of the different labels used (**5**-HRP and TCP-F).

Table 6.5. Features of the immunochemical techniques for 2,4,6-TCP analysis.

	ELISA [a]	**QFIA**	
		microplate	**LIF-microdropet**
IC_{50} (μg/L)	2.74	4.2	0.45
LOD (μg/L)	0.2 (1 nM)	0.36 (1.8 nM)	0.04 (0.2 nM)
LOD (ng)	4 x 10^{-2} ($2x10^{-13}$ mol)	$7.2x10^{-2}$ ($3,7x10^{-13}$ mol)	$1.68x10^{-4}$ ($9,7x10^{-16}$ mol)
Tracer	5-HRP	5-Fluorescein	5-Fluorescein
Procedure	multiple wash and incubation steps	homogeneous assay	homogeneous assay
Excitation	-	485 nm (20nm band)	laser, 488 nm
Volume, μL	200	200	4.2

[a] Data obtained from [259]

According to the literature, conventional fluorophores give assays with detectability values between 10^{-9} to 10^{-10}M, while enzyme-labeled immunoassays reach detectability values in the range of 10^{-10} to 10^{-11}M due to their intrinsic amplification features [189]. However, ELISA requires several time-consuming washing and incubation steps – QFIA is a homogeneous assay involving just the competitive step and no washing of the plates. It is therefore ideally suited for high-throughput screening and miniaturization.

A comparison between both QFIAs demonstrates significantly improved detectability in the LIF-microdroplet-QFIA (see **Table 6.5**). This could be a result of the superior signal-to-noise ratio accomplished with the microdroplet system, primarily because the microplate QFIA suffers higher background fluorescence. Moreover, a non-specific adsorption of the immunoreagents or certain nonpolar analytes to the microwell walls

may also occur. Background interferences due to Raman and Rayleigh scattering and impurity fluorescence are minimized when using small microdroplet volumes. In addition, the excitation source in the plate reader had a wavelength of 485 nm (band width of 20 nm full width half maximum, FWHM), while the laser excitation wavelength was 488 nm with a very narrow linewidth (recall that background plasma emission from the laser was eliminated with a special holographic prism). The induced fluorescence in the first case was detected by a photomultiplier (535 nm, filter FWHM of 25 nm), whereas in the LIF-microdroplet-QFIA we used a CCD with low readout noise. The microdroplet format ensured that all fluorophores in the sample were exposed to the laser illumination, permitting detection limits to be extended to very low concentrations of fluorophore. This could be achieved in a microplate format, in principle, if the laser beam were expanded to illuminate the entire well, at the expense of much greater elastic light scattering at the laser wavelength. Although the notch filter had a higher rejection ratio for the laser wavelength, some background would be expected to interfere with the measurements. It should be noted that the microdorplet detection scheme was arranged so that the fluorescence was observed in the plane of polarization of the incident laser beam at an angle of 90°, leading to an additional reduction in elastic scattering as described by the Mie theory of light scattering. Finally, the microdroplet approach allows detection to be performed in a volume much smaller than the volume of the standard microwells (200 μL) resulting in lower LOD. Thus, in absolute mass, the LIF-microdroplet-QFIA LOD is 1.68×10^{-4} ng while for the microplate QFIA it is 7.2×10^{-2} ng. The small absolute volume and mass that can be detected with the microdroplets could be a crucial advantage in finger prick assays where detection in very small volumes is necessary.

6.4.3. LIF-microdroplet-QFIA in urine samples

Our aim was to demonstrate that the developed LIF-microdroplet-QFIA can be applied to the urinary determination of 2,4,6-TCP. The application of homogenous fluorescence immunoassays to the measurement of analytes in untreated biological materials is usually complicated due to the presence of endogenous fluorescent compounds and other species that quench the fluorescence of the labeled probe. In general, interferences from

endogenous substances may be minimized by sample dilution. Therefore, we have evaluated the background fluorescence signal from microdroplets of diluted urine (see **Figure. 6.9.A**). With laser excitation at 488 nm, the urine has a fluorescence emission in the region of 520-545 nm that decreases with urine dilution. Furthermore, the emission of the TCP-F tracer in diluted urine does not suffer any shift and the only effect observed is the increased fluorescence intensity of TCP-F due to the presence of matrix background. It can be seen that this effect can be eliminated by urine dilution: the emission spectra of TCP-F in 50-fold diluted urine and in PBS are almost identical.

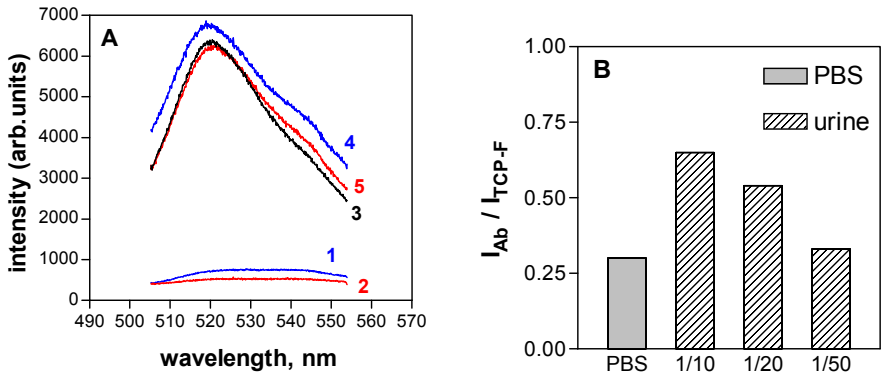


Figure 6.9. A. Urine effect on fluorescence emission measured in the LIF-microdroplet-QFIA. The graph shows the spectra that were recorded in urine diluted 20 times (1) and 50 times (2) with PBS. The spectra were obtained for a solution of 10 nM TCP-F in PBS (3), in 20 fold diluted urine (4), and in 50 fold diluted urine (5); **B.** Urine matrix effect observed as a diminution of the quenching of the fluorescence produced upon Ab binding of the TCP-F conjugate. The effect of the urine was tested after diluting the matrix 10, 20 and 50 times in PBS. The TCP-F and the Ab concentrations were 10 nM and 10 µg/mL respectively. The mixture was incubated 45 min at room temperature. The decreased fluorescence intensity is presented as a ratio between the fluorescence intensity of AF in the presence of Ab (I_{Ab}) and the fluorescence intensity of TCP-F in the absence of Ab (I_{TCP-F}).

Urine components can affect not only the fluorescence background of the sample but also the Ab-Ag interaction. The interference produced by the urine matrix in the competitive immunoreaction in the 2,4,6-TCP ELISA has been demonstrated in the previous chapters of this thesis. In the present case, we investigated the effect of interference on the immunoreaction by comparing the fluorescence quenching produced in urine (10, 20 and 50 times diluted) and in PBS buffer. The urine matrix interfered with the Ab-TCP-F

interaction resulting in a very small quenching effect at 10- and 20 fold urine dilution (see **Figure. 6.9.B**). However, a 50-fold PBS dilution of the urine gave rise to a similar fluorescence quenching effect as in the assay buffer, and thus it was used to prepare a competitive standard curve. As seen in **Figure 6.8**, the curves prepared in PBS buffer and in 50-times diluted urine are almost identical. The IC_{50} is 2.14 nM (0.42 μg/L) and the calculated LOD is 0.2 nM (0.03 μg/L) with a dynamic range between 0.41 and 15.1 nM (0.08 to 2.99 μg/L), the slope is -0.74, and the regression coefficient r^2 is 0.975. Taking into account the dilution factor applied to the urine, the LOD of the analytical method is 1.6 μg/L, which is sufficient for occupational exposure assessment studies.

In summary, a LIF-microdroplet-QFIA has been developed that has proven to be fast, highly sensitive, and simple to perform (no separation steps are needed). It has been demonstrated that this approach offers better detection limits than those of microplate immunoassays. The results reported here indicate that urine samples can be directly analyzed for 2,4,6-TCP, without any sample treatment and with enough detectability to perform biological monitoring and risk assessment of exposure to chlorophenols and other organochlorinated substances that are excreted as trichlorophenol in urine. The technology presented in this is promising regarding development of high-throughput screening and miniaturized biosensor devices. Significant improvement in precision and reproducibility could be achieved by better droplet generation (for example, using ink-jet printer micropiezo technology) and charged microdroplet focusing by electrostatic fields, resulting in a better control of the volume and droplet stability. Future work will be dedicated towards smaller volume assay development and its application to miniaturized devices (microchip). This work is the first application of a steady-state QFIA with LIF-microdroplet detection system to human urine samples, demonstrating the potential of this analytical approach for bioassays.

7. Annex I

Chromatographic analysis of halogenated phenols

With the aim to validate the immunochemical determination of chlorophenols in water and urine described in previous chapters, it was necessary to use an independent analytical method for analysis of halogenated compounds. As it was mentioned in the **Introduction**, gas chromatography (GC) with electron-capture detection (ECD) or mass spectrometry (MS) is a common tool for the analysis of halogenated phenolic compounds

in environmental and biological samples. In this annex we present the work performed to adapt both techniques (GC-ECD and GC-MS) to the quantification and identification of chlorophenols in water and urine samples. These methodologies were applied to the validation of the ELISA determination of 2,4,6-TCP in water samples (see **Chapter 3**, **Section 3.1**), in the characterization of urine samples A and B used as representative urine matrices in this thesis (see **Chapter 3**, **Section 3.4.1**), and finally in the evaluation of SPE (C_{18} and IAC) procedures used as urine clean up methods prior to ELISA (see **Chapter 4**).

The choice of the phenols to be analyzed by GC was based on the cross-reactivity of the 2,4,6-TCP ELISA method. Usually a complex mixture of congeners, ranging from monohalogenated to the heavily halogenated compounds, can be present in samples contaminated with chlorophenols that makes the chromatographic separation difficult. For our purposes it was essential to have an analytical method, which is isomer-specific and yet sensitive to the main ELISA phenolic cross-reactants (2,4,6-TCP, 2,4,5-TCP, 2,4-DBP, 2,3,4,6-TtCP and 2,4,6-TBP). The GC analysis of halogenated phenols consisted in toluene extraction from water or urine samples (directly or after SPE), followed by TMS-derivatization and ECD or MS detection. The evaluation of the analytical method (extraction and derivatization procedures, instrumental analysis) is presented in the following sections.

7.1. Studies on the derivatization of the halogenated phenols

Although the halogenated phenolic compounds are highly polar and their vapor pressures are low, a derivatization step is often used to achieve improved chromatographic performance eliminating tailing and resolution problems [154]. Reagents such as diazomethane [135], acetic anhydride [117], pentafluorobenzyl bromide [153], pentafluorobenzoyl chloride [121], heptafluorobutyric anhydride [133], and silylating reagents [417-419] have been employed to convert phenols to the corresponding

derivatives. Here we used the silylating agent *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) to transform hydroxyl groups into trimethylsilyl (TMS) ethers. For GC and MS, the addition of the TMS groups to polar compounds confers thermal and chemical stability in addition to enhanced volatility [420]. A notable advantage of BSTFA is the volatility of the by-products of the derivatization, trimethylsilyltrifluoroacetamide and trifluoroacetamide, which usually elute early in analyses, often with the GC solvent front. Heating is often used to achieve effective derivatization, but we have conducted derivatization at RT. As silylating reagents and derivatives are sensitive to the hydrolytic effects of moisture, the trimethylsilylating reaction was carried out in sealed vials directly in the toluene extract. The derivatized extracts were directly injected into the GC-ECD (MS) system.

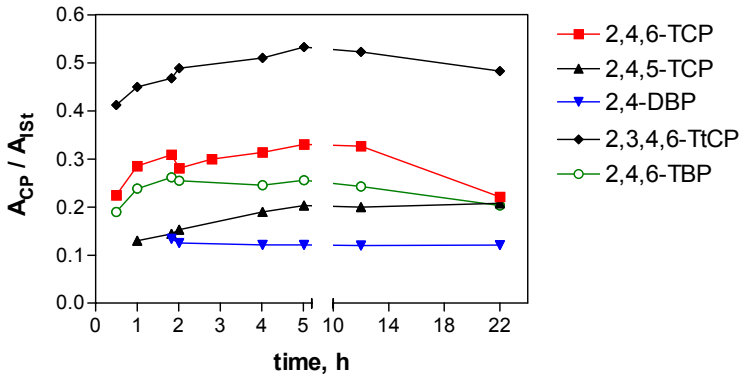


Figure 7.1. Effect of time on the silylation of a standard mixture of halogenated phenols (each at concentration 10 μg/L) with BSTFA (in excess) at RT. GC-ECD conditions are described in **Experimental Section**. ISt is 2,3,5,6-tetrachloro nitrobenzene.

Due to the negative inductive effect of chlorine atoms in the aromatic ring, the acidity of the phenolic group increases from mono- to pentachlorophenols. This effect can result in a different reactivity in the course of the derivatization procedure, and therefore the yield of products had to be controlled. In addition, in serial GC analysis of many samples, the stability of the TMS derivatives is also an important aspect. Thus, the effect of the reaction time at RT on the yields of the TMS-derivatives was examined for a standard mixture of five halogenated phenols (2,4,6-TCP, 2,4,5-TCP, 2,4-DBP, 2,3,4,6-TtCP and 2,4,6-TBP). As it is shown in **Figure 7.1** complete derivatization was achieved in 3h for

all the compounds and the derivatives were stable at RT for about 20h. In addition the silylation works equally well for the analysis of all the phenols tested. Yields for TMS ethers of the phenols on triplicate derivatizations had a RSD less than 13% at 0.5 μg/L level.

7.2. Extraction efficiency of the halogenated phenols from aqueous samples

Chlorophenols are weak acids and can be extracted by organic solvents under acidic conditions (pH<3). Various organic solvents have been reported, including hexane [117], diethyl ether [320], isopropanol/n-hexane mixture [64], etc. In our studies toluene was used because it was found to be the most efficient extraction solvent for chlorophenols from water [133] and urine samples [117].

Chromatographic analysis was used in the validation of the ELISA applied to water samples and the SPE methods applied to urine samples. It was also used in the characterization of the immunosorbent specificity in IAC-SPE. Thus, the eluting fractions containing 70% EtOH in water had to be extracted with a suitable solvent for the GC analysis. With this objective, the extraction efficiency of the five halogenated phenols from PBS and 7% EtOH/water samples (10 times diluted fractions) was evaluated after spiking at different concentration levels (see **Experimental Section** for the extraction procedure). Extraction recoveries were in the range 70-110% (see **Table 7.1**). Phenols were extracted individually and as well as in a mixture at low levels mimicking the real urinary concentrations of urine sample A (see **Section 3.4.1**). Different extraction ratios were used in order to achieve lower LOD in the urine samples. Extraction efficiency was essentially the same at extraction ratios of 1:1, 5:1, 10:1 and 30:1 PBS:toluene. According to Bengtsson et al even at water-to-toluene ratio of 100:1 the toluene extraction efficiency is the same [133]. For each case the extraction was performed in triplicate and the CV was less than 14% at all levels. These results show that the method was sufficiently precise for quantitative purposes.

Table 7.1. Extraction efficiency of halogenated phenols from

Sample	Compound	Conc., μg/L	Recovery ± SD, %
PBS	2,4,6-TCP	1	69.5 ± 10
		2.5	74± 6.7
		10	85.4 ± 5.4
		40	72 ± 2
		50	88 ± 4.3
	mixture [a]:		
	2,4,6-TCP	0.24	81± 8.4
	2,3,4,6-TtCP	0.2	107.4± 12
	2,4,6-TBP	0.1	84± 10.1
	ISt	1	64 ± 13
		2.5	68.7 ± 18
7%EtOH	2,4-DCP	8	110.9 ± 9.8
	2,4,6-TCP	8	95.2 ± 2.4
	2,4-DBP	8	81.8 ± 3.5
	2,3,4,6-TtCP	8	89.6 ± 4.4
	PCP	8	107.6 ± 7.0

PBS and 7%EtOH/water standards at extraction ratio water:toluene 5:1. Data present the result of three experiments (N=3). Extraction procedure and GC-ECD conditions are described in **Experimental Section**.[a] The extraction ratio PBS:toluene is 30:1.

It should be noted that as internal standard we used 2,3,5,6-tetrachloro nitrobenzene that has been used in chlorophenol chromatographic determination [114,147]. However, we found very low extraction recoveries for this compound with high deviation (CV=26%) (see **Table 7.1**). Although other compounds such as 2,4,6-TBP, 2,6-DBP and 2,3,6-TCP have been also used as internal standards [116,117,123], we did not use them because we needed an ISt that should not be an ELISA cross-reactant and on the other hand it should have similar behavior in the extraction procedure and finally to be well separated in the GC chromatograms from the other phenols. Considering all these requirements we decided to use 2,3,5,6-tetrachloro nitrobenzene as internal standard only in the GC measurements and to add it to the toluene extraction prior to injection in the chromatograph.

7.3. GC-ECD

We used GC-ECD analysis of chlorophenols in the validation of the ELISA applied to water samples, in C18-SPE and IAC-SPE characterization. **Figure 7.2** presents a GC-ECD chromatogram of a standard mixture of the main ELISA cross-reactants at 10 μg/L individual concentration after TMS-derivatization for 4h. As it is shown good separation of the analytes was achieved on the capillary column with a polar stationary phase (BPX35) when applying the following temperature program: 100°C (1 min) to 250°C (at 5°C/min), 250°C to 300°C (at 15°C/min) (see the rest of the conditions in the caption of **Figure 7.2**). 2,3,5,6-tetrachloronitrobenzene proved to be suitable internal standard (ISt) to monitor GC performance. The retention times of all di-, tri-, tetra- chloro- and bromophenols investigated are summarized in **Table 7.2**. Although the ECD is less sensitive to dichlorophenols by a factor of 100 compared to higher chlorinated phenols, the evaluation of the immunosorbent specificity was performed at detectable levels (concentrations higher than 20 μg/L in the toluene extract).

Table 7.2. GC-ECD –retention time of the halogenated phenols used in the specificity characterization of the IAC-SPE.

Phenolic compound	Retention time, min
2,5-DCP	7.5
2,6-DCP	7.8
2,4-DCP	7.9
3,4-DCP	8.7
2,4,6-TCP	10.6
2,3,5-TCP	11.0
2,4,5-TCP	11.2
2,3,6-TCP	11.8
2,3,4-TCP	12.9
2,6-DBP	12.4
2,4-DBP	12.6

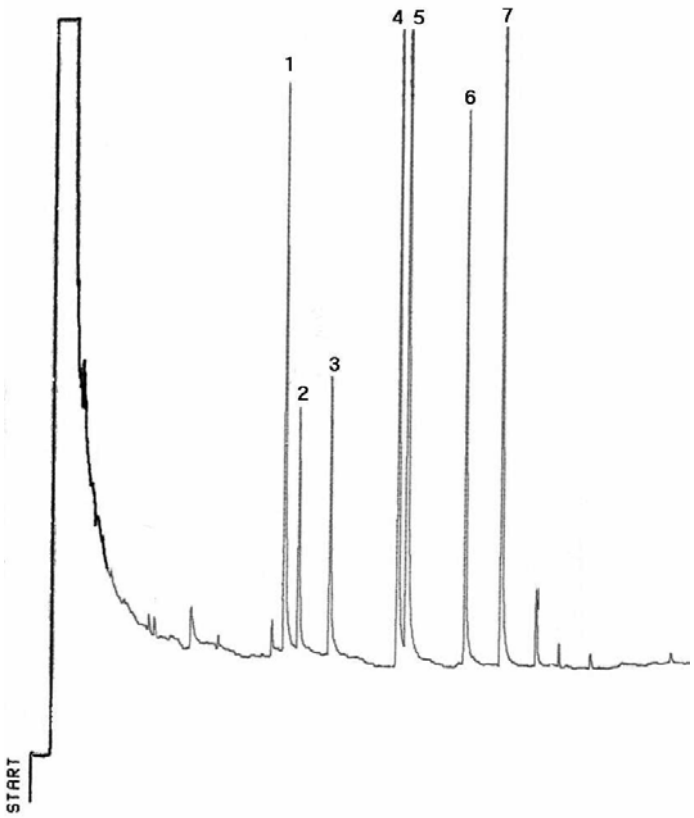


Figure 7.2. GC-ECD chromatogram of halogenated phenolic standards at 10 μg/L, derivatized with BSTFA for 3h. Peaks: 1 - 2,4,6-TCP; 2 - 2,4,5-TCP; 3 - 2,4-DBP; 4 – 2,3,4,6-TtCP; 5 – ISt; 6 – 2,4,6-TBP; 7 – PCP. Injections (1 μL, splitless) at 250°C; capillary column BPX35 (25 m x 0.22 mm i.d.), 0.25 μm film thickness; carrier gas: helium at 130 kPa (20 cm/sec); make up gas: N_2 5.0; detector temperature: 300°C; temperature program: 100°C (1 min) to 250°C (at 5°C/min), 250°C to 300°C (at 15°C/min).

Calibration curves for the ELISA cross-reactants were built using a standard mixture of the halogenated phenols in toluene at concentrations between 0.5 and 50 μg/L. The ISt was used at a concentration of 5 μg/L. Calibration graphs are obtained by plotting the quotients of the peak areas of the halogenated phenols and that of the internal standard as a function of the concentrations used (see **Figure 7.3**). The parameters of the standard curves and the detection limits for the halogenated phenols are presented **Table 7.3**.

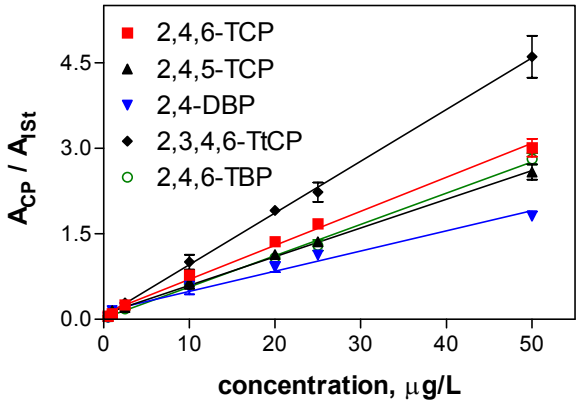


Figure 7.3. Calibration for halogenated phenols. The concentration corresponds to the phenol concentration in the toluene standard. GC-ECD conditions are described under **Figure 7.2**. ISt is 2,3,5,6-tetrachloro nitrobenzene. Each standard was prepared in triplicate and injected twice after 5h of silylation at RT.

Table 7.3. Properties of the calibration curves for GC-ECD analysis

Phenols	Calibration equation [a]	r^2	LOD [b], μg/L
2,4,6-TCP	0.299x+0.105	0.995	0.5
2,4,5-TCP	0.252x+0.092	0.998	0.5
2,4-DBP	0.177x+0.135	0.996	1
2,3,4,6-TeCP	0.453x+0.053	0.999	0.3
2,4,6-TBP	0.274x+0.021	0.997	0.5

[a] $x = m_{CP}/m_{IS}$; [b] The LOD corresponds to the phenol concentration in the toluene standard producing a signal/noise ratio = 3

The calibration curves are linear between the detection limits and 50 μg/L of the individual phenol. Low detection limits (~0.5 μg/L), which are essential for trace analysis of environmental and biological samples, were achieved using ECD for tri- and tetra-halogenated phenols. ECD response of 2,4-DBP was lower resulting in higher LOD. It should be noted that the reported LOD are related to the phenol concentration in the toluene standards. Further pre-concentration can be achieved in the extraction procedure (extraction ratio: 10:1 or higher).

7.4. GC-MS

The GC-MS technique was used for quantitative analysis and for identification of halogenated compounds in the urine extracts. As the highest sensitivity is desired to monitor phenols at trace levels, analyses were performed by mass spectrometry in the selective ion monitoring (SIM) mode monitoring positive ions. **Table 7.4** lists the analytical SIM conditions for the studied phenols.

Table 7.4. GC-MS-SIM analysis of trimethylsilyl derivatives of phenolic compounds

Parent Compound	Nominal Mw of derivative	Retention time, min	Mass Ions monitored, m/z [a]	Time monitored, min
2,4,6-TCP	268	9.55	*253*, 255, *268*	8.0 - 10.2
2,4,6-TCP	268	9.98	*253*, 255, *268*	8.0 - 10.2
2,4-DBP	322	10.45	307, **309**, **324**	10.2 - 11.2
ISt	259	11.45	203, 259, **261**	11.2 - 12.0
2,3,4,6-TtCP	400	12.64	287, **289**, **304**	12.0 - 13.2
2,4,6-TBP	302	14.05	**387**, 389, **402**	13.2 - 16.0

[a] m/z values in italics correspond to M^+; in bold correspond to $[M+2]^+$; in italics and underline are $[M-15]^+$; in bold and underline are $[(M+2)-15]^+$

To enhance sensitivity a time varied selected multiple ions mass spectral method was employed. The retention times of the phenols were between 8 and 16 min. Because of the EI fragmentation process, an $-CH_3$ group is lost. The resulting fragments show characteristic ion clustering caused by the chlorine and bromine isotopes. In general, the most abundant ion was used for ion monitoring and quantification, whereas the specific pattern was used for recognition.

Figure 7.4 shows the selected ion chromatograms for a mixture of halogenated phenolic standards in toluene and for hydrolyzed urine sample A. Chlorinated compounds in the urine extracts were identified by comparing GC-MS data of standard chlorine-containing phenols with the unknown components separated from the urine extracts. The peak assignation was based on (a) match of the mass obtained of the unknown with that of the respective chemical standard; (b) agreement of the retention time obtained of the unknown with the retention time of the chemical standard; (c) the consistency of the

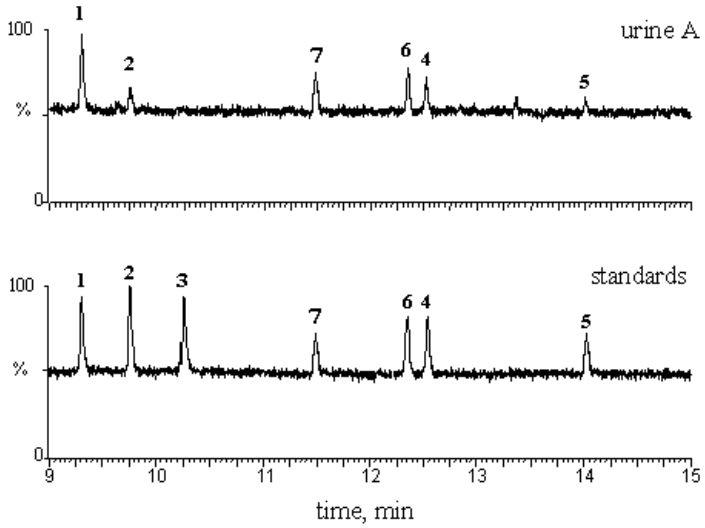


Figure 7.4. GC-MS-SIM chromatograms for halogenated standards and hydrolyzed urine smaple A. Injector: splitless at 250°C; column: HP-5MS (30 m x 0.25 mm id), 0.25 μm film thickness; carrier: helium; injection volume: 1 μL; temperature program: 100°C - 300°C (7°C/min); ion source temperature: 200°C; ionization energy: 70 eV; ion separation: quadropole rods; mass range monitored: see **Table 7.4**. Peaks: **1** – 2,4,6-TCP, **2** – 2,4,5-TCP, **3** – 2,4,-DBP, **4** – 2,3,4,6-TtCP, **5** – 2,4,6-TBP, **6** – 2,3,5,6-TtCP, **7** – ISt (m/z 203, 259, 261). The chromatogram for sample A was obtained after extraction of the urine (100 mL) with toluene (1 mL).

respective chlorinated and brominated isotopic pattern (i.e. di-,tri-, tetra-) of the standard parent ion with that obtained from the extracted ions.

In addition to qualitative analyses of the selected phenols, the described technique was also used for their quantitative analysis. To prepare the calibration curves standard solutions of the halogenated phenolic compounds were prepared in PBS 5 mL), extracted with toluene (400 μL) (PBS:toluene = 12.5:1) and sylilated. The extracts were analyzed by GC-MS using the conditions described in **Figure 7.4**. The typical standard response curves and their parameters are shown in **Figure 7.5** and **Table 7.5**. The relationship between the peak area ratio A_{CP}/A_{ISt} and the phenol concentration in the PBS sample is linear over the range 0.2 and 5 μg/L.

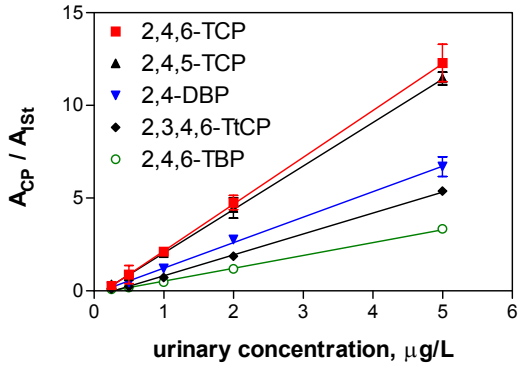


Figure 7.5. Calibration curves based on extracted halogenated phenols from PBS standards. GC-MS conditions are described in Experimental. ISt is 2,3,5,6-tetrachloro nitrobenzene. Each standard was prepared in duplicate and injected twice after 5h of silylation at RT.

Table 7.5. Calibration curves for GC-MS analysis

Phenols	Calibration equation [a]	r^2	LOD [b], μg/L
2,4,6-TCP	2.536x-0.375	0.999	0.1
2,4,5-TCP	2.355x+0.019	0.999	0.1
2,4-DBP	1.381x+0.028	0.999	0.2
2,3,4,6-TeCP	1.133x-0.322	0.998	0.2
2,4,6-TBP	0.696x-0.167	0.998	0.2

[a] x is urinary concentration, μg/L; [b] The LOD corresponds to the phenol concentration in urine sample at extraction ratio 12.5:1 producing a signal/noise ratio = 3

These type of calibration graphs were used to estimate the concentration of the halogenated phenols in the urine of the three population groups studied (see **Chapter 5**). Most of the urine samples were within this range. Samples with greater concentrations were diluted and reanalyzed. The least detectable concentration is 0.1 μg/L TCP in urine (see **Table 7.5**). For within-series imprecision values between 9.6% and 16.1% were obtained. The precision as well as the accuracy are very satisfactory with respect to the low concentrations to be determined and the sample treatment (extraction and derivatization).

8. Annex II.

Production of scFv fragments by phage display technology and their application as biorecognition elements in immunodetection systems

Owing to their high specificity, antibodies immobilized on various supports have been widely used for different purposes. In the previous chapters of this thesis we have demonstrated their application in immunoassays (ELISA, QFIA) and in immunoaffinity chromatography. Immunosensors, in which antibodies are immobilized on optical fibers,

electrodes or semiconductor chips for the detection of antigens, represent another promising application [157,166]. Some of the most important challenges in the design of novel immunosensor systems are the oriented antibody immobilization onto the transducer surface in order to increase the antigen binding capacity, the controlled formation of an antibody monolayer and the use of the minimal active recognition unit (e.g. Fv) with the aim to achieve least surface concentration [421]. Furthermore, multilayer self-assembly immobilization procedures based on electrostatic layer-by-layer deposition were incorporated in the field of the immunsensors [422-424]. These approaches would allow better control of many immunosensor parameters, such as detection limit, sensibility, reproducibility, regeneration and non-specific adsorption.

Novel antibody species with specifically designed properties for use in sensor and other applications can be generated by molecular genetic techniques quickly and at low cost. Recombinant phage display technology, developed during the past decade, has allowed the generation of a variety of antibody fragments (Fab, Fd, scFv) through their expression in bacteria derived from bacteriophage display repertoires [160,161]. Furthermore, the fusion of genetically encoded tags to antibody fragments offers an attractive solution. It was shown that fusions of the protein calmodulin with scFv fragments could be expressed by secretion from bacteria in high yield [425]. The highly acidic nature of the calmodulin tag (net charge –24 at pH 8.0) [425] could allow the ordered immobilization of the fusion protein scFv-calmodulin (scFv-CAL) on a positively charged surface of an immunosensor for the detection of a particular antigen. In this study as a model analyte was chosen the drug digoxin, a potent cardiac glycoside widely prescribed for the treatment of patients suffering from congestive heart failure, as well as some types of cardiac arrhythmias. Its narrow therapeutic range (5.10^{-4}-2.10^{-3} mg/L) requires the precisely monitoring of digoxin in human serum. Therefore, the objective of the work presented in this Annex was the production of scFv fragments with anti-digoxin specificity by phage display technology, their genetically fusion to the negative tag calmodulin and the characterization and application of the fragments obtained as biorecognition elements in immunodetection systems (ELISA, SPR immunosensor). This work was developed in the group of Dr. G.Winter at the Medical Research Council Centre for Protein Engineering –

Cambridge, UK in collaboration with Dr. E. Domínguez from University of Alcalá de Henares and Dr. I. Katakis from University Rovira i Virgili.

8.1. Production and characterization of anti-digoxin scFv antibody fragments and fusion protein anti-digoxin scFv-calmodulin (scFv-CAL)

The initial step in the production of anti-digoxin scFv antibody fragments by phage display technology is the selection of phage library with digoxin specificity (see **Figure 8.1**). We have used two synthetic libraries (DVT and NKK) constructed in the phagemid vector pIT2 encoding scFv fragments with different diversity in the CDR2 and CDR3 regions of the V_H and V_L domains (for details see **Experimental Section**, **Figure 9.4**). Both libraries were grown in *E.coli* TG1. The scFv fragments were displayed as fusions with the minor coat protein of the phage (pIII) as single polypeptide chains in which the heavy and light chain variable domains (V_H and V_L) were linked by a polypeptide linker (see **Figure 8.1.A.**). Antigen-driven selection was performed mimicking the immune system: phages displaying the scFv fragments were selected by binding to digoxin-BSA coated immunotubes. The eluted anti-digoxin phages were grown in bacterial culture and subjected to further rounds of selection to enrich affinity. The overall procedure mimics the affinity maturation process in the *in vivo* immune systems. The selected anti-digoxin phages (three rounds of selection) were grown in *E.coli* and soluble anti-Digoxin scFv fragments were expressed from individual colonies (see **Figure 8.1.B.**). The initial affinity screening was performed on ELISA and SPR directly with the bacterial supernatants containing the anti-digoxin scFv fragments isolated from 24 clones.

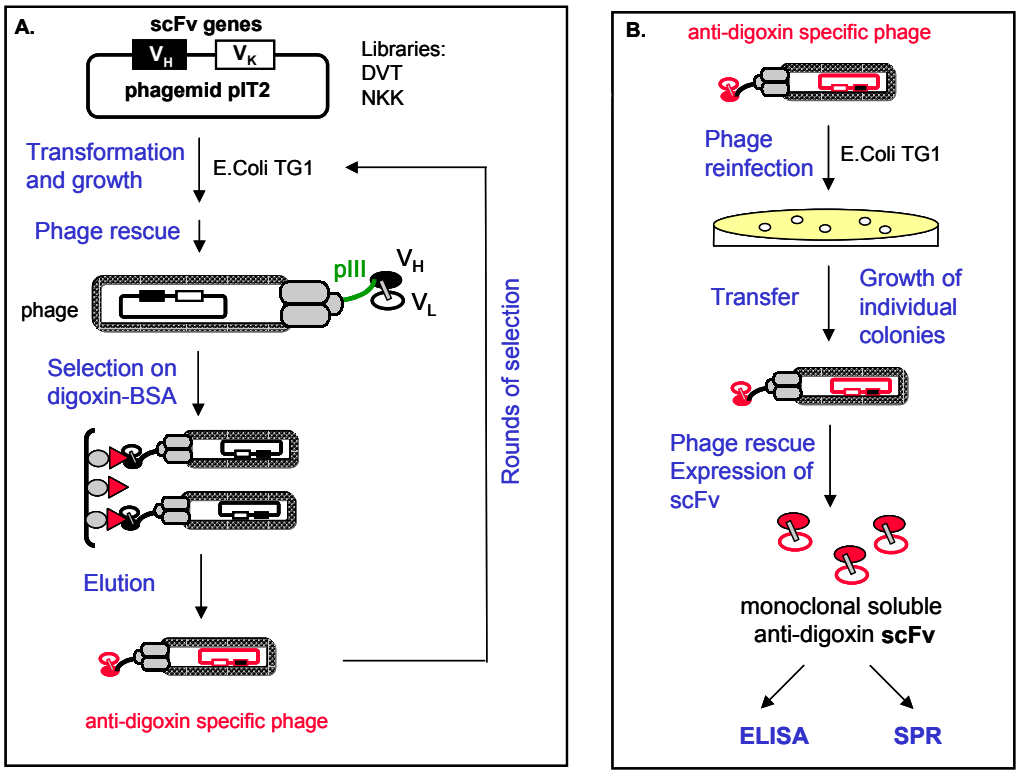


Figure 8.1. Production of anti-digoxin scFv fragments by phage display technology. **A**. Selection of phages with digoxin specificity (transformation of phagemid vector pIT2 to *E.coli*; surface display of scFv fragments; antigen driven selection and affinity maturation). **B**. Production of soluble monoclonal anti-digoxin scFv fragments.

The results are summarized in **Table 8.1**.

Table 8.1. Screening of 24 monoclonal soluble anti-digoxin scFv (bacterial supernatants)

Method	Antigen	Library	
		DVT	NKK
ELISA [a]	Digoxin-BSA	19	2
	Digoxin-HRP	12	3
SPR [b]	Digoxin-BSA	13	0
	Digoxin	5	0

[a] Number of colonies with A_{max} > 0.5 in ELISA (1D) with coating antigens Digoxin-BSA and digoxin-HRP at 25 µg/mL). [b] Number of colonies with positive binding at CM5 chips coated with Digoxin-BSA (100 µg/mL, 100 µL injected volume at pH=4) and with aldehyde activated digoxin (100 µg/mL, 100 µL injected volume)

The ELISA screening indicates that the scFv produced from phages selected from the DVT library gave higher number of positive clones against both tested coating antigens (digoxin-BSA and digoxin-HRP). The specificity of scFv binding to digoxin-BSA in ELISA (2D format) is demonstrated in **Figure 8.2.**

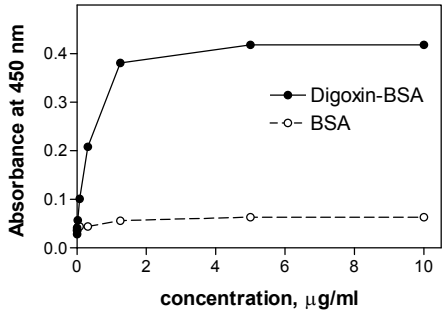


Figure 8.2. ELISA (2D experiment with plates coated with digoxin-BSA and BSA) of soluble monoclonal anti-digoxin scFv fragments (bacterial supernatant). Procedure is described the **Experimental Section**.

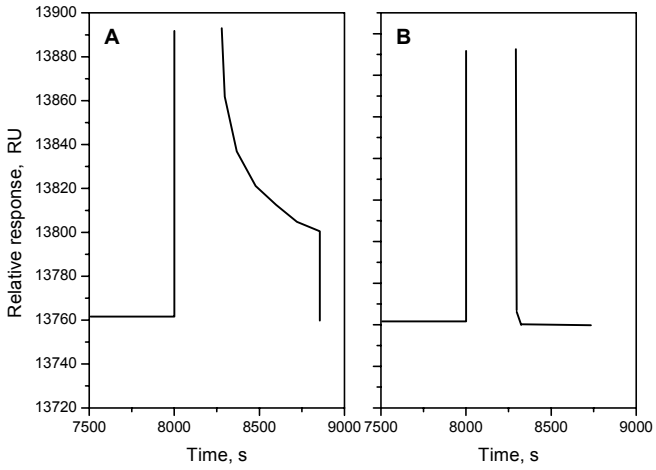


Figure 8.3. BIAcore analysis of soluble monoclonal anti-digoxin scFv fragments (bacterial supernatant). CM5 chips were coated with Digoxin-BSA (**A**) and BSA (**B**) (100 μg/mL, 100 μL injected volume at pH=4) until 13750 RUs. The increasing of ΔRU=40 RU corresponds to the binding of the anti-digoxin scFv to the immobilized antigen (digoxin-BSA). No binding to BSA is detected.

Similar result was obtained with digoxin-HRP coated plates. The clones giving positive ELISA response were checked by SPR with BIAcore system (Pharmacia Biosensor). The scFv fragments were passed over biochips immobilized with digoxin-BSA, aldehyde activated digoxin and BSA as negative control (see **Figure 8.3.**). The SPR analysis reveals some false positive ELISA binding and indicated that only scFv produced from the DVT phage library gave positive SPR-response (see **Table 8.1**). These can be explained with the higher diversity of the DVT library ($1.47x10^8$ clones with 96% insert) with respect to the NKK library ($1.37x10^8$ clones with 88% insert). The V-genes of the clones producing anti-digoxin scFv fragments with positive SPR binding were screened by PCR. The electrophoresis of the PCR products confirmed the presence of both inserts V_H and V_K with a complete nucleotide length corresponding to 935 bp. The sequence analysis (Sanger method) showed that all clones have the same DNA sequence for the V_H and V_K diversified regions. Therefore, we can conclude that only one type of monoclonal anti-digoxin scFv fragment was obtained from the synthetic DVT genomic library (pIT2 vector).

With the aim to obtain anti-digoxin scFv fragment labelled with a negative tag (calmodulin) for its electrostatic ordered immobilization on immunosensor surfaces, we have approached the genetic fusion between the anti-digoxin scFv and the protein calmodulin (see **Figure 8.4.**).

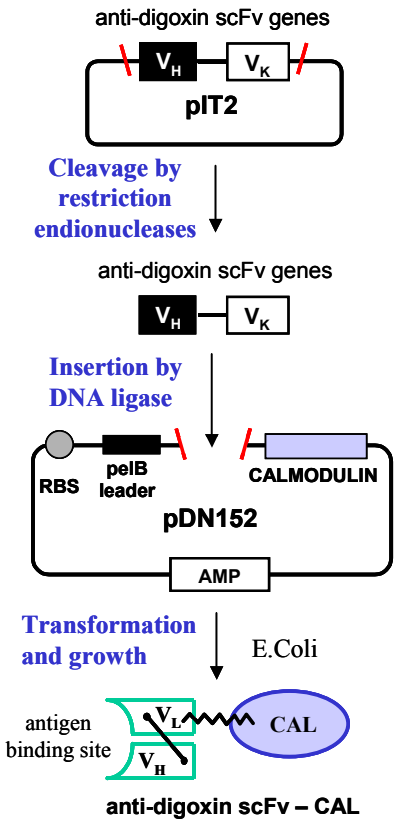


Figure 8.4. Production of the fusion protein antidigoxin scFv-CAL

The gene encoding the anti-digoxin scFv fragment was cleaved from the pIT2 vector and cloned into the calmodulin vector pDN152 [425]. The resulting vector was transformed into *E.coli* and the fusion protein anti-digoxin scFv-CAL was expressed by secretion from the bacteria. In this way the proteins were linked by a two residue polypeptide (Ala_2) between the C-terminus of the V_L domain and the N-terminus of calmodulin to place the antigen binding sites apart. The culture supernatant containing the fusion protein was assayed by ELISA using plates coated with digoxin-BSA and digoxin-HRP and it showed binding activity against digoxin. Furthermore, the fusion protein (45 kDa) was compared to the scFv fragment (28 kDa) in a PAGE electrophoresis (see **Figure 8.5.A**).

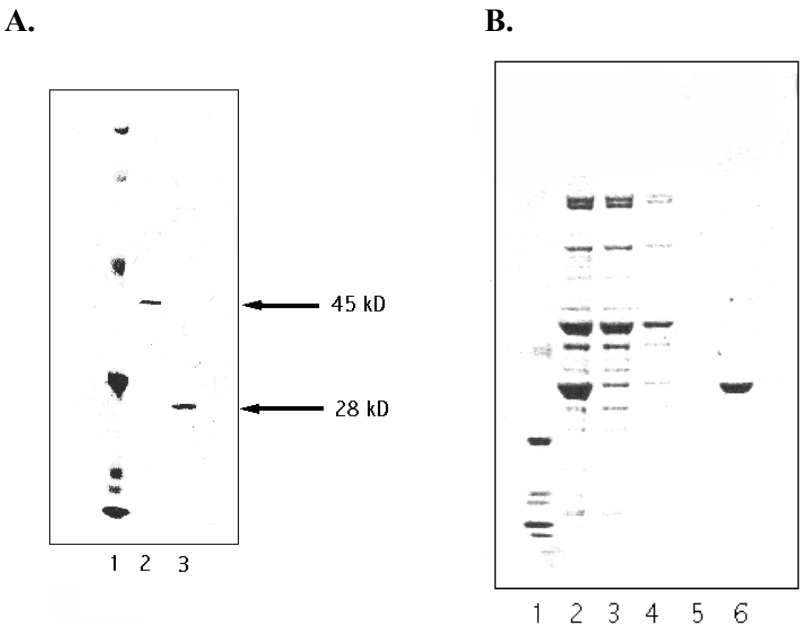


Figure 8.5.A. Detection of the obtained recombinant fragments by PAGE electrophoresis. Lane 1 – makrer; lane 2 – anti-digoxin scFv-CAL; lane 3 – anti-digoxin scFv. The gel was stained with Coomassie blue. **B.** Purification of the fusion protein anti-digoxin scFv-CAL from a bacterial supernatnat. Lane 1 – marker; lane 2 – scFv-CAL purified on DEAE Sepharose column; lane 3, 4, 5 – washings from DEAE Sepharose column; lane 6 - scFv-CAL purified on protein A column.

The last step in the recombinant antibody fragments production is their purification from the culture broth. The calmodulin tag allows the fusion protein to be purified on an anion-exchanegd resins. The fusion protein was concentrated and initially purified from the bacterial supernatant by DEAE chromatography eluting at 400 mM NaCl. As it can be observed in **Figure 8.5.B.** the obtained fraction does not represent very high degree of purification (lane 2). Thus, the scFv-CAL was further purified on protein A (lane 6). The anti-digoxin scFv fragment was directly purified on protein A.

8.2. Application of the fusion protein anti-digoxin scFv-CAL in ELISA and SPR-immunosensor for detection of digoxin

With the objective to apply the anti-digoxin scFv-CAL protein as biorecognition element in an immunosensor based on the competition between the analyte (digoxin) and the labelled analyte (digoxin-HRP), initially we have tested it in a competitive direct ELISA. The concentrations of the immunoreagents (scFv-CAL and digoxin-HRP) were previously selected in 2D-titration of the immobilized scFv-CAL with digoxin-HRP. The parameters of the competitive ELISA curve and the assay conditions are presented in **Table 8.2.**

Table 8.2. Conditions and features of the digoxin ELISA standard curve (scFv-CAL/digoxin-HRP)

Condition	Values	Parameter	Value [a]
Competition time	2 h	**A_{max}**	0.75
pH	7.2	**A_{min}**	0.03
Ionic strength	15 mS/cm	**IC_{50}, mg/L**	0.016
Tween 20	0.05%	**Dynamic range, mg/L**	1.2×10^{-4} – 0.8
		slope	0.6
		r^2	0.996

[a] The data correspond to a standard curve run in duplicate

The immunoassay dynamic range covers the therapeutic range of the digoxin (5.10^{-4} – 2.10^{-3} mg/L), which allows the quantification of the drug for toxicological control. Further evaluation of the reproducibility, precision and performance in serum samples is needed in order to apply the immunoassay for clinical purposes.

As the genetically produced fusion protein scFv-CAL was intended to be electrostatically immobilized on a positively charged surface of a multilayer immunosensor through its negative (calmodulin) tag, we have determined its isoelectric point. The results from the isoelectric focusing for scFv, scFv-CAL and calmodulin are presented in **Table 8.3**.

Table 8.3. Isoelectric points (pI) determined by IEF in agarose gel.

Protein	pI
scFv	7.1 and 8.7
ScFv-CAL	6.1 and 6.4
Calmodulin (Sigma)	3.75

It can be seen that the coupling of the calmodulin tag to the scFv produced a decrease of the isoeletric point. Therefore, the anti-digoxin scFv-CAL protein can be considered negatively charged at the usual pH working conditions (pH 7.4).

Finally, we have tested the feasibility of the multilayer immunosensor structure based on electrostatic layer-by-layer deposition. The multilayer configuration presented in **Figure 8.6**.**B** consists in gold microchip on which are successively deposited due to electrostatic interactions a negatively charged monolayer of 3-mercapto-1-propansulfonic acid (MPS), a positively charged redox polymer and anti-digoxin scFv-CAL immobilized through its negative tag. The deposition of these monolayers was monitored by SPR (BIAcore). The SPR response profile after consecutive injections of the redox polymer and the scFv-CAL protein is presented in **Figure 8.6**.**A.**

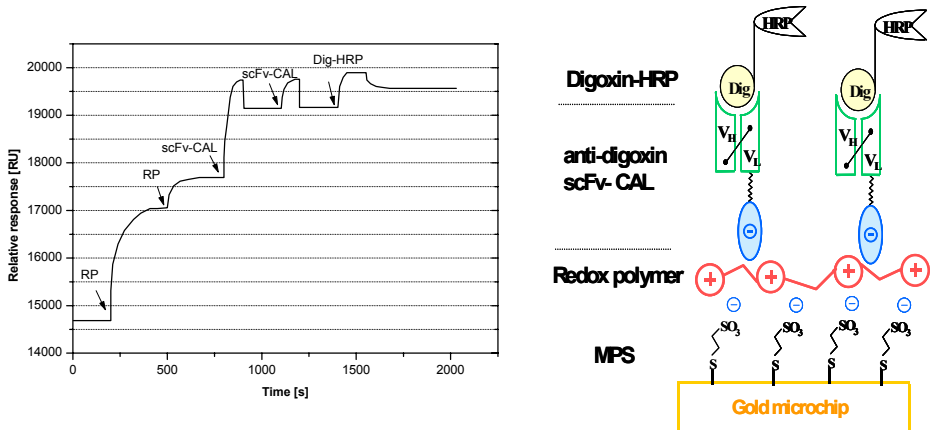


Figure 8.6. Multilayer immunsensor. **A**. SPR sensogram (Injections of positively charged redox polymer, anti-digoxin scFv-CAL and the antigen digoxin-HRP. Experimental conditions are described the **Experimental Section**); **B**. Multilayer configuration.

The formation of stable monolayers of the components and the binding of digoxin-HRP to the immobilized scFv fragment were detected. The variation of the response units (ΔRU) corresponding to each monolayer formation are summarised in **Table 8.4**.

Table 8.4. SPR multilayer formation. Data present the variation of the response units (ΔRU) corresponding to each monolayer formed.

Redox polymer	scFv-CAL	Calmodulin	Digoxin-HRP
3000	1700	-	300
3000	-	700	0

3000 RU and 1700 RU correspond to a saturation of the redox polymer and the scFv-CAL protein layers, respectively. The monolayer of scFv-CAL was stable for 10 min at flow rate 30 μL/min PBS injection. This demonstrates the stability of the electrostatic interactions between both modules: the positive polymer and the negative fragment. The immunoreaction between the immobilized scFv-CAL and the injected digoxin-HRP corresponds to 300 RU. The specificity of the signal was tested in a multilayer configuration in which the scFv-CAL was substituted by commercially available calmodulin. No binding of digoxin-HRP was detected. These results proved that an ordered and strongly absorbed layer-by-layer configuration can be built by electrostatic deposition and controlled oriented immobilization of the anti-digoxin scFv fragment via its negative calmodulin tag can be achieved. Further optimisation of layer deposition, immunoreaction parameters and development of an electrochemical immunosensor with the same architecture should be followed.

9. Experimental methods and materials

9.1. Instruments

The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). The centrifuge, model 5415D was from Eppendorf (Germany). Washing steps were

carried out using a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read at a single wavelength mode of 450 nm on a SpectamaxPlus (Molecular Devices, Synnyvale, CA USA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v2.6 (Molecular Devices) and GraphPad Prism™ (GraphPad Sofware Inc., San Diego, CA, USA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates. Loading of urine samples of volume higher than 20 mL to the HiTrap™ IAC column was performed using Akta™ Prime (Pharmacia) automated liquid chromatography system.

For the development of quenching fluorescence immunoassay (QFIA), fluorescence was read using Spectrofluorometer FluoroMax-2 (Jobin Yvon-SPEX Instruments, S.A.) and SPECTRAFluor Plus plate reader (TECAN US, Inc.). For LIF-QFIA in microdroplets the following instruments were used: TSI Model 3450 Vibrating Orifice Aerosol Generator (orifice diameter 10 μm); continuous Ar ion laser, (Coherent Innova 70, λ = 488 nm); microscope objective lens (N.A. of 0.55); imaging spectrometer (SpectraPro-150, Acton Research Corp. MA); thermoelectrically-cooled camera (TEA/CCD-521-TKMI, Princeton Instruments Inc, NJ) with a 512 x 512 pixel charge coupled device (CCD) detector; ST130 Controller (Princeton Instruments Inc, NJ); holographic laser band pass filter (Kaiser Optical Systems Inc., MI) and 488 nm holographic Raman notch filter (Kaiser Optical Systems Inc., MI).

In the production of recombinant antibody fragments by phage display technology and their characterization the following instruments were used: PCR thermal cycler (Eppendorf), DNA Sequencer 373 (Applied Biosystems), incubator (Bioblock); thermostatic bath 911 (Bioblock); oven for culture growth (Selecta), IEF (isoelectric focusing) PhastSystem™ (Pharmacia Biotech), BIAcore™ System (Pharmacia Biosensor AB, Uppsala, Sweden).

9.2. Chemicals and immunochemicals

The As 53-58 against 2,4,5-TCP were prepared in this thesis as described in Section 9.9. The As43-45 against 2,4,6-TCP were prepared previously in our group [259]. The rest of the immunochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorophenols and bromophenols for cross-reactivity studies and fluoresceinamine isomer I (5-aminofluorescein) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The proteins KLH (keyhole limpet hemocyanine), BSA (bovine serum albumin), CONA (conalbumin), OVA (ovalbumin) and HRP (horseradish peroxidase) used in the preparation of the immunoreagents, coating antigens and enzyme tracers were purchased from Sigma Chemical Co. (St. Louis, MO). Bradford Protein Assay Dye Reagent for the determination of protein concentration was obtained from Bio-Rad Laboratoties GmbH (Munich, Germany). β-glucuronidase/sulfatase (type H2 from Helix pomatia) was purchased from Sigma (121 900 β-glucuronidase U/mL and 4 100 sulfatase U/mL.

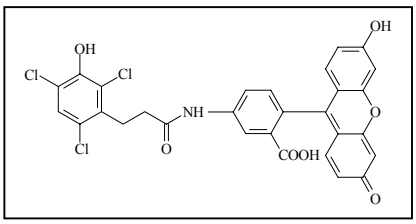
Digoxin (3β-[(*O*-2,6-didesoxi-β-D-ribo-hexopiranosil-(1-4)-*O*-2,6-didesoxi-β-D-*ribo*-hexopiranosil-(1-4)-2,6-didesoxi-β-D-*ribo*-hexopiranosil)oxy]-12β, 14-dihidroxi-5β-card-20(22)enilode) and digoxin-BSA were purchased from Biogenesis and digoxin-HRP (digoxin:HRP=5:1) from YJ Bioproducts. Calmoduline and protein A-*Sepharose*® CL-4B were purchased from Pharmacia Biotech; DEAE *Sepharose* Fast Flow, protein A-AP (alkaline phosphatase) and protein L-HRP were from Actigen Ltd. The bacterial strain *E.coli* suppressor strain TG1 for propagation of phage particles; the media for growth of cultures: 2xTY (tryptone and yeast extract) and TYE (tryptone, yeast extract and bacto-agar)-plates; antibiotics (ampicilline, kanamycin), glucose, glycerol, tripsin, 4-aminoantipirine, pNPP (p-nitrophenyl phosphate) substrate tablets and isopropyl-1-thio-β-D-galactosidase (IPTG) were purchased from Sigma. Marker ∅X 174 RF DNA/HAE III Digest was obtained from Biotechnology. Taq dyedeoxy terminator cycle sequencing kit was obtained from Applied Biosystems. Restriction enzyme Sfi I/Not I and T4 DNA ligase were purchased from Biolabs. Immunopure IgG elution buffer was from Pierce. QIAprep spin miniprep kit, QIAquick PCR purification kit and QIAquick gel extraction

kit were products of Qiagen. Sensor chip CM5 (carboxymethylated dextran matrix), pioneer chip J1 (gold surface) and amine coupling kit were obtained from BIAcore. SDS polyacrylamide phast gel was from Pharmacia and the NuPAGE™ Electrophoresis System was from Novex, San Diego, CA. Polyprep-chromatography columns were obtained from Biorad and Nunc Maxisorp immunotest tubes from Life Technologies. Helper phage KM13 for the rescue of the phagemid libraries and the primers (pHENseq and LMB3) used in the PCR screening were available from Medical Research Centre-Cambridge. The "synthetic" libraries (phagemid vector pIT2) were constructed by Dr. I.Tomlinson and the calmodulin vector pDN152 was a gift from Prof. Dr. D.Neri from MRC-Cambridge.

Hapten **5** (*3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid*) [259]; hapten **1** (*2,4,6-trichlorophenoxyacetic acid*), hapten **2** (*2,4,5-trichlorophenoxyacetic acid*), hapten **9** (*3-hydroxy-2,6-dichlorophenylacetic acid*), hapten **10** (*3-hydroxy-4,6-dichlorophenylacetic acid*) [260]; hapten **A** (*3-(2-hydroxy-3,5,6-trichlorophenyl)propanoic acid*), hapten **3** (*3-(2-hydroxy-3,5,6-trichlorophenyl)-2-propenoic acid)*, hapten **7** (*3-(4-hydroxy-3,5-dichlorophenyl)propanoic acid*) and hapten **8** (*3-(2-hydroxy-3,6-dichloropheny)propanoic acid*) [277]; hapten **11** and **12** [426] were prepared by R. Galve from our group. Hapten **4** (*2-hydroxy- 3,5,6-trichlorobenzoic acid*), was obtained from Aldrich Chemical Co. (Milwaukee, WI). Hapten **B** (*2,4,5-trichlorophenoxypentanoic acid*) was synthesized following the procedure described by Kramer *et al* [265].

The fluorescent tracers were prepared as follows:

(*3-(3-hydroxy-2,4,6-trichlorophenyl)propanoic acid*) - *fluorescein conjugate* (TCP-F)



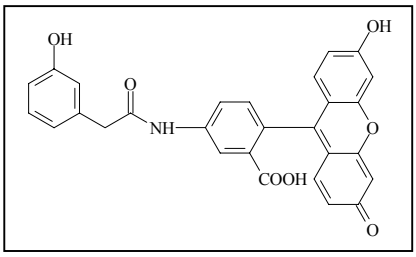
Hapten **5** (19.37 mg, 0.1 mmol) and fluoresceinamine isomer I (34.8 mg, 0.1 mmol) were dissolved in anhydrous THF (5 mL) containing 6% DMF. After adding N,N'-diisopropylcarbodiimide (DIC) (22.8 μL, 0.15 mmol) the mixture was stirred for 16 h at room temperature under an argon atmosphere. The

reaction was followed by TLC until a yellow spot (R_f=0.27) appeared on the 0.25mm pre-coated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) using toluene:ethyl acetate:acetic acid (5:5:1) as the mobile phase. The solvent was evaporated to dryness under reduced pressure and the residue was then dissolved in ethyl acetate and purified on 0.5 mm silica gel 60 F_{254} plates with the same mobile phase. The yellow band with R_f=0.73 obtained after four runs was extracted with ethyl acetate : methanol (1:1) and the extract evaporated to dryness to obtain an orange solid corresponding to the TCP-F conjugate (22.58 mg, 37.7% yield), identified according to its spectroscopic data on a Varian Unity-300 (Varian Inc., Palo Alto, CA, USA) spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C).

^{1}H NMR (300 MHz, CD_3OD) δ: 2.68 (t, *J* = 8.4 Hz, 2H, -CH_2CONH-), 3.32 (t, *J* = 8.4 Hz, 2H, PhCH_2-), 6.55 (dd, J_1 = 9 Hz, J_2 = 2.4 Hz 2H, FNH_2), 6.67 (d, *J* = 2.4 Hz, 2H, FNH_2), 6.68 (d, *J* = 9 Hz, 2H, FNH_2), 7.14 (d, *J* = 8.4 Hz, 1H, FNH_2), 7.38 (s, $1H_{Ar}$ meta, hapten 5), 7.85 (dd, J_1 = 8.4 Hz, J_2 = 1.9 Hz, 1H, FNH_2), 8.3 (d, *J* = 1.8 Hz, 1H, FNH_2).

^{13}C NMR (75 MHz, CD_3OD) δ: 28.53 (t), 35.81 (t), 103.6 (d), 111.9 (s), 114.4 (d), 116.7 (d), 121.8 (s), 124.7 (s), 125.4 (s), 126.3 (d), 127.5 (d), 129.3 (d), 130.5 (d), 137.2 (s), 141.7 (s), 147.0 (s), 150.6 (s), 154.7 (s), 159.9 (s), 171.5 (s), 173 (s).

***3-hydroxyphenylacetic acid - fluorescein conjugate* (HOPh-F)**



3-hydroxyphenylacetic acid (15.2 mg, 0.1 mmol) and fluoresceinamine isomer I (34.8 mg, 0.1 mmol) were dissolved in anhydrous THF (3 mL) containing 10% DMF. After adding N,N'-diisopropylcarbodiimide (DIC) (22.8 μL, 0.15 mmol) the mixture was stirred for 4 h at room temperature under an argon atmosphere. The reaction was followed by TLC until a yellow spot (R_f=0.3) appeared on the 0.25mm pre-coated

silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) using toluene:ethyl acetate:acetic acid (5:5:1) as the mobile phase. The solvent was evaporated to dryness under reduced pressure and the residue was then dissolved in ethyl acetate and purified on 0.5 mm silica gel 60 F_{254} plates with mobile phase dichloromethane:methanol:acetic acid (19:1:0.1). The yellow band with R_f=0.23 obtained after five runs was extracted with ethyl acetate : methanol (1:1) and the extract evaporated to dryness to obtain an orange solid corresponding to the HOPh-F conjugate (21 mg, 40 % yield), identified according to its spectroscopic data on a Varian Unity-300 (Varian Inc., Palo Alto, CA, USA) spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C).

^{1}H NMR (300 MHz, CD_3OD) δ: 3.66 (s, 2H, -CH_2CONH-), 6.55 (dd, J_1 = 8.7 Hz, J_2 = 2.4 Hz 2H, FNH_2), 6.6 (d, J = 2.1 Hz, 2H, FNH_2), 6.69 (ddd, J_1 = 8.1 Hz, J_2 = 2.4, J_3 = 0.4, 1H_{Ar}, *para*, hapten) 6.74 (d, J = 9 Hz, 2H, FNH_2), 6.85 (d, 2H_{Ar} *ortho*, hapten), 7.14 (dd, J = 8.1 Hz, 1H, FNH_2), 7.19 (t, J_1 = 8.1 Hz, 1H_{Ar} *meta*, hapten), 7.87 (dd, J_1 = 8.4 Hz, J_2 = 1.9 Hz, 1H, FNH_2), 8.27 (d, J = 1.8 Hz, 1H, FNH_2).

^{13}C NMR (75 MHz, CD_3OD) δ: 44.7 (t), 103.6 (d), 112.6 (s), 115 (d), 115.6 (d), 116.9 (d), 117.6 (d), 121.3 (d), 126.6 (d), 127.1 (d), 130.6 (d), 130.8 (d), 132.2 (s), 137.8 (s), 141.6 (s), 144.3 (s), 155.4 (s), 158.7 (s), 159.9 (s), 165.1 (s), 171.8 (s), 172.7 (s).

9.3. Buffers

PBS is 10 mM phosphate buffer, 0.8 % saline solution and unless otherwise indicated the pH is 7.5. Borate buffer is 0.2 M boric acid-sodium borate pH 8.7. Coating buffer is 50 mM carbonate-bicarbonate buffer pH 9.6. PBST is PBS with 0.05 % Tween 20. Citrate buffer is a 40 mM solution of sodium citrate pH 5.5. The substrate solution contains

0.01% TMB (tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer. The water used was deionised, filtered and purified on a Milli-Q Reagent Grade Water System from Millipore.

9.4. Other materials

The dialysis membranes (12-14000 Da) used have 0.81 mL/cm capacity, diameter of 10 mm and width of 16 mm. Polystyrene microtiter plates (96 wells) used in the ELISA experiments were purchased from Nunc (Maxisorp, Roskilde, DK). Microtiter plates (96 wells) used in the Bradford protein assay were purchased from Dynatech (St. Peter Port, Guernsey, Channel Islands). The precision pipettes of different volume and the multichannel pipette (50-1200 μL) used in HTS-IAC-SPE are Eppendorf production and the multichannel pipette (25–200 μL) used in ELISA is from Micronic Systems (Lelistad, Holland).

HiTrap™ desalting column (5 mL bed) prepacked with Sephadex®G-25 Superfine (Pharmacia Biotech, Uppsala, Sweden) was used for desalting of the purified IgG fraction of As45. Human serum ultrafiltration for matrix effect studies was performed with filters (10 kDa cut-off, Millipore). A Sep-Pak® Plus C_{18} cartridge (Waters, S.A.) (360 mg nominal weight of sorbent, 10-15 mg analyte capacity, maximum sample volume 300 mL, stable in the range pH 3-8) was used for urine clean-up. For IAC-SPE, HiTrap NHS activated Sepharose™ columns (1 mL bed) were purchased from Pharmacia Biotech (Uppsala, Sweden). The gel is based on highly cross-linked agarose beads with 6 atoms spacer arms attached to the matrix by epichlorohydrine and activated by N-hydrohysuccinimide (NHS). The substitution level is approximately 10 μmol NHS-groups/mL gel. For HTS-SPE-IAC NHS-activated Sepharose® 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden) was used. It is highly cross-linked 4% agarose matrix with a ligand density 16-23 μmol NHS/mL drained medium, mean particle size 90 μm and pH stability 3-13.

VersaPlate™ 96-Well SPE System (Varian, Palo Alto, CA, USA) consists of a 96-well baseplate, removable 96 empty tubes and a vacuum manifold set. The vacuum manifold was connected to a water pump. The vacuum controller (mechanical gauges) was purchased from Hucoa-Erlöss. VersaPlate™ accessories, such as disposable waste reservoir, cartridge removal tool, 20 μm pore frits, 96 glass vials (0.75 mL) in a collection rack, 96-well microplate Teflon-coated silicone rubber seal, sealing tape pads, and sealing caps, were purchased from Varian.

9.5. Molecular modeling and theoretical calculations

Molecular modeling was performed using the Hyperchem 4.0 software package (Hyperube Inc, Gainesville, FL, USA). Theoretical geometries and electronic distributions were evaluated for 2,4,5-trichlorophenol and the haptens using semiempirical quantum mechanics MNDO [273] and PM3 [427] models. All the calculations were performed using standard computational chemistry criteria. Theoretical calculations regarding pKa values were carried out using the ACD/p*K*a 1.2 software package (Advanced Chemistry Development Inc., Toronto, ON, Canada) at the Department of Analytical Chemistry (University of Lund, Sweden).

9.6. Preparation of protein conjugates

9.6.1. Mixed anhydride (MA) method – general protocol

Tributylamine (11.6 μL, 44 μmol) and isobutylchloroformate (6.4 μL, 48 μmol) were added dropwise under Ar atmosphere to a solution of the hapten (40 μmol) in anh. DMF (300 μL) in an ice bath. The reaction took place for 30 min at RT under stirring. The activated hapten was added dropwise to a solution of the protein (10 mg) in 1.8 mL borate buffer (pH=8.7). The coupling reaction took place under stirring for 4h at room temperature (see **Figure 9.1**). The conjugates were dialyzed against PBS 10 mM (4 times

x 5L) and H_2O (1 time x 5L), lyophilized and stored at –40ºC. Working aliquots (1mg/mL, PBS 10mM) were kept at 4ºC.

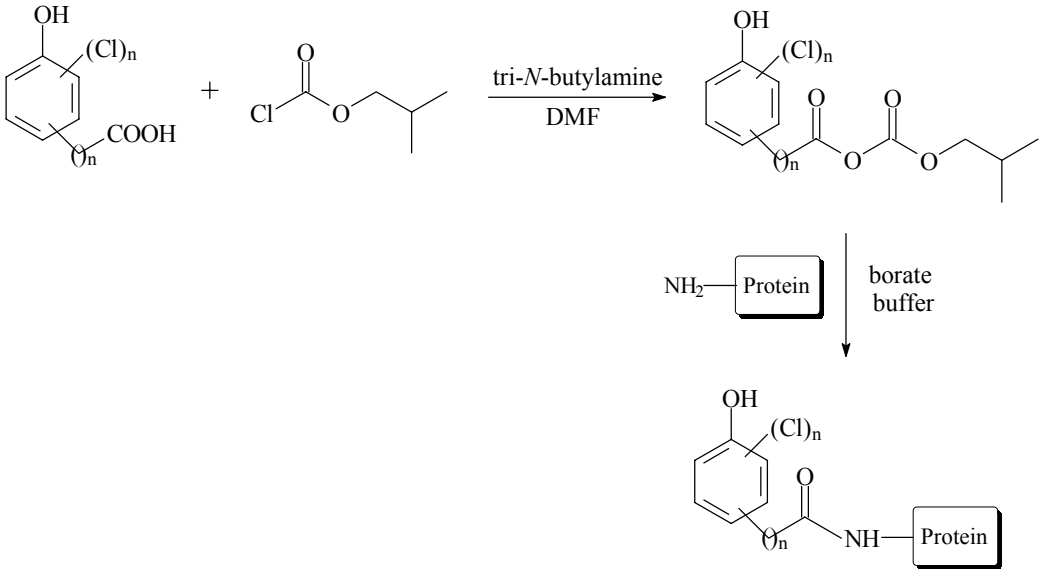


Figure 9.1. Mixed anhydride method

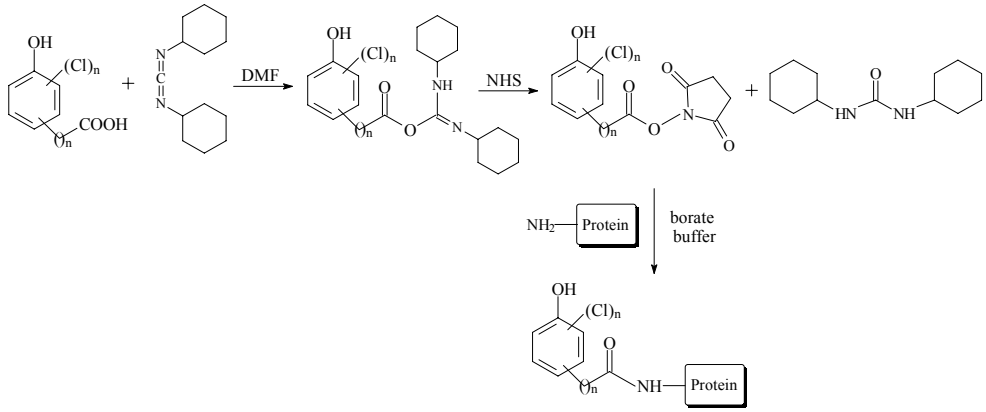


Figure 9.2. Active ester method used for the ET conjugation

9.6.2. Active ester (AE) method – general protocol

The hapten (10 μmol) was reacted with NHS (*N*-hydroxysuccinamide, 5.7 mg, 50 μmol) and DCC (dicyclohexylcarbodiimide, 20.6 mg, 100 μmol) in anh. DMF (200 μmol) under stirring for 1h at room temperature. The suspension formed was centrifuged at 10000 rpm for 10 min and the supernatant was added drop wise to a solution of the protein (HRP, 2 mg or BSA, OVA and CONA, 10 mg) in 1.8 mL borate buffer. The coupling reaction took place under stirring for 2h at room temperature (see **Figure 9.2**). The conjugates were dialyzed against PBS 10 mM (4 times x 5L) and H_2O (1 time x 5L), lyophilized and stored at –40ºC. Stock solutions of the obtained ETs (1 mg/mL) were prepared in PBS/saturated $(NH_4)_2SO_4$ (1:1) and stored in aliquots under argon atmosphere at 4ºC. Working aliquots of the coating antigens (1 mg/mL, PBS 10mM) were kept at 4ºC.

9.7. Bradford protein assay

Standard curves (1–40 μg/mL) for each protein (BSA, CONA, OVA) were prepared in PBS 10 mM. These standards and the samples of CAs (in serial dilutions) were placed in a microplate (160 μL/well) in triplicate and then Bradford Protein Assay Dye Reagent was added (40 μL/well). After 15 min the absorbance at 595 nm was measured.

9.8. Hapten density analysis

Hapten densities of BSA and OVA conjugates were determined by matrix-assisted laser-desorption ionization time-of-fly mass spectrometry (MALDI-TOF-MS) by comparing the molecular weight of the standard protein (BSA or OVA) with that of the respective conjugates. The MALDI-MS (matrix-assisted laser desorption ionization mass spectrometer) used for analyzing the protein conjugates was a Perspective Biosystems Time of Flight (TOF) Mass Spectrometer Voyager-DE ^{TM} RP equipped with a laser unit which operates with an intensity of 2800. The instrument is controlled by a

BioSpectrometry™ Workstation provided with the software Voyager-DE™-RP (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA, USA) and GRAMS/386™ (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corporation (Salem, NH, USA). MALDI spectra were obtained by mixing 2 μL of the matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg/mL in CH_3CN/H_2O 70:30, 0.1% TFA) with 2 μL of a solution of the conjugates or proteins (10 mg/mL in CH_3CN /H_2O 70:30, 0.1% TFA). Hapten density (δ) is calculated according to:

$$\delta = \frac{Mw(\text{CA}) - Mw(\text{Protein})}{Mw(\text{hapten})},$$

where Mw(CA) is the molecular weight of the coating antigen, Mw(Protein) is the molecular weight of the protein used for the conjugation, and Mw(hapten) is the molecular weight of the hapten.

9.9. Polyclonal antisera

Three female New Zealand white rabbits (As53-55) were immunized with **A**–KLH and other three rabbits (As56-58) were immunized with **B**-KLH. Evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the antisera to microtiter plates coated with A–BSA and B-BSA. After an acceptable antibody titer was observed, the animals were exsanguinated and the blood was collected on vacutainer tubes with a serum separation gel. Antiserum was obtained by centrifugation and stored at -40°C in the presence of 0.02% NaN_3. The antisera (As) were used in ELISA without further purification. Working aliquots were stored at 4ºC.

Polyclonal antisera (As43, As44 and As45) against 2,4,6-TCP were obtained in our group by immunization of female New Zealand white rabbits with hapten 5 conjugated to KLH by the mixed anhydride method [259].

9.10. Development of an ELISA for 2,4,5-TCP

Non-competitive immunoassays were used to select immunoreagents and to establish the most appropriate concentrations. The objective of the experiments called "1D" was to select those immunoreagents (ETs, CAs, ans As) able to react giving enough response under the immunoassay conditions. The objective of the experiments called "2D" was to establish the most appropriate concentrations for competitive assays and to evaluate antibody avidity versus ETs and CAs.

The competitive immunoassays used in this thesis were used to measure the analyte. The shape and features of the calibration curves were used to optimize the immunoassay conditions. In these assays the standard curves were fitted to a four-parameter logistic equation according to the following formula:

$$y = (A-B/[1-(x/C)^D])+B,$$

where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve.

In this work we have used direct and indirect ELISA formats.

9.10.1. Direct ELISA

General protocol

Microtiter plates were coated with the respective dilution of the As in coating buffer (100 µL/well) overnight at 4ºC. The following day the plates were washed 4 times with PBST (300 µL/well) and the ET (or ET and analyte) solutions prepared in PBST were added and incubated for 30 min at RT. The plates were washed again 4 times with PBST and the substrate solution (100 µL/well) was added and incubated 30 min at room temperature. The enzymatic reaction was stopped by adding 4 N H_2SO_4 (50 µL/well). Finally, the plates were read at absorbance 450 nm.

A. Non-competitive assays

No analyte was added. The ETs were added to the coated plates appropriately diluted in PBST (100 μL/well).

- Experiment 1D

For each ET (haptens **A**, **1**-**5** and **7**-**12**) microtiter plates were coated with each antisera (As53-58, 1/1000 dilution) in different columns. On the next day the plates were washed and the ETs were added to each column in serial dilutions (1/500 to 1/32000, stock solution 0.5 mg/mL). The plates were processed as it is described in the general protocol.

- Experiment 2D

The avidity of the As53-As58 versus the enzyme tracers was determined by measuring the binding of serial concentrations (2 to 0.030 μg/mL in PBST, 100 μL/well) of each ET (**2**-, **5**-, **A**-HRP) to microtiter plates coated overnight at 4ºC with twelve different dilutions (1/1000 to 1/1024000 in coating buffer, 100 μL/well) of each of As. Last column of the plate was reserved to test non-specific absorbance of the ET to the microtiter plate. The plates were processed as it is described in the general protocol.

B. Competitive assays

To the coated plates the analyte was added (100 000 to 0.025 nM in PBST, 50 μL/well) followed by the ETs (appropriately diluted in PBST, 50 μL/well).

9.10.2. Indirect ELISA

General protocol

Microtiter plates were coated overnight at 4ºC with the appropriate concentration of the CA in coating buffer (100 μL/well). The plates were washed 4 times with PBST and a solution of the As appropriately diluted in PBST (or a solution of the analyte standrads

followed by the solution of the As) was added and incubated for 30 min at RT. Then the plates were washed again and a solution of goat anti-rabbit IgG coupled to horseradish peroxidase (antiIgG-HRP) in PBST (1/6000) was added to the wells (100 μL/well) and incubated for 30 min at room temperature. The plates were washed again and the substrate solution (100 μL/well) was added. Color development was stopped after 30 min at RT with 4N H_2SO_4 (50 μL/well) and the absorbance was read at 450 nm.

A. Non-competitive assays

No analyte was added. The CAs were added to the coated plates appropriately diluted in PBST (100 μL/well).

- Experiment 1D

For each As, each column of a microtiter plate was coated by a solution of CA in coating buffer at 1 μg/mL concentration (100 μL/well). After washing the As was added in serial dilutions (from 1/1000 to 1/64000 in PBST; 100 μL/well). Next the general protocol was followed.

- Experiment 2D

The avidity of the different As53-As58 for the coating antigens was determined by measuring the binding of serial dilutions (from 1/1000 to 1/64000 in PBST; 100 μL/well) of each As to microtiter plates coated overnight at 4ºC with twelve different concentrations (from 10 to 0.005 μg/mL in coating buffer; 100 μL/well) of the **1**-**5** and **7**-**12** -BSA, -CONA and –OVA conjugates. The plates were processed as described above. Optimal concentrations for coating antigens and As dilution were chosen to produce around 0.7 to 1 units of absorbance in 30 min. One column of the plate was reserved to test non-specific binding of the As to the microplate. One raw of the plate served to test non-specific binding of the antiIg-HRP to the CA.

B. Competitive assays

Analyte standards (100 000 to 0.025 nM, prepared in PBS), cross-reactants or samples were added to the coated plates (50 µL/well) followed by the corresponding antisera appropriately diluted in PBST (50 µL/well).

9.10.3. Indirect ELISA for 2,4,5-TCP (As53/7-OVA)

Microtiter plates were coated with 7-OVA (AE) (1:2.5) (0.6 µg/mL in coating buffer, 100 µL/well) overnight at 4 °C. The following day the plates were washed with PBST (4 times, 300 µL/well). 2,4,5-TCP standards (400 to 0.04 nM, prepared in PBS), cross-reactants or samples were added to the coated plates (50 µL/well) followed by the sera As53 (1/1000 in PBST, 50 µL/well). The plates were processed as described above.

9.10.4. Optimization and evaluation of the indirect ELISA for 2,4,5-TCP (As53/7-OVA)

Preincubation time: The As and the 2,4,5-TCP standard solutions were incubated under two different conditions (overnight at 4°C and 0 h at RT) and, subsequently, added to the antigen coated plates. The plates were processed as already described.

Length of competitive step: The mixture of As and analyte standards were incubated during different periods of time (10, 20, 40, 60 and 120 min) at RT on the same antigen coated plate. The plates were processed as already described.

Effect of Tween 20: PBST solutions containing different concentrations of Tween 20 (2.5; 0.5; 0.05; 0.025; 0%) were used to dilute the As and prepare different 2,4,5-TCP standards that were run simultaneously in the competitive ELISA.

Effect of pH: PBS solutions were prepared with pH values ranging from 2.5 to 10.5 units. These solutions were used to prepare 2,4,5-TCP standard curves and the As

solution and employed for the competitive immunoassay. The curves with the different pH were all measured in the same ELISA plate and the experiments was repeated in two separate plates. The absorbances were adjusted to a four-parameter equation and the features of the resulting ELISAs were compared.

Effect of ionic strength: PBS solutions at different concentrations (0, 5, 10, 20 and 50 mM corresponding to conductivities of 0, 10, 19, 35, 80 mS/cm respectively) were prepared, their conductivity measured and used to prepare standards and to dilute the As. The solutions were employed in the above described ELISA and the calibration curves obtained with each PBS solution were compared after adjusting them to a four-parameter equation.

Effect of ethanol: PBS solutions containing different concentrations of EtOH (v/v) (50%; 25%; 10%; 0%) were used to prepare standard curves and were run simultaneously in the competitive ELISA according to the procedure described above.

Specificity Studies: Stock solutions of different phenolic compounds (2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,6-dichlorophenol, 3,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,4-trichlorophenol, 2,3,5-trichlorophenol, 2,3,6-trichlorophenol, 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol, pentachlorophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, 2-bromo-4-chlorophenol, 2,4,6-tribromophenol, pentabromophenol) were prepared (1 mM in DMSO) and stored at 4°C. Standard curves were prepared in PBS (10 000 to 0.04 nM) and each IC_{50} determined in the competitive experiment described above. The cross-reactivity values were calculated according to the following equation:

$$CR\% = \frac{IC_{50}(2,4,5-TCP)}{IC_{50}(phenolic\_compound)} \times 100$$

Precision and Accuracy: PBS solution was spiked with 2,4,5-TCP at several concentration levels. Analyses of the samples were performed using the above-described ELISA in triplicates in three separated plates in three different days.

9.11. ELISA for 2,4,6-TCP (As43/8-BSA)

According to [277] microtiter plates were coated with 8-BSA(*1:5*) in coating buffer (0.625µg/mL, 100 µL/well) overnight at 4°C covered with adhesive plate sealers. The following day the plates were washed with PBST (4 times, 300 µL/well). 2,4,6-TCP standards (1000 to 0.625 nM, prepared in PBS) or samples were added to the coated plates (50 µL/well) followed by the sera As43 (1/2000 in PBST, 50 µL/well). The plates were processed as described above.

9.12. Sample Treatment Methods

9.12.1. Water samples

Drinking and well water samples were collected and their conductivity and pH values were measured. In order to adjust these parameters, samples were buffered by adding 10% (v/v) of 10x PBS.

9.12.2. Milk samples

Homogenized skimmed milk was obtained by a local supermarket. It contained 31 g/L proteins, 1.2 g/L Ca^{2+} and fat ≤ 5 g/L. It was stored refrigerated and used prior to the manufacturer´s expiration date.

Protein removal by alkaline precipitation: A solution of 5N NaOH was added dropwise to a stirred milk sample until pH=12. The white precipitate formed was removed by centrifugation (12000 rpm, 5 min) and the supernatant was collected. It was diluted 5 fold by 2x PBS and the pH was adjusted to 7.5 using 5N HCl. Further milk dilutions (1/10, 1/50 and 1/100) were prepared with 2x PBS. All the samples were vortexed vigorously between dilutions.

Ca^{2+}- precipitation by Na_2SO_4: Na_2SO_4 was added to a stirred to the milk sample until final concentration 2 g/L. The $CaSO_4$ precipitate formed was centrifuged (12000 rpm, 5 min) and the supernatant filtered through Whatmann paper. The filtrate was split in two parts. One part was treated with NaOH (protein removal) as described above and the other split was used to prepare serial dilutions in 2x PBS until final milk dilution 1/100. All dilutions had the pH and conductivity of the buffer.

9.12.3. Human serum

Lyophilized-pooled human sera was purchased from Sigma Chemical Co. (St. Louis, MO) and reconstituted with 5 mL milliQ water following the supplier instructions. The serum was stored at -4°C until use. Several sample treatments were assayed as follows:

Protein removal by heat: Serum (1 mL) was diluted two times with water and heated on a sand bath (65ºC) for 30 min. After cooling it at 4ºC it was centrifuged (12000 rpm, 5 min) and the white precipitate formed was removed. The supernatant was diluted in PBS until a final serum dilution 1/20.

Alkaline protein precipitation: KOH was added to a stirred serum sample (0.5 mL) until 0.2M KOH final concentration. The solution was stirred for 30 min. The precipitate formed was removed by centrifugation (12000 rpm, 5 min) and the supernatant collected. The pH was adjusted to 7.5 using 5N HCl. Further serum dilutions were prepared with PBS.

Acidic protein precipitation by trichloroacetic acid (TCA): Trichloroacetic acid (50 mg/mL) was added dropwise under magnetic stirring to human serum (0.5 mL) at 1:4 v/v ratio for 5 min. The precipitate was formed immediately and it was removed by centrifugation (12000 rpm, 5 min). The supernatant was diluted two-fold times in PBS and pH was adjusted to 7.5 by 5N NaOH. Thus the final solution was a 1/10 serum dilution. Further serum dilutions were prepared in PBS.

Acidic precipitation by perchloric acid: Aqueous perchloric acid (6%) was added dropwise under magnetic stirring to human serum sample (0.5 mL) until a 1:1 v/v ratio for 5 min. The precipitate was formed immediately and it was removed by centrifugation (12000 rpm, 5 min). The supernatant was diluted 2-fold times in PBS and the pH was adjusted to 7.5 by 5N NaOH. The final solution was a 1/5 serum dilution containing 1.2% perchloric acid. Further serum dilutions were prepared in PBS.

Ethanol deproteinization: Absolute ethanol was added dropwise under magnetic stirring to human serum sample (1 mL) until a 1:1 v/v ratio for 5 min. The precipitate formed was removed by centrifugation (12000 rpm, 15 min) and the supernatant was diluted two-fold times in PBS. Thus, the final solution was a 1/4 serum dilution containing 25% EtOH. Further serum dilutions were prepared with 25% EtOH/PBS.

9.12.4. Human urine

Sample A was collected over 2 days from a healthy male volunteer and then mixed to have a large and homogeneous urine sample. Sample B is pooled urine from different individuals (tested negative for specific drugs of abuse, 0.1%NaN3) and it was purchased by Bio-Rad Laboratories, Irvine, USA. Urine samples (C, D, E, F, G, H, I) were obtained (morning void) from persons with not known occupational exposure. Urine samples collected from petrochemical workers living in town A, service workers and population living in vicinity of a municipal incinerator (town B) were kindly supplied by the Instituto

de Higiene y Seguridad en el Trabajo in Barcelona. The urine sample used in the QFIA was a pool from different healthy individuals (24 h void). All urine samples were aliquoted and stored at -30°C.

Creatinine determination: The concentration of creatinine was determined by the Jaffé method, in which creatinine reacts with alkaline picrate to form a coloured complex. The intensity of the colour is measured at 520 nm. The clinical method NºSM4-0141 for Technicon RA® Systems [428] was adapted to a microplate assay determination: a solution of 0.4N NaOH was added to the plate (100 µL/well) followed by the urine samples (1/100 diluted in water, 50 µL/well). After 5 min at 25°C a solution of picric acid (2 g/L, 100 µL/well) was added and gently mixed. The absorbance was read immediately (~1 min) at 520 nm (A_{520} = A1) and after 5 min it was measured again (A_{520} = A2). As standard 1 mg/mL creatinine solution was used (A_{St}). The creatinine concentration was calculated according to:

$$C(mg\,\text{creatinie/ml urine}) = \frac{A2 - A1}{Ast2 - Ast1}$$

Hydrolysis (Deconjugation step): Glucuronides and sulfates of chlorophenols were cleaved by acidic, enzymatic or alkaline hydrolysis of urine samples.

Acid hydrolysis: concentrated H_2SO_4 (0.52 mL) was added to the urine sample (10 mL) until 1M H_2SO_4 final concentration and heated on a sand bath for 1h at 100ºC. After cooling the sample was neutralized (pH=7.5) by adding NaOH and centrifuged.

Enzymatic hydrolysis: urine (3 mL), 1M acetate buffer (pH=5.2, 1 mL) and β-glucuronidase/sulfatase (300 µL) were mixed and incubated for 17h at 37°C. The pH was finally adjusted to 7.5 with 1N NaOH.

Alkaline hydrolysis: A solution of 15M KOH (2 mL) was added to urine (10 mL) and heated on a sand bath for 30 min at 100ºC. After cooling the sample was neutralized (pH=7.5) by adding concentrated H_2SO_4 and centrifuged to eliminate the precipitated salts and/or proteins.

9.13. ELISA performance in environmental and biological samples

9.13.1. Matrix effect studies

Standard curves of 2,4,5-TCP and/or 2,4,6-TCP were prepared in water samples, in cow milk, in human serum and in human urine samples treated as described above. The parallelism of the standard curves was studied by comparing the calibration curve features with those obtained in the assay buffer. For human serum samples deproteinized with ethanol the standard curve was run in 25% EtOH/PBS for the 2,4,5-TCP ELISA and in 12% EtOH/PBS for the 2,4,6-TCP ELISA. The competitive ELISAs were run following the protocols described in **Section 9.10.4** and **Section 9.11** respectively.

9.13.2. Recovery studies

Water, urine and serum samples treated as described above were spiked at different concentration levels with 2,4,5- and/or 2,4,6-TCP and applying the dilution factors needed to eliminate the corresponding matrix effect were measured by the ELISA.

9.14. Development of SPE procedures for urine analysis by ELISA

9.14.1. C_{18}-SPE procedure

A Sep-Pak® Plus C_{18} cartridge (Waters, S.A.) was conditioned by passing MeOH (5 mL) followed by 0.1% aqueous acetic acid (5 mL). Acidified samples (10 mL, pH=3.5) (PBS standards, spiked and non-spiked urine) were then loaded into the column. The cartridge was washed with 5 mL of 50:50 MeOH:H_2O (0.1% acetic acid) and 2,4,6-TCP eluted in 5 mL of MeOH:H_2O (0.1% acetic acid) containing different concentration of MeOH (65%,

75%, or 85%). All the steps were performed at a flow rate of 1 mL/min. Fractions of 1 mL were collected, diluted 10 times (to the initial sample volume) with PBS and split in two parts for 2,4,6-TCP ELISA (see potocol in **Section 9.11**) and GC-ECD analysis. The C_{18} cartridge was regenerated by washing with 30 mL MeOH.

9.14.2. IAC-SPE procedure

A. Immunosorbent preparation

Antibody purification: Polyclonal antiserum 45 was purified by 35% $(NH_4)_2SO_4$ precipitation as described [429]. The precipitated IgG fraction was restored with 10 mM PBS and dialized against 0.5 mM PBS with three buffer changes and one with MilliQ water. The aqueous solution was finally freeze-dried and stored at -30°C until use.

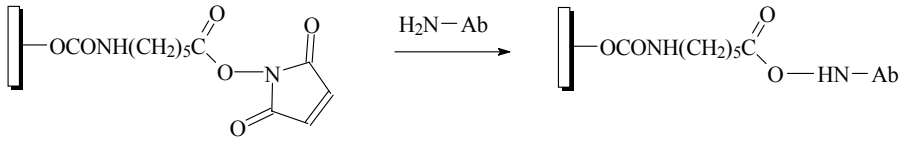


Figure 9.3. Antibody coupling to the NHS-activated Sepharose.

Antibody immobilization: Subsequently the IgG (Ab45) was covalently coupled to the NHS-activated Sepharose column as recommended by the manufacturer (Pharmacia) (see **Figure 9.3**). Briefly, a solution of IgG (10 mg/mL, 1mL) in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH=8.3) was injected onto the HiTrap column at a flow rate of 1 mL/min. The column was then left to stand 25 min at room temperature and washed with three column volumes of coupling buffer. The excess active groups that have not coupled to the ligand were deactivated by alternate (6 mL each) and repetitive (3 cycles) injections of 0.5 M ethanolamine (0.5 M NaCl, pH=8.3) and 0.1 M acetate (0.5 NaCl, pH=4) buffers. Finally, the column was washed with 2mL 10 mM PBS buffer and stored at 4°C in 0.05 M Na_2HPO_4 (pH=7) containing 0.1% NaN_3 until needed.

B. Determination of antibody coupling efficiency

The coupling efficiency to the HiTrap column was estimated as the difference between the total amount of IgG initially loaded onto the column and the amount of IgG found in the eluted buffer after the coupling took place. The IgG concentration in both solutions was determined by UV measurement at λ=280 nm. Previously, the collected solution after coupling of IgG to the column was purified from the co-eluting NHS groups absorbing at the same wavelength as proteins using a HiTrap™ desalting column prepacked with Sephadex®G-25 Superfine.

C. Evaluation of analyte desorption conditions

Glycine-HCl elution: 0.05M glycine-HCl (pH=3) was used for analyte elution. To each eluted fraction (1 mL) a basic aqueous solution (0.2M NaOH/1.8M NaCl) was added (87 μL) resulting in pH=8.6 and conductivity of 15 mS/cm. The 2,4,6-TCP concentration in each fraction was determined by ELISA using a standard curve run in glycine-HCl/NaOH/NaCl (pH=8.6, 15 mS/cm). Previously the accuracy of the ELISA method in this buffer system was evaluated using 2,4,6-TCP standards prepared in 0.05M glycine-HCl (pH=3) and treated as eluted fractions.

Ethanol elution: The optimum elution conditions were investigated using different concentrations of EtOH (10%, 20%, 30%, 50%, 70%) in PBS after loading the column with 2,4,6-TCP standards prepared in PBS. The 2,4,6-TCP concentration in each eluting fraction was determined by ELISA using a standard curve run in the corresponding percentage of EtOH in PBS. Blanks and washings were evaluated from a curve prepared in PBS.

D. General IAC-SPE procedure

The IAC process cycle consists of sample loading, washing, eluting and regenerating. The column was brought to room temperature and washed with 5 mL PBS. Then was preconditioned with 5 mL of 70% EtOH (or 0.05M glycine-HCl, pH=3) and 5 mL PBS. After loading the sample (spiked PBS or urine) the column was washed with 5 mL PBS (or 20% EtOH). The bound analyte was eluted with 70% EtOH (or 0.05M glycine-HCl, pH=3). Subsequently, the column was regenerated with 10 mL PBS. All the steps were performed at flow rate of 1 mL/min and at RT. Eluted fractions were diluted 10 times with PBS in the case of ethanol elution (or adjusted to pH=8.6 and conductivity 15 mS/cm in the case of glycine-HCl elution) and analyzed by ELISA and/or by GC-ECD.

E. Analyte binding capacity of the IAC column

The capacity of the column was determined by loading PBS samples 10 mL with three levels of 2,4,6-TCP: 1.2, 0.5 and 0.05 μg. After washing away the unbound fraction, the bound fraction was eluted (E) following the general IAC procedure (see **Section 9.14.D**), diluted 10 times with PBS and the concentrations were measured by ELISA and evaluated from a standard curve run in 7% EtOH/PBS buffer. The collected loading (L) and washing (W) fractions were determined using a standard curve run in PBS.

9.14.3. Matrix effect studies on the 2,4,6-TCP ELISA after SPE clean-up of human urine

Standard curves of 2,4,6-TCP were prepared in the corresponding buffer (8.5% MeOH/0.1% AcOH/PBS or 7% EtOH/PBS) and in the combined eluted fractions obtained in the C_{18}-SPE and IAC-SPE of the urine respectively. The competitive ELISA was run according the protocol described in **Section 9.11** to compare the parallelism of the urine standard curves to the calibration curves run in the corresponding buffer.

9.14.4. Recovery studies on the SPE procedures

Non-hydrolyzed and hydrolyzed urine samples were spiked with 2,4,6-TCP and processed according to the SPE (C_{18} or IAC) procedures described above (**Section 9.14.1** and **Section 9.14.2.D** respectively). After 10-fold dilution with PBS buffer the recoveries were determined by ELISA using 2,4,6-TCP standards prepared in the same medium (8.5% MeOH/0.1%AcOH/PBS for C_{18} SPE or 7% EtOH/ PBS for IAC).

9.15. Development of a HTS-IAC-SPE procedure for efficient urine clean-up

9.15.1. Immunosorbent preparation

The IgG (Ab45) was covalently coupled to the NHS-activated Sepharose 4 Fast Flow as recommended by the manufacturer (Pharmacia) (see **Figure 9.3**). Coupling was performed using 24 mL of Sepharose suspension. All washing steps were performed with filtration funnel under vacuum (water pump). The procedure per 1 mL is briefly described as follows. The Sepharose suspension (1 mL) was washed with cold 1 mM HCl (12 mL). The drained gel (0.5 mL) was transferred to a tube and mixed with the solution of the purified IgG (20 mg/mL, 0.5 mL) in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH=8.3). The suspension was left shaking (100 rpm) for 3h at room temperature. After coupling took place, the Sepharose was washed with 3 mL of coupling buffer. Further, it was washed 3 x 1 mL 0.5 M ethanolamine buffer (0.5 M NaCl, pH=8.3), followed by 3 x 1 mL 0.1 M acetate (0.5 NaCl, pH=4) buffer and again with 3 x 1 mL ethanolamine buffer. The non-reacted NHS-groups were blocked for 1h at room temperature with ethanolamine buffer. After 3 cycles of alternate washing with ethanolamine and acetate buffers the gel was washed 3 x 1 mL 10 mM PBS buffer. Finally, it was reconstituted to the initial suspension volume (1 mL) and stored at -4°C in 0.05 M Na_2HPO_4 (pH=7) containing 0.1% NaN_3 until needed. The Ab coupling efficiency was estimated as described in **Section 9.14.B**.

9.15.2. Mini-IAC column preparation

Mini-IAC columns (0.2 mL bed volume) were prepared by loading 0.4 mL coupled Sepharose suspension into the empty columns. The suspension should be very well mixed before use! The columns were packed by suction under vacuum (water pump) washing them with PBS. Once the gel drained, a frit (20 μm pore) was loaded manually over it to protect the column bed. 96 columns were packed in this way and inserted into the VersaPlate base plate. Finally, the VersaPlate 96-Well SPE System (Varian) was assembled.

9.15.3. Analyte binding capacity of the mini-IAC columns

The capacity of the column was determined by loading PBS samples (6 mL) with four 2,4,6-TCP levels: 0.75, 0.5, 0.1 and 0.05 μg. After washing away the unbound fraction, the bound fraction was eluted (see the general HTS-IAC SPE procedure, **Section 9.15.5**), diluted 10 times with PBS and the concentrations were measured by ELISA and evaluated from a standard curve run in 7% EtOH/PBS buffer.

9.15.4. Evaluation of desorption conditions of the mini-IAC columns

The optimum elution conditions were investigated using different concentrations of EtOH in the elution buffer (10%, 20%, 30% and 70%) after loading the column with 2,4,6-TCP standards prepared in PBS. The 2,4,6-TCP concentration in each elution fraction was determined by ELISA using standard curve run in the corresponding percentage of EtOH in PBS.

9.15.5. General HTS-IAC-SPE procedure

HTS-IAC-SPE procedures were performed with the VersaPlate 96-Well SPE System, which consists of vacuum manifold set equipped with a vacuum controller and water pump. All steps of the IAC cycle were performed under gentle low vacuum maintaining flow-rate in the range 1 - 2 mL/min. The vacuum manually controlled allowed the different solvents (buffers, samples) to pass through the IAC-extracton columns: the columns should be kept wet after conditioning and loading steps and dried after the washing and elution. To protect the immunosorbent for being dried at the conditioning and loading steps the vacuum was stopped as soon as the first columns were empty. All liquid loadings were done manually using eight-channel electronic pipette (Eppendorf). During sample loading, washing and regeneration steps waste was collected in a disposable reservoir. Before elution the waste reservoir was replaced with a collection rack of 96 glass vials (0.75 mL). Following the elution step, the extracts were diluted with PBS and kept at 4°C sealed with 96-well microplates silicone rubber seal. When not in use, the VersaPlate 96-Well IAC-SPE assembly was stored sealed with caps at 4°C in PBS containing 0.1% NaN_3.

The HTS-IAC-SPE cycle consists of sample loading, washing, eluting and regenerating. The columns were brought to room temperature and washed with 5 mL of PBS. Then were preconditioned with 1.2 mL of 70% EtOH and 1.2 mL of PBS. After loading the sample (spiked PBS or urine) the columns were washed with 1.2 mL of PBS or 20%EtOH. The bound analyte was eluted with 0.6 mL 70% EtOH. Subsequently the columns were regenerated with 1.2 mL of PBS. Eluted fractions were diluted 10 times with PBS and analyzed by ELISA curve run in 7%EtOH/PBS and/or by GC-MS.

9.15.6. Validation of the HTS-IAC-ELISA method using real urine samples

In the validation studies were used 117 urine samples from different individuals (workers from petrochemical industry, workers from service sector, and population living close to

an incinerator. Each sample (12 mL) was hydrolyzed with KOH (see **Section 9.12.4**, Alkaline hydrolysis) and purified in duplicate following the general protocol of HTS-IAC (see **Section 9.15.5**). The 2,4,6-TCP-IR equiv. were determined by ELISA (see **Section 9.11**) and the halogenated phenols (2,4,6-TCP, 2,4,5-TCP, 2,3,4,6-TtCP, 2,4-DBP were 2,4,6-TBP) analyzed by GC-MS (see **Section 9.17.4**).

9.16. Quenching Fluorescence Immunoassay (QFIA)

9.16.1. LIF-microdroplet-QFIA

Stock solutions of hapten **5** - fluorescein conjugate (**TCP-F**) and 2,4,6-TCP were prepared in methanol. All working solutions were prepared in 10 mM PBS buffer. The fluorescence emission from microdroplets of TCP-F was determined in standard PBS solutions (laser excitation wavelength at 488 nm, emission wavelength of 520 nm). For antibody quenching studies a standard PBS solution of TCP-F (final concentration 10 nM) was mixed with different concentrations of Ab solution in PBS (Ab43, Ab44 and Ab45). The reaction mixture was incubated at room temperature for 55 min. For kinetics estimation the fluorescence emission was measured every 5 min. Prebleed (preimmune) Ab was used as a control for the specificity of the quenching effect. For data presentation, the maximum fluorescence intensity (at 520 nm) measured in the presence of Ab (I_{Ab}) was divided by the maximum fluorescence intensity corresponding to TCP-F emission (I_{TCP-F}) in the absence of Ab.

9.16.2. QFIA Competitive Procedure

PBS solutions of 2,4,6-TCP standards (10^{-5} - 10^{-11}M) and the TCP-F (10 nM, final concentration) and Ab43 (2,5 μg/mL, final concentration) were mixed and incubated for 45 min at room temperature. The steady-state quenched fluorescence intensity (I_{TCP}) measured at 520 nm was used to construct the standard curve. The I_{TCP}/I_{TCP-F} values were

plotted against the 2,4,6-TCP concentration and fitted to a four-parameter logistic equation. Mean fluorescence responses correspond to 5 replicates. The IC_{50} and the limit of detection (LOD) were determined as the analyte concentration giving 50% and 90%, respectively, of the maximal quenching. The dynamic (linear) range corresponds to 20% - 80%.

In urine matrix effect studies pooled urine was used after centrifugation (5000 rpm, 10 min) and pH adjustment to pH=7.5. Urinary Ab quenching and competitive QFIA were performed in urine diluted with PBS (1/10 – 1/100).

9.17. Chromatographic analysis

9.17.1. Preparation of standards of halogenated phenols

Stock solutions containing each of the phenols at a concentration of 1 g/L were prepared in ethanol. Solutions in concentrations between 0.1 and 100 μg/L were prepared by further dilution with PBS (water). These working standards were treated in the same way as the urine samples (C_{18}, IAC, or extracted with toluene) and determined by GC-ECD or GC-MS after derivatization. Stock standard solutions for quantitative analysis of the main halogenated phenols and the ineternal standard (2,3,5,6-tetrachloronitrobenzene) were prepared in toluene at concentration 1 g/L. Further dilutions were performed in toluene. Calibration curves for tri, tetra chlorophenols and di-, tri-bromophneols were set up in the range 0.5 to 50 μg/L using working standards prepared in toluene for GC-ECD and using extracted standards prepared in PBS for GC-MS.

9.17.2. Phenol extraction and TMS-derivatization

Halogenated phenols were extracted from acidified (pH=3) PBS standards and urine samples (directly or after C_{18}-SPE or IAC-SPE clean up) in toluene by agitating the

mixture on an automatic shaker at 300 rpm for 50 min (PBS-to-toluene extraction ratio = 5:1, 12:1, 100:1). After separation of the phases by centrifugation, 100 µL of the toluene extract was silylated by adding 2 µL *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and incubated for 3 h at room temperature. Then the internal standard 2,3,5,6-tetrachloronitrobenzene was added to a concentration of 5 µg/L. The TMS-derivatives were then analyzed by GC-ECD or by GC-MS. If the measured values were above the liner range of the calibration graphs the toluene extracts were diluted with toluene and injected again. Calibration standards were analyzed with each analytical series. A reagent blank (derivatized as samples) was also analyzed in each analytical series.

9.17.3. GC-ECD detection

Injections (1 µL, splitless) were made on a HP 5890A gas chromatograph equipped with HP 7673A autosampler and with a ECD detector ($^{63}_{28}$Ni), and HP 3396 Series II integrator (recorder). A capillary column BPX35 (35% Phenyl(equiv.)Polysilphenylene-siloxane) (SGE Europe Ltd, UK) was used 25 m x 0.22 mm i.d. x 0.25 µm (film thickness). GC conditions were set as follows: temperature program: 100°C (1 min) to 250°C (at 5°C/min), 250°C to 300°C (at 15°C/min). Injection temperature was 250°C, detector temperature was 300°C. He was the carrier gas employed at 130 kPa (20 cm/sec). The make up gas was N_2 5.0.

9.17.4. GC-MS analysis

For the GC/MS analysis, injections (1 µL) were splitless (48 sec) with solvent delay (5 min). A HP-5MS (cross-linked 5% Ph Me siloxane, 30 m x 0.25 mm id x 0.25 µm (film thickness)) column was used. The ion source temperature was set at 200°C, and He was the carrier gas employed at 1 mL/min. The ionization energy was 70 eV. GC conditions were as follows: temperature program: 100°C - 300°C (7°C/min); injector temperature, 250°C; and the ions 253/255/268 (2,4,6-TCP and 2,4,5-TCP), 307/309/324 (2,4-DBP);

387/389/402 (2,4,6-TBP); 287/289/304 (2,3,4,6-TtCP); 203/259/261 (ISt) were monitored in the SIM mode. The mass spectrometer was operated in the Time Varied Selected Ions Monitoring Mode (i.e. with different group ions monitored for various time specific absolute intervals) (see **Table 7.4**). For the SCAN mode, the mass range explored was 45-550. All data is reported as m/z (relative intensity). Great care was taken to ensure that the standards as well the extracts were run under identical conditions for purposes of identification as well as quantification.

9.18. Production and application of recombinant antibody fragments (scFv, scFv-CAL) as recognition elements in immunodetection systems

9.18.1. Production of anti-digoxin scFv fragments by phage display technology

A. Selection of phage library with digoxin specificity

The construction and selection of library of phages displaying scFv fragments has been previously described by Marks *et al*. [430]. Here we have used two "synthetic" libraries (DVT and NKK) constructed in the phagemid vector pIT2 (see **Figure 9.4**) based on a single human framework for V_H and Vκ domains with different side chain diversity incorporated at the following positions in the antigen binding site (in total 18 aminoacid residues): for V_H: CDR2 (H50, H52, H52a, H53, H55, H56, H58) and CDR3 (H95, H96, H97, H98); for Vκ: CDR2 (L50, L53) and CDR3 (L91, L93, L94, L96). The DVT library contained $1.47x10^8$ clones and has 96% insert and the NNK library contained $1.37x10^8$ clones and 88% insert. Both libraries were stored as *E.coli* harbouring the phagemid. Library growth and phage production was realised following the protocols described in [431]. Both phagemid libraries were used in parallel and were subject to three rounds of selection against the antigen using immunotubes coated with digoxin-BSA. Each round of

selection comprises a cycle of binding of phage to the immobilized antigen, washing, elution of the bound phage by trypsin, and propagation of the enriched phage ready for the next round [432].

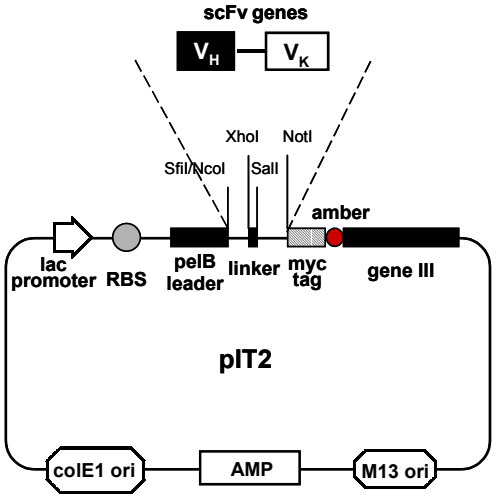


Figure 9.4. Phagemid vector pIT2 for the expression of scFv. RBS-ribosome binding site, PelB – leader sequence, amp – ampicilline resistance gene, myc tag – enables detection with anti-myc mAb, amber codon – enables switching between soluble scFv expression induced by IPTG (*E.coli* non-supressor strain, read as a glutamine) and scFv fragments displayed on the surface of the phage by fusion to the minor coat protein pIII after rescue with helper phage (*E.coli* supressor strain, read as stop codon).

B. Screening of soluble anti-digoxin scFv fragments produced from the selected phages and sequencing of clones

Monoclonal soluble scFv fragments were produced after infection of *E.coli* TG1 with the selected phages from both libraries following the procedure detailed in [432]. The bacterial supernatants containing the soluble anti-digoxin scFv fragments were screened for binding to the antigen by ELISA and Surface Plasmon Resonance (SPR).

ELISA: Plates were coated overnight with digoxin-BSA, digoxin-HRP and as negative controls with BSA and HRP (concentration range 25-0.001 μg/L in PBS, 100 μL/well), washed 3 times with PBST and blocked with 2%Tween 20-PBS (200 μL/well) for 2h. After washing the plates were incubated with the bacterial supernatant (100 μL/well) for 90 min at RT. After washing the binding of the scFv to digoxin-BSA (or to BSA) was detected with protein L-HRP (1:5000), which recognizes the Vκ chain of the fragment. TMB (100 μL/well) was used as substrate and the absorbancse were read at 450 nm. In the case of digoxin-HRP immunorecognition, protein A-AP, which recognizes the V_H chain was used followed by pNPP substrate addition (100 μL/well) and the absorbances were read at 405 nm.

SPR: SPR measurements were performed on the BIAcore system (Pharmacia Biosensor). The antigens (Digoxin-BSA and BSA) were immobilized to the CM5 sensor chips by chemical coupling (NHS/EDC) through their amine groups using the Amine Coupling Kit (Pharmacia Biosensor AB) and digoxin was coupled directly after activation in the form of dialdehyde following the recommended instructions of Pharmacia Biosensor AB [433]. The supernatants containing the anti-digoxin scFv fragments were injected (sample volume 30 μL) over the antigen-coated chips at constant flow rate of 10 μL/min. After scFv binding the antigen coated chips were regenerated by 100 mM HCl for 1 min.

Clones found to give positive SPR signal were screened by PCR using primers LMB3 and pHENseq (25 cycles, at 55°C) and "fingerprinted" with restriction enzymes to identify different clones [430]. Heavy and light chains were sequenced [434] using Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems 373A DNA sequencer. The sequenced clones were analyzed with the program Mac Vector 3.5 (IBI Kodak, CT).

9.18.2. Production of the fusion protein anti-digoxin scFv-calmodulin (scFv-CAL)

The genes encoding the anti-digoxin scFv fragment were digested from the vector pIT2 using the restriction endonucleases (Sfi1/Not1) and were subcloned into the Sfi1/Not1 sites of the expression vector pDN152 using T4 DNA ligase [425]. The ligation of the genes encoding the anti-digoxin scFv fragment and the protein CAL were verified by PCR screening. The new vector (anti-digoxin scFv-CAL) was transformed into *E.coli* TG1 for antibody soluble expression. The obtained fusion protein was checked on ELISA and in PAGE electrophoresis.

9.18.3. Expression and purification of anti-digoxin scFv and scFv-CAL

Large cultures of TG1 harboring the obtained vectors encoding anti-digoxin scFv and anti-digoxin scFv-CAL, respectively, were grown and the antibody fragment and the fusion protein were expressed in presence of IPTG following the detailed protocols described in [425,432]. The culture supernatants containing the proteins were purified by ion exchange (DEAE chromatography) and further by affinity chromatography (protein A) [432]. The degree of purification was checked by PAGE electrophoresis. The purified anti-digoxin scFv and anti-digoxin scFv-CAL were pre-concentrated by ultrafiltration using CentriPrep (Amicon), stored in PBS and their concentration was determined with BCA Protein Assay Kit (Pierce).

9.18.4. Characterization and application of the recombinant antibody fragments in ELISA and immunosensors

A. ELISA

Microtiter plates were coated overnight at 4°C with the anti-digoxin scFv-CAL in an appropriate dilution (1/320) determined by experiments 2D (100 µL/well). The following

day the plates were washed with PBST (5 times, 300 μL/well) and different concentrations of digoxin standards (250 to 2.10^{-5} mg/L, prepared in PBST, 50 μL/well) were added followed by the digoxin-HRP solution in PBST (1/12800, 50 μL/well). After 2h of incubation at RT the plates were washed again with PBST. The substrate solution was added (100 μL/well) and the enzymatic reaction was stopped after 10 min at RT with 4 N H_2SO_4 (50 μL/well). The absorbance was read at 450 nm.

B. Isoelectric focusing

The isoeletric points of the recombinant proteins were determined using Electrophoresis IEF Phast System (Pharmacia) according to the procedure described in Phast System™ Separation technique File N100 of Pharmacia. PhastGel IEF calibration kit 3-10 was used.

C. Multilayer construction in SPR

The monolayer of 3-mercapto-1-propansulfonic acid (MPS) was formed by incubation of chip J1 (BIAcore) in 1 mM ethanol MPS solution for 1h. Then the chips were washed with ethanol and water to remove excess of MPS. Consecutive injections of a positively charged rdeox polymer, scFv-CAL (calmodulin) and digoxin-HRP were performed at flow rate 20 μL/min using MilliQ water as carrier. The concentration of the reagents and the injection volumes are presented in **Table 9.1**.

Table 9.1. Experimental conditions for multiplayer formation

Compound	Concentration, μg/mL	Injection volume, μL
Redox polymer	20	100
scFv-CAL	10	30
calmodulin	10	30
Digoxin-HRP	5	50

10. Conclusions

1. An indirect ELISA for 2,4,5-TCP has been developed with an IC_{50} of 0.23 μg/L and a LOD of 0.053 μg/L after rational design of the immunizing hapten chemical structure, and the investigation of a battery of homologous and heterologous competitor haptens. Precision study performed with buffer samples showed that the within and between assay coefficients of variation were below 12%. Accuracy studies demonstrated very good linear correlation (r^2=0.999, slope 1.07) between spiked and measured 2,4,5-TCP in buffer samples.

2. Computer assisted theoretical and molecular modeling studies have provided objective and reliable criteria to design an appropriate immunizing hapten to raise antibodies against

2,4,5-TCP, as it has been demonstrated by the experimental results regarding the avidity of the antisera raised for the target analyte. The theoretical data indicated that, independently from the pH of the media, haptens **A** and **C** mimicked very well the behaviour of the target analyte regarding geometric and electronic properties. In contrast, hapten **B** demonstrated a completely different behavior that was especially evident when the pH was higher than the pKa value of the target analyte. In agreement with this, the antisera raised against hapten **B** was not able to rend any competitive assay, while the antisera raised against hapten **A** provide several useful immunoassays with sufficient detectability and sensitivity for environmental and biological monitoring.

3. It exists a correlation between the ELISA detectability and the competitor hapten heterology as it has been demonstrated by the molecular modeling and theoretical studies made with a battery of 11 different haptens and the IC_{50} values achieved on the corresponding ELISAs obtained with the antisera raised. For the case of the 2,4,5-TCP, the best competitive immunoassays were achieved with competitor haptens with high homology with the target analyte. Thus, haptens **5** and **7,** that showed very similar electronic distribution at the assay pH, provided with As53 assays with higher detectability than other haptens with a higher degree of heterology. In spite of this we should not forget other factors such as the hapten density of the biomolecule conjugates, their tertiary structure or the protein nature.

4. The conjugation degree of the competitors have a real influence on the ELISA features resulting in an improvement of the detectability when coating antigens with a low hapten density are used on indirect ELISA formats. In contrast, the conjugation method employed does not seem to have a direct effect on the 2,4,5-TCP immunoassay detectability. Hence, the a LOD of 0.053 μg/L was accomplished using **7**-OVA (1:2.5), prepared by the AE method, with a hapten density of 10 mols/mol of protein.

5. Immunoassays for phenolic compounds are strongly affected by the ionic strength of the media and by the pH as demonstrated in the evaluation made in this thesis of the behavior the 2,4,5-TCP ELISA and the results previously obtained in our group with other immunoassays for phenolic substances [259,260,289,291]. The 2,4,5-TCP immunoassay

parameters remain constant in media with pH values between 6.6 and 10.5 and conductivity values higher than 30 mS/cm. However at pH values lower than 5.5 the assay is totally inhibited and a marked increase of the IC_{50} value is produced below 20 mS/cm.

6. The specificity studies performed with the 2,4,5-TCP immunoassay plus the results previously obtained in our group with ELISAs for other organochlorinated compounds [1,435] suggest that the greater recognition of homologous brominated compounds (halogen atoms are placed at the same positions) could be a general behavior of these type of antibodies. Regarding the specific recognition of other substances, the results indicate that these should be phenol-like compounds containing in their structure at least two chlorine atoms, at *ortho* and *para* positions, and one hydrogen atom in *meta* position.

7. Distinct immunoassays are differently affected by the non-specific interferences of the sample matrices. For the case of complex matrix samples such as human serum and urine, the 2,4,5-TCP ELISA demonstrated to be less affected by undesirable matrix effects than the 2,4,6-TCP ELISA.

8. Most of the undesirable matrix effects caused by complex samples on the performance of the trichlorophenol immunoassays can effectively be eliminated after simple sample treatment procedures as it has been demonstrated by the study of the parallelism of standard curves prepared in different matrices, the accuracy studies and the comparison of the results obtained by chromatographic methods. However, for certain applications the necessary detectability can be compromised. Direct immunochemical analysis of drinking water can be performed after just buffering the sample reaching LOD values of 0.07 μg/L and 0.18 μg/L for 2,4,5-TCP and 2,4,6-TCP, respectively. Human serum could be analyzed after protein precipitation with absolute ethanol and further dilution of the supernatant with PBS achieving LOD values of 0.8 μg/L and 4.6 μg/L, respectively for 2,4,5-TCP and 2,4,6-TCP. Non-hydrolyzed urine samples can be analyzed after sample dilution with buffer with LOD of 0.26 μg/L for 2,4,5-TCP and 17.5 μg/L for 2,4,6-TCP. The same sample treatment applied to hydrolyzed urine samples resulted in LOD values about 100 times worse that those obtained in the assay buffer media. Finally, milk samples completely inhibited both

immunoassays and all the simple treatment procedures tested until now have not been able to remove the interferences causing this effect.

9. The matrix effects caused by urine samples from different individuals affect immunoassay performance in a different extent. Therefore, accurate quantitative analysis is only possible, if samples are sufficiently diluted to diminish idiosyncratic differences, or if interferences are removed by other sample treatments, such as solid-phase extraction. The creatinine concentration of the urine appears to be a good indicator of the extent of the matrix effect produced by particular urine.

10. C_{18}-SPE could be an effective clean-up method to remove not all, but an important fraction of the nonspecific interferences present in both hydrolyzed and non-hydrolyzed urine, prior to the analysis of trichlorophenols by ELISA. The established C_{18}-SPE-ELISA method allows accurate quantification of free chlorophenols in non-hydrolyzed urine and total (free and conjugated) chlorophenols in hydrolyzed urine samples with a LOD at about 4 μg/L for both types of urines.

11. The SPE based on immunosorbents prepared by covalently coupling of antibodies to a highly cross-linked agarose, is an effective clean-up method to remove all non-specific interferences present in both hydrolyzed and non-hydrolyzed urine samples prior to the analysis of trichlorophenols by ELISA or by GC-MS (ECD).

12. The selectivity of the immunosorbents can be modulated by the working protocol used. A class-specific clean-up procedure, where 2,4,6-TCP and other congeners of the same family are retained, can be performed if a washing step with PBS is employed. However by increasing the ethanol content in this buffer until a 20%, a selectivity similar to that of the 2,4,6-TCP ELISA method can be achieved.

13. The immunosorbents prepared in this thesis have sufficient capacity to effectively extract 2,4,6-TCP from real urine samples allowing the biological monitoring of occupationally and non-occupationally exposed persons. Thus, the quantitative immunochemical analysis of

alkaline hydrolyzed urine can be performed in the range 1 - 20 μg/L 2,4,6-TCP urinary concentrations by the single column IAC-ELISA method and in the range 0.3 - 30 μg/L by the HTS-IAC-ELISA.

14. The 2,4,6-TCP immunosorbents can be used for many cycles with real urine samples without a significant loss of their efficiency. The capacity of the single IAC column was not affected adversely for more than 50 cycles and that of the mini-columns used in the HTS-IAC - for up to 35 analyses. The columns can be efficiently regenerated by washing with the elution buffer followed by PBS.

15. The HTS-IAC-ELISA method (Versaplate 96-well SPE system) developed in this work is efficient, consistent and reliable. Its main advantages are the significant increase in sample throughput, the decrease of urine sample volume required for the assays and the absence of dilution in the clean-up step. It allows the processing of at about 100 samples/day with both inter- and intra-assay precision (% CV) lower than 20% except in the case when the measurements take place at the ELISA limit of detection. The validation of the HTS-IAC-ELISA method by GC-MS using 117 urine samples from different individuals shows an excellent correlation (r^2=0.912, slope 1.14) between the 2,4,6-TCP-IR equivalents estimated by both techniques.

16. A preliminary biomonitoring of the general population of Cataluña using the HTS-IAC-ELISA method revealed that the urinary chlorophenol levels are either lower or of the same magnitude as in the general population of other countries (Germany, US). The HTS-IAC-GC-MS analysis revealed the presence of organochlorine compounds in almost 100% of the persons studied. The reported values will serve as a basis against which to compare concentrations in subjects who may have been occupationally exposed to halogenated phenols or toxic compounds that metabolize to them.

17. A comparison between three population groups (petrochemical workers, services workers and population living close to an incinerator) demonstrated that the highest values (median, 95%, maximum) of 2,4,6-TCP-IR equivalents determined by HTS-IAC-ELISA were

detected for the incinerator group followed by the petrochemical workers and finally by the services workers. However, according to the HTS-IAC-GC-MS studies the average excretion of 2,4,6-TCP in urine was highest for the petrochemical workers (0.48 μg/g), followed by the general population living in vicinity of the incinerator (0.26 μg/g) anf lowest for the services workers (0.13 μg/g). The urinary levels of brominated phenols (2,4-DBP and 2,4,6-TBP) were highest for the incinerator vicinity population followed by the services workers and finally by the petrochemical workers. These results suggest that the general population living in the surroundings of the incinerator suffers higher exposure to these compounds, although the source is uncertain. Although the results shown here draw a tendency on the potential risk or level of exposure of these population groups, we can not make any definitive conclusion because much more information and criteria should be taken into consideration. However, these results and the comparison between ELISA and GC-MS methods prove the potential usefulness of the procedure described and developed in this thesis.

18. The LIF-microdroplet-QFIA is a novel biodetection system that combines the selectivity of immunoassays and the sensitivity of laser-induced fluorescence detection in microdroplets (d = 58.5 μm). The method has proven to be fast, highly sensitive, and simple to perform (no separation steps are needed). This approach offers better detection limits than those of microplate immunoassays (LOD of LIF-microdroplet-QFIA is 0.04 μg/L 2,4,6-TCP vs LOD in microplate-QFIA is 0.36 μg/L 2,4,6-TCP).

19. This work is the first application of a steady-state QFIA with LIF-microdroplet detection system to human urine samples, demonstrating the potential of this analytical approach for bioassays. Urine samples can be directly analyzed for 2,4,6-TCP, without any sample treatment (just 50-fold sample dilution) and with enough detectability (LOD 1.6 μg/L 2,4,6-TCP) to perform biological monitoring and risk assessment of exposure to chlorophenols and other organochlorinated substances that are excreted as trichlorophenol in urine.

11. Resumen

11.1. Introducción

El vertido de contaminantes al medio ambiente ha incrementado considerablemente durante las dos últimas décadas. Hace algunos años, la Comunidad Europea (CE) publicó la llamada “lista negra” que incluye 132 sustancias peligrosas (Directiva 76/464/CEE) cuyo vertido al medioambiente debe ser vigilado. Esta lista contiene diferentes compuestos organohalogenados entre los que se encuentran diversos clorofenoles. En

1996, la CE promulgó una nueva directiva que aumenta el numero de contaminantes cuyo vertido debe ser controlado, además de recomendar el establecimiento de programas para la vigilancia de determinados sectores industriales como es el del papel, el textil y el de las refinerías entre otros (Directiva 96/61/CE). Sin embargo, además del impacto medioambiental, el uso abusivo de productos químicos también afecta el estado de salud de la población. Los efectos varían desde una simple irritación debido a una intoxicación de tipo accidental hasta el desarrollo de cánceres específicos tras una exposición continuada a dosis aparentemente insignificantes. La exposición real (dosis interna) y el riesgo para la salud pueden ser evaluados mediante el ***control biológico*** (*biological monitoring*) que se define como la medida y valoración de estos agentes con los que la población entra en contacto, o de sus metabolitos, bien en tejidos, secreciones, productos de excreción aire aspirado o cualquier combinación de ellos [5-7].

Con el fin de proteger la salud de la población diferentes agencias gubernamentales de Europa y Estados Unidos han establecido cuales deben ser los límites máximos de exposición a productos particulares, referidos a la concentración medioambiental permitida (OEL, occupational exposure limits) o a los niveles que ya realmente han penetrado en el organismo del individuo potencialmente expuesto (BLV, biological limit value) antes de ocasionar un efecto adverso[16,20,21]. Además en las últimas directivas (Directiva 98/24/EC, Directiva 2000/39/EC), la unión europea insta a los empresarios a establecer los programas de control necesarios para proteger la salud de sus empleados. Ello implica la necesidad de proveer al mundo empresarial y especialmente a los médicos laborales de técnicas sencillas que permitan un control eficaz tanto del medioambiento y como del grado de exposición de los individuos.

Los métodos analíticos habituales para el control ambiental y biológico se basan en la utilización de técnicas cromatográficas (GC/MS, HPLC/MS, etc.) [7,23,24] que la mayoría de las veces no alcanzan los límites de detección necesarios, invierten un tiempo demasiado elevado para cada análisis y exigen una preparación previa de cada muestra con el fin de concentrar el analito y purificar la muestra. Frente a esto, las técnicas inmunoquímicas se caracterizan por permitir el análisis simultáneo y directo de

numerosas muestras de una manera sencilla y rápida ya que una de sus características principales es su elevada detectabilidad y especificidad. Así pues la UE y la EPA apoyan el desarrollo de nuevas estrategias analíticas que permitan una mayor eficacia de la vigilancia ambiental y biológica [26]. En los últimos años las técnicas inmunoquímicas han abierto paso en su aplicación en el campo del control biológico [25,26].

Las ***técnicas inmunoquímicas*** de análisis se caracterizan por su elevada detectabilidad, especificidad y rapidez (más de cien muestras pueden ser procesadas simultáneamente). Se basan en la utilización de ***anticuerpos específicos*** para detectar un determinado compuesto. El método empleado para la detección de esta reacción de reconocimiento molecular da lugar a una variedad de técnicas inmunoquímicas como los ensayos inmunológicos de tipo ELISA (*Enzyme-Linked Immunosorbent Assay*), la cromatografía de inmunoafinidad (IAC, *ImmunoAffinity Chromatography*) o los inmunosensores [26,157,185,196,198-200]. El bajo coste y la fácil utilización de este tipo de técnicas analíticas hacen que su utilización pueda ser asequible a medianas y pequeñas empresas, para el control de sus vertidos. Igualmente pueden constituir excelentes herramientas para una vigilancia eficaz de la exposición a estos tóxicos por parte de ciertos grupos de riesgo, mediante el análisis de estos compuestos en sus fluidos biológicos. En los últimos años se han desarrollado varios métodos inmunoquímicos para la detección de varios biomarcadores en orina [7,26,28,29,30, Knopp, 1995 #1846,176-184]. También se han desarrollado varios métodos inmunoquímicos para la determinación de compuestos organoclorados (DDT, PCP, 2,4-D, 2,4,5-T. PCBs, PCDDs, 2,4-DCP) en muestras ambientales y fluidos bilógicos [212,213,217,222,231-233,245,249,257,258].

Los ***triclorofenoles*** son un grupo de contaminantes de origen industrial que llegan al medioambiente debido a su masiva utilización como intermedios sintéticos en numerosos procesos industriales [31]. Además por si mismos se han usado exhaustivamente como pesticidas y bactericidas. Una de sus aplicaciones más conocidas ha sido su utilización como agentes conservantes de la madera y los productos textiles. Por otro lado se ha detectado la formación de triclorofenoles en procesos de combustión en presencia de iones cloruro y en los procedimientos de blanqueado y desinfección que todavía utilizan

hipoclorito o productos derivados [32,35,36]. A pesar de que en los últimos años su producción en los países desarrollados se ha reducido, los clorofenoles siguen siendo uno de los contaminantes del medio ambiente. Se han detectado en aguas de ríos [38-40], lagos [41], agua de bebida [49-51], aguas subterráneas [47,48], sedimentos [42-45], peces [40,46].

Los triclorofenoles (2,4,5- y 2,4,6-TCP) son algunos de los 5 clorofenoles calificados por sus significantes efectos toxicológicos y cancerígenos [65]. Tienen también efectos mutagénicos [66]. La exposición ocurre a través de su absorción por la piel, la inhalación o ingestión de productos contaminados. Además de los clorofenoles, otros compuestos organoclorados se metabolizan y se excretan en la orina como clorofenoles en forma de glucurónidos y sulfatos [63,64]. El nivel de triclorofenoles en la orina puede ser usado como un importante indicador de una exposición a diferentes compuestos organohalogenados (HCB [80], HCH [74,78,79], dioxinas, herbicidas derivados del ácido cloropfenoxyacético [77], insecticidas organofosforados [75,76], etc.). Adicionalmente los triclorofenoles son los precursores directos de la formación de dioxinas, por lo que muchos autores han coincidido en denominarlos predioxinas [81,106]. Por otro lado, todos los productos derivados de triclorofenoles contienen dioxinas como impurezas. En la literatura existen opiniones contradictorias sobre el tema de la relación entre la exposición a clorofenoles y dioxinas y su excreción en orina y sangre y los efectos adversos relacionados con esto [103,110,111]. A pesar de eso existen numerosos ejemplos que ponen en evidencia que la exposición a clorofenoles, herbicidas de tipo clorofenoxiácido y otros derivados producen un elevado nivel de dioxinas en el suero [35,83,107-109].

La exposición laboral sucede mediante inhalación o contacto dermal en ambientes contaminados. Tal es el caso de trabajadores en plantas químicas [83,105,109], aplicadores de pesticidas o fungicidas [83] así como trabajadores de incineradoras industriales [36,113] y serrerías [108]. Los niveles detectados varían entre 0.2 y 850 μg/L según el sector industrial. También se ha probado la existencia de una exposición de la población general a través del medio ambiente contaminado en varios países: Alemania

[121,123,124], USA [122], España [118,128], etc. El valor de referencia establecido para 2,4,5- y 2,4,6-TCP en orina en Alemania en base de los niveles encontrados en la población general es 4.5 μg/L (5 μg/g creatinina) [36,81].

11.2. Objetivos

El trabajo realizado en esta tesis ha tenido los siguientes objetivos:

1. Desarrollo de un inmunoensayo enzimático de tipo ELISA para la detección de 2,4,5-TCP.
2. Evaluación y validación de los inmunoensayos (ELISAs) para 2,4,5-TCP y 2,4,6-TCP en muestras ambientales y biológicas.
3. Desarrollo de un método de purificación de muestras de orina mediante extracción en fase sólida para el análisis de 2,4,6-TCP mediante ELISA (SPE-ELISA).
4. Desarrollo de un método HTS-IAC-SPE y su validación mediante su aplicación al control biológico de una serie de grupos representantes de la población general en Cataluña.
5. Desarrollo de un inmunoensayo de fluorescente con detección inducida por laser en microgotas (LIF-microgotas-QFIA).

11.3. Resultados

11.3.1. Desarrollo de un ELISA para 2,4,5-TCP

Uno de los pasos cruciales en el desarrollo de una técnica inmunoquímica es el diseño del hapteno empleado como inmunógeno, ya que la estructura química del mismo influye directamente en la especificidad y selectividad de los anticuerpos resultantes y en consecuencia en las características del ensayo en sí. Se han propuesto tres haptenos diferentes como posibles inmunógenos considerando que dichos compuestos habían de conservar la estructura química del 2,4,5-TCP en la medida de lo posible (**Figura 11.1**).

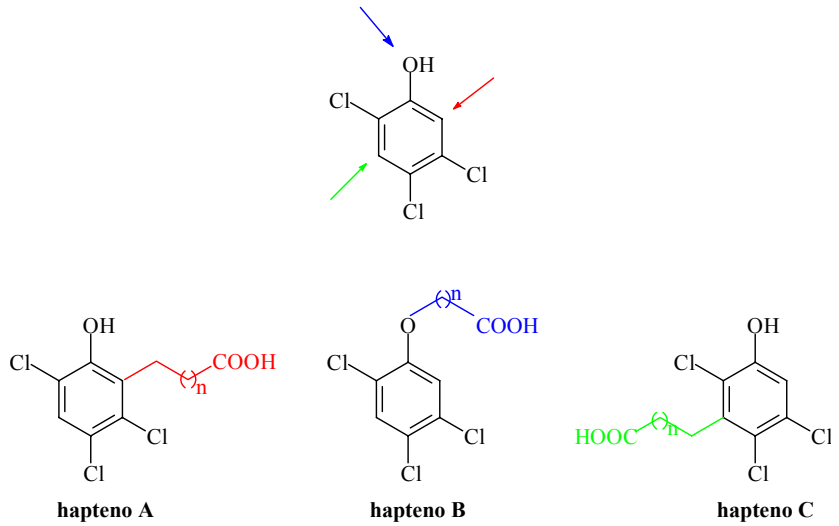


Figura 11.1. Estructura química de los tres haptenos propuestos como inmunogenos. Las flechas indican el lugar en que se ha introducido la modificación en la estructura química del analito 2,4,5-TCP.

El efecto de la introducción del brazo espaciador en la conformación espacial y la distribución electrónica de la molécula, se ha evaluado mediante la optimización de la conformación de los tres haptenos hasta un mínimo energético, usando un software de simulación molecular. Se ha tenido en cuenta también el equilibrio ácido-base (pKa, entalpía de desprotonación) de los compuestos fenólicos en medio acuoso. Los estudios semiempíricos de modelización molecular demostraron que los haptenos A y C guardaban mayor semejanza con el analito en cuanto a su geometría molecular, distribución de las cargas puntuales y carácter ácido de la molécula. Se han considerado también los esfuerzos necesarios para la síntesis de dichos haptenos y se ha estimado que la introducción del brazo espaciador en posición *meta* necesaria para el hapteno C sería difícil. Por lo tanto, para comprobar los resultados de los estudios teóricos se han generado anticuerpos contra los haptenos A (As53, 54, 55) y B (As56, 57,58).

En el desarrollo de los ensayos inmunoenzimáticos se han ensayado 11 competidores con diferente grado de heterología en la longitud y la posición relativa del brazo espaciador y en el número y la posición de los átomos de cloro respecto al inmunógeno usado (véase

Figura 11.2.). En el formato de ELISA directo en condiciones no competitivas se ha observado reconocimiento sólo para los trazadores enzimáticos homólogos (hapteno A-HRP/As53-55 y hapteno B-HRP/As56-58) o quasihomólogos (hapteno 5-HRP/As55). El mejor ELISA directo competitivo se obtuvo con la combinación hapten 5-HRP/As55 con los siguientes parámetros: A_{max} = 0.94, IC_{50}=15.84 μg/L 2,4,5-TCP, pendiente = -0.97, r^2 = 0.971.

Debido a la insuficiente detectabilidad del formato directo se ha desarrollado un ELISA indirecto. Inicialmente se ha hecho un *screening* (formato no competitivo) de 33 antígenos de tapizado formados por los haptenos de diferente estructura fenólica conjugados a BSA, CONA, OVA frente los seis anticuerpos. El mejor reconocimiento se ha observado para las combinaciones homólogas y quasihomólogas (haptenos 5, 8/As53-55, haptenos 1,2,11,12/As56-58). La afinidad de los As53-58 hacia los haptenos más reconocidos se ha evaluado en formato indirecto no competitivo (2D) donde se han elegido las concentraciones de los inmureactivos (antígeno de tapizado y As) para obtener una señal A_{max} entre 0.7 y 1. Bajo estas condiciones se han evaluado los inmunoensayos competitivos. Ninguna de las combinaciones de los As56-58, generados contra el hapteno B, dieron ensayos competitivos tal como ya se esperaba de los estudios de modelización molecular. Con los As53-55 se han conseguido 7 combinaciones con valores de IC_{50} menores de 5 μg/L. Al igual que en el formato directo, los mejores ensayos competitivos se consiguieron con los competidores que representan mayor homología con el analito respecto a la distribución electrónica (haptenos 5 y 7/As53,54). Se ha puesto en evidencia que la detectabilidad de los ensayos competitivos está afectada por varios factores, tales como la estructura de la molécula (geometría y carga), el método de conjugación empleado en la preparación de los inmunógenos y antígenos de tapizado, la densidad de haptenos conjugados, el tipo de proteína empleada. Un estudio teórico sobre el grado de heterología en la distribución de carga y la geometría de los competidores respecto al analito ha constatado con datos objetivos que, efectivamente, los mejores ensayos competitivos se han conseguido con algunos de los competidores con mayor grado de homología. Igualmente, se ha demostrado que la detectabilidad de los ensayos mejora con

la disminución del grado de conjugación de los competidores alcanzando mejores valores en el caso de 3 ó 5 moléculas de hapteno por molécula de proteína (véase **Tabla 11.1**).

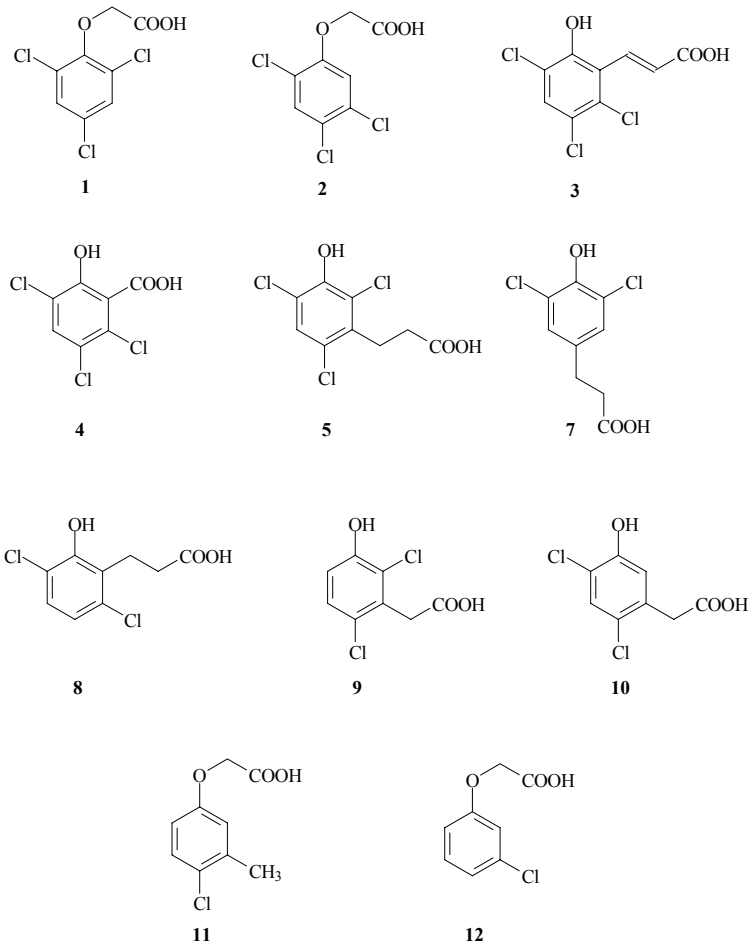


Figura 11.2. Estructuras químicas de los compuestos empleados como competidores

Tabla 11.1. Effecto de la densidad de haptenos del competidor 7-OVA(AE) sobre los parámetros del inmunoensayo (**As53/7-OVA**)

Hapteno:proteína [a]	δ [b]	% conjug.	A_{max}	A_{min}	Pendiente	IC_{50} [c]	r^2
2:1	n.d.[e]		0.94	0.06	-1.20	0.9	0.990
1:1	12	60	0.77	0.03	-1.24	0.57	0.990
1:2.5	**10**	**50**	**0.72**	**0.01**	**-1.13**	**0.29**	**0.990**
1:5	3	15	0.65	0.02	-1.00	0.21	0.990
1:10	3	15	0.74	0.02	-1.20	0.23	0.990

[a] Relación molar hapteno:proteína usada en la reacción de conjugación. [b] Densidad de haptenos (δ) está expresada en mol de hapteno por moles de proteína y está determinada de MALDI-TOF-MS. [c] IC_{50} está expresada en µg/L.

Entre los ELISAs competitivos indirectos se ha seleccionado como mejor combinación **As53/7-OVA(1:2.5)**. Se ha estudiado el efecto de varios factores fisicoquímicos (tiempo de incubación, pH, fuerza iónica, Tween 20) sobre los parámetros de este inmunoensayo. Se ha demostrado que una etapa de preincubación del analito con el anticuerpo o una variación del tiempo de competencia no producen un efecto significativo sobre la detectabilidad del ensayo. El ELISA funciona mejor cuando el valor del pH corresponde a medio neutro o básico. A valores de pH inferiores de 6 y superiores de 10 el ensayo está inhibido. La fuerza iónica del medio tiene un importante efecto sobre la detectabilidad del analito: un aumento de la conductividad de 10 a 30 mS/cm produce una mejora en la detectabilidad de tres veces. Finalmente, concentraciones bajas de detergente (menores de 0.05%) favorecen la detectabilidad del inmunoensayo.

Tabla 11.2. Condiciones óptimas y características del inmunoenasayo **As53/7-OVA(1:2.5)**

Condición	Valores	Parámetros	Valores [a]
Tiempo de preincubación	0 min	**A_{max}**	0.838±0.020
Tiempo de competencia	30 min	**A_{min}**	0.031±0.009
pH	7.5	**IC_{50}, µg/L**	0.231±0.011
Fuerza iónica	20 mS/cm (20 mM PBS)	**Rango lineal**	0.093±0.004 to 0.725±0.106
Tween 20	0.025%	**Pendiente**	1.399±0.070
		LOD, µg/L	0.053±0.004
		r^2	0.995±0.001

[a] Los valores están extraídos de la ecuación de cuatro parámetros usada para ajustar la curva estándar y corresponden a la media de nueve curvas de calibración realizadas en tres días diferentes. Cada curva se ha realizado en dúplica.

En **Tabla 11.2** están resumidas las condiciones óptimas del inmunoensayo y los correspondientes parámetros de la curva de calibración.

La especificidad del inmunoensayo As53/7-OVA fue evaluada respecto a 27 compuestos (clorofenoles, bromophenoles y anisoles) con estructura similar al analito. Se ha demostrado que el ensayo presenta un alto grado de selectividad, aunque se observa cierta reactividad cruzada con algunos clorofenoles (2,3,4,6-TtCP – 18.9%, 2,4,6-TCP – 17%) y bromophenoles (2,4,6-TBP – 27.7%, 2,5-DBP – 15%, 2,4-DBP – 10.5%). El reconocimiento está directamente relacionado con la presencia de dos átomos de Cl situados en *ortho* y en *para* y un hidrógeno en *meta*. Se ha confirmado también la importancia del grupo fenólico para el reconocimiento del analito por el anticuerpo (2,4,5-TCA – 0.29%). La reactividad cruzada observada con algunos compuestos fenólicos deberá ser considerada al aplicar el inmunoensayo a muestras reales.

Finalmente se ha caracterizado la precisión del ELISA para la detección de 2,4,5-TCP. Los coeficientes de variación CV (inter-ensayo) varían entre 5.1 y 15% y los CV (intra-ensayo) varían entre 10.9 y 20% para un intervalo de concentraciones comprendidas entre 0.1 y 1.5 μg/L. El estudio de exactitud realizado mediante el análisis de muestras ciegas preparadas en medio acuoso demostraron una excelente correlación ($y=1.07x-0.21$, $r^2=0.999$) entre los valores estimados mediante el ELISA y los valores con que se habían dopado las muestras.

11.3.2. Evaluación del efecto matriz de muestras ambientales y biológicas sobre la determinación inmunoquímica de 2,4,5-TCP y 2,4,6-TCP.

Una de las limitaciones de las técnicas inmunoquímicas es el denominado efecto matriz, el cual origina falsos positivos como consecuencia de las condiciones y componentes del medio que se analiza. De este modo, las muestras reales ambientales y biológicas son matrices complejas donde se encuentran un gran número de sustancias que podrían

interferir con el inmunoensayo para clorofenoles. Con el objetivo de analizar chlorophenoles en muestras reales se ha evaluado el efecto matriz de varios tipos de muestras ambientales (agua) y biológicas (leche, suero y orina) sobre los parámetros de dos inmunoensayos: el ELISA para 2,4,5-TCP desarrollado en el capítulo anterior (As 53/7-OVA) y el ELISA para 2,4,6-TCP (As43/8-BSA) desarrollado por R.Galve de nuestro grupo. Nuestro objetivo ha sido comprobar si los inmunoensayos son capaces de medir TCP en aguas considerando las exigencias de la UE sobre los valores máximos permitidos de pesticidas en aguas potables (0.1 μg/L para el compuesto individual y 0.5 μg/L para la concentración total) (Directiva 80/778/CCE). Para el control biológico hemos considerado oportuno estudiar el comportamiento de los ensayos inmunoquímicos de TCP en leche, suero y orina. Nos hemos propuesto establecer un procedimiento que exija un tratamiento mínimo de la muestra y que el inmunensayo sea capaz de alcanzar límites de detección adecuados para el estudio de la población general: 12 μg/L para el serum (según los valores de referencia de los niveles de PCP en suero), 1 μg/g materia grasa para la leche (según los valores de referencia de pesticidas organoclorados) [20] y 1 μg/L para la orina (según los niveles de referencia establecidos para la población general [75,81,121,123]).

11.3.2.1. Muestras de agua

Se han considerado dos tipos de muestras de agua: agua de bebida y agua del pozo. Se ha demostrado que una correcta determinación inmunoquímica de 2,4,6- y 2,4,5-TCP se puede conseguir solo con tamponar las muestras de agua con PBS para asegurar que los valores de pH y salinidad de la muestra sean los adecuados para el buen funcionamiento del inmunoensayo. La exactitud del método se ha estudiado con muestras de agua de bebida dopadas con 2,4,5-TCP. La recuperaciones obtenidas fueron entre 92 y 105.3% para niveles entre 0.2 y 5 μg/L 2,4,5-TCP. La validación del ELISA mediante la comparación de los resultados obtenidos tras el análisis de las mismas muestras por GC-ECD (los clorofenoles han sido extraídos con tolueno de las muestras acuosas y silanizados) ha resultado en una correlación lineal entre los dos métodos muy buena

(y=1.26x-4.27, r^2=0.993). Los parámetros característicos para la detección de muestras de agua por el ELISA de 2,4,5-TCP y 2,4,6-TCP están presentados en **Tabla 11.3**.

Tabla 11.3. Concentraciones detectables en muestras de agua (μg/L) por los inmunoensayos de 2,4,5- y 2,4,6-TCP

Parámetros	2,4,5-TCP	2,4,6-TCP
LOD	0.07 ± 0.01	0.18 ± 0.03
IC_{50}	0.32 ± 0.02	1.13 ± 0.36
Rango lineal	0.13 ± 0.01 - 1± 0.15	0.29 ± 0.05 – 3.12± 0.68

Los parámetros presentados se han extraido de las curvas estándares preparadas en agua de bebida tamponada.

Los LOD para los dos inmunoensayos son inferiores a los LOD de los métodos estándares cromatográficos aceptados por la EPA para estos compuestos (ej. 0.64 μg/L 2,4,6-TCP) y muy cercanos a los requerimientos de la UE en lo que se refiere a la contaminación de las aguas potables con residuos de clorofenoles.

11.3.2.2. Muestras de leche

El ELISA de 2,4,5-TCP fue evaluado en muestras de leche desnatada. A pesar de que el pH y la salinidad de la muestra de leche fueron ajustados como los del tampón PBS, el inmunoensayo quedaba completamente inhibido al introducir este tipo de muestras. Como posibles causas se podrían considerar la presencia de proteínas, grasa y la alta concentración de iones de calcio, etc. Se han probado varios tratamientos de la leche, tales como precipitación de las proteínas en medio básico (NaOH) y la precipitación de los iones de calcio en forma de $CaSO_4$, pero hasta el momento no se ha conseguido solucionar este problema.

11.3.2.3. Muestras de suero humano

El efecto matriz del suero sobre los inmunoensayos para la detección de 2,4,5-TCP y 2,4,6-TCP consistía en un efecto de desplazamiento de las curvas estándares hacia concentraciones más altas de analito siendo paralelas a la curva del tampón. Este efecto podría atribuirse a los altos niveles de albúminas presentes en el suero. Se han utilizado

diferentes procedimientos para la eliminación de las albúminas: ultrafiltración, precipitación alcalina (KOH), ácida ($HClO_4$, ácido tricloracético), precipitación térmica (65°C) (véase **Figura 11.3**).

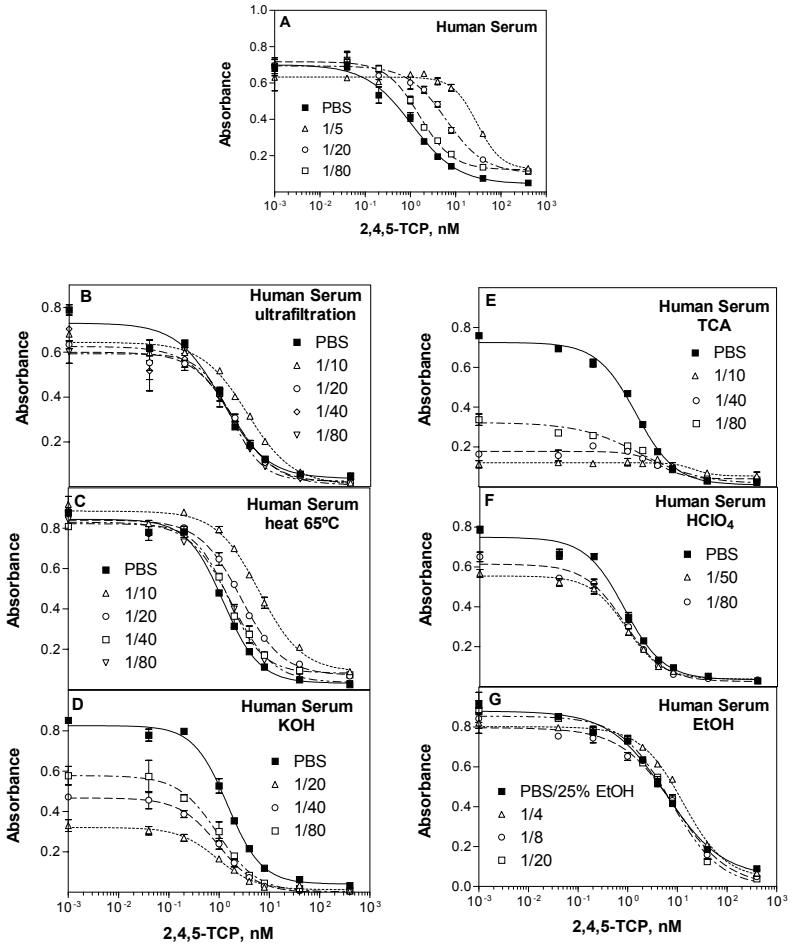


Figura 11.3. Efectos matriz del suero sobre el ELISA para 2,4,5-TCP. **A,** Suero sin tratar; **B**. Ultrafiltración; **C**. Precipitación térmica a 65C; **D**. KOH; **E.** ácido tricloroacético; **F**. ácido perclórico; **G**. etanol absoluto.

Aunque en algunos casos el efecto matriz fue eliminado en gran parte, el análisis de muestras dopadas con varias concentraciones de 2,4,5-TCP ha demostrado que las recuperaciones del analito eran menores de 50%. Finalmente, la precipitación de las albúminas del suero por disolventes orgánicos (EtOH absoluto) ha permitido una mínima dilución del sobrenadante (1/8) para una correcta cuantificación del 2,4,5-TCP. Con este

procedimiento se han conseguido recuperaciones comprendidas entre 76 y 98% para muestras dopadas con 3.2 - 32 μg/L 2,4,5-TCP. El procedimiento de precipitación de las proteínas con EtOH ha sido menos efectivo en el caso del ELISA de 2,4,6-TCP y una dilución de 32 veces ha sido necesaria para eliminar el efecto matriz observado dando lugar a un LOD más elevado. Los parámetros característicos para la detección de 2,4,5- y 2,4,6-TCP en muestras de suero están resumidos en **Tabla 11.4**.

Tabla 11.4. Concentraciones en muestras de suero (μg/L).

Parámetros	**2,4,5-TCP** [a]	**2,4,6-TCP** [b]
LOD	0.80 ± 0.16	4.576
IC_{50}	6.46 ± 0.256	85.12
Rango lineal	1.2 ± 0.144 - 40 ± 6.962	15.04 - 288.8

[a] Los valores presentados están obtenidos multiplicando los parámetos de la curva estándar en 1/8 suero por 8. El suero está precipitado con EtOH. [b] Los valores presentados están obtenidos multiplicando los parámetos de la curva estándar en 1/32 suero por 32. El suero está precipitado con EtOH.

Como se desprende de lso resultados que aparecen en la tabla, el método finalmente establecido permitiría la aplicación de ambos inmunoensayos en el control biológico y/o en la realización de estudios toxicológicos.

11.3.2.4. Muestras de orina

Para la evaluación del ELISA para 2,4,5-TCP y 2,4,6-TCP en muestras de orina se han utilizado dos tipos de muestras de personas no expuestas a clorofenoles (población general): muestra A (creatinina 1.8 g/L) y muestra B que presenta un *pool* de orinas de diferentes individuos (creatinina 0.8 g/L). Ya que se ha demostrado que la población general se encuentra frecuentemente expuesta a clorofenoles no es de extrañar que exista un cierto nivel de clorofenoles en la orina [75,81,121,123]). Por este motivo, se llevó a término una caracterización previa de las muestras para así determinar la posible presencia inherente en estas muestras (A y B) de cloro- y bromofenoles (2,4,5-TCP, 2,4,6-TCP, 2,3,4,6-TBP, 2,4-DBP, 2,4,6-TBP) que, al producir cierta reactividad cruzada en los inmunoensayos, podrían interferir con los estudios destinados a evaluar el efecto matriz. Para ello se ha desarrollado un método analítico independiente basado en GC-

ECD y GC-MS para la determinación de chloro- y bromofenoles. En breve, el procedimiento está basado en la extracción de los clorofenoles de la fase acuosa (PBS, orina) con tolueno y su posterior derivatización con BSTFA. Se han optimizado las condiciones de la extracción y la silanización respecto al tiempo y la temperatura para varios niveles de 2,4,6-TCP. La recuperación de muestras dopadas con 1 μg/L y 10 μg/L 2,4,6-TCP es del 70 y 85%, respectivamente, con una SD del 10%. Siguiendo este procedimiento las muestras de orina A y B (no hidrolizadas e hidrolizadas) se han analizado por GC-MS y se ha detectado la presencia de varios cloro- y bromo-fenoles. Teniendo en cuenta la reactividad cruzada de estos compuestos en los ELISAs y su concentración en la muestras de orina se han estimado los equivalentes de immunoreactividad correspondientes (véase **Tabla 11.5**).

Tabla 11.5. Niveles de clorofenol expresados como TCP equivalentes de inmunoreactividad

Equivalentes totales de immunoreactividad	**orina A**		**orina B**	
	hidrolizada	**no hidrolizada**	**hidrolizada**	**no hidrolizada**
2,4,6-TCP-IR equiv.	**0.72**	0.16	**1.12**	0.24
2,4,5-TCP-IR equiv.	**0.155**	0.021	**0.097**	0.033

Los LOD del 2,4,6- y 2,4,5-TCP ELISA son 0.17 μg/L y 0.053 μg/L respectivamente.

Considerando los límites de detección de los inmunoensayos se ha estimado que las muestras de orina no hidrolizada se podrían considerar como una "matriz blanca", mientras que las muestras hidrolizadas podrían presentar cierta interferencia específica por lo que los niveles de compuestos crossreactantes determinados debían ser considerados en la evaluación del efecto matriz.

Para evaluar el efecto de la orina sobre los inmunoensayos se han comparado en paralelo las curvas estándares de 2,4,6-TCP (2,4,5-TCP) preparadas en PBS y en la orina (no hidrolizada e hidrolizada por varios métodos) diluida con PBS. Los valores del pH y de la conductividad de las diferentes diluciones han sido ajustadas dentro del rango funcional del inmunoensayo. **Tabla 11.6** resume el efecto matriz observado para todos los casos estudiados.

Tabla 11.6. Resumen del efecto matriz de orinas hidrolizada y no hidrolizada sobre los ELISAs para 2,4,5-TCP y 2,4,6-TCP.

Tratamiento	Muestra de orina	ELISA	
		2,4,5-TCP	**2,4,6-TCP**
No hidrolizada	**A**	1/100	> 1/100
	B	1/5	1/100
Hidrólysis ácida (c.H_2SO_4)	**A**	n.d.	> 1/128
	B	> 1/100	1/200
Hidrólysis enzimática	**A**	n.d.	> 1/200
(β-glucuronidasa/sulfatasa)	**B**	n.d.	> 1/200

El efecto matriz está expresado como la dilución de la orina necesaria para alcanzar el comportamiento de la curva estándar en condiciones de tampón.

Como se puede ver una misma muestra (ej. muestra B, no hidrolizada) tiene diferente efecto matriz sobre los dos inmunoensayos estudiados: para el caso de 2,4,5-TCP se necesita una dilución de la muestra de 5 veces y para el 2,4,6-TCP 100 veces. Muestras de orina no hidrolizada dopadas a varios niveles de 2,4,5-TCP se han medido por el ELISA después de una simple dilución y las recuperaciones obtenidas fueron entre 96% y 109% con CV entre 7.2 y 12.7%. En **Tabla 11.7** están presentados los niveles de 2,4,5-TCP y 2,4,6-TCP que se podrían detectar en orina no hidrolizada mediante los dos inmunoensayos trás una simple dilución d e la muestra.

Tabla 11.7. Concentraciones en muestras de orina no hidrolizada (μg/L).

Parámetros	2,4,5-TCP [a]	2,4,6-TCP [b]
LOD	0.26 ± 0.02	17.5 ± 2.7
IC_{50}	1.28 ± 0.06	113.2 ± 36.1
Rango lineal	0.52 ± 0.05 - 5.30 ± 0.85	28.8 ± 4.5 - 311.7 ± 67.9

[a] Los valores presentados están obtenidos multiplicando los parámetros de la curva estándar en 1/5 orina por 5. La orina está diluida con PBS. [b] Los valores presentados están obtenidos multiplicando los parámetros de la curva estándar en PBS por 100.

Considerando los valores de referencia (4.5 μg/L) establecidos en Alemania para la población no expuesta [81], podríamos concluir que este método podría ser aplicado para la determinación de 2,4,5-TCP en orina de personas no expuestas sin ningún tratamiento. Por otro lado, el mayor efecto matriz observado para el inmunoensayo de 2,4,6-TCP permitiría sólo la evaluación de los niveles de 2,4,6-TCP en trabajadores con exposición laboral suficientemente alta. Sin embrago, para la vigilancia de 2,4,6-TCP en la población general sería necesario una purificación previa de la muestra.

En el caso de las orinas hidrolizadas la inhibición de los inmunoensayos ha sido mayor que en el caso de las muestras no hidrolizadas. Los niveles de clorofenoles detectados por GC-MS no pueden justificar el efecto de inhibición de la A_{max} loque lleva a la conclusión de la presencia de interferencias no específicas. Cabe destacar, y así puede observarse en la tabla, que muestras de orina de diferentes individuos con diferente concentración de creatinina han presentado un efecto matriz diferente, por lo que se pone en evidencia la necesidad de desarrollar un método de tratamiento de muestra tanto para orinas hidrolizadas como no hidrolizadas. Antes de abordar este tema, se ha explorado la posibilidad de eliminar el efecto matriz desarrollando los inmunoensayos no en el tampón PBS sino directamente en la orina B de origen comercial y considerada como una muestra estándar. Lamentablemente se ha observado de nuevo una variabilidad del efecto matriz para muestras de orina de diferentes individuos. Esto impide aplicar los inmunoensayos como un método general (universal) para la detección de triclorofenoles en muestras de orina sin previa purificación de la muestra.

11.4. Desarrollo de métodos de extracción en fase sólida SPE-ELISA para la detección de clorofenoles en orina

Para la purificación de la orina se han estudiado dos métodos de extracción en fase sólida: fase reversa e inmunoextracción. El objetivo ha sido i) conseguir una extracción eficiente del analito 2,4,6-TCP de la muestra de orina que sea compatible con el método ELISA; ii) que se adapte fácilmente al formato de 96 pocillos del las placas de ELISA para así mantener la eficiencia del método de screening; iii) que permita alcanzar los límites de detección deseados para la monitorización biológica de la población general (alrededor de 1 μg/L).

11.4.1. Fase reversa (C_{18})-ELISA

La extracción en fase sólida de C_{18} permite aislar el análito de la muestra en función de las diferentes polaridades del analito y la matriz. El procedimiento de purificación se ha evaluado inicialmente con tampón PBS y posteriormente con muestras de orina (dopada y sin dopar con 2,4,6-TCP) usando minicolumnas de tipo Sep-Pak ®. La concentración del analito en todas las fracciones recogidas ha sido determinada por ELISA después de una dilución (1/10) en PBS y/o por GC-ECD. Se ha determinando el perfil de elución del 2,4,6-TCP empleando diferentes concentraciones del MeOH en el tampón de elución (véase **Figura 11.4**). Como se puede observar la purificación de la orina en fase reversa

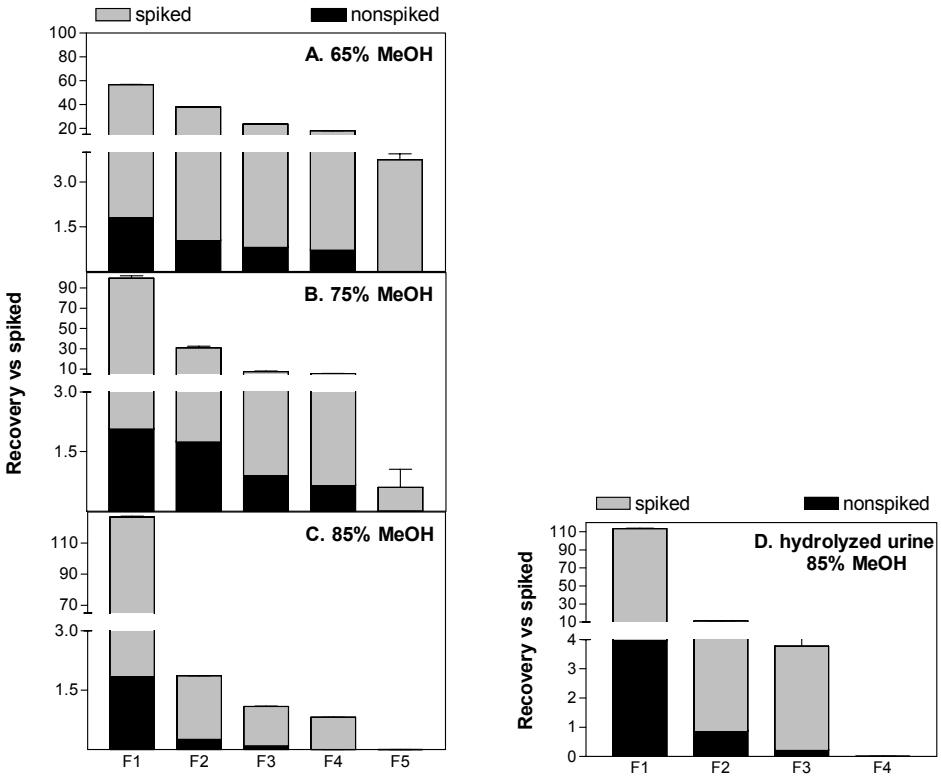


Figura 11.4. Perfil de elución de C_{18}-SPE clean-up de orina A (dopada y no dopada) para varias concentraciones de MeOH en el tampón de elución.Orina no hidrolizada: (**A**) 65% MeOH, (**B**) 75% MeOH, (**C**) 85% MeOH; Orina hidrolizada: (**D**) 85% MeOH. La concentración de cada fracción está determinada por ELISA y la recuperación está calculada respecto a la cantidad dopada.

permite una extracción completa del analito (recuperaciones alrededor de 100%) en 3 mL en el caso de elución con 65% MeOH, en 2 mL – 75% MeOH y en 1 mL – 85% MeOH. No obstante la orina no dopada también ha producido una respuesta positiva en el ELISA que podría ser debida a interferencia específicas y/o no específicas. Deberíamos anotar que la respuesta de muestras de orina dopadas no ha sido equivalente en las diferentes diluciones seriadas. Esta observación junto con el hecho de que la cantidad total (μg) de equivalentes de immunoreactividad de 2,4,6-TCP (2,4,6-TCP-IR equiv.) extraidos de la orina no dopada superan la correspondiente cantidad evaluada por GC-MS, nos ha llevado a la conclusión de que junto con el analito co-eluyen interferencias no-específicas. Se ha tenido en consideración que la co-elución de componentes interferentes ha sido menor en el caso de elución con tampón 85% MeOH/PBS cuando el analito puede ser recogido en una sola fracción de 1 mL.

Se ha evaluado también el efecto matriz de la orina purificada por C_{18} (veáse **Figura 11.5.**). Tal como puede observarse en la **Figura 11.5**, es necesaria una dilución de 1/25 para poder determinar 2,4,6-TCP en la orina. Podemos concluir que la introdución de esta etapa de purificación mejora el límite de detección de la técnica, ya que ahora es posible medir orina diluida tan solo 25 veces lo que supone un límite de detección alrededor de 4 μg/L. La extracción en fase sólida permite mejorar la detectabilidad del 2,4,6-TCP en la orina 6 veces respecto al inmunoensayo directo. Este límite de detección permitiría el control biológico de personas expuestas laboralmente. No obstante, es necesario una

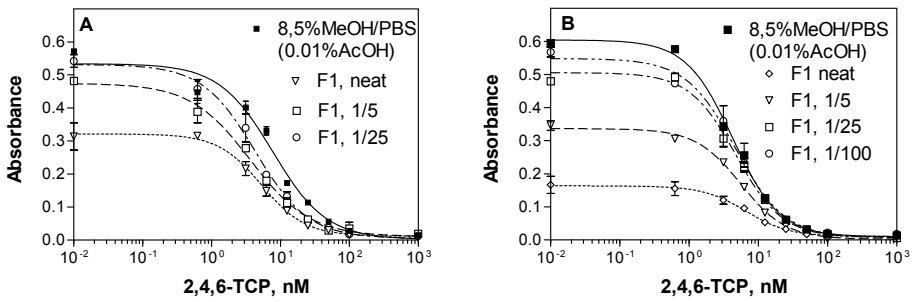


Figura 11.5. Efecto matriz de la orina A después de purificación por C_{18}-SPE. (**A**) no hidrolizada, (**B**) hidrolysis ácida. El cartucho de C_{18} está cargado con 10 mL de orina, lavado con 50% MeOH, y eluído con 85% MeOH. La fracción F1 está diluída con PBS hasta 10 mL. Las diluciones superiores se han echo en 8.5% MeOH/0.01% AcOH/PBS.

purificación más efectiva de la orina para la evaluación de los niveles de triclorofenol en la población general.

11.4.2. IAC-SPE-ELISA

La purificación de la orina mediante cromatografía de inmunoafinidad (IAC) permite una purificación específica y por lo tanto más eficaz para la eliminación del efecto matriz observado. Inicialmente el procedimiento de IAC-SPE fue estudiado usando una columna Hi-Trap™ de una capacidad de 1 mL. La columna de inmunoafinidad se ha obtenido mediante la inmovilización de anticuerpos policlonales (fracción de IgG obtenida por purificación del antisuero As45) sobre un soporte de Sepharosa NHS-activada con 94 ± 0.98% (N=3) de eficiencia de acoplamiento. Esto corresponde a una capacidad teórica de 1.2 ± 1.22 μg (6.27 ± 6.14 nmol) 2,4,6-TCP (N=3) considerando la cantidad de Ab acoplado y asumiendo que el 10% de las IgG policlonales son específicas, que la unión Ab-Ag es bivalente y que 50% de las IgG no son accesibles debido a su orientación no efectiva y/o a un impedimento estérico.

El procedimiento del análisis IAC-ELISA se ha optimizado respecto a varios parámetros: condiciones de elución, volumen de la muestra cargada a la columna, tipo de muestra (orina no hidrolizada e hidrolizada) y nivel de carga. Inicialmente se ha probado una elución con tampón glicina (0.05M Glicina-HCl, pH=3) para tres niveles de carga (97%, 40% y 4% de la máxima capacidad de la columna). Se ha observado que el perfil de elución depende del nivel de carga produciendo una banda muy ancha para cargas pequeñas (4%). Debido a esto se ha buscado otro sistema de elusión más efectivo como podría ser la utilización de un disolvente orgánico como el EtOH. Inicialmente se ha estudiado el efecto de la concentración del EtOH en el tampón de elución con estándares de 2,4,6-TCP en PBS. Se ha demostrado que el analito empieza a eluir con un 30% EtOH/PBS y que 3 mL de 70% EtOH son suficientes para su extracción completa (recuperaciones superiores al 90% para los tres niveles de carga 4%, 40% y 97%). Por lo

tanto, se ha escogido este sistema para la elusión efectiva del analito del inmunosorbente cargado con muestras de orina.

Mediante GC-ECD se ha evaluado la especificidad de la columna de inmunoafinidad respecto a 15 fenoles clorados (mono-, di-, tri-, tetra- sustituidos) bajo diferentes condiciones de carga (individual y en mezcla equimolar; en PBS y orina) y tampón de lavado (PBS y 20% EtOH/PBS). Se ha observado que el lavado de la columna de inmunoafinidad con PBS da lugar a una extracción menos específica (todos los isómeros de TCP, TtCP y PCP se retienen por la columna en más de 80%) que podría encontrar aplicación como una IAC-SPE selectiva de clase de clorofenoles de muestras de orina previa de su cuantificación por técnicas inmunoquímicas o por cromatografía de gases. Una extracción más selectiva se consigue mediante un lavado con 20%EtOH/PBS. En este caso los principales co-eluyentes son 2,3,4,6-TtCP, 2,4,6.TBP y 2,4-DBP, que son precisamente los crossreactantes principlaes del ELISA para la detección de 2,4,6-TCP. La selectividad parecida de los anticuerpos usados en la IAC (As45) y el del ELISA (As43) se debe al hecho de que fueron generados contra un mismo inmunógeno. La especificidad de la columna ha sido considerada al evaluar el efecto matriz de la orinas purificada por IAC-SPE. El efecto matriz de orinas (muestras A y B, hidrolizadas y no hidorlizadas) están presentadas en la **Figura 11.6** donde se observa que la utilización del inmunosorbente consigue eliminar en una gran medida el efecto matriz de la orina hidrolizada y no hidrolizada.

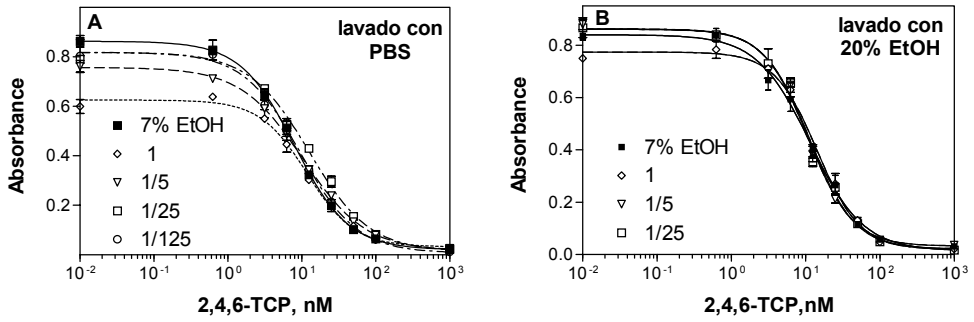


Figura 11.6. Efecto matriz de la orina A hidrolizada con ácido purificada por IAC-SPE sobre el ELISA de 2,4,6-TCP. **A.** lavado con PBS; **B**. 20%EtOH. La elución es con 70% EtOH. La fracción recogida de 3 mL ha sido diluída con PBS 10 veces. Las diluciones consecutivas se han hecho en 7% EtOH/PBS.

Un análisis del extracto por GC-ECD y la posterior confirmación de los compuestos fenólicos por GC-MS ha revelado que la ligera inhibición de la A_{max} , todavía visible al introducir una etapa de lavado con un 20% de EtOH, se debe a interferencias específicas, i.e. al hecho de que las muestras de orina usadas no presentan una "muestra blanca". Por lo tanto, puede considerarse que la eliminación del efecto matriz procedente de interferencias no específicas, es prácticamente total al usar inmunosorbentes.

La recuperación de la IAC del 2,4,6-TCP de muestras de orina depende del volumen de la muestra a purificar y del nivel del dopaje. Como se puede ver en **Figura 11.7** para orina no hidrolizada el volumen de ruptura es más de 50 ml para una carga de 5 ng 2,4,6-TCP (4% nivel de carga de la columna) y unos 2 ml para niveles más altos (500 ng TCP, 40%). En general la recuperación del TCP de muestras de orina hidrolizada ha sido más baja que la de no hidrolizada para un volumen y nivel de carga constantes. Para niveles de carga bajos (4%) la recuperación en la orina hidrolizada por ácido está alrededor del 50% al introducir 30 mL como volumen de muestra, y para niveles de carga superiores (40%) el volumen de ruptura es sólo 5 mL.

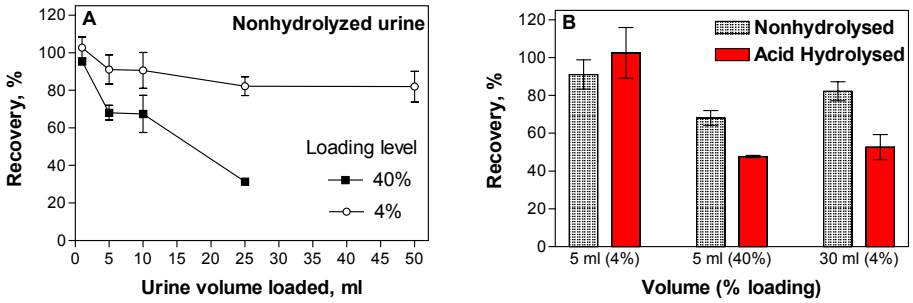


Figura 11.7. Efecto del volumen y la carga de la muestra sobre la recuperación del IAC-SPE (lavado con PBS y elución con 70% EtOH. La máxima capacidad de la columna corresponde a 1.2 μg 2,4,6-TCP.

En base de estos resultados se ha seleccionado 10 mL como un volumen adecuado de muestra que permita asegurar la extracción del TCP con una recuperación superior al 80%. El efecto de la carga sobre la recuperación de orinas no hidrolizada e hidrolizada

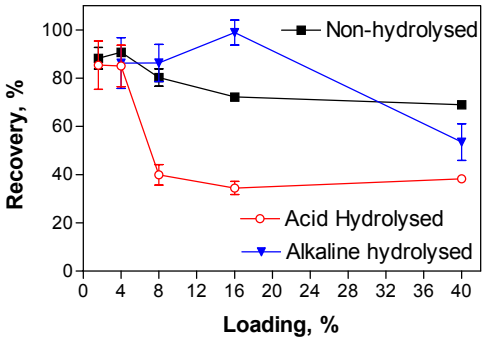


Figura 11.8. Efecto del nivel de carga sobre la recuperación de IAC-SPE de orinas hidrolizadas y no hidrolizadas (volumen de la muestra 10 mL, lavado con 20% EtOH y elución con 70% EtOH).

por ácido (H_2SO_4) y base (KOH) está presentado en **Figura 11.8**. Excelentes recuperaciones se han obtenido para los tres tipos de orina hasta un nivel de carga de 4%. La columna de inmunoafinidad puede extraer eficazmente 200 ng 2,4,6-TCP (16% de carga) de orinas no hidrolizada y hidrolizada en medio básico cuando el volumen de muestra es de 10 mL. A pesar de esto la capacidad de la columna para orina hidrolizada en medio ácido es inferior: 50 ng 2,4,6-TCP (4% de carga). Una comparación de las posibles pérdidas de 2,4,6-TCP durante el proceso de hidrólisis (alcalina y ácida) ha demostrado que ambos métodos producen una recuperación del TCP similar (alrededor de 75-80%), por lo tanto la hidrólisi de la orina en medio básico sería el método de elección para el caso de un programa de control biológico.

La validación del método IAC-ELISA por GC-ECD ha demostrado una correlación excelente para orina hidrolizada y no hidrolizada en el intervalo 2 - 20 μg/L. El protocolo fue aplicado a muestras de orina procedentes de diferentes individuos obteniéndose recuperaciones entre 80% y 115%, lo que demostró la versatilidad del método desarrollado. Finalmente se ha de mencionar que una misma columna de inmunoafinidad ha sido estable después de 60 ciclos de análisis.

Concluyendo, el protocolo optimizado de IAC-ELISA consiste de los siguientes etapas: 1. hidrolizar la orina; 2. introducir 10 mL de muestra en el inmunosorbente; 3. lavar con

5mL de 20% EtOH (para el caso de un análisis total de clorofenoles, un lavado con PBS es suficiente); 4. eluir con 3 mL de 70% EtOH y 5. diluir las fracciones en PBS 10 veces (7% EtOH, final) y analizarlas por el ELISA. El LOD del método IAC-ELISA está determinado por el LOD del ELISA en 7% EtOH/PBS (0.2 μg/L), los factores de la recuperación de la inmunoextracción y de la etapa de hidrólisis y finalmente del hecho de que bajo las condiciones del procedimiento establecido la muestra inicial se diluye 3 veces. Para casos en los que se necesite disminuir aún más el LOD, se ha comprobado que una dilución de tan solo 5 veces de las fraciones de elusión (14% EtOH final), también permitirían el análisis por ELISA. En **Tabla 11.8** se reúnen los LOD y los niveles máximos que se pueden detectar cuantitativamente (recuperación superior a 70%) para cada tipo de orina.

Tabla 11.8. Rango de concentraciones para una determinación cuantitava de 2,4,6-TCP en diferentes orinas por el método de IAC-SPE-ELISA.

Orina	LOD [a], μg/L	MCM [b], μg/L
No hidrolizada	0.66	50
Hidrólisis ácida	0.83	5
Hidrólisis básica	0.99	20<MDC<50

[a] $LOD_{7\%EtOH/PBS}$=0.2 μg/L está corregido con las correspondientes recuperaciones de la extracción IAC y hidrólisis. [b] MCM (máxima concentración medida con recuperación >70%)

2,4,6-TCP libre se puede determinar con un LOD de 0.66 μg/L 2,4,6-TCP-IR equiv.en 10 mL de orina no hidrolizada. La orina no hidrolizada puede ser extraida eficazmente hasta unos 50 μg/L de 2,4,6-TCP. Para orina hidrolizada el LOD es alrededor de 0.83 μg/L 2,4,6-TCP-IR equiv. para hidrólisis ácida y 1 μg/L 2,4,6-TCP-IR equiv.para hidrólisis básica. 2,4,6-TCP puede ser extraído cuantitativamente en muestras de orina con un contenido en TCP entre 0.83 y 5 μg/L en orina hidrolizada por ácido y entre 1 y 20 μg/L en orina hidrolizada por KOH. Para concentraciones mayores de 20 μg/L el análisis debería ser repetido después de una dilución de la orina.

11.5. Desarrollo de un método HTS-IAC-SPE para la detección de clorofenoles en orina

El procedimiento de IAC-SPE-ELISA está limitado principalmente por el tiempo necesario para la purificación de una muestra de orina. Ya que el análisis clínico y el control biológico exigen un análisis rápido y eficaz de un gran número de muestras se ha propuesto de adaptar el protocolo de IAC-SPE a un formato de 96 pocillos (*high throughput screening, HTS*), lo que permitiría la cuantificación simultánea de los extractos de orina por ELISA en el mismo formato. Para ello se ha usado el sistema comercial Versaplate 96-Well SPE (Varian, Palo Alto). Como soporte se ha usado Sepharosa NHS-activada, a la cual se ha acoplado covalentenmente el anticuerpo Ab45 en *batch*. Se han empaquetado las 96 columnas con 200 μL (suspensión) del inmunosorbente preparado y han sido caracterizadas de una manera similar al descrito anteriormente respecto der su capacidad, las condiciones de elución y de lavado, el efecto del EtOH, la estabilidad, etc., utilizando el sistema Versaplate™ conectado al vacío mediante un regulador como sistema de elusión. La capacidad máxima de cada columna (lecho 0.2 ml) es de 0.5 μg (0.25 nmol) 2,4,6-TCP (CV% = 4.7, N=10). Las columnas fueron estables hasta después de 40 ciclos de uso. Un lavado con 20% EtOH se puede realizar sin pérdidas del analito retenido a un nivel de carga de 10% de la máxima capacidad de la columna (50 ng 2,4,6-TCP, 6 mL, 8 μg/L). A niveles de carga más elevados (40%) correspondientes a 200 ng 2,4,6-TCP (32 μg/L) se ha observado que 6.8% del analito puede eluir en las fracciones de lavado con el 20% de EtOH. Estos niveles de TCP son bastante elevados y, en principiio, solo se habrían de encontrar en grupos con un alto riesgo de exposición. En base a este comportamiento se ha decidido usar 20% EtOH.

El protocolo establecido para el IAC-SPE-ELISA del apartado anterior fue adaptado a volúmenes menores: 6 mL carga de muestra de orina; 1.2 mL de lavado con 20% EtOH; 0.6 mL elución 70% EtOH/PBS, regeneración de la columna con 1.2 mL de PBS. Las fracciones recogidas se diluyen 10 veces con PBS y se pueden analizar por ELISA o por GC-ECD (GC-MS). Siguiendo el protocolo HTS-IAC-ELISA se ha evaluado la precisión

y la exactitud de la extracción por 96 columnas con controles de tampón PBS y orina hidrolizada (KOH) a tres niveles de concentración (0.7, 2, 8 μg/L 2,4,6-TCP). La recuperación del analito (superior a 80%) y los CV% (inter-ensayo 17-23% (N=24); intra-ensayo 6-11% (N=72)) demuestran que el método HTS-IAC-ELISA permite una correcta cuantificación de 2,4,6-TCP en muestras de orina y que es consistente y fiable.

En **Tabla 11.9** están resumidas las características de los dos métodos (IAC-SPE-ELISA y HTS-IAC-ELISA). El método HTS-IAC-ELISA ofrece la ventaja de un tratamiento de muestras por día mucho mayor que en el caso del uso de una columna. Se pueden procesar 100 muestras/día con un LOD alrededor de 0.3 μg/L 2,4,6-TCP-IR equivalentes. La otra ventaja del HTS-IAC-ELISA es el LOD más bajos debido al hecho de que no es necesario diluir la muestra de orina después de la purificación.

Tabla 11.9. Características del análisis de 2,4,6-TCP en orina mediante IAC-ELISA y HTS-IAC-ELISA

Parámetros	IAC-ELISA	HTS-IAC-ELISA
volumen de muestra	10 mL	6 mL
clean-up	dilución 1/3	no se diluye
velocidad del IAC	96 muestras/ **8 días**	96 muestras/ **1 hora**
velocidad del análisis total	96 muestras/ **9 días**	96 muestras/ **1 día**
LOD [a], μg/L	0.99	0.3
LOQ [b], μg/L	1.4	0.55
MCM [c], μg/L	20<MDC<50	30
% CV [d]	5.1-18.4% para 5-30 μg/L	17.4 – 22.9% para 0.7 – 8 μg/L
Número de falsos positivos	0	0
Número de falsos negativos	0	0

[a] El límite de detección esta evaluado según el LOD del ELISA ($LOD_{7\%EtOH/PBS}$=0.2 μg/L, correspondiente a 90% de la señal de cero concentración) y las correspondientes recuperaciones de la extracción IAC y la hidrólisis. [b] El límite de cuantificación está evaluado según el LOD del ELISA ($LOQ_{7\%EtOH/PBS}$=0.37 μg/L, correspondiente a 80% de la señal de cero concentración) y las correspondientes recuperaciones de la extracción IAC y la hidrólisis. [c] MCM (máxima concentración medida con recuperación >70%). [d] El coeficiente de variación (% CV) corresponde a interday análisis.

El método HTS-IAC-SPE-ELISA ha sido validado por GC-MS analizando 117 muestras de orina hidrolizada de diferentes individuos. Se han determinado los niveles de 2,4,6-TCP, 2,4,5-TCP, 2,4-DBP, 2,3,4,6-TtCP y 2,4,6-TBP por análisis de GC-MS y se han calculado los correspondientes equivalentes de inmunoreactividad de 2,4,6-TCP según la

reactividad cruzada del inmunoensayo. Considerando los 2,4,6-TCP-IR equiv. se ha obtenido una correlación muy buena entre los resultados obtenidos por GC-MS y por ELISA ($y=1.14x-0.21$, $r^2=0.912$).

Finalmente el método se ha aplicado al control biológico de tres grupos de población: trabajadores en oficinas de servicio, trabajadores en una industria petroquímica y población vecina a una incineradora de residuos municipales. Los resultados del análisis por el método HTS-IAC-SPE-ELISA están presentados en **Tabla 11.10**.

Tabla 11.10. Niveles de 2,4,6-TCP-IR equivalentes en orina de tres grupos de población.

Población	N	Mean±SD	Min	5%	25%	50%	75%	95%	Max
Trabajadores en oficinas	41	1.33±0.78	0.42	0.61	0.85	1.13	1.44	2.67	4.69
Trabajadores en petroquímica	43	1.90±1.68	0.18	0.65	0.92	1.33	2.02	5.68	8.06
Vecinos a una incineradora	33	3.86±4.36	0.59	0.78	1.54	2.19	3.93	14.47	22.69

Las concentraciones están determinadas por HTS-IAC-ELISA y expresadas en μg/g creatinina. El LOD es 0.17 μg/g creatinina.

Se puede observar que los valores característicos de la distribución estadística (la media, 50%, 95% y el valor máximo) son más bajos para los trabajadores de oficinas seguidos por los de la industria petroquímica y finalmente con niveles más elevados son los vecinos a la incineradora de residuos. Es difícil explicar estos resultados ya que no disponemos de información sobre el riesgo real de exposición a compuestos organoclorados de los tres grupos, tampoco de sus hábitos particulares y su estado de salud. Hay que destacar que las incineradoras se consideran como fuentes de emisión de clorofenoles, hexaclorobenceno, hidrocarburos poliaromáticos y dioxinas [374,436], a pesar de que la exposición interna real y los efectos sobre la salud de la población son todavía cuestión de controversias [35,102,123,375,376,378,437].

Los niveles de cloro- y bromophenoles determinados por GC-MS están presentados en **Figura 11.9**. La excreción de 2,4,6-TCP se observa en el grupo de trabajadores petroquímicos, a pesar de que los niveles determinados por nosotros son similares con los valores encontrados para la población general de la misma cuidad [118]. La excreción de

2,3,4,6-TtCP es similar para los tres grupos estudiados. Es sorprendente el mayor nivel de fenoles bromados (2,4-DBP, 2,4,6-TBP) en el grupo de vecinos a la incineradora, a pesar de que la fuente de compuestos oragonobromados puede ser relacionada con la contaminación del agua potable, de la comida y/o el medio ambiente en general debido al uso de agentes ignífugos [315,316]. Finalmente, cabe destacar que los niveles de triclorofenoles en las orinas de los grupos estudiados son menores de los valores de referencia establecidos en Alemania [81] y Estados Unidos [75].

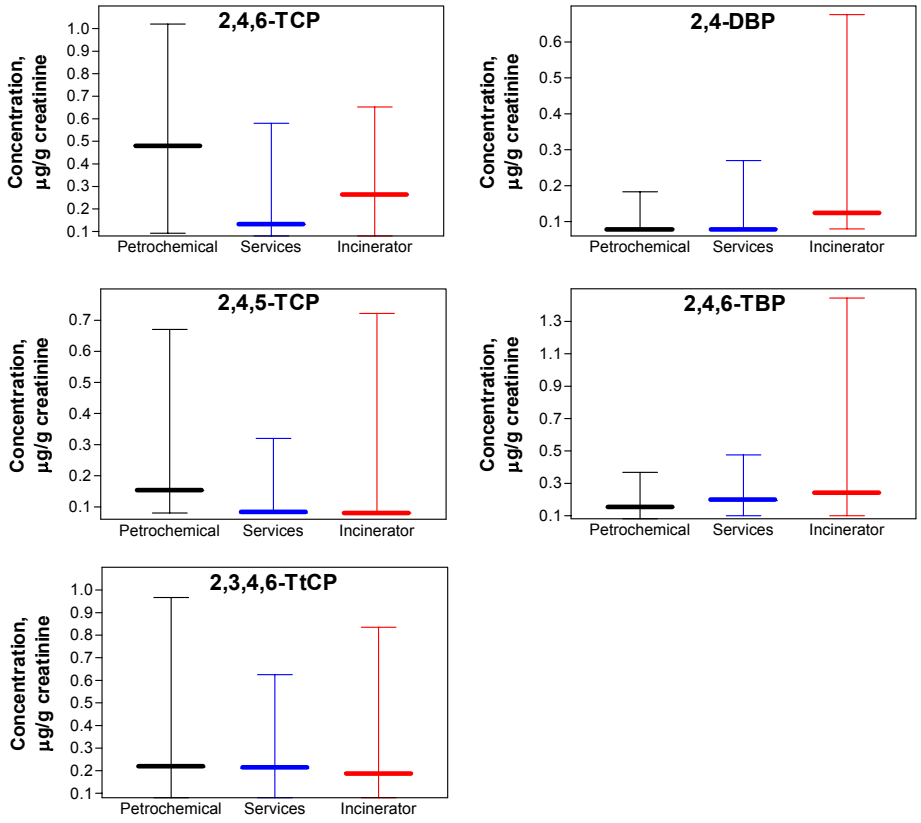


Figure 11.9. Descripción de las concentraciones cloro- y bromo- fenoles en la orina de trabajadores de la petroquímica, de oficinas y de vecinos de una incineradora. Las marcas superiores corresposnden al 95% de la distribución, las del medio al valor medio y las inferiores al 5%. Las concentraciones están determinadas por HTS-IAC-GC/MS y expresadas en µg/g creatinina. Los límites de detección son 0.08 µg/g creatinina para TCPs y 0.16 µg/g creatinina para DBP, TtCP y TBP.

11.6. Desarrollo de un inmunoensayo de quenching con fluorescencia inducida por láser con detección en microgotas (LIF-QFIA en microgotas)

La fluorescencia inducida por láser en microgotas (volumen alrededor de pL) permite la detección de compuestos fluorescentes a nivel molecular [382,383]. Una de las ventajas más importantes relacionadas con la detección en microgotas es la reducción del ruido de fondo que conduce a límites de detección mucho más bajos que las técnicas de análisis en muestras de volumen grande. Con estos antecedentes se ha propuesto demostrar que es posible realizar un fluoroinmunoensayo de *quenching* con detección de la fluorescencia inducida por láser en microgotas. Este trabajo se ha realizado en colaboración con los grupos de Dr. I. Kennedy y Dr. B. Hammock de University of California - Davis.

En primer lugar se ha sintetizado un conjugado entre el marcador fluorescente fluoresceinamina y el hapteno 5 (TCP-F). El producto fue purificado por capa fina y caracterizado por RMN. Las propiedades ópticas del conjugado fluorescente han sido caracterizados inicialmente en un espectrofotómetro estándar, donde se determinaron la longitud de onda de máxima excitación (490 nm) y emisión (520 nm).

La detección de la fluorescencia inducida por laser (Ar, 488 nm) en microgotas se realizó mediante un dispositivo formado por un generador de microgotas, un láser y un espectrómetro acoplado a una CCD cámara . Las microgotas generadas tenían un diámetro de 58 μm (volumen 104 pL). Se ha construido una recta estándar de la intensidad de la fluorescencia en función de la concentración del trazador TCP-F. Se ha estimado un límite de detección absoluto correspondiente a $5x10^{-10}$M (0.5 nM o 5.2 x 10^{-20} mol TCP-F) por microgota.

Se ha estudiado la cinética del *quenching* de la fluorescencia del trazador TCP-F debido a su unión con el anticuerpo específico (As43) para varias concentraciones de anticuerpo. Se ha determinado también que el tiempo de equilibrio de la reacción inmune es 45 min. Mediante una titulación del As43, se ha determinado la concentración de 2.5 μg/mL

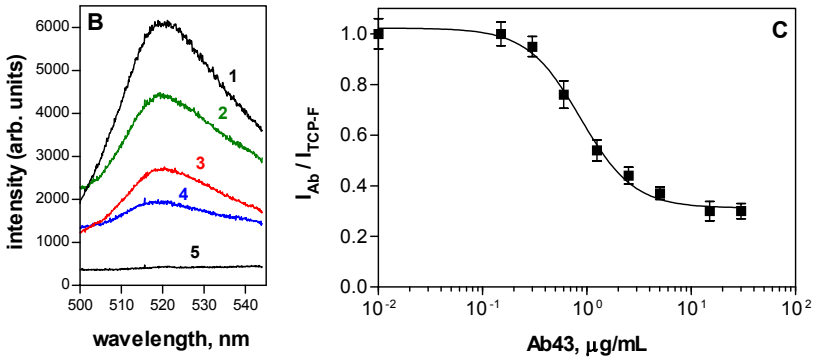


Figura 11.10. B. Espectro de emisión de la fluorescencia del trazador TCP-F (10 nM) (1) y su *quenching* por varias concentraciones de Ab43 en 10 mM PBS: 0.6 µg/mL (2), 2.5 µg/mL (3), 15 µg/mL (4); 15 µg/mL Ab43 en ausencia de TCP-F (5). **C**. Curva de titulación del trazador TCP-F y Ab43. Los reactantes han sido incubados por 45 min. La reducción en la intensidad de la fluorescencia está presentada como la relación entre la fluorescencia de TCP-F en presencia de Ab43 (I_{Ab}) y la fluorescencia de TCP-F sin Ab (I_{TCP-F}).

necesaria para observar unos 80% de *quenching* de la señal máxima de fluorescencia del trazador (véase **Figura 11.10**). Bajo las condiciones seleccionadas se ha realizado la detección de 2,4,6-TCP en las microgotas mediante un fluoroinmunoensayo homogeneo competitivo: a concentraciones bajas de analito, el competidor TCP-F está unido al anticuerpo y por lo tanto se observa *quenching* de su fluorescencia; una concentración más alta de 2,4,6-TCP provoca el aumento de la intensidad de la fluorescencia debido a la presencia de competidor libre (véase **Figura 11.11**).

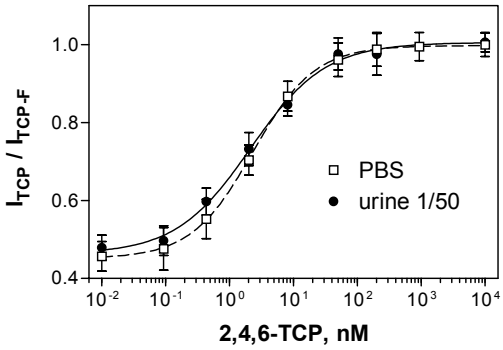


Figura 11.11. Curva de calibarción del ensayo LIF-QFIA para 2,4,6-TCP en microgotas de tampón PBS y orina diluida 50 veces en tampón.

Los parámetros de la curva de calibración del fluroinmunoensayo en microgotas de tampón PBS están comparados con el correspondiente ensayo realizado en placa de 96 pocillos y también con el ensayo ELISA basado en los mismos inmunoreactivos (As43, hapetno 5 como competidor) (veáse **Tabla 11.11**).

Tabla 11.11. Parámetros de los dos inmunoensayos (ELISA y LIF-QFIA) para 2,4,6-TCP.

	ELISA [a]	**QFIA**	
		microplaca	**LIF-microgotas**
IC_{50} (μg/L)	2.74	4.2	0.45
LOD (μg/L)	0.2 (1 nM)	0.36 (1.8 nM)	0.04 (0.2 nM)
LOD (ng)	4 x 10^{-2} ($2x10^{-13}$ mol)	$7.2x10^{-2}$ ($3,7x10^{-13}$ mol)	$1.68x10^{-4}$ ($9,7x10^{-16}$ mol)
Trazador	5-HRP	5-Fluoresceina	5-Fluoresceina
Procedimiento	lavados e incubaciones	ensayo heterogéneo	ensayo homogéneo
Excitación	-	485 nm (20nm band)	láser, 488 nm
Volumen, μL	200	200	4.2

[a] – los datos son de [259]

La significante mejora en la detectabilidad del QFIA en microgotas se debe sobre todo a una relación señal-ruido mayor, ya que el fluoroinmunoensayo en microplaca sufre mayor ruido de la señal fluorescente. También se debería considerar la posible adsorción inespecífica de algunos inmunoreactantes o analitos no polares a los pocillos. Los pequeños volumenes de las microgotas minimizan las interferencias en la señal de la fluorescencia debido a la dispersión de *Raman y Rayleigh*. Además, el uso del láser como fuente de excitación y la cámara CCD para la detección de la señal emitida permiten alcanzar niveles de detección tan bajos.

El fluoroinmunoensayo fue realizado también en microgotas de muestras de orina. El efecto matriz debido al ruido de fondo procedente de la fluorescencia de los componentes de la orina y debido a la inhibición de la interacción Ag-Ab ha sido eliminado mediante una dilución de 50 veces, lo que determina un LOD del método analítico de 1.6 μg/L 2,4,6-TCP en orina. Este nivel de detectabilidad permite la utilización de este método en estudios de la evaluación de la exposición laboral a productos halogenados.

La técnica de LIF-QFIA-microgotas podría ser la base para el desarrollo de nuevos sensores ópticos miniaturizados. Este trabajo es la primera aplicación de un ensayo QFIA con detección en microgotas a muestras de orina demostrando el potencial de esta técnicas para el desarrollo de nuevos sistemas de detección bioanalíticos.

11.7. Producción de fragmentos de anticuerpo anti-digoxina (scFv) por el método de *phage display* como elementos de bioreconocimiento en inmunosensores

La exposición de fragmentos de anticuerpos en la superficie de bacteriófagos filamentosos y la posterior selección del fago con el antígeno deseado ha proporcionado una manera eficaz de producir fragmentos de anticuerpos de bibliotecas genómicas y también ha impulsado el diseño de novedosos fragmentos recombinantes [160,161]. Estas técnicas permiten crear fragmentos de Abs con propiedades que aseguren su inmovilización ordenada sobre la superficie de los inmunosnesores. La fusión genética de marcajes (*tag*) con fragmentos de cadena simple (scFv) de anticuerpos ha sido expresada con un buen rendimiento a través de la secreción de bacterias [425]. En este contexto se ha propuesto producir una proteína de fusión entre el *tag* calmodulina y un fragmento scFv de anticuerpo anti-digoxina con el objetivo de ser aplicada como elemento de bioreconocimineto de un inmunosensor de configuración modular. La naturaleza ácida de la calmodulina (carga neta –24 a pH=8) aseguraría la inmovilización orientada del fragmento scFv por fuerzas electrostáticas sobre la superficie del inmunosensor. Se ha elegido como analito la digoxina dado su elevado interés clínico y farmacológico. La digoxina es un fármaco cardíaco cuyo estrecho rango terapéutico ($5x10^{-4}$ – $2x10^{-3}$ mg/L) exige su monitorización exacta y rápida en suero con el fin de establecer una posología adecuada. Este trabajo d eha realizado en el grupo de Dr. G. Winter de Medical Research Council, Centre for Protein Engineering (Cambridge, UK) y en colaboración con el grupo de Dra. E.Domínguez de la Facultad de Farmacia, Universidad de Alcalá de Henares y Dr. I. Katakis de Universidad Rovira y Virgili.

Para la selección de scFv con especificidad anti-digoxina se han usado dos bibliotecas "sintéticas" de formato *phagemid/scFv* (~10^8 clones) basadas en *single human framework* para los dominios V_H y V_k con diversidad de las cadenas laterales DVT y NNK en ciertas posiciones de las regiones determinantes de complementaridad (CDR2, CDR3). Las bibliotecas fueron objeto de tres ciclos de selección en inmunotubos derivatizados con digoxina-BSA. Cada ciclo de selección consiste en la unión del fago al antígeno inmovilizado, la elución del fago unido y la propagación del fago seleccionado para un siguiente ciclo [431]. Se han inducido fragmentos scFv solubles de colonias aisladas de bacterias infectadas por los fagos seleccionados. Los sobrenadantes bacterianos que contienen scFv monoclonales se han ensayado para su especificidad de unión al antígeno por ELISA usando placas tapizadas de digoxina-BSA y digoxina-HRP y por resonancia de plasmones de superficie (*Surface Plasmon Resonance,* SPR). Unión positiva se ha observado sólo para los fragmentos scFv producidos de fagos seleccionados de las bibliotecas con diversidad DVT. Los V-genes de los clones positivos se han amplificado mediante la reacción en cadena de la polimerasa (PCR) y su secuencia de nucleótidos se ha determinado por el método del terminador de la cadena (Sanger). El análisis secuencial ha mostrado que todos los clones positivos tienen la misma secuencia de DNA para los dominios V_k y V_H.

Para añadir el gen de calmodulina se ha clonado el gen que codifica el fragmento scFv anti-digoxina en el vector pDN152 [425]. La fusión se ha verificado a través de *PCR screening*. La proteína de fusión (CAL-scFv) se ha expresado mediante la secreción de la bacteria y su especificidad de unión a la digoxina se ha comprobado por ELISA. La fusión también se ha confirmado con electroforesis en gel de poliacrilamida (la fusión CAL-scFv (45 kD); el fragmento scFv (28 kD). La proteína CAL-scFv se ha purificado del caldo de cultivo mediante cromatografía DEAE de intercambio iónico. Para conseguir una purificación mejor la proteína eluída fue purificada adicionalmente con cromatografía de proteína A *Sepharose*.

Con la proteína obtenida CAL-scFv se ha desarrollado un ELISA competitivo para la detección de digoxina (veáse **Tabla 11.12**). El rango lineal del ensayo cubre el rango

terapéutico de la digoxina ($5 \times 10^{-4} - 2 \times 10^{-3}$ mg/L) lo que permitiría su aplicación para un control toxicológico.

Tabla 11.12. Parámetros del ELISA (scFv-CAL/digoxina-HRP) para la detección de digoxina

Condiciones	Valores	Parámetro	Valores
Tiempo de competencia	2 h	**A_{max}**	0.75
pH	7.2	**A_{min}**	0.03
Conductividad	15 mS/cm	**IC_{50}, mg/L**	0.016
Tween 20	0.05%	**Rango lineal, mg/L**	$1{,}2.10^{-4} - 0.8$
		pendiente	0.6
		r^2	0.996

Finalmente, se ha estudiado la incorporación de los fragmentos producidos en una configuración de multicapas [424]. La deposición *layer-by-layer* de un polímero redox y del fragmento CAL-scFv ordenadamente adsorbidos sobre chips de oro ha sido monitorizada por SPR. Se ha detectado la formación de monocapas estables de cada uno de los componentes de la configuración modular propuesta (Au derivatizado con carga negativa/polímero redox con carga positiva/scFv-CAL/digoxina-HRP). Estos resultados preliminares demostraron la viabilidad de la configuración propuesta para la inmovilización ordenada de fragmentos de anticuerpo que presentan la mínima unidad necesaria para el reconocimiento analítico. La optimización de la deposición de las monocapas y la construcción de un inmunosensor electroquímico serían los próximos objetivos a conseguir.

11. 8. Conclusiones

1. Durante el trabajo de investigación de la presente tesis doctoral se ha desarrollado un inmunoensayo enzimático ELISA para la detección de 2,4,5-TCP. Basándose a estudios semiempíricos de modelización molecular se han elegido dos haptenos como inmunogenos para la producción de anticuerpos específicos contra 2,4,5-TCP. Los resultados experimentales en los inmunoensayos confirmaron los estudios teóricos.

2. En formato no competitivo (directo e indirecto) de los inmunoensayos estudiados se ha observado un mejor reconocimiento para las combinaciones homólogas y quasihomólogas. El mejor inmunoensayo ha sido As53/7-OVA(1:2.5) con densidad de hapteno igual a 10 mol hapteno/mol de proteína. Su LOD es de 0.053 μg/L y su IC_{50} es de 0.231 μg/L.. Se ha puesto en evidencia que la detectabilidad está afectada por varios factores, tales como la estructura de la molécula (geometría y carga), el método de conjugación empleado en la preparación de los antígenos de tapizado, la densidad de haptenos en los antígenos de tapizado, el tipo de proteína empleada. Se ha demostrado que los mejores ensayos competitivos se han conseguido con los competidores con mayor grado de homología respecto al analito. Se ha demostrado también que la detectabilidad de los ensayos mejora con la disminución del grado de conjugación (densidad de hapteno) alcanzando mejores valores en el caso de 3 ó 5 moléculas de hapteno por molécula de proteína.

3. El inmunoensayo As53/7-OVA es estable entre pH de 6.6. y 10.5. La detectabilidad del inmunoensayo mejora con el aumento de la conductividad del medio. Se han establecido como condiciones óptimas las siguientes condiciones: tiempo de competencia 30 min; pH=7.5; salinidad 20 mS/cm; concentración del Tween 20 de 0.025%. El inmunoensayo es muy específico y su selectividad es relacionada directamente con la presencia del grupo fenólico, dos átomos de cloro (en posiciones *orhto* y *para* del anillo aromático) y un átomo de hidrógeno en posición *meta*. Los compuestos con mayor reactividad cruzada son 2,4,6-TCP, 2,3,4,6-TtCP 2,4-DBP y 2,4,6-TBP.

4. Como muestras ambientales se ha estudiado agua potable. Es necesario tamponar la muestra antes de analizarla por ELISA. Se han conseguido LOD de 2,4,5-TCP de 0.07 μg/L y para 2,4,6-TCP de 0.18 μg/L lo que permite la evaluación d ela contaminación de agua de bebida según las exigencias de la UE. El análisis de TCP en muestras de agua tiene una precisión y exactitud muy buena.

5. La determinación de 2,4,5- y 2,4,6-TCP en muestras de leche por los ELISAs respectivos sin ningún tratamiento de la muestra no es posible debido a una fuerte

inhibición. Precipitación de las proteínas y los iones de calcio presentes en la leche no ha mejorado el funcionamiento de los ensayos. Para el análisis de clorofenoles en leche sería necesario desarrollar un método de previa purificación de la muestra para eliminar el efecto matriz observado.

6. La evaluación de los dos inmunoensayos en suero humano ha demostrado que el mejor tratamiento de la muestra es la precipitación de las proteínas del suero con etanol absoluto. Una simple centrifugación para eliminar las proteína precipitadas y la posterior dilución del sobrenadante con PBS son el único tratamiento necesario antes del análisis de los clorofenoles por ELISA. Los LOD conseguidos son 0.8 μg/L 2,4,5-TCP y 4.6 μg/L 2,4,6-TCP. Los inmunoensayos podrían ser aplicados en estudios toxicológicos y control biológico de la población general.

7. Los dos inmunoensayos se han evaluado en muestras de orina caracterizadas por su presencia de cloro- y bromofenoles que podrían presentar una interferencia específica. Se ha observado una inhibición de la señal máxima en las muestras de orina no hidrolizada e hidrolizada que se puede eliminar con una dilución apropiada de la muestra. Este procedimiento permite analizar con buena exactitud muestras de orina no hidrolizada con LOD de 0.26 μg/L 2,4,5-TCP y 17.5 μg/L 2,4,6-TCP. Por lo tanto, el inmunoensayo para 2,4,5-TCP podría ser aplicado en la monitorización biológica de la población general, pero el de 2,4,6-TCP se podría usar sólo en el caso de la evaluación de población con alto riesgo de exposición . El efecto matriz en muestras de orina hidrolizada es mayor y es debido a interferencias específicas y no específicas. Se ha observado que muestras de diferentes individuos han ejercido un efecto matriz diferente sobre los dos inmunoensayos. Debido a la limitada detectabilidad de los inmunoensayos y la variabilidad del efecto matriz para los diferentes individuos ha sido necesario desarrollar un método de purificación de la orina para poder analizarla por ELISA.

8. La introducción de una extracción en fase sólida por C_{18} previa al análisis de 2,4,6-TCP en muestras de orina ha eliminado una gran parte de las interferencias inespecíficas. Una dilución de 25 veces es necesaria para la correcta determinación de 2,4,6-TCP. Esto ha

resultado en una mejora del LOD del método que en este caso sería alrededor de 4 μg/L 2,4,6-TCP.

9. El observado efecto matriz de la orina fue eliminado combinando ELISA con una previa purificación mediante extracción de inmunoafinidad (IAC). El procedimiento de IAC-ELISA fue evaluado y optimizado respecto a varios parámetros como tipo de la hidrólisis, volumen de la muestra, condiciones de elución, etc. Las columnas son estables hasta 55 ciclos. 2,4,6-TCP en orina no hidrolizada se puede determinar con un LOD de 0.66 μg/L y un MCD de 50 μg/L. Orina hidrolizada (ácido) se puede cuantificar correctamente en el rango 0.83 – 5 μg/L y orina hidrolizada por KOH – en el rango 1-20 μg/L. El clean-up de iAC puede ser usado también con una técnica de análisis GC-MS (GC-ECD). La técnica inmunoquímica desarrollada se podrá aplicar en la monitorización de bajo coste de la exposición ocupacional a clorofenoles y otros contaminantes halogenados.

10. Se ha desarrollado un IAC en formato de 96 columnas (HTS-IAC-ELISA). Las ventajas principales del método son el aumento de la velocidad del análisis (100 muestras/día) y la mejor detectabilidad (LOD 0.3 μg/L 2,4,6-TCP; LOQ es de 0.55 μg/L). No se observan falsos positivos y negativos.

11. El método HTS-IAC-ELISA ha sido validado por GC-MS y aplicado en un estudio previo de monitorización de tres grupos de poblaciones. Los niveles de triclorofenoles determinados en la población de Ctaluña no exceden los niveles establecidos como valores de referencia en otros países (Alemania, USA).

12. Se ha desarrollado un novedoso sistema de inmunodetección basado en la fluorescencia inducida por láser en microgotas. El método tiene una detectabilidad mejor que el fluoroinmunoensayo realizado en micropaca y del ELISA correspondiente. Una dilución d ela orina permite el análisis de muestras de orina con LOD de 1.6 μg/L. El método desarrollado es la base para el desarrollo de un biosensores ópticos miniaturizados.

13. Se ha realizado el diseño dirigido de una proteína de fusión entre la calmodulina y un fragmento scFv anti-digoxina, seleccionado de bibliotecas sintéticas de V genes humanos. La nueva proteína producida podría aplicarse como elemento de bioreconocimiento en un novedoso inmunosensor de digoxina permitiendo la inmovilización ordenada del fragmento del anticuerpo. Con relación a eso será necesario un estudio de las propiedades de la proteína CAL-scFv como afinidad, especificidad, estabilidad, etc.

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List of publications originated from this thesis

[1] R. Galve, **M. Nichkova**, F. Camps, F. Sánchez-Baeza, M.-P. Marco Development and evaluation of an immunoassay for biological monitoring of chlorophenols in urine as potential indicators of occupational exposure, *Anal. Chem.* 74 (2002) 468-478.

[2] **M. Nichkova**, R. Galve, M.P. Marco, Biological monitoring of 2,4,5-trichlorophenol (I): Preparation of antibodies and development of an immunoassay using theoretical models, *Chemical Research in Toxicology* 15 (2002) 1360-1370.

[3] **M. Nichkova**, R. Galve, M.P. Marco, Biological monitoring of 2,4,5-trichlorophenol (II): Evaluation of an enzyme-linked immunosorbent assay for the analysis of water, urine, and serum samples, *Chemical Research in Toxicology* 15 (2002) 1371-1379.

[4] **M. Nichkova**, J. Feng, F. Sánchez-Baeza, M.P. Marco, B.D. Hammock, I.M. Kennedy, Competitive quenching fluorescence immunoassay for chlorophenols based on laser-induced fluorescence detection in microdroplets, *Anal. Chem.* 75 (2003) 83-90.

[5] **M. Nichkova**, M.-P. Marco, Immunochemical Analysis of Trichlorophenols in Urine Samples after Solid-Phase Extraction. *Anal. Chim. Acta*, submitted

[6] **M. Nichkova**, M.-P. Marco, Development and Evaluation of an IAC-ELISA procedure for Analysis of Chlorophenols in Human Urine. *J. Chromatogr. A*, submitted

[7] **M. Nichkova**, M.-P. Marco, Development of HTS-IAC-ELISA for urinary detection of chlorophenols, in preparation