# Development of a Bioelectronic Tongue -Applications for Wastewater Analysis

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2006

*Till Hjalmar*

”Forskning är att försöka förstå vad man inte vet mer vet jag inte, men jag forskar på det...”

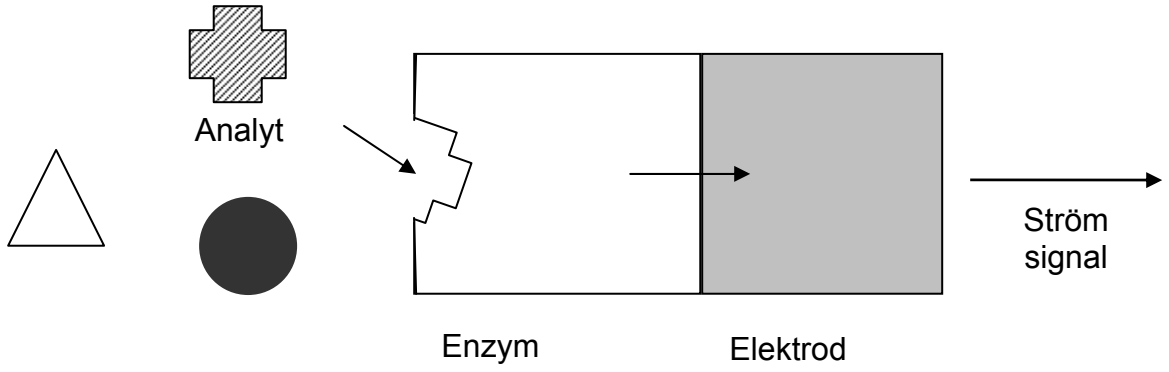
RA

# POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Kemisk analys handlar om att identifiera ämnen i olika typer av prov. Detta kan göras kvantitativt, där man bestämmer hur mycket man har av ett eller flera ämnen, eller kvalitativt, där man tar reda på vilket eller vilka ämnen som finns i provet. En analytisk kemist arbetar med att utveckla och förbättra olika analysmetoder. Biosensorer och elektroniska tungor är analytiska verktyg baserade på funktioner som finns i biologiska system, och är den typ av system som använts i arbetet med denna avhandling.

## Biosensorer

En biosensor är ett mätverktyg där ett biologiskt igenkänningselement (t.ex. enzym, cell, vävnad eller receptorer) är kopplat till en signalöverförare (i vårt fall en elektrod) som omvandlar den biologiska signalen från det ämne som mäts i ett prov (analyten) till en signal man kan mäta i form av en ström (amperometri) eller en spänning (potentiometri). Figur 1 visar principen för hur den biosensor vi arbetat med fungerar.



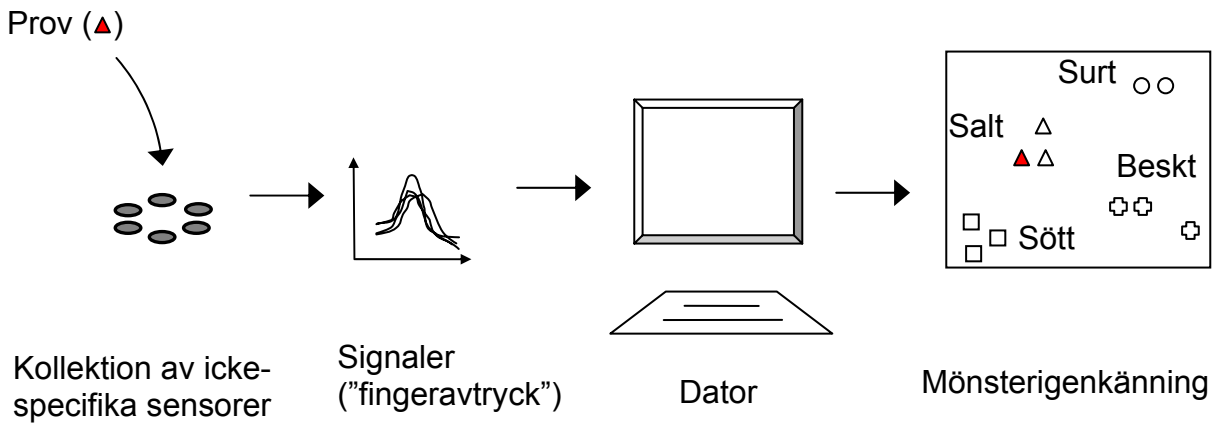

*Figur 1. Mätprincip för en amperometrisk enzymmodifierad elektrod.*

En katalysator är ett ämne som påskyndar en kemisk reaktion utan att själv förbrukas. Till exempel har de flesta bilar idag en katalysator som omvandlar giftiga ämnen till mindre giftiga ämnen innan de släpps ut som avgaser. Den biologiska varianten av katalysator kallas enzym. Förutom att de

påskyndar reaktioner som aldrig annars skulle kunna ske så är enzym specifika. Det är endast de ämnen som passar in i enzymets ”ficka” (som en nyckel passar i ett lås (se Figur 1)), som omvandlas. De flesta enzymbaserade biosensorer är tillverkade för att mäta ett enda ämne och många kommersiella biosensorer finns för mätning av t.ex. sockerhalten i blodet. De enzymelektroder som använts i denna avhandling mäter snarare grupper av likartade ämnen och kan delas upp i två olika typer: oxidoreduktaser och hydrolaser. Båda används i miljöanalys för mätning av olika miljöfarliga ämnen, där de förstnämnda är känsliga för fenoliska ämnen och de sistnämnda för pesticider (växtbekämpningsmedel). När man mäter i starkt förorenade avloppsvatten kan det finnas andra ämnen än de man vill mäta som ger upphov till falska signaler. Även andra fenomen kan tänkas ske såsom ämnen som sätter sig på enzymet och blockerar möjligheten för analyten att sätta sig i enzymfickan. I sådana fall försöker man skydda biosensorn genom att sätta på ett membran eller någon form av polymer som gör att icke önskvärda ämnen inte kommer i kontakt med enzymet. En annan strategi är att ta till vara alla de reaktioner som sker i provet och betrakta signalerna från en eller flera biosensorer som ett fingeravtryck av det aktuella provet. Ett mätinstrument som då kan användas är en elektronisk tunga.

## Elektroniska tungor

När man känner igen en smak så är det celler som sitter i smaklökarna på tungan som får en signal som överförs via nervceller till hjärnan. I hjärnan bearbetas signalen och man känner igen smaken som sur, söt, salt eller besk. Det talas ibland även om en femte smak, umami, som kan karaktäriseras som behaglig. En elektronisk tunga försöker imitera den biologiska och kan kortfattat beskrivas enligt Figur 2. Den elektroniska tungans igenkänningssystem utgörs av ett antal icke specifika elektrodtyper där flera ämnen kan registreras av samma elektrod. Vidare vill man gärna att samma ämne ger upphov till signal på flera olika elektroder på tungan. Detta för att få fram ett mönster, ett fingeravtryck, som speglar ett så brett spektrum av ämnen som möjligt. De uppmätta signalerna förs vidare över till den konstgjorda hjärnan, en dator som har ett program för mönsterigenkänning. Många av dessa program arbetar enligt principen att ta fram dold information som sorteras ut ifrån ett uppmätt komplext signalmönster. Den dolda informationen kan vara kvalitativ, t.ex. gruppering av ämnen utifrån hur de smakar, eller kvantitativ, bestämning av hur mycket man har av ett visst ämne i sitt prov.




*Figur 2. Principen för en elektronisk tunga. Exempel på igenkänning av smaker där ett salt prov karaktäriseras utifrån det ”fingeravtryck” som registrerats av sensorerna.*

## En bioelektronisk tunga för analys av avloppsvatten

I mitt arbete har en bioelektronisk tunga konstruerats baserat på enzym som är känsliga för fenoler och pesticider. Syftet har varit att använda denna inom miljöanalys och då främst för mätningar i avloppsvatten. Det finns många bra exempel där elektroniska tungor har använts för detta ändamål. Dock är det svårt att med dessa mäta vilka effekter ett visst avloppsvatten har på omgivningen och människan dvs. hur toxiskt (giftigt) vattnet är.

Toxicitet kan bestämmas på många olika sätt och utslaget på dessa tester kan variera. Gemensamt är dock att testerna utförs på någon form av biologisk organism och att analyserna tar relativt lång tid att utföra. Om biosensorer kopplas till en elektronisk tunga skulle därför även avloppsvattnets toxiska effekter kunna registreras. Vi har med vår biologiska tunga i experiment kunnat karaktärisera avloppsvatten från en pappersmassaindustri som renats i olika steg. Dessutom har vi kunnat visa att det finns starka samband mellan det signalmönster ett prov ger upphov till från en kollektion av biosensorer och toxicitet (samt även andra icke-biologiska parametrar). Med denna bioelektroniska tunga har man alltså ett mycket snabbare och billigare mätinstrument som kan utvärdera innehållet i avloppsvatten. Det är också möjligt att detta instrument skulle kunna användas för att effektivt sortera ut avvikande avloppsvatten som kan skickas vidare till mer utförlig analys med mer konventionella inarbetade analysmetoder.

## Comments on my own participation

**Papers I, III, IV and manuscript VII:** I carried out most of the experimental work and data processing. I was responsible for the writing of the manuscripts.

**Paper II:** I planned and supervised the experimental work. I processed some of the data and wrote the manuscript.

**Paper V and VI:** Minor contribution in planning of the experiments, interpretation of data and in the manuscript writing process.

**The following related papers have been omitted from the thesis because of the nature of the material (A) and the extent of my contribution (B, C):**

A. **Direct Heterogeneous Electron Transfer of Theophylline Oxidase.**
A. Christenson, E. Dock, L. Gorton and T. Ruzgas
*Biosens. Bioelectron.*, 20 (2004) 176

B. **Amperometric Sensors Based on Tyrosinase-Modified Screen-Printed Arrays**
S. Sapelnikova, E. Dock, T. Ruzgas and J. Emnéus
*Talanta*, 61 (2003) 473

C. **Screen-Printed Multienzyme Arrays for Use in Amperometric Batch and Flow Systems**
S. Sapelnikova, E. Dock, R. Solná, P. Skládal, T. Ruzgas and J. Emnéus
*Anal. Bioanal. Chem.*, 376 (2003) 1098

# Development of a Bioelectronic Tongue

## Applications for Wastewater Analysis

This thesis is based on the following papers, referred to in the text by their Roman numerals

I. **Effect of Interfering Substances on Current Response of Recombinant Peroxidase and Glucose Oxidase–Recombinant Peroxidase Modified Graphite Electrodes.**
E. Dock, A. Lindgren, T. Ruzgas and L. Gorton
*Analyst*, 126 (2001) 1929

II. **Multivariate Data Analysis of Dynamic Amperometric Biosensor Responses from Binary Analyte Mixtures – Application of Sensitivity Correction Algorithms.**
E. Dock, J. Christensen, M. Olsson, E. Tønning, T. Ruzgas and J. Emnéus
*Talanta*, 65 (2005) 298

III. **Screen-printed Carbon Electrodes Modified with Cellobiose Dehydrogenase: Amplification Factor for Catechol vs. Reversibility of Ferricyanide.**
E. Dock and T. Ruzgas
*Electroanalysis*, 15 (2003) 492

IV. **A Steady-State and Flow-Through Cell for Screen-Printed Eight-Electrode Arrays.**
E. Dock, A. Christenson, S. Sapelnikova, J. Krejci, J. Emnéus and T. Ruzgas
*Anal. Chim. Acta*, 531 (2005) 165

V. **Amperometric Screen-Printed Biosensor Arrays with Co-Immobilised Oxidoreductases and Cholinesterases.**
R. Solná, E. Dock, A. Christenson, M. Winther-Nielsen, C. Carlsson, J. Emnéus, T. Ruzgas and P. Skládal
*Anal. Chim. Acta*, 528 (2005) 9

VI. **Chemometric Exploration of an Amperometric Biosensor Array for Fast Determination of Wastewater Quality.**
E. Tønning, S. Sapelnikova, J. Christensen, C. Carlsson, M. Winther-Nielsen, E. Dock, R. Solná, P. Skládal, L. Nørgaard, T. Ruzgas and J. Emnéus
*Biosens. Bioelectron.*, 21 (2005) 608

VII. **An Amperometric Bioelectronic Tongue for Simultaneous Determination of Toxicity, COD and BOD in Wastewater.**
E. Dock, E. Tønning, J. Christensen, M. Winther-Nielsen, C. Carlsson, R. Solná, P. Skládal, L. Nørgaard, J. Emnéus and T. Ruzgas
*Manuscript*

# Abbrevations

| | |
|---|---|
| GC | gas chromatography |
| MS | mass spectrometry |
| HPLC | high performance liquid chromatography |
| TYR | tyrosinase |
| AChE | acetylcholinesterase |
| BChE | butyrylcholinesterase |
| CDH | cellobiose dehydrogenase |
| POD | peroxidase |
| HRP | horseradish peroxidase |
| SBP | soybean peroxidase |
| GOx | glucose oxidase |
| COD | chemical oxygen demand |
| BOD | biological oxygen demand |
| TOC | total organic carbon |
| SPE | screen-printed electrode |
| WWTP | wastewater treatment plant |
| CV | cyclic voltammetry |
| RDE | rotating disk electrode |
| FIA | flow injection analysis |
| PCA | principal component analysis |
| PC | principal component |
| MLR | multiple linear regression |
| PLS-R | partial least squares regression |
| ANN | artificial neural networks |
| RMSEP | root mean square error of prediction |

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# 1 INTRODUCTION

This thesis describes the analytical concepts of electronic tongues and biosensors, and how these techniques can be combined into a bioelectronic tongue for application in wastewater analysis. The introduction starts with a short background about wastewater monitoring in an attempt to clarify the potential advantages of using bioelectronic tongues, and ends with a summary of the work that is the basis for this thesis.

## 1.1 Background – Wastewater Analysis

Monitoring of complex wastewater with a composition that can vary from time to time put rather specific demands on the analytical methods employed. Classical analytical techniques, e.g. solid-phase extraction followed by GC or HPLC and their combination with MS [1, 2], generally provide the most reliable quantitative information about wastewater components. Unfortunately, the majority of these methods are time-consuming and expensive, and thus, the number of samples that can be analyzed is limited.

To measure and compare the quality of wastewater effluents, global organic pollution parameters have traditionally been used [3], e.g. biological oxygen demand (BOD), chemical oxygen demand (COD) and total organic carbon (TOC). However, some of the standard procedures to determine these global parameters are also time-consuming (BOD generally takes five days to determine) or are unable to differentiate between biodegradable and non-biodegradable material (COD and TOC).

An increasing number of low-cost and rapid analytical screening techniques have been developed in recent years that can give preliminary information about the quality of wastewater. Such techniques are helpful for identifying positive samples in a large sample population for more thorough subsequent characterization by classical analysis methods. In this context the application of different sensor systems [4] gives promising alternatives, since they can be used *in situ*, i.e. directly inside the process line [5], which makes it possible to continuously monitor the effluent from wastewater treatment plants (WWTP). The use of several sensors coupled together in an array increases the analyte window. Very popular are electronic noses (gas sensors) [6] and electronic tongues (liquid

sensors) [7], which are array systems comprising a set of non-specific sensors. Their responses are processed by appropriate software for pattern recognition. These systems have been used for qualitative discrimination of wastewater samples to monitor changes in wastewater composition as well as for quantitative analysis where the sensor array signals have been correlated to for example COD and BOD.

The global organic pollution parameters, however, do not give much information about the toxicity of a sample. To obtain information about toxic effects, a number of bioassays have been developed in which test organisms are exposed to various doses of pollutants [8-10]. In this thesis results from two types of bioassays have been used as reference values: the Microtox® test based on luminiscence inhibition of the freeze-dried marine bacteria *Vibrio Fischeri* strain NRRL B-11177 according to ISO 11348-3 (1998), and the growth inhibition test with the freshwater alga *Pseudokirchneriella subcapita* according to ISO8692 (1989). To obtain a clear understanding of the sample toxicity a battery of different assays is usually needed. Many of the standard toxicity assays take a long time to perform with several dilution steps included in the analysis procedure. An alternative to these bioassays could be biosensors [11, 12]. A biosensor consists of a biorecognition element coupled to a transducer that converts the biological signal to a physical one, e.g. a current. The advantages of biosensors include for example that they can be mass-produced at low cost and that they can be used as fast on-line screening tools. Therefore, in this thesis a bioelectronic tongue was assembled from an array of biosensors, resulting in a multi-sensing device for fast and simultaneous determination of several wastewater characteristics including COD, BOD and toxicity.

## 1.2 Summary of the Work

The main part of this doctoral thesis is based on work that was carried out in a project financed by the European Commission, *Intelligent Signal Processing of Biosensor Arrays using Pattern Recognition for Characterization of Wastewater: Aiming towards Alarm Systems* (INTELLISENS: QLK3-2000-01481).

The aim of my work was to develop a measurement system (steady-state or flow-injection analysis) using screen-printed eight-electrode arrays modified with different enzymes for amperometric analysis of wastewater. The obtained array responses were further treated with multivariate methods

to (i) classify effluents from WWTPs according to the quality indicators "normal", "alert", "alarm" or "untreated", and (ii) in parallel to determine the correlation against reference parameters such as toxicity, COD and BOD.

The contents of this thesis can be summarised according to the following: Chapter 2 concerns amperometric biosensors, how they are constructed and how they have been used in environmental applications; Chapter 3 handles the electrochemical methods used to characterise screen-printed electrodes (chronoamperometry and cyclic voltammetry) and amperometric measurements using steady-state or flow-injection analysis; Chapter 4 gives an overview of different types of array systems used mainly under hydrodynamic conditions and a more detailed explanation of the principles of the electronic tongue; Chapter 5 handles chemometric or multivariate methods used for sensor systems as well as pre-processing methods to overcome unavoidable drift problems; Chapter 6 gives an overview of reported amperometric/voltammetric bioelectronic tongues and concludes with results from our own biosensor systems; and Chapter 7 concludes the work and gives some future perspectives in this field. Finally follows a compilation of six already published papers and one manuscript, all briefly summarised below:

Paper I represents a background work to the subject treated in this thesis. Native and recombinant forms of horseradish peroxidase, HRP, were immobilised on a graphite electrode. The aim was to develop a fully selective amperometric biosensor for hydrogen peroxide. Thus, different well-known interfering substances that can be found in blood were tested with the biosensors; however none of the biosensors could be classified as interference-free. Many of the phenolic substances present in blood (e.g. dopamine and acetaminophen) are HRP substrates and gave a clear response. However, one important observation was that clear variations in peak shape of the responses could be seen when comparing different substances. This pointed to the more sophisticated idea that it might be possible to differentiate between analyte mixtures using multivariate data analysis based on differences in peak shape.

In work reported in Paper II a tyrosinase modified graphite electrode was used to determine catechol and 4-chlorophenol in a mixture using a flow-injection system. The whole peak responses were processed using multivariate methods such as principal component analysis (PCA) and partial least squares regression (PLS-R). A pre-processing method was also introduced that compensated for drift

due to (i) shift in baseline and (ii) decay in the responses of the biosensor with time. It was also shown that it is possible to validate responses from a newly prepared biosensor with a multivariate calibration model performed with another biosensor of the same kind prepared in exactly the same way. The results indicate that even unstable biosensors can be used for continuous on-line measurements – if an appropriate correction method for drift compensation is used.

Paper III describes a method to determine whether a screen-printed carbon-based electrode is suitable for adsorption of the enzyme cellobiose dehydrogenase, CDH (and hopefully other enzymes). It was shown that a correlation could be found between the electrochemical reversibility of the $FeCN_6^{3-}/FeCN_6^{4-}$ couple and the sensitivity for catechol at a CDH modified electrode. The material at a screen-printed electrode surface consists of a mixture of non-conductive and conductive parts. The main idea is that a higher fraction of the conducting electrode area facilitates the transfer of electrons between CDH and the electrode, and thus, increased biosensor sensitivity is obtained. Different pretreatment methods including polishing or electrochemical cleaning in $H_2SO_4$ or $Na_2SO_3$ solutions before enzyme immobilization also improved the sensitivity for catechol at the CDH sensor.

In Paper IV an amperometric cell for radially distributed screen-printed eight-electrode arrays is described and in addition a technique using spraying of carbon ink directly on screen-printed platinum is introduced. The amperometric cell can be used for steady-state as well as flow-injection measurements with errors of less than 5%, showing that the main error was due to differences between the manufactured arrays. The produced sprayed electrodes worked well for catechol detection with immobilised HRP and may in some cases be seen as an alternative to screen-printed electrodes.

Paper V is about immobilisation of enzymes (oxidoreductases and cholinesterases) on screen-printed arrays consisting of five carbon and three platinum working electrodes distributed radially around a printed Ag/AgCl reference electrode. Mainly cross-linking with glutaraldehyde was employed for enzyme immobilisation. The developed array was tested for model compounds containing either phenols or pesticides. Measurements were also carried out in real samples obtained from the pulp & paper and pesticide industries. The multivariate method PCA was used to simplify the discrimination between wastewater samples from the two different sources.

Paper VI describes a system comprised of eight screen-printed electrodes in a radial array modified respectively with immobilised horseradish peroxidase (HRP)- two electrodes, tyrosinase (TYR) – two electrodes, acetylcholinesterase (AChE) – two electrodes, butyrylcholinesterase (BChE) – one electrode, leaving one platinum electrode left unmodified. The array was used for steady-state measurements of a pulp & paper wastewater effluent from a WWTP. A pre-processing procedure (similar to the one described in Paper II) was introduced to compensate for different types of drift. The wastewater samples were also analysed in parallel with reference methods for changes in e.g. toxicity, global organic parameters (COD, TOC and BOD) and physical parameters (pH and conductivity). A PCA score plot of the corrected responses showed that changes in the effluent quality from the WWTP process could be followed gradually using the sensor array. The response could be described by a set of formal characteristics spanning from "alarm" to "normal" character. It was shown that it was mainly the HRP sensor that was responsible for the realised differentiation of samples. Moreover, mainly the unmodified platinum electrode was responsible for effective differentiation between untreated samples and samples treated in the WWTP.

In Manuscript VII the results presented in Paper VI are followed up with the aim of determining the correlation between sensor signals and parameters such as COD, BOD and toxicity using the multivariate method partial least squares regression (PLS-R). The number of pulp & paper wastewater samples treated in the WWTP process was expanded and two more biosensors based on the enzymes soybean peroxidase (SBP) and cellobiose dehydrogenase (CDH) were added to the array system and the measurements were performed with flow-injection analysis. Best correlations were found between array responses and toxicity determined by the Microtox® test. In some cases the array signals even showed good correlation to COD and BOD.

# 2 AMPEROMETRIC BIOSENSORS

A biosensor can be defined as a device that combines a biological recognition element (e.g. enzymes, whole tissues, cells, antibodies or DNA) with a transducer (e.g. electrochemical, optical, calometric or piezoelectric) that converts the biological reaction to a measurable physical signal [13, 14], see Figure 2.1. In case of electrochemical transducers, these can be further subdivided according to the detection principle: potentiometric, amperometric, conductometric, or different combinations and modifications of these [15, 16].

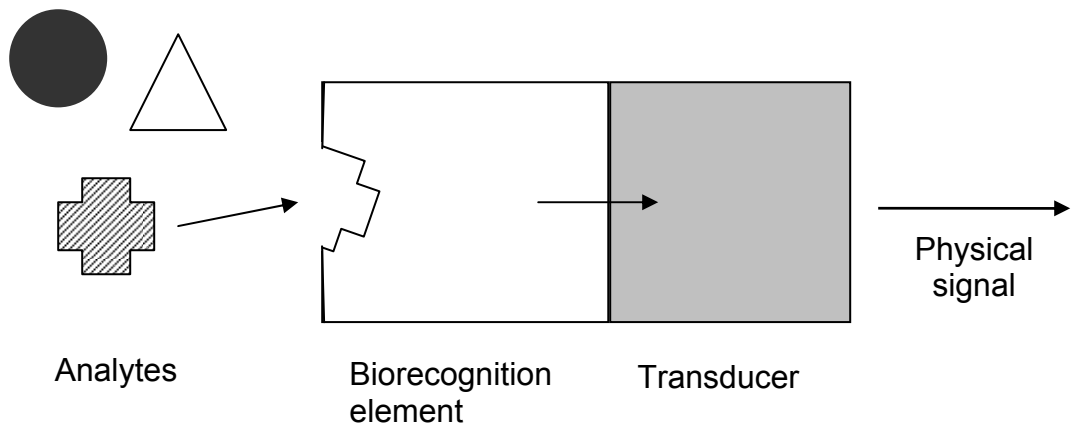


*Figure 2.1. Classical example of the biosensor detection principle.*

The work presented in the publications included in this thesis was conducted with biosensors based on amperometric detection. The simpliest amperometric system consists of two electrodes: a working electrode and a reference electrode (e.g. SCE or Ag/AgCl reference electrodes). During measurements the potential between these electrodes is kept constant. The detected current is directly proportional to oxidation or reduction of electroactive species at the working electrode. More general is the use of a three-electrode system to avoid that current passes through the reference electrode and thus changing the reference potential. In this case the applied potential is adjusted between the working and the reference electrode whereas the current flows between the working and an additional auxiliary electrode. A more thorough review of the used measurement techniques based on amperometric (and voltammetric) detection can be found in chapter 3. Among the advantages of using amperometric detection techniques are for example that the used electrodes and analytical

systems are relatively cheap, and that electrodes can be mass produced in a reproducible way, using screen-printing technology [17, 18]. The main drawback is, however that other compounds than the target analyte(s) present in a sample solution can contribute to the obtained amperometric signal. Immobilisation of biological material on the electrode is thus a common way to reduce the effect from interfering compounds and thereby to improve the selectivity for a specific analyte [19].

## 2.1 Amperometric Biosensors in Environmental Analysis

Several reviews about biosensors for environmental monitoring have been published [10-12, 20, 21] and many commercial devices are now available on the market where the detection principles are mostly either electrochemical or optical [11]. Biosensors for environmental analysis can be divided in two classes [10]: (i) biosensors for a single or a group of target compounds; ii) biosensors for measurements of global effects. Both classes can be built as amperometric biosensors with different biorecognition elements, e.g. enzyme based biosensors [16], whole cell biosensors [22], DNA biosensors [23] and immunobiosensors [24]. In this work only enzyme-based sensors have been used and, thus, a more detailed discussion about these types of biosensors will be included here.

### 2.1.1 Enzyme based biosensors

Enzymes are the reaction catalysts of biological systems [25]. With a small exception all enzymes are highly specialised proteins that have an extraordinary catalytic power, often far greater than that of synthetic catalysts. They are generally highly specific for the substrate (the molecule that is enzyme-catalysed to form a product) and can work under mild conditions.

Mainly, two types of enzymes have been used as the biorecognition element in our work: i) *oxidoreductases* that catalyze reactions where transfer of electrons is central, see Papers 1-VI and manuscript VII, and ii) *hydrolases* that transfer functional groups to water (Papers V-VI and manuscript VII).

## Biosensors based on oxidoreductases

For environmental water analysis oxidoreductases are used to detect phenolic compounds [26-34]. The mechanism for detection of phenols relies on mediated electron transfer [35]; the mechanism used for construction of the sensors in this work (Papers I-VI and Manuscript VII). Mediated electron transfer is illustrated in Figure 2.2 a and is similar for electrodes modified with the enzymes tyrosinase (TYR) [36, 37], peroxidase (POD) [38] and laccase [36]. It is possible to construct single-analyte sensors as well as more general group selective sensors based on this mechanism [27]. At the applied potential (-50 mV vs. Ag/AgCl), the actual enzymes are in their native state. The reaction cycle begins with that the first enzyme substrate, $H_2O_2$ for POD and $O_2$ for the other two, binds to the active site of the enzyme, whereby the enzymes are converted to their oxidized forms. An enzyme-catalysed oxidation starts where the second substrate, e.g. phenol, binds to the enzyme. The enzyme is reduced back to its reduced form leaving an oxidised phenolic product, which in many cases is a quinone. The quinone is reduced back to its phenolic structure by receiving two electrons from the electrode surface resulting in a current, which is detected. The phenol can then again be oxidised by the enzyme. An advantage with the described electrode mechanism based on phenol recycling is that the current due to phenol is amplified. Oxidation of phenols directly on a bare electrode surface asks for significantly higher working potentials with very high risk for interfering responses. The enzyme cellobiose dehydrogenase (CDH) [39] can also be used for phenol detection [34] in presence of the enzyme substrate cellobiose. The native state of the enzyme is in an oxidative state and the mechanism (see Figure 2.2 b) is opposite to the one explained in Figure 2.2 a. The detected current is based on initial electrocatalytic oxidation of phenols to a corresponding quinone, which subsequently is enzymatically reduced back to the phenol, thereby requiring a higher applied potential (+300 mV vs. Ag/AgCl). When using CDH modified electrodes amplification factors can be calculated defined as the current due to the CDH catalysed phenol oxidation divided by the direct oxidation of the same phenol (without cellobiose present). The amplification factor can be used to evaluate how suitable different electrode materials are for constructing CDH biosensors, as was done for screen-printed carbon electrodes in Paper II.

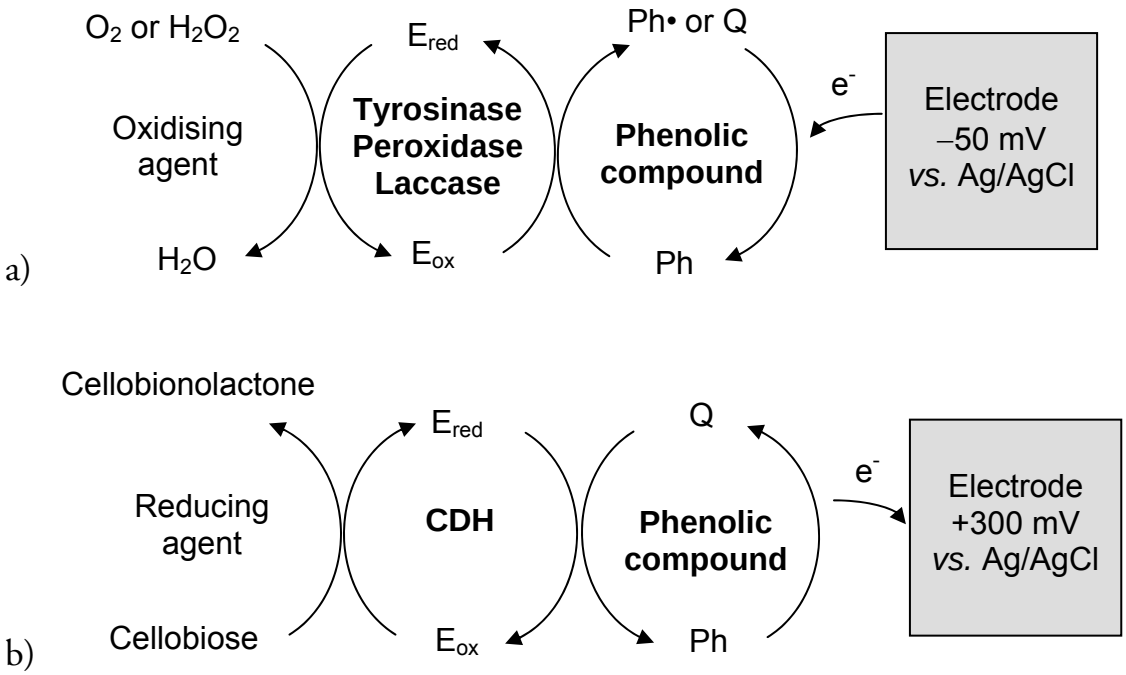


*Figure 2.2. Electrode mechanism based on enzyme and electrochemical reactions with a phenolic compound as substrate/analyte for a) tyrosinase, peroxidase or laccase, and b) cellobiose dehydrogenase (CDH). $E_{ox}$ and $E_{red}$ are the oxised and the reduced form of the enzyme, respectively. Ph is the phenol, Ph• its radical and Q the corresponding quinone.*

The enzyme substrate for POD biosensors, hydrogen peroxide, is an unstable compound that can react with other components in a solution. A way to avoid addition of hydrogen peroxide directly in the sample to be measured is to co-immobilise the POD with an enzyme that can produce hydrogen peroxide, e.g. glucose oxidase (GOx) ([40], and Papers I, V and Manuscript VII). The mechanism of GOx is presented in Figure 2.3. In this system the substrate for GOx, glucose, instead needs to be present in the measurement solution. Other solutions without addition of an enzyme substrate in the sample are possible. Chang et al [41] presented an elegant alternative using a disposable sensor based on co-immobilised tyrosinase and HRP. In this case, sufficient hydrogen peroxide for HRP is formed on the electrode surface from electrochemical reduction of oxygen. Moreover, the immobilised HRP helps to speed up the recycling process of the detected phenol and it was suggested that the enzyme co-immobilisation improved the electrode sensitivity compared with using tyrosinase alone as the phenol-sensitive element.

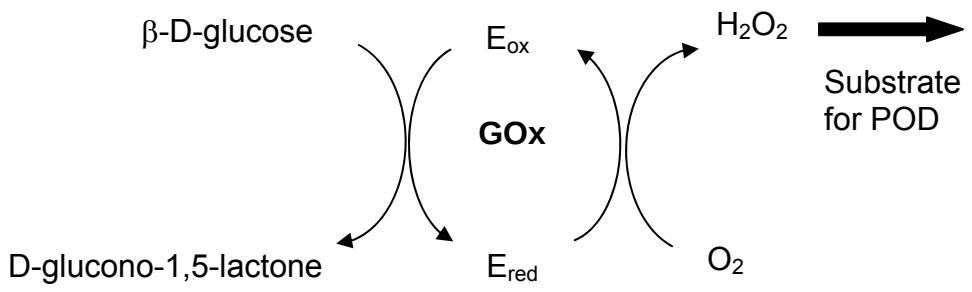


*Figure 2.3. Mechanism of glucose oxidase (GOx) catalysed production of $H_2O_2$ serving as a substrate for peroxidase (POD) at a bienzyme electrode.*

Even though biosensors modified with oxidoreductases generally utilise the catalytic properties to detect target compounds, situations can also be found where the substrate instead inhibits the biosensor by a non-specific mechanism [42]. Others have pointed that heavy metals can serve both as activators or inhibitors of the enzyme activity [43]. More complex response patterns are thus common when biosensors are used in real dirty wastewater samples where the biorecognition element can be affected by interferences in the matrix giving rise to inhibition, activation, adsorption, etc [44]. One approach to overcome such effects is to use adequate sample pretreatment techniques and separations with LC [20]. Another approach is to see the biosensor response as a pattern (or fingerprint) that characterises the actual sample (Paper V). With multivariate tools, such as PCA, hidden information and trends can be found for responses obtained from complex samples.

### Biosensors based on hydrolases

Two of the most investigated amperometric hydrolase biosensors (that also have been used in this work, see Papers V-VI and Manuscript VII) are based on the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [45, 46]. The target analytes are organophosphate and carbamate pesticides that selectively inhibit cholinesterase modified electrodes [47, 48]. The general inhibition mechanisms have been described elsewhere [49]. An example of a cholinesterase inhibition based amperometric sensor is presented in Figure 2.4. If a salt of either acetyl- or butyryl thiocholine is added to the measurement solution, an enzyme catalysed hydrolysis reaction starts. The product,

thiocholine, is further oxidised at the electrode giving a detectable current. In the presence of a pesticide, the cholinesterase is inhibited resulting in a decrease of the detected oxidation current. One drawback with the presented inhibition of cholinesterases is that the binding is irreversible. Thus, disposable electrodes have been used in many of the latest publications about cholinesterase sensors for pesticide determinations [50-52]. However, it is in many cases possible to reuse the sensor for a new inhibition step as long as a part of the enzyme activity remains. The cholinesterase activity can also be affected by other pollutants such as heavy metals, fluorides etc. Thus, cholinesterase sensors have also been used as a more general indicator of the pollution level (Papers V-VI and Manuscript VII).

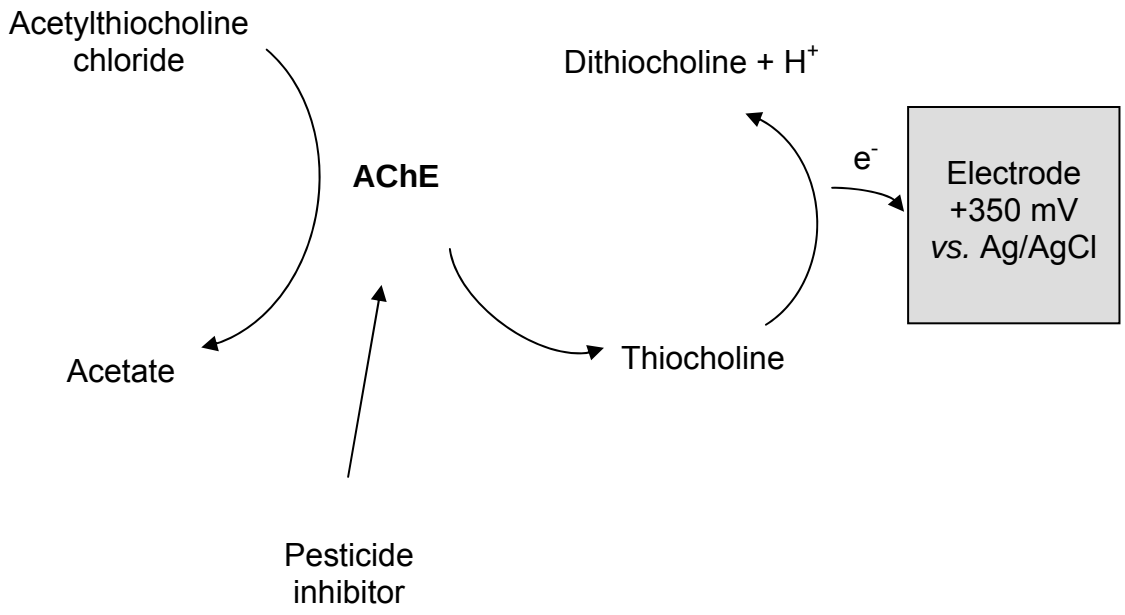


*Figure 2.4. Schematic picture of a pesticide inhibition at a cholinesterase modified electrode.*

### 2.1.2 Whole-cell biosensors

Whole cells as the biorecognition element in biosensors involve a complex living system [22]. Most of the used whole cell biosensors are based on bacterial cells but other species such as yeast [53] are also common. Whole cell biosensors have been seen as a low-cost alternative to electrodes modified with purified enzymes and in the literature many examples of amperometric whole cell biosensors specific against a single- or a group of substrates (e.g. phenols [54], benzene [55], ammonia [56] and nitrate [57]) have been reported. However, due to many reaction pathways, the whole cell sensors have also been utilized frequently to determine more general parameters. Many publications can be found about microbial sensors for determination of BOD [58, 59]. The detection principle is mainly based on measuring oxygen depletion due to respiration of the immobilised microbial organism. Whole-cell sensors can also be used to determine toxicity [9, 60], where one well-known device is the amperometric Cellsense® system, which uses *Escherichia coli* bacterial cells for rapid ecotoxicity determinations [61].

### 2.1.3 DNA biosensors

Since the toxic actions of numeric pollutants can be related to DNA damage (e.g. carcinogens and mutagens), there is a growing interest in creating DNA biosensors for environmental monitoring [23, 62]. DNA biosensors using amperometric or voltammetric techniques can be found for detection of e.g. heavy metals [63] and s-triazine derivatives [64]. Lucarelli et al [65] demonstrated that a voltammetric DNA biosensor can be used as a rapid tool for toxicity screening in real water samples with promising correlation to the commercial toxicity test Toxalert®100.

### 2.1.4 Immunobiosensors

The detected biological recognition in immunobiosensors is due to the affinity of an antibody (Ab) for its corresponding antigen (Ag). Applications are mainly found in analysis of single target compounds [20, 66]. Immunobiosensors are usually very sensitive, but do not exhibit catalytic activity, i.e. the binding is generally irreversible and the sensors are more suited for 'one-shot' detection [24]. Amperometric detection is common to use with immunobiosensors for determination of, e.g. atrazine [67], polycyclic aromatic hydrocarbons (PAHs) [68] or the hormone 17-β-estradiol

[69]. However, the formed Ab-Ag complex is in many cases not enough electrochemically active to be detected directly by amperometry and thus, to amplify the obtained signal, an enzyme label is necessary such as HRP [67, 70] or alkaline phosphatase (AP) [69].

## 2.2 Design of Amperometric Biosensors

The design of a biosensor is highly dependent of what kind of biorecognition element that is used and how it should be applied. Desired properties such as high sensitivity, selectivity, long-term stability, short response time, easy-to-use etc. are highly dependent of the chosen electrode material as well as the immobilisation procedure. Thick-film biosensors are planar constructions well suited for mass-production and also are the types of sensors that have been used frequently in this thesis and will be described in a separate paragraph.

### 2.2.1 Electrode materials

In this work was carbon the main electrode material. Noble metals have also been used as a component in thick-film sensors presented in section 2.2.3. Both types of materials have desired electrode properties such as high electrical conductivity, wide potential range, and no or very low chemical reactivity.

#### Carbonaceous material

Carbonaceous materials have been widely used for construction of electrochemical biosensors due to their good electrical conductivity, stability and low cost, as well as the presence of various functional groups (e.g. OH- or COOH-) that can be used to link a biorecognition element, either by adsorption (weak inter-molecularly forces) or by stronger covalent attachments [71]. Pretreatment of the electrode surface (polishing, electrochemical cleaning, heating) increases the amount of oxygen containing functional groups at the electrode surface. Various types of carbon electrodes are available that differ in structure and degree of order: graphite, carbon black, carbon fibres, glassy carbon etc. [71-73]. Newer carbon based electrode materials are for example nanotubes that have unique properties such as high electrical conductivity, high chemical stability and extremely high mechanical strength [74]. The bulk properties vary from one type of carbonaceous material to another due to the

size and the orientation of the carbon particles [73]. These differences affect the electrode performance such as electrode kinetics and background currents.

Carbon composite electrodes consist of a conductive phase (carbon) and an inert non-conductive phase. One advantage with composite electrodes is that the biorecognition element and other components (e.g. mediators or stabilisers) can be incorporated into the composite mixture [75, 76]. The biosensor surface can then easily be renewed by polishing (solid composite electrode) or by just wipe away the old surface (carbon paste electrode). Another advantage is that carbon composite electrodes can be easily combined in arrays by screen-printing.

In Paper I and II the results were obtained using solid graphite electrodes. Carbon composite material was used as thick-film sensors for the work presented in Paper III-VI and Manuscript VII.

**Noble metals**

Noble metals are good electrode materials with non-corrosive properties and high electrical conductivity. The electrodes are susceptible to fouling effects but can be reactivated by a suitable pre-treatment. Biosensors have often been prepared by trapping the biorecognition element on the electrode surface under a membrane [14, 77, 78]. Very popular are especially gold electrodes where the biomolecule is bound to the surface via self-assembled monolayers (SAM) [77-79]. Nobel metal nanoparticles as electrode material are an increasing research area offering several benefits over macroelectrodes: enhancement of mass transport, catalysis, high effective surface area and control over the electrode microenvironment [80].

In this work noble metal particles (gold and platinum) were used in composite materials deposited on thick-film electrodes (Paper IV-VI and Manuscript VII).

### 2.2.2 Immobilisation techniques

Important factors in the development of immobilisation techniques are how to stabilise and tether the biomolecules to the electrode surface. Several strategies are available and these can mainly be

divided into four different classes [14, 24, 81]: physical or chemical adsorption at a solid surface; covalent binding to a surface; physical entrapment; and cross-linking between molecules.

### Physical or chemical adsorption at a solid surface

Physical adsorption is usually due to van der Waals forces, ionic binding or hydrofobic forces. In case of chemisorption the binding is due to sharing or transfer of electrons. The main advantage with these methods is the simplicity and that they can be performed under mild conditions. Drawbacks such as desorption and denaturation due to structural changes of the immobilised material is common when physical parameters, e.g. pH or temperature, in the environment are changed. Electrodes modified with adsorption technique have been used in the work described in Paper I and Paper III based on the enzymes HRP and CDH, respectively.

### Covalent binding to a surface

Stronger binding than in the case of adsorption is obtained when functional groups of the biomolecule (e.g. amino, carboxyl or thiol groups etc.) are covalently linked to functional groups at an electrode surface that first have been activated with for example carbodiimide [71]. The covalent binding has the advantage that the biomolecule is generally strongly immobilised to the electrode surface and thus, unlikely to come loose from the surface during measurements. Moreover, due to the variety of functional groups available for covalent binding, disadvantages depending on that the active site of the biomolecule is involved in the linkage can be avoided [24].

### Physical entrapment

Biomolecules can be physically entrapped behind permselective membranes (e.g. poly(ester-sulfonic acid) or perfluorosulfonic acid) [82, 83] or immobilised in a polymeric network [84-86], which can be non-conducting (e.g. polyacrylamide) or conducting (e.g. polypyrrole or osmium redox hydrogels). The advantage with physically entrapped biorecognition elements is that the electrode is protected against fouling and interferences, and that the linear responses range is extended. The main drawback is the risk for enzyme leaking. Poly(ester-sulfonic acid), Eastman AQ 55D, was used to stabilise the immobilisation of tyrosinase at a solid graphite electrode, described in Paper II.

**Cross-linking between molecules**

Cross-linking of biological components can often reduce the problem of biomolecule leakage. Covalent linkages can be formed between the biorecognition element and the electrode as well as between two biomolecules. One of the far most used reagents for cross-linking at electrode surfaces is glutaraldehyde (Papers V-VI and Manuscript VII). The disadvantages being that it is difficult to control the reaction, large amounts of biological material are required and that large diffusional barriers to the transport of the biological substrates may lead to retarded interactions.

### 2.2.3 Thick-film biosensors

Thick-film techniques are versatile and permit the integration of sensors and electronic circuitry [87]. These types of sensing devices have been extremely popular for mass production of disposable biosensors [18, 88, 89]. Furthermore, the sensors are also easy to handle and can be used in portable systems. For example, many of the commercial devices for fast glucose determination in blood are based on amperometric systems using thick-film sensors modified with the enzyme GOx [90]. In wastewater analysis, the Cellsense® system, used to determine toxicity, is another example of a commercial tool using thick-film technology [61]. Other recently published examples of amperometric thick-film biosensors for measurement in real water samples can be found in the literature, e.g. to screen for BOD [91], toxicity [60] or heavy metals [92] as well as to determine spiked concentrations of phenols [93] or 17-β-estradiol [69].

Thick-film sensors are manufactured with screen-printing technology (thereby also the name screen-printed electrode, SPE) in a planar design with a layer-by-layer procedure. A general description of screen-printing for sensor production is shown in Figure 2.5 [88]. The steps described in Figure 2.5 are eventually repeated, depending on the design of the final sensing element. The deposited film is baked to cure and to drive the solvents from the applied paste. The film based on the working electrode consists often of a paste mixture of a conductive material (such as graphite or metal powder), a binding agent (e.g. cellulose acetate, powdered glasses or epoxy) and a solvent (e.g. cyclohexanone or ethylene glycol). Different configurations of enzyme modified SPEs have been described, and these can be divided in three groups [94]: i) multiple-layer deposition (biological deposition by hand or electrochemically), ii) screen-printing of composite inks or pastes using two or

more steps (biological deposition by screen-printing), and iii) one-step deposition layer or biocomposite strategy. The use of biocomposite inks that can be deposited on the substrate in one-step simplifies the fabrication of the thick-film sensor. However, better results have been observed with sensors based on manual deposition of enzyme than in sensors where the biological layer is screen-printed [94]. In the same paper it is also concluded that the best results were obtained when the sensors were modified with enzyme manually together with the cross-linker glutaraldehyde. In the work behind the articles presented in this thesis, manual immobilisation of the enzymes has been performed on the SPE, either by adsorption without any additional binding element (Papers III and IV) or by cross-linking with glutaraldehyde (Papers V-VI and Manuscript VII).

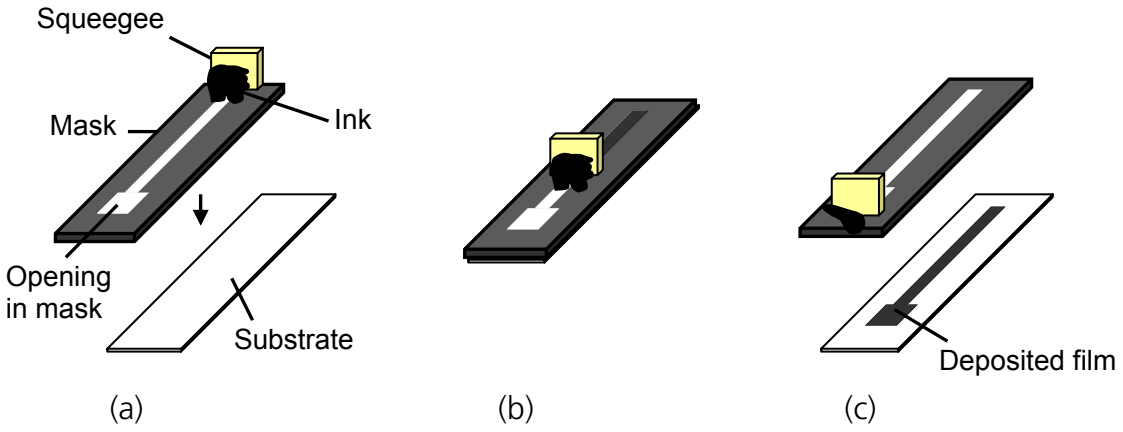


*Figure 2.5. Schematic overview of a screen-printing process: (a) A mask is placed on a substrate generally made of ceramic or PVC material. (b) A conductive film is printed through the mask on the substrate. (c) The deposited film is left on the substrate after the screen printing mask is lifted.*

The main drawbacks with thick-film sensors are related to the lack of compatibility between the different materials, and between the materials and the manufacturing process [88]. Negative effects can also occur in the contact between the electrode and the immobilised biorecognition element since the obtained screen-printed working electrode surface contains spots with electroactive area based on the conductive component as well as non-conductive parts from the binder. Commercial carbon based SPEs were studied with the purpose to find products which are better suited to realise

the mechanism of phenol recycling at enzyme modified electrodes (Paper III). The results pointed to that with a higher fraction electroactive material (i.e., graphite) on the printed area, the better the phenol recycling, as determined by the amplification factor for catechol when the electrode was modified with CDH. It was also shown that electrode pretreament (e.g. electrochemical cleaning in $Na_2CO_3$ or $H_2SO_4$ [95-97] or polishing with fine emery paper [98]) before enzyme immobilisation resulted in a higher amplification factor indicating that a more electroactive area was received after the pretreatment process, as also observed by other authors [73, 97].

Screen-printed electrodes (SPEs) based on both noble metals (Papers IV-VI and Manuscript VII) carbon (Papers III-VI and Manuscript VII) have been used for construction of biosensors in this thesis. As a complement to screen-printing, a method based on spraying the working electrode material on a screen-printed platinum electrode was introduced, showing good enzyme immobilisation properties (Paper IV). This deposition procedure is similar to the ink-jet printing technique that have been used for enzyme deposition [99, 100].

# 3 ELECTROCHEMICAL MEASUREMENTS

The electrochemical measurements in this work are based on detection of a current, either by using a constant potential (amperometry) or measuring the current as a function of the potential (voltammetry) [101]. Both electrochemical techniques, i.e. amperometry and voltammetry, are conducted using two or three electrode systems (as described in chapter 2). For an electrochemically reversible electrode reaction, where the oxidised (O) and the reduced (R) form of a redox couple is at equilibrium (1),

$$\mathrm{O} + ne \rightleftharpoons \mathrm{R} \qquad (1)$$

the electrode potential, $E$, is described by Nernst equation (2).

$$E = E^{0'} + \frac{RT}{nF}\ln\frac{[\mathrm{O}]}{[\mathrm{R}]} \qquad (2)$$

$E^{0'}$ is the formal electrode potential, $R$ is the gas constant, $T$ is the temperature, $n$ is the number of electrons involved in the electrode reaction, $F$ is the Faraday constant, and, [O] and [R] are the concentrations of the oxidised and the reduced specie, respectively. In amperometric and voltammetric measurements the equilibrium is disturbed by applying an electrode potential ($E_{appl}$) that differs from the equilibrium potential. If $E_{appl}>E$, then the electrode surface promotes an oxidation of R. The opposite happens if $E_{appl}<E$.

The detected electrode current is dependent on how the analyte is transported to the electrode surface. The modes of possible mass transfer are [101]:

(i) *Migration*. Movement of charged species under the influence of an electric field (a gradient of electrical potential). This type of mass transfer is usually negligible at high concentration of salts of an inert supporting electrolyte.

(ii) *Diffusion*. Movement of species under the influence of a gradient of a chemical potential (a concentration gradient). The two methods chronoamperometry and cyclic

voltammetry, based on diffusion–controlled currents, have been used to characterise the surface at screen-printed electrodes (see section 2.2.3).

(iii) *Convection*. Stirring or hydrodynamic transport. Movement of species due to convection. All amperometric measurements in this work have been performed under convection.

This chapter summarises and describes the electroanalytical techniques used during my work with this thesis.

## 3.1 Chronoamperometry

Consider that a potential step is applied from a value where no redox reactions occur of an actual redox pair (1) to a value remarkably lower than the formal potential of the same redox couple or equilibrium potential. A reduction reaction starts immediately leading to depletion of the analyte near the electrode surface. The concentration gradient that builds up promotes transfer of new species from the bulk to the electrode by diffusion. A diffusion layer is formed that grows if no convection is used. If the electrode is planar and the diffusion is linear then the decrease in the electrode current is described by the Cottrell equation [102] (3):

$$i(t) = \frac{nFAD_O^{1/2}C_O}{\pi^{1/2}t^{1/2}} \qquad (3)$$

where $i$ is the diffusion controlled current, $t$ is the time, $A$ is the electrode area, $D_O$ is the diffusion coefficient and $C_O$ the bulk concentration of the oxidised specie. $F$ and $n$ have the same meaning as in equation (2). The above described electrochemical method is called *chronoamperometry*.

As was mentioned in section 2.2.3 the SPE surface in thick-film sensors consists of a mixture of conductive and non-conductive areas. In many cases the surface can be seen as an array of closely packed microelectrodes [103-106], and if this is the case, it is possible to determine the fraction of the conductive area of the SPE surface using chronoamperometry. How this is performed is easily explained by considering the diffusion pattern at the SPE when a chronoamperometric measurement is performed (Figure 3.1). In the very beginning of the potential step, the analyte (redox couple) experiences linear diffusion to the individual conductive spots (or microelectrodes), Figure 3.1a, and

the measured current follows the Cottrell equation (3). A little bit later radial diffusion starts, which is a very typical behaviour at microelectrodes, Figure 3.1b. After a while the diffusion layers from each individual conductive spot overlap. The diffusion is finally again linear and the Cottrell current can be determined for the entire electrode area, Figure 3.1c. If the Cottrell current (i) is plotted against $1/t^{1/2}$ , the fraction of electroactive area of the SPE can be estimated from the ratio of the slope at a very short time domain when individual diffusion occur at each electroactive spot (Figure 3.1a) to the slope at a long time domain when diffusion is obtained at the whole electrode surface (Figure 3.1c). This procedure has been used to compare different SPE based on carbon to find the electrode that has the highest possible electroactive area (Paper III). The model shall be seen as a rough approximation. If the electroactive spots are too far away from each other (e.g. a few hundred µm) no overlap will occur [107] and the determined Cottrell current will not represent the entire electrode surface. Furthermore, another factor that can affect the entire calculated electrode area is the roughness of the surface, e.g. overlap that normally should occur at a planar surface can be hindered by a rough electrode surface [103].

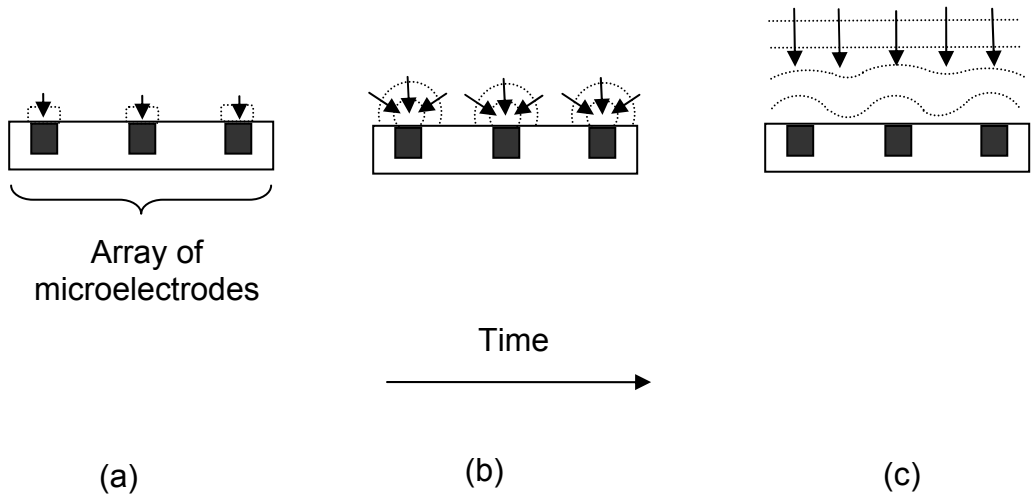


*Figure 3.1. Diffusion profile at an array of microelectrodes from linear diffusion at each single electrode (a), to radial diffusion (b), and, to finally linear diffusion again due to overlap of diffusion layers.*

## 3.2 Cyclic Voltammetry

Cyclic voltammetry (CV) is the most widely used voltammetric technique [101, 108]. The power of CV lies in the diagnostic strength due to the easiness in evaluating reaction kinetics, formal redox potential of a redox couple, adsorption effects etc. In CV the potential is linearly sweeped between two levels (forward and back) with a defined speed. Figure 3.2a shows a typical CV sweep going from a level lower than the formal potential ($E^{0'}$) of a redox couple O/R (1), i.e. the region where reduction occurs, to a level higher than $E^{0'}$ where an oxidation process is possible. When the potential reaches the highest level of the applied potential the sweep direction is changed towards a lower potential until the starting potential is reached again. The obtained voltammogram, showing the current as a function of the potential, can be seen in Figure 3.2b. In the beginning only a background current (a non-faradaic current) flows until the electrode potential reaches the vicinity of $E^{0'}$ where an oxidation of R to O starts. The oxidation current increases with increasing potential until the surface concentration of R drops. The current then decreases due to limitations in mass-transfer since the concentration gradient for R decreases with time, lowering the diffusion of R to the electrode surface. A peak in the opposite direction will occur when the potential is scanned towards a lower potential. If a redox process is electrochemically reversible the equilibrium between [O] and [R] is reached near the electrode surface at any potential during the CV sweep. The system can then be described by Nernstian behaviour (equation 2). The distance (ΔE) between the oxidation peak ($E_{ox}$) and the reduction peak ($E_{red}$) is then 0.059/$n$ V at 25°C (if the redox species are in solution). For a reversible system the formal potential can easily be determined as $E^{0'}=(E_{ox}+E_{red})/2$. If electrochemical reactions are kinetically hindered ΔE is higher than for the reversible systems and varies with sweep rate. For microelectrodes where radial diffusion dominates [109], the obtained voltammogram has a sigmoidial shape.

CV was used to characterize the heterogeneous surface of different SPEs consisting of conductive and non-conductive areas (Paper III). The reversibility was evaluated with one of the most popular redox couples, i.e. $FeCN_6^{3-}/FeCN_6^{4-}$. Moreover, CV measurements with very low sweep rates were also used to determine if the electrode in some sense behaved as a microarray.

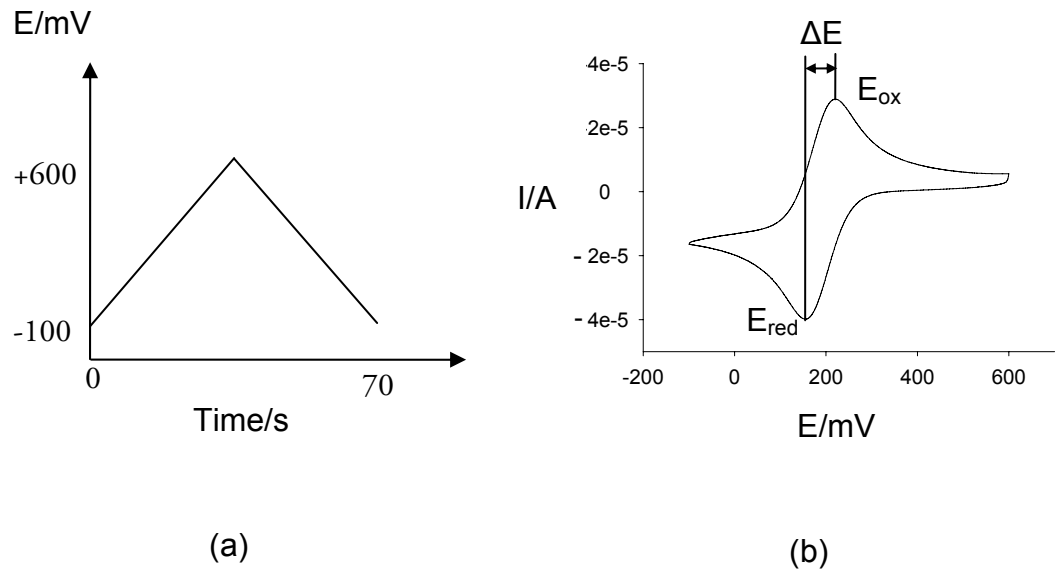


*Figure 3.2. The figure shows a) a typical potential sweep in cyclic voltammetry at 20 mV/s, and b) the obtained voltammogram for the* $FeCN_6^{3-}/FeCN_6^{4-}$ *redox couple at a screen-printed platinum electrode.*

## 3.3 Hydrodynamic Methods

In amperometric hydrodynamic methods the mass transfer is due to convection (e.g. stirring the bulk solution, rotating an electrode or using a flow system) [101]. The diffusion layer is then limited by convection and the measured current (*i*) depends on the mass transfer of an analyte to the electrode according to equation (4) (shown for the case of a reduction):

$$i = \frac{nFAD_O C_O}{\delta} \qquad (4)$$

The thickness of the diffusion layer is described by δ when a steady-state has been reached. The other parameters have the same meaning as in equation (3). Equation (4), however, only deals with the steady-state mass transport problem. How the current are related to parameters such as flow rates, rotation rates, solution viscosity etc. is estimated for each specific hydrodynamic method. The advantages with hydrodynamic techniques are that a steady-state is obtained very quickly and

measurements can be performed with high precision. In this chapter the amperometric hydrodynamic methods are divided into batch and flow techniques.

### 3.3.1 Hydrodynamic batch methods

In batch systems, either the electrode or the solution can be in motion. The most convenient and widely used system is the rotating disk electrode (RDE), used mainly for the determination of kinetic parameters of a redox reaction [101]. A simple variant of a hydrodynamic batch measurement is to mix the solution with help of a magnetic stirrer (Paper III). However, to ensure uniform stirring of the measurement solution at an array of electrodes more sophisticated approaches are sometimes needed. In this work a system for steady-state measurements was developed, making use of radially distributed screen-printed eight-electrode arrays where a rotating rod was placed closely over the array surface during measurements (Paper IV). More of this can be read in chapter 4.1.2.

### 3.3.2 Flow systems

Another way to perform hydrodynamic amperometric measurements is to use a flow system. An important application of flow cells is as detectors in liquid chromatography [101]. One of the most common designs is the wall-jet flow-through cell [110-112] in which the flow is directed perpendicular to the electrode surface, as shown in Fig. 3.3. Physical factors such as low dead volume or optimized positioning of the electrode in the cell are necessary to receive satisfactory results, and even more so if array electrodes are to be used. The developed steady-state cell for screen-printed eight-electrode arrays was also used for flow-injection analysis (FIA) by simply drilling a hole and incorporating a flow tube in the rotating rod (Paper IV), see chapter 4.1.2. With FIA, developed by Ruzicka et al [113], flow measurements can easily be automated. The main principle is that a continuous flow of an appropriate liquid carrier (an electrolyte in electrochemical measurements) is pumped through the analytical cell. A volume of a sample containing the analyte is injected directly into the flow via an injection valve. When the plug of sample reaches the detector a peak-shaped current is registered. The peak width is usually due to both dispersion (dilution) of the sample as it passes through the FIA system and kinetic limitations as the analyte interacts with the sensing layer of the sensor (detector). FIA have been used in experiments described in Papers I, II, IV and VII.

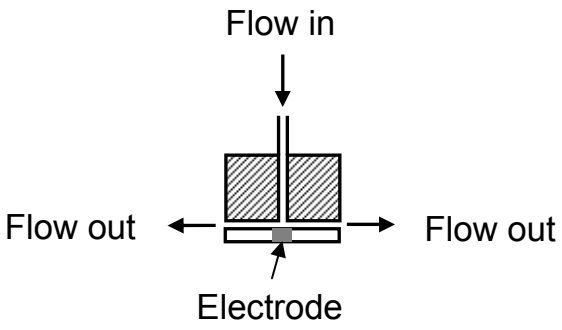


*Figure 3.3. A wall-jet flow-injection cell.*

# 4 ELECTROCHEMICAL ARRAYS IN LIQUID MEDIA

Electrochemical sensors can be used as tools for fast measurements in a sample without prior sample pretreatment. If several different sensors are arranged together in an array, the number of different analytes that can be detected simultaneously can be increased [114]. In case of need for low-cost multi-sensing devices that can be used directly on-line or even in-line for rapid analysis, electrochemical arrays are one of the most promising techniques to use [5]. The drawback of using arrays instead of single electrodes is mainly the higher complexity of the signal processing and technical design/construction of the detection device. Special instrumentation with the possibility to apply different potentials on individual electrodes in the array and more complex software for data recording is generally required [115]. The compatibility between different electrodes and the measurement media can be unfavourable (especially in case of using different biosensors, which often work best in rather narrow pH intervals) etc. Furthermore, the sensors are in many cases sensitive towards changes in the environment such as temperature or flow rate [6]. Two types of approaches are recommended to deal with these effects: either working under fixed experimental conditions or measuring the external parameters to numerically compensate for their change. For automatic measurements with electrochemical arrays (e.g. continuous monitoring of WWTP effluents) the choice of a flow-injection analysis setup is a good alternative [113]. The electrode array configuration and the cell construction are then important factors that must be carefully designed to obtain equal or at least reproducible hydrodynamics over the electrodes, which will be discussed in more detail below. Moreover, a summary related to the growing area of electrochemical microarrays and the principles of electronic tongues will also be discussed.

## 4.1 Arrays in Flow Systems

Electrochemical dual electrode systems have been widely used as detectors in HPLC [116, 117]. One of the first multi-channel devices (more than two independently controlled working electrodes) was reported in 1982 by Matson et al, [118], using up to 16 separate coulometric flow-cells in series (each working electrode had its own reference and auxiliary electrode, controlled by a separate potentiostat). Two frequently used array geometries, serial or radial distribution of electrodes, will be overviewed below. Other geometries, e.g. parallel arrangements, can be found for some of the

mentioned array constructions. Since all these configurations include serial configurations they are also discussed under paragraph 4.1.1.

### 4.1.1 Serial configurations

Many array configurations for flow systems have been arranged just in series [119-125], Figure 4.1a. The advantage with an array using a serial electrode distribution is that approximately equal flow and sensitivity are obtained for each sensor in series, however, one drawback is changes in the flow-injection peak shapes due to increased dispersion when a sample injection zone is pumped further through the flow channel [119]. The difficulty in maintaining potentiostatic control over the entire serial array due to ohmic drop has also been reported for an amperometric system based on glassy carbon electrodes [126]. This effect can be reduced using an individual auxiliary electrode in close proximity to each working electrode in the array [121] or using a single large auxiliary electrode that is situated close to all the working electrodes [123]. Furthermore, in systems using serial array configurations electrochemical crosstalk can occur, i.e. a product obtained at an upstream electrode can cause a response at a downstream electrode. This was utilised in a positive manner in dual electrode systems to improve selectivity; oxidation products at the upstream electrode were monitored in a reductive mode at the downstream electrode [116]. However, in most situations electrochemical crosstalk is a problem. One such example is the use of enzyme-based biosensors, where the common enzyme product, hydrogen peroxide, is transported from one biosensor to the other sensors, causing interference on the total response [124].

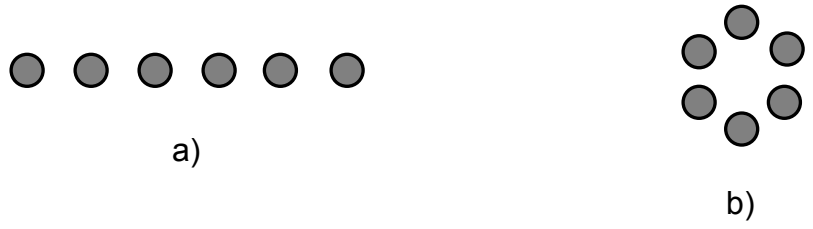


*Figure 4.1. Array geometries: a) serial b) radial*

### 4.1.2 Radial configurations

Undesired electrochemical crosstalk, common in serial array configurations, can be avoided by using radial distribution of the electrodes in an array having an inlet flow in the middle and perpendicular to the electrode surfaces as shown in Figure 3.3 [127-129]. The electrode surfaces that are in contact with the measurement solution are generally circular in this type of configurations, but a selective array system without crosstalk has also been developed using a split-disk array consisting of eight sector electrodes [130]. Drawbacks with the mentioned types of radial array configurations are that the sensitivity is lower compared to using a single electrode of the same size. Furthermore, the positioning of the array in the analytical cell must be exact to obtain equal hydrodynamics over all electrodes.

In this work a combined amperometric steady-state and flow-through cell has been developed for radial eight-electrode arrays produced by screen-printing technology (Paper IV), see Figure 4.2 a and b. Also shown are flow-injection peaks from the developed array system based on reduction of $Fe(CN)_6^{3-}$, Figure 4.2 c. This configuration allows flow-injection measurements according to the wall-jet principle described in section 3.3.2. The inlet flow occurs via a rotating rod that is placed over the planar array surface.

Other types of radial flow systems have been described where the flow is in the same line as the electrode surfaces [131,132]. Both these systems have been used as electronic tongues.

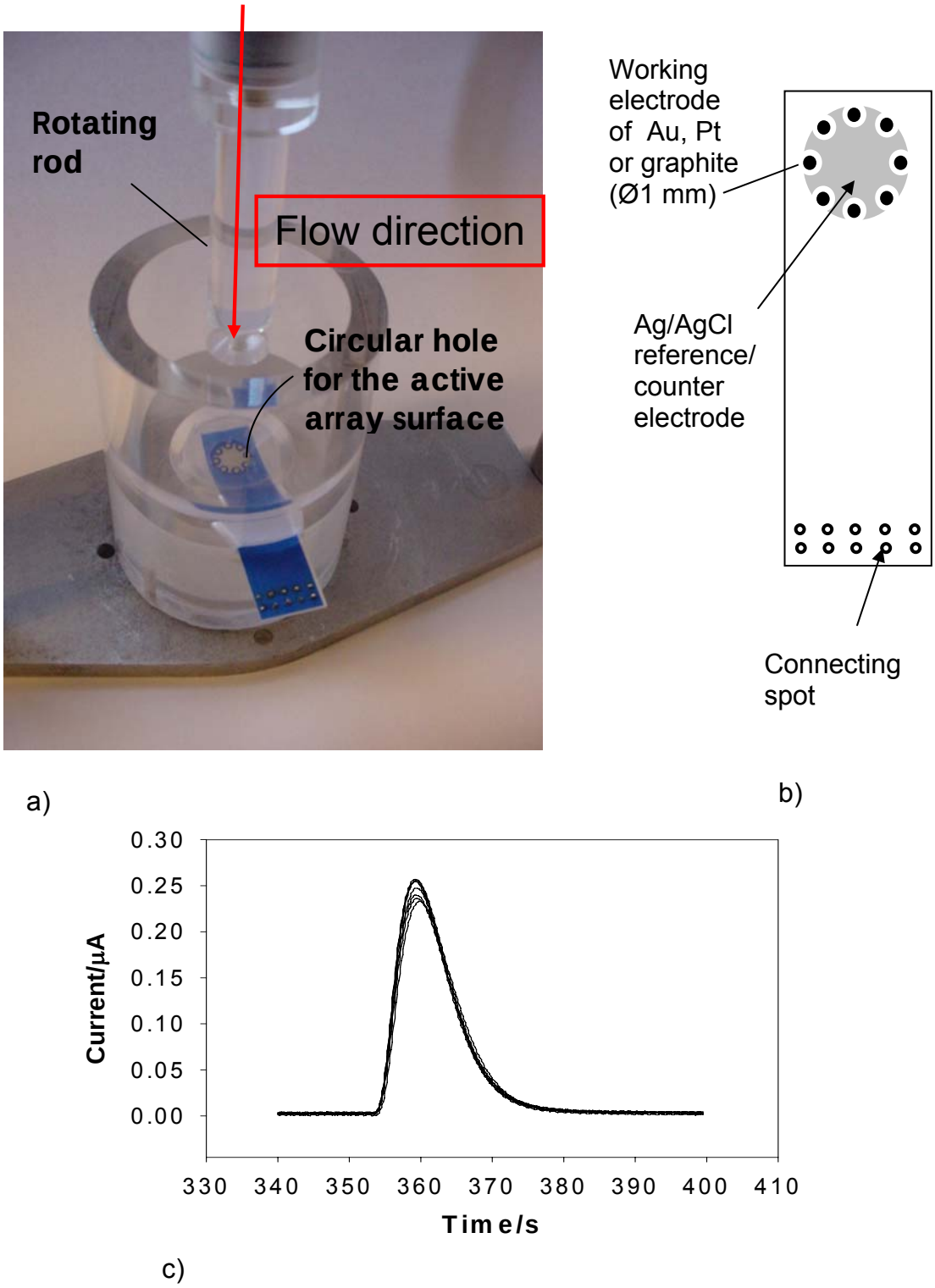


*Figure 4.2. An electrochemical cell (a) for screen-printed eight-electrode arrays (b) that can be used both for flow-injection as well as steady-state measurements. An example of flow-injection responses from 0.2 mM* $Fe(CN)_6^{3-}$ *(at -50 mV vs. Ag/AgCl) using this cell can be seen in (c).*

## 4.2 Microarrays

Microarrays differ from their macroscopic counterparts in several ways. Advantageous properties of using arrays built of microelectrodes are among others [101, 133]: i) increased mass-transport due to radial diffusion (giving a faster response at the electrode), ii) increased signal to noise ratio due to reduced double-layer capacitance since the electrode surface is smaller, and iii) reduced ohmic drop. The current output from a single microelectrode is very low but can easily be amplified by the design of multiple electrode arrays [107]. A special case being interdigitated electrode arrays (IDA) where the current output can be amplified by redox cycling of an analyte between a pair of microbands [134]. Depending on the distance between the microelectrodes in the array, the current output is based on ideal multiple responses of a microelectrode (loosely packed electrodes) or macroelectrode behaviour (closely packed electrodes) as described in section. When employed in a flow-cell, lower flow rate fluctuation effects are generally observed when using a microarray. Moreover, by decreasing the size of the flow-cell, the inherent dead volume can be reduced [135]. Some examples of microarrays used in flow systems can be found in [136-138]. One drawback with miniaturization of sensor systems are that their overall performance is usually worse compared with their macroscopic counterpart [139], e.g. directed immobilisation of functional proteins on individual microscopic regions is still a challenge [133].

A working electrode manufactured by thick-film technology can generally be classified as a microarray assembly with randomly distributed electroactive spots. Such a microarray assembly structure was confirmed for screen-printed carbon based electrodes described in Paper III.

## 4.3 Electronic Tongues

In the human taste system various substances are sensed by cells collected in the taste buds on the tongue. Electrical signals are sent along nerve fibres to the brain, where the taste is recognized. An artificial “electronic tongue” tries to mimic the biological system and can be described as a detector based on an array of non-specific sensors where the obtained signals are further transferred to the “brain”, a computer containing a multivariate data analysis software to resolve and interpret the often complex response patterns. Figure 4.3 shows a schematic presentation of an electronic tongue [140]. Often the detection principles used in electronic tongues are electrochemical (potentiometric [141,

142] or voltammetric [143]). Traditionally, sensors have been constructed to possess a high specificity against a single analyte. In electronic tongues the opposite is favoured: poorly selective sensors increases the possibility to extract structural information from measurement data by the help of multivariate data analysis [17]. In most cases it is the simultaneous contribution from several different sensors that enables multivariate modelling of a property that can be qualitative, based on discrimination of samples according to e.g. different tastes [142] or stages in water clean-up processes [7], or quantitative, e.g. the determination of tartaric acid and other components in wine [141]. The first attempts to design electronic tongues were done in the late 1980's [144, 145]. Since then many articles can be found about electronic tongues mainly for application in food analysis but also for the environmental area [146-148].

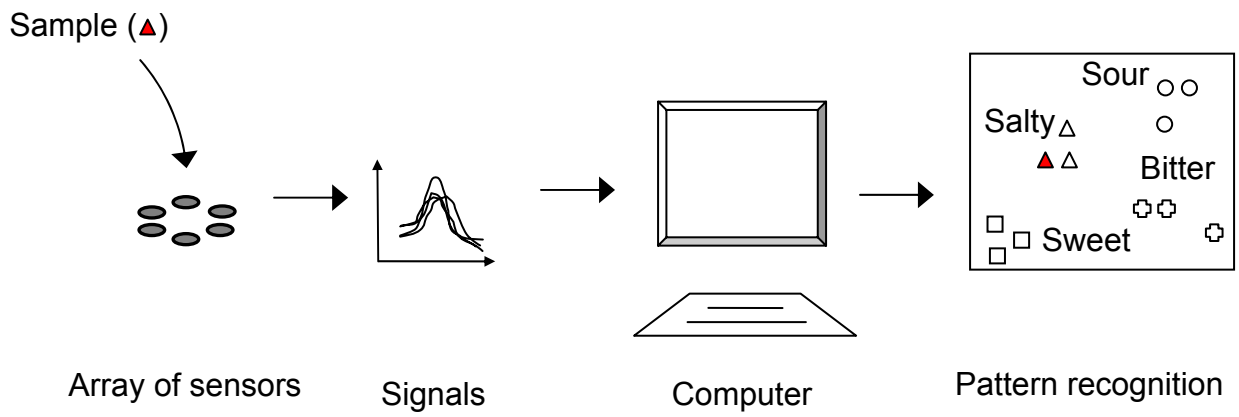


*Figure 4.3. Principle of an electronic tongue. An example is shown where the obtained signals from a salty sample are classified with a pattern recognition method.*

# 5 MULTIVARIATE DATA ANALYSIS

The term 'Chemometrics' was proposed in 1972 to describe procedures for mathematical and statistical interpretation of chemical data [149]. This chapter focuses on one of the chemometrics areas, specifically, on multivariate data analysis tools for pattern recognition using data from non-specific sensor arrays. The output from such a sensor array system can be rather complex and a direct interpretation is seldom possible. The obtained data can be seen as a sum of the *structural information* (which correlates to the searched property) and the *noise* (which describes everything else like instrumental noise, drift etc.) [150]. The structural information will at first be hidden in the raw data but can with multivariate data analysis be separated from the noise part. Among the most common multivariate methods to handle sensor array data are principal component analysis (PCA) and partial least squares regression (PLS-R). Another interesting new direction in analysis of sensor data is the use of multiway methods. Some information is also provided about artificial neural networks (ANN), a chemometric method using the learning processes in the human brain as model. However, before doing any multivariate analysis of sensor responses, pre-processing must be performed on the raw data to reduce the influence from noise that otherwise may totally obscure underlying structural information. A well-planned experimental design [150] prior to carrying out any experimental work, can in many cases, simplify the pre-processing procedure and the results obtained from multivariate data analysis will be more reliable.

The data matrix ($\mathbf{X}$) of sensor array responses to be used in PCA and PLS-R is two-dimensional and is generally arranged in an unfolded form where all sensor response variables for each single sample are placed after each other in a row in $\mathbf{X}$, as shown in Figure 5.1a. In multiway analysis (in this case three-way analysis), however, is the data arranged three-dimensionally with directions describing samples, responses and sensors (Figure 5.1b).

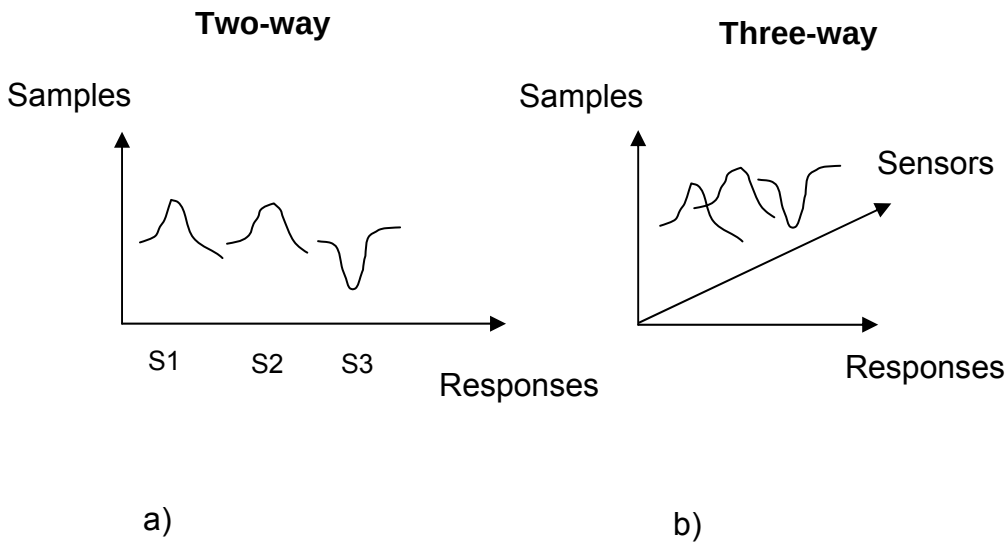


*Figure 5.1. Graphical presentation of sensor array responses a) unfolded in two-ways (where the responses from the sensors, S1-S3, are placed after each other in a row), and b) three-way (where the sensors are described in a third direction).*

## 5.1 Principal Component Analysis

In principal component analysis (PCA), a large multivariate data matrix is reduced into a matrix of smaller dimensions without losing any structural information [151]. The response data matrix (**X**) is built of objects (samples) and variables (sensor responses). The new matrix is a product of score vectors (**t**) and loading vectors (**p**) containing a simplified distribution pattern of the objects and the variables, respectively. PCA is mathematically described as follows.

$$\mathbf{X} = \mathbf{T}\mathbf{P}^{T} + \mathbf{E} \qquad (5)$$

Here **E** is the residual matrix containing the noise. The main idea with PCA is that the most structural information can be found in the same direction as the largest variation in **X**. This direction will comprise the first axis in the new coordinate system and is called the first principal component (PC1). The second principal component (PC2) is perpendicular to PC1 and is found in the same direction as the second largest variation in **X** and so on. This process can be repeated but usually only few components are needed to visualise hidden structural information and relations in data. A graphical presentation of PCA from three to two dimensions can be found in Figure 5.2. The data

points are projected onto the PCs to define the scores, i.e. the scores are the coordinates. The loadings describe how much the axes (or variables, as this is the same) in the old coordinate system contribute to each PC building up the new coordinate system. PCA is a useful method to qualitatively describing how samples and sensor responses are related to each other, using score and loading plots, respectively. Several examples where PCA has been used are found in Papers II, V and VI, and Manuscript VII.

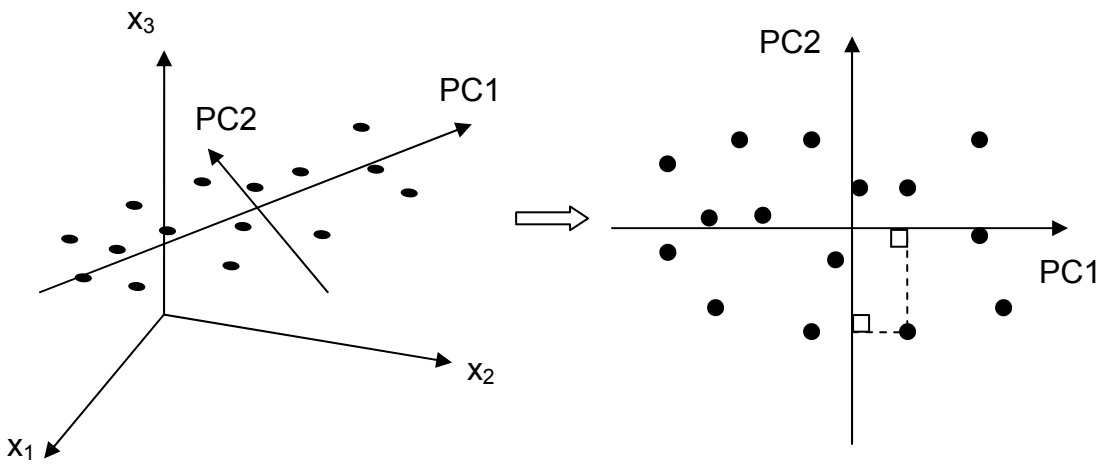


*Figure 5.2. Graphical presentation of PCA. The three-dimensinal response data (**X**) is decomposed to a two dimensions by PCA. The scores are the coordinates in the new coordinate system built of principal components (PCs).*

## 5.2 Partial Least Squares Regression

Partial least squares regression (PLS-R) is a multivariate calibration method that models how two data matrices, **X** and **Y**, are related to each other by regression [152, 153]. In case of sensor arrays, **X** often contains the measured responses whereas **Y** represents the exact concentrations of the samples. The aim with multivariate calibration methods is to solve equation (6), where **b** contains the calibration model parameters and **E** constitutes the residual matrix:

$$\mathbf{Y} = \mathbf{Xb} + \mathbf{E} \qquad (6)$$

In PLS-R, PCA models are calculated for the **X**- and **Y**-data, but not independently of each other. Properties such as maximum covariance between the two blocks as well as minimized residuals are obtained by using an inner relation based on interchanging of score vectors between the **X**- and **Y**-space during calculation. PLS-R is often used to find how a developed method is correlated to a standard method. The obtained PLS-R model can be validated with new samples (**X**), a so-called test set. The output is predicted y-values often validated with the average prediction error: the root mean square error of prediction (RMSEP), equation (7):

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{n}(y_p - y_m)^2}{n}} \qquad (7)$$

Here $y_p$ are the predicted values, $y_m$ are the measured values, and $n$ is the number of samples. If no additional test sets have been measured, cross-validation [154] is an alternative method for validation of an obtained PLS-R calibration model. Some samples are then excluded from the calibration and used for prediction. This is repeated until all samples have been used once. Paper II and Manuscript VII show results where PLS-R has been used for data analysis.

## 5.3 Multiway Analysis

Multivariate data analysis performed on unfolded two-dimensional data matrices (Figure 5.1a shows an example obtained from sensor array data) generally results in overfitted models. A solution to overcome such effects is to analyse the data when it is arranged in three ways. An example of how data from a sensor array can be arranged in three ways is exemplified in Figure 5.1b. A corresponding method for PCA in multiway analysis is PARAFAC [155]. The data cube ($\underline{\mathbf{X}}$) is decomposed in three loading matrices reflecting the samples, responses and the sensors, respectively. Advantages with PARAFAC compared to PCA are that the obtained solution is more robust and easier to interpret. Furthermore, a shift in either the sensor or sample mode, a not unusual event when dealing with data from sensor arrays, can be handled in a more efficient manner using the closely related method PARAFAC2 [156-158]. The multiway calibration method, N-PLS (multilinear PLS) [159], is an extended version of the two-way unfolded PLS. Models obtained with N-PLS are superior to

corresponding models based on the unfolded version. This is due to stabilization of the composition, which potentially gives increased interpretability and better predictions.

Our data were analysed with N-PLS. The obtained correlations against toxicity and COD were similar to results from two-way PLS. However, only few examples can yet be found about analysing sensor data with multiway methods [158, 160]. One of the reasons is that the user needs to have broad knowledge about linear algebra and should also be experienced in working with MatLab [161]. However, three-way PLS has recently become possible to perform in the commercial multivariate software Unscrambler (CAMO, Trondheim, Norway).

## 5.4 Artificial Neural Networks

The algorithms in artificial neural networks (ANN) model the learning and recognition processes in the human brain. The network structure is built of a number of units, neurons, that are arranged in parallel to form (usually three) layers: an input neuron layer, a hidden neuron layer(s) and an output neuron layer [162]. Each neuron in the hidden layer receives the same inputs (e.g. sensor responses). The sum of each input multiplied by a weight plus a bias is then passed through a transfer function (that can be linear or non-linear) to produce a result for that neuron. The neurons in the hidden layer further communicate with the output layer, which shows the results that can be qualitative (e.g. classifications) or quantitative (e.g. concentrations). An advantage with ANN is that even non-linear data can easily be modeled. However, usually a multitude of samples are needed to build up the network resulting in complex and often overfitted models. ANN have not been used in the work reported in this thesis but the method is very commonly employed when processing sensory signals.

## 5.5 Data Pre-Processing

Data pre-processing is frequently used to reduce variances derived from, e.g. shift in baseline, noisy data, etc., as well as to reduce and arrange data according to which multivariate method should be used. Many methods are possible such as mean-centering, scaling, smoothing, transformation, etc. This chapter discusses common pre-processing strategies dealing with data from sensor arrays: variable selection and drift correction.

### 5.5.1 Variable selection

Consider a flow-injection response that is built of 800 data points. For an array of eight sensors this resolves into 800 points × 8 = 6400 points. If 50 samples have been analysed the total amount of points is 6400 × 50 = 320000. As can be seen, the amount of data increases rapidly, and in many cases variable selection or reduction is necessary to perform on sensor array data. A simple strategy is to choose regularly placed data points along the detected responses ([163] and Papers II, VI and Manuscript VII). A risk with this method is, of course, if data points important for the results obtained from multivariate analysis are excluded. In some cases enough information is obtained by using, e.g. only the peak heights in further analysis. Other examples of how variables have been selected are when (i) the sensor responses were fitted with exponential functions and the obtained parameters were used as input variables in the multivariate software [164], or (ii) the RMSEP from a multivariate calibration model was used as the selection criteria for further analysis with ANN [165]. In both cases the results based on variable selection were at least as good as if the original data had been used in the chemometric model.

### 5.5.2 Drift correction

Systematic errors in sensor array systems commonly originate from drift, especially if measurements are performed over an extended time period. A multivariate model can be totally destroyed if there is too much drift in data since it is mainly the drift that is then modelled instead of the desired properties. The source of drift can be rather complex and be due to changes in the environmental conditions like temperature or pH, as well as to ageing or fouling of the sensors. A simple way to correct for drift is to adjust the baselines for each sensor in the array to the same level. However, additional corrections are needed if the sensor signal magnitudes have changed during analysis. A frequently used method for correcting drift due to decreased sensor sensitivity is multiplicative drift correction (MDC). In MDC the sample responses are corrected through multiplication by factors derived from an algorithm calculated from a curve fitting of references detected regularly during the analysis. However, a high signal-to-noise ratio is required when using MDC since there is otherwise a risk for errors due to inexact curve fitting. In another common method, multiplicative component correction (MCC), the drift correction is based on multivariate methods (PCA and PLS-R) [166-168]. The main idea with MCC is that the drift has a preferred direction in the multivariate space.

The correction is carried out by subtraction of the drift direction component of reference responses from the data. The method generally works well as long as the drift direction does not coincide with structural information in the data.

Drift correction (baseline correction and variants of MDC) has been applied in the work reported in all articles dealing with multivariate analysis, included in this thesis (Papers II, V-VI and manuscript VII).

# 6 APPLICATIONS OF BIOSENSOR ARRAYS

Several array systems have been reported consisting of different biosensors, where each separate sensor has been constructed to be as selective as possible against a single substrate [121, 169, 170]. This reflects a traditional view on how a biosensor should be used. The selectivity for many biosensors is, however, not always achieved, especially in cases when one carries out measurements in complex samples. Strategies to improve the selectivity of a biosensor include for example the application of membranes on the sensor that block interfering compounds [82] or the use of recombinant or mutant forms of the same enzyme that are less sensitive to interfering compounds ([171] and Paper I). A totally opposite approach in respect to the traditional view of biosensors is to actually take advantage of non-specificity. In this approach, the responses from biosensor arrays are regarded as a fingerprint of a sample. With multivariate tools for pattern recognition (discussed in chapter 5) the structural information hidden in the usually complex response patterns can be visualised. Such a system, consisting of biosensor array hardware equipped with chemometric analysis software, can then be denoted as a bioelectronic tongue. Furthermore, the possibility to vary biorecognition elements, immobilisation methods, electrode materials or applied potentials in amperometric biosensors increases the amount of analytes that can be detected. An amperometric biosensor system can then be tuned according to the kind of sample to be measured.

## 6.1 Overview of Reported Bioelectronic Tongues

Papers reporting design and applications of amperometric or voltammetric bioelectronic tongues are listed in Table 1. Most of the applications are found in food or environmental analysis. A majority of the publications presented in Table 1 concern simultaneous quantitative determination of analytes in synthetic binary or ternary mixtures [132, 163, 172-186]. The correlation coefficient is usually high (often around 0.99) and the relative prediction errors are in most cases less than 10%. The multivariate processing of data has been performed on responses from array systems built of different biosensors as well as on single sensors using the whole peak response, which can vary for different analytes. An example of how a substrate determination reliability using PLS-R on whole chronoamperometric data instead of regression on a single data point from the same data can also be found in Table 1 [172]. A similar idea was proposed for detection of formate using a polypyrrole

formate dehydrogenase sensor with ANN [173]. When it comes to real samples, quantitative determination is much less secure. An analysis method based on an enzyme array for detection of biogenic amines in food samples was correlated to high performance liquid chromatography measurements of the same samples with a correlation coefficient of 0.854 [174]. Moreover, binary mixtures of paraoxon and carbofuran showed prediction errors of 8% and 10% in synthetic samples using an array of four different AChE sensors whereas in spiked real samples the errors were 44% and 27% for the same compounds [175]. Few reports can be found about amperometric biosensor systems for qualitative characterisation, but examples include identification of microorganisms [176, 177] in synthetic samples or real sample analysis based on quality evaluation of peaches and nectarines [178].

*Table 1 Reported biosensor systems used together with chemometric methods for pattern recognition.*

| Measurements | Biological recognition elements | Detection principle | Chemometrics | References |
|---|---|---|---|---|
| Quantitative determination of mixtures of paraoxon and carbofuran in synthetic and spiked wastewater samples | Four types of AChE sensors | Amperometric | ANN | [175, 179] |
| Quantitative determination of ternary phenol mixtures | TYR membrane sensors | Amperometric | ANN | [180] |
| Quantitative determination of ternary phenol mixtures | One TYR sensor | Voltammetric | ANN | [132] |
| Quantitative determination of binary phenol mixtures | Two sensors, TYR and laccase | Amperometric | PLS-R | [181] |
| Quantitative determination of mixtures of glucose and ethanol | Two sensors, GOx and microbial | Amperometric | MLR* | [182] |
| Quantitative determination of mixtures of glucose and ethanol | Two microbial sensors | Amperometric | ANN | [183] |

*Table 1 continued*

| Measurements | Biological recognition elements | Detection principle | Chemometrics | References |
|---|---|---|---|---|
| Quantitative determination of binary and ternary mixtures of acetate, gluconate, L-threonine, L-serine, L-lactate and L-succinate | One microbial sensor | Amperometric | PLS-R | [163, 184, 185] |
| Quantitative determination of ascorbic acid and glucose in synthetic samples and fruit juice | Three types of GOx sensors | Linear sweep voltammetry | ANN | [186] |
| Quantitative determination of propionaldehyde | One aldehyde dehydrogenase sensor | Chronoamperometry | PLS-R | [172] |
| Quantitative determination of formate | One formate dehydrogenase sensor | Amperometric | ANN | [173] |
| Quantitative determination of histamine, tyramine and putrescine in food samples | Four sensors: diamine oxidase, monoamine oxidase, tyramine oxidase + blank | Chronoamperometry and steady-state | ANN | [174] |
| Qualitative evaluation of peaches and nectarins | Malic enzyme, GOx, fructose dehydrogenase | Amperometric | PCA | [178] |
| Qualitative identification of microorganisms | Ten different lectins bound to cell surface lipopoly-saccharide | Chronocoulometry | PCA | [176, 177] |

* Multiple linear regression (MLR) is a multivariate calibration method.

## 6.2 Bioelectronic Tongues Used in This Work

A summary of the results from the amperometric biosensor systems used in this work together with multivariate data analysis methods can be found in Table 2. Described in Paper II is how multivariate data analysis can be used to compensate for long-term drift. In addition, it was also shown that a calibration model from one sensor can be used to predict sample concentrations measured with a second sensor. The other two papers (V and VI) and manuscript VII report detection of analytes in real complex wastewater samples with screen-printed eight-electrode biosensor arrays. These measurements were performed in an electrochemical cell that can be used for steady-state as well as flow-injection analysis (Paper IV). The array is controlled by an eight-channel potentiostat that registers current responses to be processed with multivariate data analysis for qualitative as well as quantitative evaluation of the measured samples. Figure 6.1 shows an overview of the above-described bioelectronic tongue. Good correlations of sample responses against toxicity and COD, presented in manuscript VII, show the potential of using this type of device for fast determination of several environmental parameters simultaneously.

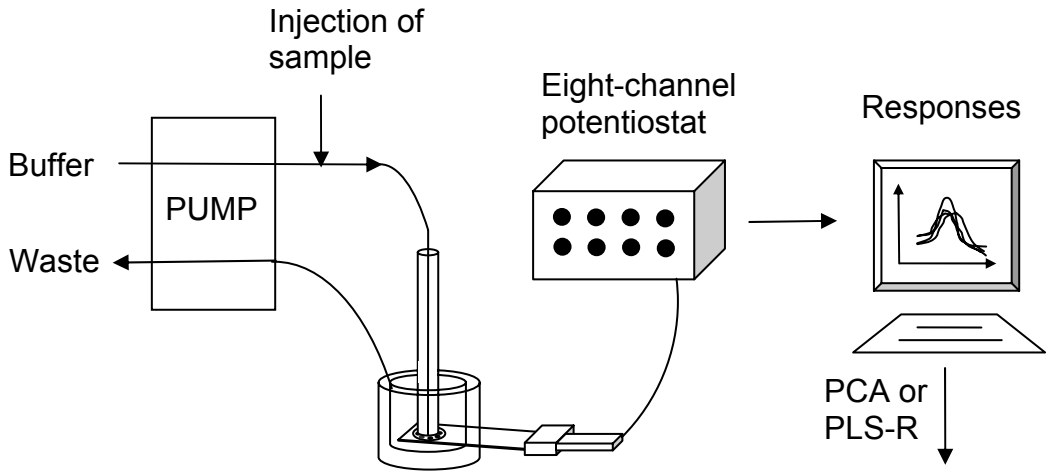


*Figure 6. The bioelectronic tongue used in our measurements based on FIA.*

*Table 2 Overview of the amperometric biosensor systems used together with multivariate analysis presented in this thesis.*

| Measurements | Biological recognition elements | Chemometrics | Papers |
|---|---|---|---|
| Quantitative determination of synthetic binary mixtures of *p*-Cresol and 4-Chlorophenol | One tyrosinase sensor | PLS-R | Paper II |
| Qualitative classification of wastewater samples from two different industries | Eight-electrode array based on oxidoreductases and hydrolases | PCA | Paper V |
| Qualitative differentiation of CTMP wastewater samples treated in a WWTP | Eight-electrode array based on oxidoreductases and hydrolases | PCA | Paper VI |
| Quantitative determination of toxicity, COD and BOD in CTMP wastewater treated in a WWTP using multivariate calibration on biosensor data | Eight-electrode array based on oxidoreductases and hydrolases | PLS-R | Manuscript VII |

# 7 CONCLUSIONS AND FUTURE PERSPECTIVES

The final prototype of the bioelectronic tongue for wastewater analysis has been constructed by focusing efforts on development of three critical parts of the analytical system: design of the biosensor array, construction of an electrochemical measurement cell, and implementation of effective signal processing of responses from the array.

Regarding the *design of a biosensor array*, the first challenge was to find suitable screen-printed electrodes enabling acceptable modification and functionalisation of the electrode surfaces by redox enzymes and hydrolases. We have shown that an electrochemical method (chronoamperometry) can be used to determine the electroactive electrode area of screen printed electrodes (the area not covered by insulating binder), which is important for realising the recycling mechanisms of cellobiose dehydrogenase modified electrodes as well as other phenol oxidising enzymes such as peroxidases and tyrosinases. During optimisation of biosensors a traditional cross-linking of enzymes at electrode surface with glutaraldehyde appeared to be a sufficient and adequately robust electrode modification protocol.

The choice of a radial configuration of the eight screen-printed electrodes in the array was motivated by the desire to limit cross-reactivity between the different electrodes in the array. This, however, demanded the creation of a special *electrochemical measurement cell* ensuring equal hydrodynamics at the position of each electrode in the array. Forced mixing of the solution with a mixing (rotating) rod centered above the array electrode was exploited for this purpose and was proven to be sufficient for obtaining reproducible signals from the electrodes in the array.

The last part of this work was focused on *signal processing of responses from the array* with multivariate data analysis. With the developed bioarray system it has been possible to characterise wastewater during different purification stages in a wastewater treatment plant as well as to correlate the obtained signals with parameters such as COD and toxicity. Drift problems were common when working with the biosensor array. To avoid systematic errors due to drift, a number of procedures have been proposed and tested for pre-processing of data before running the multivariate analysis.

The main advantage with the presented bioelectronic tongue lies in the rapid characterisation of complex samples and in the fact that several pollution and toxicity parameters can be determined simultaneously. The large number of existing individual biosensors available for environmental analysis also opens the possibility to construct new bioarray systems suitable for a wide range of samples that have to be analysed. The largest drawback with bioelectronic tongues, however, is the complexity of the overall analytical system. Continuous efforts must therefore be made to improve the robustness of the analytical technique as well as to develop effective pre-processing methods that can be used before or during multivariate processing of the sensor signals. However, if the analytical system develops to become more robust, few other analytical screening alternatives will be able to compete with the bioelectronic tongue in rapid characterisation or determination of chemical and biological parameters in complex, real-life samples.

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