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CRANFIELD BIOTECHNOLOGY CENTRE

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Development of a gas-phase biosensor for exposure measurement

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PhD Thesis

Abstract

This thesis describes the development and evaluation of a formic acid biosensor. The aim was to evaluate the possibility of developing a simple, inexpensive and specific gas-phase biosensor, which can be used for chemical exposure measurements.

Although there are methods for the sampling and analysis of many of the chemicals with threshold limit values, there are few exposure measurements done in occupational environments. Since such measurements are important to make accurate assessments of workers' exposure to hazardous chemicals, there is a need for simple and fast measurement methods.

The amperometric biosensor described is based on the enzymatic reaction between formic acid and formate dehydrogenase (FDH) with NAD^+ as a cofactor and Meldola's blue as mediator. An effective way to immobilise the enzyme, cofactor and Meldola's blue on screen-printed, disposable, electrodes was found to be in a mixture of glycerol and phosphate buffer covered with a membrane. The storage stability was investigated for up to 20 days and no significant decrease in response was found over this time.

The formic acid biosensor was evaluated using multivariate methods. Using experimental design, eleven factors that could influence the performance of the biosensor were examined. The response matrixes consisted of six parameters (steady state currents at three different formic acid concentrations and response rates during changes in formic acid concentrations) describing the performance of the biosensor. The data were evaluated using a combination of principal component analysis (PCA) and multiple linear regression (MLR). To confirm the conclusions from the PCA-MLR, partial least square (PLS) was also used. The most important factor for the biosensor performance was found to be the enzyme concentration. Using the information from the multivariate analyses the optimum

operation conditions for the biosensor were determined. The steady state currents were increased by 18 – 30 % and the initial two response rates were increased by 47 – 89 % compared with biosensors that had not been optimised.

The biosensor was also evaluated in regard to selectivity for formic acid in the presence of interfering compounds. It was found that acetic acid had a minor influence on the biosensor but that it was insensitive to methanol and formaldehyde. The use of the biosensor, as a monitoring device in an industrial setting was also evaluated, and it was found to perform satisfactorily. The field investigation did not show any signs of interference problems, and the limit of detection for the biosensor was calculated at 0.03 mg/m³.

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Notations

Abbreviations

AA	acetic acid
AcDH	alcohol dehydrogenase
ACGIH	American Conference of Governmental Industrial Hygienists
AdDH	aldehyde dehydrogenase
ANOVA	analysis of variance
AOD	alcohol oxidase
ATD	automated thermal desorption
<i>C1, C2, C3</i>	current responses (in the multivariate evaluation)
CE	capillary electrophoresis
cfu	colony forming units
CV	coefficient of variation
DCIP	2,6-dichloroindophenol
DMF	dimethylformamide
DNP	2,4-dinitrophenylhydrazine
<i>E</i>	electrochemical potential (in the multivariate evaluation)
EAG	electroantennography
FA	formic acid
<i>FDH</i>	concentration of FDHr (in the multivariate evaluation)
FDH	formate dehydrogenase (in general)
FDHr	formate dehydrogenase (recombinant)
FDHs	formate dehydrogenase (standard)
Fo	formaldehyde
FoDH	formaldehyde dehydrogenase
GC	gas chromatography
<i>Gly</i>	concentration of glycerol (in the multivariate evaluation)
GPES	general purpose electrochemical software
HPLC	high performance liquid chromatography
HSE	Health and Safety Executive
IC	ion chromatography
ISFET	ion-sensitive field-effect transistor
IUPAC	International Union of Pure and Applied Chemistry
<i>KCl</i>	concentration of KCl (in the multivariate evaluation)
LDH	lactate dehydrogenase
<i>MB</i>	concentration of Meldola's blue (in the multivariate evaluation)
MB^+	oxidised form of Meldola's blue
MBH	reduced form of Meldola's blue
Me	methanol
MLR	multiple linear regression
<i>NAD</i>	concentration of NAD^+ (in the multivariate evaluation)
NAD^+	nicotinamide adenine dinucleotide, oxidised form
$NADH$	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidised form
$NADPH$	nicotinamide adenine dinucleotide phosphate, reduced form
NIOSH	National Institute for Safety and Health

NIPALS	nonlinear iterative partial least squares
ORP	olfactory receptor proteins
OSHA	Occupational Safety and Health Administration
OVAT	one-variable-at-the-time
PC	principal component
PCA	principal component analysis
PE	polyethylene
<i>pH</i>	pH of the phosphate buffer (in the multivariate evaluation)
<i>PhC</i>	concentration of phosphate buffer (in the multivariate evaluation)
PID	photo ionisation detectors
PLS	partial least square
POD	peroxidase
PPO	polyphenol oxidase
PTFE	polytetrafluoroethylene
<i>R1, R2, R3</i>	rate responses (in the multivariate evaluation)
<i>Rh</i>	relative humidity (in the multivariate evaluation)
SOD	sulphite oxidase
SWEA	Swedish Work Environment Authority
SWEDAC	Swedish Board of Accreditation and Conformity Assessment
<i>T</i>	temperature of the biosensor (in the multivariate evaluation)
TMA	trimethylamine
TLV	threshold limit value
UV	ultraviolet
<i>v</i>	amount of the glycerol solution (in the multivariate evaluation)

Symbols

A_o	area of the diffusion tube orifice
A_e	electrode area
C	capacitance
C_c	concentration
C_0	vapour concentration at the collector surface
C_x	vapour concentration outside the diffusive sampler
D	diffusion coefficient
d	diameter of the orifice
dn/dt	rate of diffusion
dE/dt	potential change over time
i_p, i_c	current
L	length of the diffusion path
n	number of electrons transferred in the reaction
R	resistance
S	sampling rate
U	potential
v	scan rate
V_{input}	input potential
V_{output}	output potential
α	level of significance
$\partial c/\partial x$	concentration gradient of a compound in a diffusion tube

Publications

The thesis is partially based on the following publications:

Sandström, K. J. M. & Turner, A. P. F. (1999). Biosensors in air monitoring. *J. Environ. Monit.* **1**: 293-298.

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Sandström, K. J. M., Newman, J., Sunesson, A.-L., Levin, J.-O. & Turner, A. P. F. (2000). Amperometric biosensor for formic acid in air. *Sens. Actuators, B* **70**: 182-187.

Sandström, K. J. M., Carlson, R., Sunesson, A.-L., Levin, J.-O. & Turner, A. P. F. (2001). Multivariate evaluation of factors influencing the performance of a formic acid biosensor for use in air monitoring. *Analyst* **126**: 2008-2014.

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1 Introduction

1.1 Aim

The aim of this thesis was to investigate the possibility of developing an analyte selective and direct reading sensor that was suitable for exposure measurements in air. The particular focus was on the development and evaluation of an enzyme biosensor for formic acid.

1.2 Formic acid

Formic acid is the shortest of the carboxylic acids and constitutes melting and boiling points of 8.4 and 100.7°C, respectively, and a vapour pressure of 4.7 kPa at 20°C. It has an important role in the conservation of animal feed as it can be used instead of other more environmentally hazardous chemicals (e.g. antibiotics). Therefore, an increase in production of formic acid is expected (Engelmark Cederborg, 2001). Exposure to formic acid is principally a problem for farmers during silage making where it is used in large amounts (Liesivuori and Kettunen, 1983). It has also been measured in various other locations such as the atmosphere (Kawamura *et al.*, 1985), museum environments (Gibson *et al.*, 1996; Gibson *et al.*, 1997a), and exhaust emissions (Kawamura *et al.*, 1985; Spicer *et al.*, 1991).

1.2.1 Biological effects

Since formic acid is an acid it gives rise to irritation in respiratory systems and eyes for exposed persons. The toxicological effects of formic acid have been reviewed (Liesivuori and Savolainen, 1991). Exposure to formic acid leads to accumulation of acid in the body and it acts as an inhibitor of the cytochrome oxidase complex in the mitochondria. Optical nerves, brain, heart and kidney were found to be particularly sensitive. Formic acid poisoning can not only be caused by exposure to formic acid but also by exposure to methanol, which is metabolised into formic acid in the body. Indoor air guidelines for low-molecular-weight carboxylic acids have been proposed where the effects of exposure are divided into four categories (Nielsen *et al.*, 1998):

1. annoyance due to odour
2. sensory irritation (eye and nose)
3. health effects (all other non-genotoxic effects)
4. genotoxic carcinogen effects

The proposed guideline value for health effects from formic acid was 0.3 mg/m^3 . The sensory irritation exposure limit estimate was 2 mg/m^3 and the estimated odour threshold was 53 mg/m^3 . For comparison, the 8 hour exposure limits for occupational environments has been set at 9.4 mg/m^3 (5 ppm) by the American Conference of Governmental Industrial Hygienists (ACGIH) in the USA (ACGIH, 2001), and by the Health and Safety Executive (HSE) in the UK (HSE, 2001), and at 5 mg/m^3 (3 ppm) by the Swedish Work Environment Authority (SWEA) in Sweden (Arbetskyddsstyrelsen, 2000).

One way to determine the exposure of formic acid is by biological monitoring. It has been performed by measuring the excretion of formic acid in urine at different times after exposure. The urinary formic acid excreted after 30 hours has been correlated to the measured formic acid vapour concentration (Liesivuori *et al.*, 1992). However, formic acid excreted in urine also may be an indicator of exposure to methanol, as mentioned earlier.

1.2.2 Methods used today

1.2.2.1 Analysis

A review concerning the determination of low-molecular-weight carboxylic acids was recently published (Dabek-Zlotorzynska and McGrath, 2000). The main areas that were covered were ambient environments and vehicle emissions. The concentrations of carboxylic acids were found to be low or sub-ppb for rural areas, and somewhat higher in urban areas. The collection of carboxylic acids during sampling was mainly based on alkaline-coated materials, e.g. glass fibre filter impregnated with potassium hydroxide. However, it had been found that

these sampling methods give interferences due to side reactions on the alkaline-treated media. Since formic acid and other low-molecular-weight carboxylic acids are soluble in water, it is often convenient to use water as an extraction media. Other extraction methods are also used especially when acids are derivatised for analysis using gas chromatography (GC). Most of these extraction methods do not involve formic acid but rather acetic acid and higher acids.

The extraction methods are of course dependent on which analytical method will be used. The most commonly used analytical methods are GC and ion chromatography (IC). Capillary electrophoresis (CE) has lately become a popular method for analysing carboxylic acids. GC often achieves low detection limits and also has the advantage that it can easily be connected to a mass spectrometer for better qualitative information. However, GC usually requires derivatisation, which complicates the procedure, and many of the GC methods also seem to exclude formic acid.

IC has a more straightforward approach when it comes to extraction of the samples. The media for extraction is usually water and the samples can be directly injected into the IC system. Using IC in combination with suppressed conductivity detection also achieves lower detection limits than using UV-detection, which has traditionally been used.

When using CE as an analytical method it is also favourable to use water as an extraction media. Since CE is a fairly new technique it has not yet been widely adopted as a method to use in routine analysis. However, it does have the advantage that it uses a very small (nanolitre) sample size.

1.2.2.2 Sampling

The National Institute for Safety and Health (NIOSH) and the Occupational Safety and Health Administration (OSHA) in the USA have produced standard methods for sampling and analysing formic acid (OSHA, 1993; NIOSH, 1994).

The NIOSH method utilises silica based adsorption tubes for sampling and has been evaluated for sampling formic acid for between 1 and 24 l air samples at sampling rates of 50 to 200 ml/min. The samples are desorbed in boiling water for 10 minutes. Analysis is performed using suppressed IC with a conductivity detection and a 2.5 mM Na₂B₄O₇ eluent. The NIOSH method was the base of the reference method for analysing formic acid in this thesis; modifications made to the method are mentioned in Chapter 2.

The OSHA method uses charcoal tubes for sampling instead of silica tubes but otherwise the methods are quite similar. The recommended air volume and sampling rate are set to maximum of 48 l and 200 ml/min, respectively.

Only one diffusive sampler for formic and acetic acid has been found in the literature. It is based on the Palmes diffusion tube and is validated and used for monitoring formic and acetic acids in museum cabinets (Gibson *et al.*, 1997b; Gibson *et al.*, 1997c). It consists of an open tube with a NaOH/glycerol impregnated filter at one end. The acids diffuse through the tube and are collected on the filter. Since the sampling rates for this sampler was quite low (1.02 ml/min for formic acid and 0.88 ml/min for acetic acid) the sampling was performed over a period of two weeks. The analyses were performed with IC and the results showed that there were substantial amounts of formic and acetic acid emitted from show room cases based on wood.

1.3 Exposure monitoring

Exposure monitoring can be used to assess workers chemical exposure and, thereby, to establish if governmentally determined guidelines are followed. It can also be an efficient tool when incorporated into companies' quality assurance policies and regular internal controls. Data from these kinds of measurements are also necessary to improve epidemiological studies of occupational exposures and for risk assessment (particularly for detecting low risks) (Ulfvarson, 1995;

Kauppinen, 1996). Therefore, sampling and analysis methods must be simplified in order to encourage more measurements to be performed in the workplace.

1.3.1 Personal exposure measurements

Personal exposure measurements can be performed as part of exposure monitoring. The main reason for this is to achieve as accurate an estimation of the chemical exposure as possible. For instance, if we compare personal exposure measurements with stationary measurements it is logical to assume that stationary methods require a large number of assumptions and calculations in order to assess a person's exposure level. Also, they likely require a thorough investigation of the workplace in question and how the work is carried out, how long certain procedures take, how the air flows in the building, the number and length of breaks, etc. Therefore, the results from such measurements may contain a large number of uncertainties. However, the results from personal exposure measurements are directly related to the amount of chemicals to which the person has been exposed.

Personal exposure measurements are well suited for occupational environments where the risk of exposure to chemicals is much higher than, for example, in home environments. However, they can of course be used in many types of environments, and the problems in most cases lie in finding the right sampling strategy depending on the purpose for which it is to be used.

Personal exposure measurements are performed by measuring chemical compounds in the breathing zone, that is as close to the mouth as possible. There are a number of ways to perform these measurements. Methods used for exposure measurements, personal or otherwise, are usually based on a two-step procedure - sampling followed by analysis. The sampling step can either be performed by using active sampling or by using diffusive sampling devices. The diffusive samplers have the advantage of being less invasive to the workers' performance

during sampling. The sampling is then followed by an analysis, which is typically performed using high performance liquid chromatography (HPLC) or GC.

1.3.1.1 Active sampling

Active (pumped) sampling utilises a pump to draw an air sample through an adsorbent tube or a filter on which the analyte is collected. Since pumps are used a method to determine the correct airflow through the sampling tubes or filters is also required. This is usually done by measuring the flow in the beginning and at the end of the sampling period by using some type of flow meter, e.g. rotameter. Therefore, more equipment has to be transported to the sampling site during active sampling. However, it does have the advantage that the flow rate, through the sampling tube, can be varied depending on the sampling time and the estimated concentrations that are measured. The possibility of high sampling rates also yields shorter sampling times. Another advantage is that the flow rate and thereby the rate of collection of analytes is known. When using diffusive sampling, individual uptake rates have to be determined to make accurate assessments of the concentrations of the sampled compounds.

1.3.1.2 Passive sampling

Passive (diffusive) sampling uses the technique of diffusion instead of pumps to collect samples. Research concerning diffusive sampling has been performed for decades, and there are a number of different types of diffusive samplers. Below are a few examples.

- *Palmer diffusion tube* (Figure 1.1a) is based on a cylindrical tube with a coated filter in the bottom of the tube (Gibson *et al.*, 1997b).
- *GMD sampler* (Figure 1.1b) has a glass fibre filter impregnated with 2,4-dinitrophenylhydrazine (DNP) which reacts with aldehyde to give a compound easily analysed using HPLC-UV (Levin and Lindahl, 1994). Other coating materials have also been used for other compounds.

- *Perkin Elmer ATD diffusive sampler* (Figure 1.1c) contains an adsorbent in which the sampled compounds are adsorbed and then analysed using automated thermal desorption (ATD)-GC (Sunesson *et al.*, 1999).
- *SKC 530-series* (Figure 1.1d) uses a micro-porous membrane as diffusion barrier (no longer commercially available).

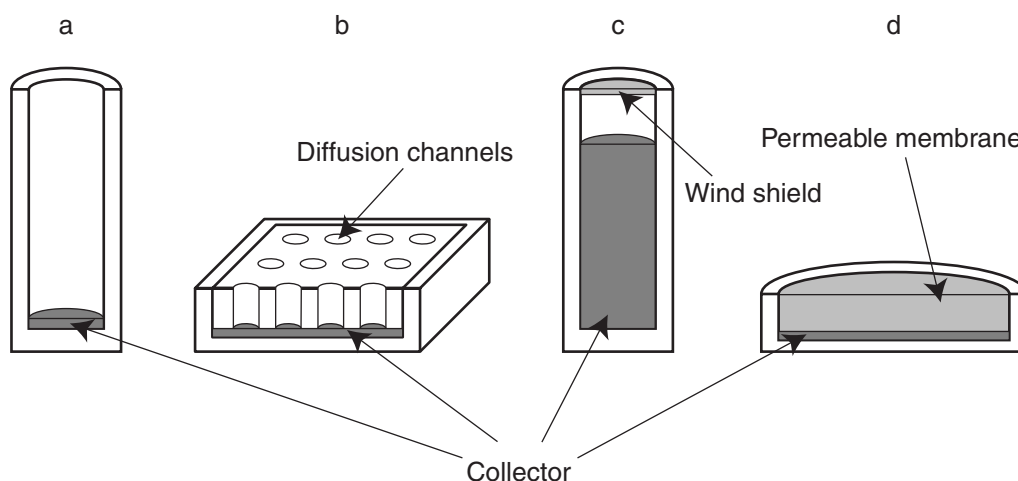


Figure 1.1: Schematic pictures of different types of diffusive samplers. *Palmes* diffusion tube (a), GMD sampler (b), ATD diffusive sampler (c) and SKC 530-series (d).

The theoretical calculation of the rate of diffusion, dn/dt (mol/s) is given by Fick's first law of diffusion:

$$\frac{dn}{dt} = -DA_o \frac{\partial c}{\partial x} \quad (1.1)$$

where D is the diffusion coefficient for the compound (m^2/s), A_o is the area of the diffusion tube (m^2) and $\partial c/\partial x$ is the concentration gradient of the compound in the diffusion tube (mol/m^4).

Since the samplers mentioned above are shaped like cylindrical tubes and since the concentration within a tube falls linearly, when the concentrations on each side of the tube are C_x and C_0 (mol/m³) the following equation applies:

$$-\frac{dc}{dt} = \frac{C_x - C_0}{L} \quad (1.2)$$

Combining equation (1) and (2) gives:

$$\frac{n}{t} = DA_o \frac{C_x - C_0}{L} \quad (1.3)$$

$C_x - C_0$ implies diffusion through a tube from one concentration to another. However, for the diffusive samplers the compound is collected at the end of the tube which yields $C_0 = 0$ and thereby:

$$\frac{n}{t} = DA_o \frac{C_x}{L} = SC_x \quad (1.4)$$

where $S = DA_o/L$ is the sampling rate, usually recalculated and expressed in ml/min.

The sampling rate is thereby dependent on the area of the tube orifice and the length of the diffusion path (L). To increase the sampling rate the diameter of the orifice (d) has to be increased and/or the diffusion path has to be shortened. If the orifice is completely opened there is a limit to how low the L/d ratio can be before uncontrolled air movement influences the sampling rate. This limit is approximately 3 (Lautenberger *et al.*, 1980) but can be overcome by using the techniques employed by the examples above: a large number of diffusion channels (GMD) or windshields; such as a metal grid (Perkin Elmer). Another alternative is to use a gas permeable membrane to control the mass transport into

the sampler (SKC 530). To determine the sampling rate of a diffusive sampler, a comparison with another validated sampling method is conducted. The sampling rate can be calculated theoretically but this would require ideal conditions, which cannot be achieved in a real sampling situation. The determination of the sampling rate is, therefore, determined empirically. The difference between the theoretical and the empirical value of the sampling rate can be attributed mainly to the equilibrium between air and the collector (see Figure 1.2). Ideally the concentration of the analyte is zero at the surface of the collector, which is the case when reactive compounds are sampled with chemisorption (Levin and Lindahl, 1994). However, when the analyte does not react or adsorb instantly at the surface, the concentration will be greater than zero. The equilibrium at the surface is dependent on, for example, the type of collector, the analyte and the temperature. In practice it could be seen as different sampling rates when an analyte is sampled with the same type of sampler but with different types of adsorbents (Sunesson *et al.*, 1999).

Today, diffusive sampling has become an important tool for assessing occupational exposures to chemical compounds. Most recently it has also become a tool to map outdoor environments for air-borne chemicals. Diffusive samplers can easily be placed at different sites in an area of interest, e.g. town centre or countryside. The importance of diffusive sampling is also expressed by the international conferences dedicated explicitly to this subject (Berlin *et al.*, 1987). At the conference “Measuring air pollutants by diffusive sampling” in Montpellier, France in 2001 a large number of the presentations dealt with environmental mapping of air pollutants by diffusive sampling. This trend is not surprising since the ease of use associated with this sampling technique does not restrict it to occupational environments. The future might even see samplers that will make it possible for the general public to assess their chemical exposure.

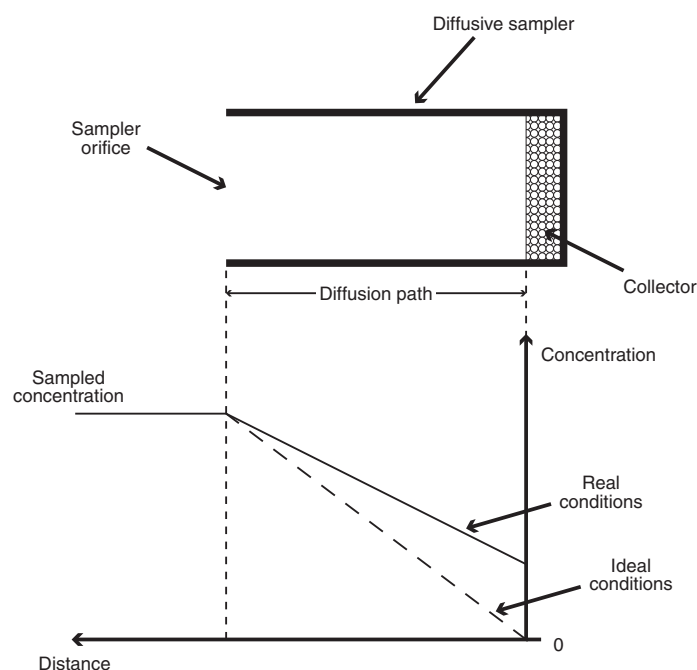


Figure 1.2: *Diffusive sampler. The difference between the ideal and the real conditions is caused by the equilibrium between air and collector close to the collector surface. Since the concentration of a compound is more than zero, close to the surface, it is difficult to determine the sampling rate of a diffusive sampler theoretically. Hence, an empirical determination is required.*

However, the methods used today based on diffusive sampling do have a small drawback, as does the active sampling method. As mentioned above, these methods are generally two step procedures that require separate sampling and analysis. Therefore, when the samplers are analysed in a separate procedure, both the cost and the time between the sampling and the result from the analysis are increased.

To avoid this drawback direct-reading instruments can be used. There are a number of direct-reading instruments that are suitable for exposure measurements, e.g. photo ionisation detectors (PID) and electrochemical sensors. However, these instruments use techniques that can not measure different compounds selectively, e.g. PID detects mostly organic vapours and gases that can be ionised by photo

ionisation. The electrochemical sensors have better selectivity than the PID but still there are a number of compounds that can give an electrochemical response (Siekmann and Kleine, 1992).

In order to perform sampling as easily as with diffusive sampling, and to receive the response quickly as with a direct-reading instrument, an obvious possibility would be to combine the two approaches. However, this does not overcome the problem of non-selectivity. There have been attempts to produce “portable” GCs aimed at getting highly selective and portable instruments. However, so far these have proved too invasive to the workers performance to be used on a larger scale. Another way to achieve selectivity in a direct reading sampler is to harness the selectivity of biological systems, i.e. construct a biosensor.

1.4 Biosensors

The concept of a biosensor has been defined by the International Union of Pure and Applied Chemistry (IUPAC) as an integrated receptor-transducer device, which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element (Thévenot *et al.*, 1999). Research concerning biosensors has been performed since the 60s with the first paper published in 1962 by Clark and Lyons (Clark Jr. and Lyons, 1962). The paper described the use of glucose oxidase to convert the concentration of glucose into a measurable concentration of oxygen, which was measured with an oxygen electrode. Much of the earlier biosensor research focused on medical applications, and sensors for self-assessment of blood glucose have been particularly successful in a commercial sense. Today, the principal focus for research is still medicine, but other fields, such as the food industry (White and Turner, 1997), the environment (Turner and Bilitewski, 2000) and defence against chemical and biological warfare (Iqbal *et al.*, 2000) also receive considerable attention. Biosensors have also been developed to be used in different media, e.g. aqueous, organic and gas phases.

1.4.1 Electrochemical biosensors

Electrochemically transduced biosensors are widely used and have been thoroughly investigated over the years. Voltammetry is an electrochemical method that implies control of the voltage and measurement of the resulting current. Amperometry is probably the most commonly used and simplest form of voltammetry. This means that the voltage is held at a constant value while the current is measured. Other voltammetric techniques, e.g. linear sweep, cyclic and square wave voltammetry, are commonly used to characterise biosensor systems.

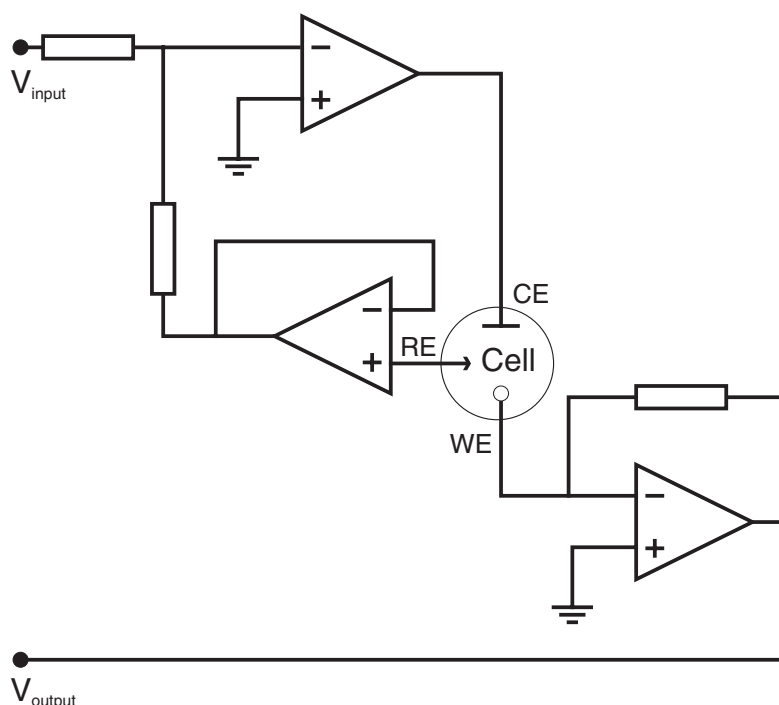


Figure 1.3: Basic potentiostat circuit where CE is the counter electrode, RE is the reference electrode and WE is the working electrode.

The equipment needed for amperometric measurements is a simple potentiostat (Figure 1.3), where the potential across the electrochemical cell is the input potential (V_{input}). Since the operational amplifier at the working electrode is used as a current follower the output (V_{output}) is proportional to the resulting current.

The applied voltage causes oxidation or reduction to take place at the electrode surface.

One of the most useful techniques in the design and development of electrochemical biosensors is cyclic voltammetry. The reversibility of potential mediators used in biosensor construction can be readily deduced from the separation between anodic and cathodic peak potentials, i.e. $\Delta E_p = 59/n$ mV for a totally reversible process. In such cases the magnitudes of the anodic and cathodic peak currents is the same i.e. $i_{pa}/i_{pc}=1$. The peak current resulting from the Faradaic process is given by the Randles-Sevcik equation:

$$i_p = (2.69 \times 10^5) n^{3/2} A_e C_c D^{1/2} \nu^{1/2} \quad (1.5)$$

where i_p is the current, n is the number of electrons transferred in the reaction, A_e is the electrode area, C_c is the concentration in the cell, D is the diffusion coefficient and ν is the scan rate.

The resulting current is also affected by how the ions behave close to the electrode surface. This effect causes a so called “electrical double layer” and has the behaviour of a capacitor according to:

$$i_c = C \frac{dE}{dt} \quad (1.6)$$

where C is the capacitance and dE/dt is the potential change over time.

1.4.1.1 Manufacturing

A commonly used method for manufacturing electrochemical electrodes for biosensors is screen-printing. It is conventionally used in the graphics industry but has become an important way of producing electrode systems for biosensors. The technique promotes low cost and mass production of electrodes, which also

enables the electrodes to be disposable. Epoxy or vinyl resin base inks are deposited on plastic base materials in a number of steps to achieve the desired design. Other materials are also used, e.g. aluminium oxide ceramic base materials combined with boro- or aluminosilicates. Other ways of manufacturing electrochemical sensors for biosensors are electrochemical deposition, polymerisation, photolithography and nano techniques (Zhang *et al.*, 2000).

1.4.2 Optical biosensors

Biosensors based on transduction using electromagnetic radiation are called optical biosensors. It is another large group of biosensors and utilises a number of different optical phenomena, e.g. light absorption, luminescence, evanescent field, surface plasma resonance. This type of biosensors also has a promising future due to modern fabrication techniques. This renders possible for inexpensive and small optical biosensors (Gauglitz G., 2000). Other types of transduction systems are for instance piezoelectric, thermal and magnetic transducers.

1.4.3 Biosensors in air monitoring

Many attempts have been made to produce biosensors with characteristics suitable for air monitoring, and research shows that they are important tools that can successfully be used for this purpose. A biosensor designed for air monitoring was first published in 1974 in a paper by Goodson and Jacobs (Goodson and Jacobs, 1974). It was a sensor for toxins that inhibit the cholinesterase enzyme. The sensor was operated by pumping air along with a reagent solution through an electrochemical cell with an enzyme pad between two electrodes. The pad contained cholinesterase, which is inhibited by some chemicals. If the air sample contained a cholinesterase inhibitor, competition between the reagent and the inhibitor occurred. Since the reagent produced an easily oxidised product after reaction with cholinesterase, and the inhibitors did not, the measured potential increased when an inhibitor was present. The sensor was produced for both air and, with some modifications, water sampling; it could be used for 8 hours without changing the enzyme pad.

1.4.3.1 Methane

Another early biosensor for air monitoring used a reactor containing immobilised methane-consuming micro-organisms to measure methane in air. The technique was described by Okada and co-workers in two similar articles (Okada *et al.*, 1981; Karube *et al.*, 1982). The sample gas was pumped through the reactor and on to an oxygen electrode. A reference reactor was also used to monitor the oxygen concentration in a reactor without sample. Microbial metabolism of methane requires oxygen resulting in a decreased concentration of dissolved oxygen, which was monitored by the oxygen electrode. The sensor had a response time of 1 minute and was said to give a constant response for 20 days. The minimum concentration of methane in air that could be detected was calculated at 13.1 μM and the linear range was up to 6.6 mM. No interfering agents were examined as the micro-organisms were said to use methane as their only source of energy.

1.4.3.2 Carbon monoxide

An enzyme-based carbon monoxide sensor was described by Turner and co-workers (Turner *et al.*, 1984; Turner *et al.*, 1985). The biosensor was based on the oxidation of carbon monoxide (CO) to carbon dioxide (CO₂) by the enzyme carbon monoxide oxidoreductase. CO oxidoreductase was placed on a conducting gel and covered with a membrane. The conducting gel consisted of graphite, mediator and liquid paraffin, and was in contact with a platinum electrode. The gas permeable membrane was used to keep the enzyme at the surface and to make it possible for carbon monoxide to pass through to the enzyme. 1,1'-dimethylferrocene was used as a mediator and was oxidised at the electrode surface at 150 mV versus Ag/AgCl. The electron flow of the system can be seen in Figure 1.4. The amperometric response reached a steady-state current in less than 15 s but the current output from the device decreased by 12 % per hour. Most of the experiments reported, however, were performed in solution.

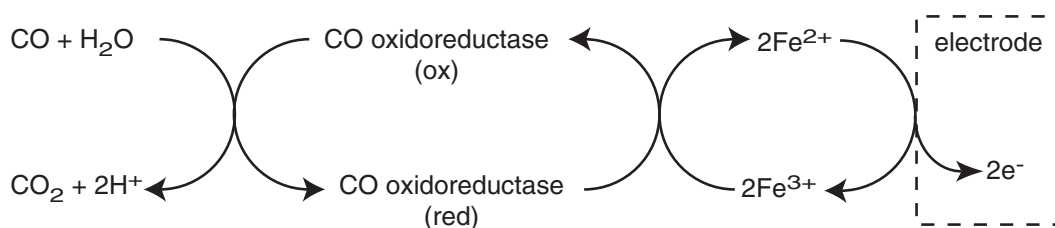


Figure 1.4: *Electron flow diagram of the CO biosensor.*

1.4.3.3 Formaldehyde

Monitoring formaldehyde is of great importance since it is widely used in industry. It is also a known irritant and carcinogen. In 1983, Guilbault described a biosensor for the determination of formaldehyde in air using formaldehyde dehydrogenase (FoDH) coated on a piezoelectric crystal (Guilbault, 1983). The piezoelectric crystal technique has been widely used in the construction of biosensors and is based on crystals that oscillate at a certain frequency when they are exposed to an alternating voltage. In air, if the mass on the crystal changes, a shift in the frequency can be observed. Biological material can thereby be attached to the surface to create a piezoelectric biosensor and the change in, or adsorption to, the biological material monitored by measuring the frequency shift. However, the oxidation of formaldehyde to formic acid catalysed by FoDH necessitated the presence of NAD⁺ and reduced glutathione. According to Guilbault, the stability for this device was 3 days or 100 analyses but this could be increased to 10 days if the enzyme and cofactors was chemically bound to the crystal surface. However, this was not recommended since the crystal in this case would not be reusable. The response to formaldehyde in air was linear from 10 ppb to 10 ppm. The test atmosphere was generated by syringe injection of a known volume of gas into a controlled airflow, and validated by formaldehyde sampling tubes and fluorometric analysis. The biosensor was specific to formaldehyde. No significant interference was seen from other aldehydes or alcohols. It is notable that no further development of this biosensor has occurred. However, other researchers have used FoDH in devices to monitor formaldehyde in air.

FoDH was one of the enzymes used in the diffusion badges developed by Rindt and Scholtissek (Rindt and Scholtissek, 1989). They used various enzymes lyophilised onto sinterglass rods put into vessels containing buffer-reagent solutions and covered with gas permeable membranes. The diffusion badges contained buffer solutions to overcome the problem of drying. Since the device was constructed from two parts, the glass rod with enzyme and the vessel with buffer-reagent solution, they could be stored separately. The enzyme could be stored dry, which increased the storage time. In addition to formaldehyde, the compounds analysed using this type of construction were hydrogen peroxide, acetaldehyde and ethanol and the enzymes used were diaphorase, aldehyde dehydrogenase (AdDH), alcohol dehydrogenase (AcDH) and horseradish peroxidase, respectively. The reaction between analytes and enzymes caused a dye to change colour. This colour change was documented photographically and the colour was stable for several hours after exposure. The gas mixtures, used to test the badges, were generated with a perfusion vessel at controlled temperatures and verified with different types of enzymatic reactions. The constant flow of reagent-buffer to the top of the device not only kept it moist but also concentrated the enzyme at the top of the glass rod, because the water slowly but constantly evaporated through the gas permeable membrane. The technique used in this device was later developed into a commercial product called Bio-Check F (Drägerwerk AG, Lübeck, Germany) (Rindt *et al.*, 1992). The Bio-Check F is used for quantitative measurements of formaldehyde and requires visual comparison with a colour code that is received with the device.

In 1996, three biosensors for monitoring formaldehyde in air were described. Hämmerle and co-workers described a biosensor based on an electrochemical cell divided into two parts by a dialysis membrane to prevent migration of the enzyme (Hämmerle *et al.*, 1996). FoDH was put on the working electrode and prior to sampling the cell was filled with electrolyte containing cofactor and mediator. Since the electrolyte was not added until the time of sampling the device could be

stored for a long time. The device was tested in a controlled atmosphere by measuring the equilibrium gas-phase above an aqueous formaldehyde solution and the limit of detection was determined to 0.3 ppm. A linear response was achieved up to 6 ppm using steady state measurements and the range was improved when initial rate data was used. The device could also be used for 7 hours without any loss of activity.

Another biosensor for formaldehyde was described by Dennison and co-workers who utilised enzymes and cofactors immobilised in a reversed micelle medium on screen-printed electrodes (Dennison *et al.*, 1996). The biosensor used FoDH for the analysis of formaldehyde but a similar construction was also used to analyse alcohols with AcDH. The reoxidation of NADH to NAD^+ was measured amperometrically at 0.8 V versus Ag/AgCl. The reversed micelle medium was used to prevent water loss as the silicone oil acted as a barrier against evaporation. The biosensor was found to be suitable for gas-phase sensing when it was tested in controlled atmospheres. Formaldehyde permeation tubes and ethanol diffusion vials, connected to a gas rig, were used to create the atmospheres. The gas concentrations were calibrated using Dräger tubes or by measuring the loss of sample gravimetrically. The linearity of the biosensors were estimated to be 1.3 ppb-1.2 ppm for formaldehyde and 50-250 ppm for ethanol and the biosensor could be stored for 60 hours at 4°C without a decrease in response.

An ion-sensitive field-effect transistor (ISFET) was used by Vianello and co-workers in their biosensor for formaldehyde measurement (Vianello *et al.*, 1996). The ISFET monitors H^+ produced when formaldehyde is oxidised by FoDH with NAD^+ as a cofactor. The formaldehyde was removed from the atmosphere by pumping air through a glass coil along with an aqueous solution. The solution dissolved the formaldehyde and acted as a carrier of the formaldehyde to the ISFET. A membrane containing FoDH covered the ISFET and the solution containing formaldehyde was transferred directly to the surface. The enrichment factor of this sampling technique was determined at 8000 times but there were

some problems with the immobilisation of the enzyme, which complicated the evaluation of the sensor.

1.4.3.4 Ethanol

Detecting ethanol in air has an important application in analysing breath alcohol. Barzana and co-workers developed a device, which changes colour when it is exposed to ethanol (Barzana *et al.*, 1989). The detection was based on a visual observation of a colour change. This gives a crude indication of the amount of alcohol in the breath. For quantification, the device was tested using a densitometer for a more precise determination of ethanol vapour. The device was constructed by adding alcohol oxidase (AOD), peroxidase (POD) and 2,6-dichloroindophenol (DCIP) to microcrystalline cellulose. The ethanol was oxidised by AOD, and acetaldehyde and hydrogen peroxide were produced. The hydrogen peroxide reacted with POD and at the same time the reduction of DCIP caused a colour change in the device. To make the device simple and fast to use it was optimised to give a sharp colour change after 1 minute if the ethanol concentration was over the legal limit for driving. Since AOD has the ability to oxidise formaldehyde, it can also be used to detect this compound. However, for this application both methanol and ethanol would be serious sources of interference.

A sensor for determination of alcohol and sulphur dioxide in air was described by Matuszewski and Meyerhoff (Matuszewski and Meyerhoff, 1991). It was mainly constructed for continuous electrochemical detection of hydrogen peroxide (H_2O_2). By dissolving gaseous H_2O_2 into a buffer solution using coiled tubing with an internal buffer flow, the H_2O_2 concentration could be measured when the buffer was pumped over an electrochemical cell. By adding an enzyme reactor containing H_2O_2 -producing enzymes, such as AOD or sulphite oxidase (SOD), prior to the electrochemical cell, dissolved alcohol or sulphur dioxide could be detected. To increase the sensitivity of the continuous flow measurements a stopped-flow approach was investigated. The buffer flow through the coiled

tubing was stopped for a certain time, which accumulated the compounds of interest during sampling. The ethanol and sulphur dioxide atmospheres were generated with a permeation tube and a commercial gas emitter, respectively, and diluted with air. The limit of detection was calculated for sulphur dioxide as 0.50 ppb with continuous flow and 0.15 ppb for 2 minutes at stopped-flow, and for ethanol as 1.0 ppb with continuous flow and 0.5 ppb for 2 minutes at stopped-flow. After storage for 2 weeks the SOD reactor lost more than 50 % of its activity while the AOD reactor kept its activity for more than a month.

Mitsubayashi and co-workers constructed another type of a biosensor for analysis of ethanol in air (Mitsubayashi *et al.*, 1994). A reaction cell consisting of both gas- and liquid-phase compartments separated by a diaphragm membrane was used in the sensor. AOD was immobilised in a cross-linked acrylamide gel and placed on a Clark-type oxygen electrode and covered with a polycarbonate membrane. The ethanol atmosphere in the test chamber was generated by a gas generator connected to a computer controlled mass flow system and the calculated atmosphere was compared with a commercially available semiconductor gas sensor. According to Mitsubayashi and co-workers the biosensor measured ethanol down to 0.357 ppm and had a linear response from 1.57 to 41.5 ppm for steady state measurements and from 15.7 to 1242 ppm for maximum response slope measurements. The biosensor response decreased with time. After 4 days, the output was 25 % of the initial response. Interferences were only measured for a few compounds and did not include any of the compounds known to react with AOD (methanol, propanol, formaldehyde etc.). This construction was also developed to monitor trimethylamine and acetaldehyde as described later.

In 1995, another biosensor for ethanol vapour was developed by Park and co-workers (Park *et al.*, 1995). This sensor, which was also constructed mainly for measuring breath alcohol, used AcDH and NAD^+ immobilised on screen-printed electrodes with a mixture of hydroxyethyl cellulose, ethylene glycol and carbon powder. Ethanol reacted with the enzyme and at the same time the NAD^+ was

reduced to NADH. NAD^+ was then regenerated at the electrode surface from NADH. This amperometric regeneration of NAD^+ was carried out at a potential of 0.65 V versus Ag/AgCl. The storage stability was dependent on the amount of enzyme in the biosensor. However, it could be stored more than 35 days if the AcDH/ NAD^+ ratio was over 6. The sensor had a linear response up to 250 ppm and the vapour was generated by bubbling nitrogen through an ethanol solution. Preliminary tests were performed with people mainly to investigate if there were any interfering compounds in human breath. Consequently no interference was found.

Another disposable ethanol biosensor was also developed (Yee *et al.*, 1996). To increase the precision the biosensor was based on a screen-printed electrode consisting of four parallel working electrodes (three active and one inactive), one counter electrode and one reference electrode. The active electrodes consisted of AcDH, NAD^+ , hydroxyethyl cellulose and carbon powder while for the inactive electrode bovin serum albumin replaced the AcDH. In order to activate the biosensors from their dry state they were dipped in a buffer solution. Thereafter it took approximately five seconds to perform the measurements. Considerably better reproducibility was reported for the multi-working electrode biosensor when compared with a simple differential-type electrode biosensor.

In 1999, Park and co-workers published another biosensor aimed at monitoring breath alcohol (Park *et al.*, 1999). It was a differential screen-printed biosensor consisting of one active and one inactive working electrode combined with a Ag/AgCl reference electrode. As for the biosensor described above, the active electrode consisted of immobilised AcDH with NAD^+ as cofactor and the inactive electrode consisted of immobilised bovin serum albumin. The biosensor was activated by dipping in buffer solution, and measurements were performed by reading the steady state current after approximately 20 seconds. A breath alcohol simulator was used to prepare the ethanol concentrations and a GC method used

to determine the concentrations. The biosensor was said to give a good response over the range of 0.02 to 0.1 % (calculated as blood alcohol concentration).

(Williams and Hupp, 1998) developed a sensor based on sol-gel encapsulated AcDH for the analysis of alcohols and aldehydes in air. The AcDH was immobilised in the sol-gel with the cofactors NAD^+ and NADH and placed in cuvettes. Fluorescence from NADH was measured with a spectrophotometer. Since the enzymatic reaction was based on the fact that the reaction between alcohol and AcDH with NAD^+ as cofactor gave the corresponding aldehyde and NADH in a reversible reaction, it would be possible to detect both alcohol and aldehyde. It also could be possible to regenerate the sensor after exposure to one species by exposing the sensor to the other species. The device was tested by gas-phase exposure to ethanol-containing gasoline and human breath containing ethanol. In both cases a detectable difference to ethanol-free control samples was achieved.

A bacterial biosensor for the determination of ethanol vapours has been described by Reshetilov and co-workers (Reshetilov *et al.*, 1998). This biosensor was also constructed to measure ethanol, glucose, glycerol and xylose in solutions. In the design used to measure ethanol vapour, the bacteria were immobilised on chromatographic paper and attached on the surface of a Clark electrode. Ethanol concentrations were measured down to 20 mg/m^3 when the steady state current was read after approximately 5 minutes.

A patented biosensor, originally developed for estimation of alcohol contents in saliva, has been used to monitor ethanol as an indicator of low O_2 levels in packaged food products (Smyth *et al.*, 1999). A disposable biosensor strip with immobilised AcDH, POD and a chromagen, had to be wetted before use, and was then exposed for 15 seconds. The colour change in the strip was then measured with an external chromatometer. Although it had a separate sampling and analysis, the strip also had a short sampling time and could detect about 1 Pa of

ethanol partial pressure. A GC method was used to determine the ethanol concentrations and real samples consisted of fresh-cut vegetables in modified atmosphere packages.

1.4.3.5 Phenol

Phenol is a chemical widely used in industry and exposure to it is known to cause irritations. Air monitoring of phenol is therefore very important. Saini and co-workers investigated the possibility of using biosensors to monitor phenol in air (Saini *et al.*, 1995). An interdigitated microband electrode was chosen as the transducer. Polyphenol oxidase (PPO) was immobilised on the electrode in two different materials - Nafion and tetrabutylammonium toluene-4-sulphonate. Various electrochemical techniques were used to investigate the device with respect to parameters such as thermodynamics and kinetics.

In regard to health and safety monitoring, Dennison and co-workers further developed the biosensor for phenol (Dennison *et al.*, 1995). Their device was constructed by immobilising PPO on a gold microelectrode using a glycerol-based gel. The phenol vapour reacts with the enzyme and the product (catechol) takes part in a redox recycling reaction at the electrode surface. The authors reported that the good sensitivity was achieved partly by this recycling of the catechol/quinone redox couple. The limit of detection was estimated to be 29 ppb and the response was linear up to 13 ppm, both at 40 % relative humidity. The phenol atmosphere was generated with a phenol high-emission permeation tube mixed with humidified air. The phenol concentration was verified with a method using an impinger to trap phenol and analysing it spectrophotometrically. Since glycerol is hygroscopic it had the ability to maintain the water content of the gel. The glycerol gel was also particularly suitable for phenol analysis because of its ability to concentrate phenol in the biosensor.

In two papers Kaisheva and co-workers described a biosensor for monitoring phenol in both liquid- and gas-phase. The first paper (Kaisheva *et al.*, 1996)

mainly described the performance of the sensor in liquid-phase but preliminary experiments in gas-phase were also described. The second paper (Kaisheva *et al.*, 1997) described experiments performed in gas-phase investigating *p*-cresol and 4-chlorophenol vapours. The enzyme used in the sensor was tyrosinase, which catalyses both the reaction of phenol to catechol and the reaction of catechol to *o*-quinone. The electrochemical reduction of *o*-quinone back to catechol then produced a measurable signal at the electrode. The sensors were evaluated in the gas-phase over aqueous sample but the gas-phase concentrations were not calculated. However, linear calibration curves were achieved in the range of 5×10^{-7} to 10^{-4} M for phenol, 5×10^{-5} to 5×10^{-3} M for *p*-cresol and 5×10^{-4} to 5×10^{-2} M for 4-chlorophenol for the aqueous standards. The sensor was also capable of being stored for 20 days without losing its activity.

1.4.3.6 Pesticides and other hazardous chemicals

Monitoring of pesticides has been of interest over a long period since they are highly toxic and widely used. Biosensors for monitoring pesticides have mainly been developed for liquid phase although some sensors have been described for gas-phase monitoring. Ngeh-Ngwainbi and co-workers used antibodies against parathion (a known pesticide) attached to a piezoelectric crystal to monitor parathion in air (Ngeh-Ngwainbi *et al.*, 1986). The response to parathion was fast, usually 1-2 minutes, and the time to return to baseline was 2-5 minutes. The linearity of the device was shown to be in the range of 2-35 ppb. However, since there was a problem with the generation of parathion at higher concentrations, experiments were only performed up to an analyte concentration of 35.5 ppb. The test atmosphere was generated by bubbling carrier gas through a trap containing liquid sample. The vapour-saturated carrier gas was then diluted with pure carrier gas and the concentration of the sampling atmosphere was verified with GC. Some interferences were seen from other pesticides but with lower responses. It took between 3 and 20 times more of the different interferents to give the same response as parathion. The lifetime of the crystals was approximately one week. After a week the response decreased rapidly.

Non-specific adsorption of compounds to antibody coated piezoelectric crystals was addressed and utilised by Rajakovic and co-workers (Rajakovic *et al.*, 1989). They used piezoelectric crystals coated with different proteins (valproic acid antiserum, parathion antibody, IgG and bovine serum albumin) and exposed these to atmospheres containing different hazardous compounds (valproic acid, o-nitrotoluene, toluene, parathion, malathion and disulfoton). The results showed that there was a higher sensitivity to the three pesticides (parathion, malathion and disulfoton) compared to valproic acid, o-nitrotoluene and toluene. The pesticides also adsorbed better to an uncoated crystal. This was explained by the ability of organosulphuric compounds to chemisorb strongly to metal surfaces. However, this does not explain the higher sensitivity of the sensors towards pesticides. The paper by Rajakovic and co-workers described the problems of non-specific adsorption to antibodies when used in gas-phase monitoring and demonstrated that it was important to consider these kinds of interactions; it also demonstrated that it was possible to construct non-specific antibody biosensors for air monitoring.

Another simple device is the C-probe film badge described by Case and Crivello (Case and Crivello, 1990). This device required visual observation and might be suitable as a hazard indicator. The device was described as a biological layer between a film base and a layer of dye. The chemical agents reacted with the biological layer and were converted into active intermediates that triggered a colour change in the dye. The badge was said to respond to 130 organic and inorganic compounds with high correlation to carcinogenic hazards. It could be stored for three months and the sampling time was up to 8-15 hours.

A whole-cell biosensor was developed by (Gil *et al.*, 2000). The cells used were recombinant bioluminescent *Escherichia coli* harbouring a plasmid-borne lux gene and immobilised in agar. This causes the micro-organisms to emit light during their metabolism, and by disturbing the metabolism with toxic gases the

emission of light will decrease. Hence, the biosensor would respond to general gas toxicity and not necessarily just to the concentration of a certain gas. Benzene was used as an example of a toxic gas and it was shown that the decrease in bioluminescence corresponded well with the concentration of benzene. The benzene vapour atmospheres were generated by injecting known amounts of benzene solutions into a test chamber and the concentrations were then determined using GC. The biosensor could be stored for up to one month at 4°C and it was operational for at least 200 minutes. However, it would also have been relevant to test the biosensor for other toxic gases to ensure that it discriminates between toxic and non-toxic gases.

In three papers, Albery and co-workers described an inhibited enzyme electrode. The three papers deal with (a) a theoretical model for an electrochemical sensor measuring the inhibition of the enzyme activity (Albery *et al.*, 1990b), (b) the kinetics of the cytochrome c and the cytochrome oxidase enzyme systems (Albery *et al.*, 1990c) and (c) a description of an application where the sensor was used to analyse HCN and azide ion in liquid-phase and H₂S in gas-phase (Albery *et al.*, 1990a). The sensor was based on the inhibition of the enzyme, cytochrome oxidase. H₂S inhibited the enzymatic reaction producing a decrease in the current from the gold electrode. In gas-phase H₂S could be measured down to 1 ppm and the linearity was said to be good up to 20 ppm.

Naessens and Tran-Minh described a whole-cell biosensor that can be used to monitor organic compounds in both vapours (Naessens and Tran-Minh, 1998b) and aerosols (Naessens and Tran-Minh, 1998a). The sensor used a Clark oxygen electrode to monitor the oxygen produced during the photosynthesis of immobilised micro algae. When the algae were exposed to the organic compounds, as vapour or aerosol, the photosynthesis was inhibited and a decrease in oxygen was measured. The algae were flash lit with an external light source for 1 minute every 5 minutes to start the photosynthetic process. Methanol was used as an example of a gaseous compound and the sampling was carried out in a

thermostated cell with a gas/liquid equilibrium. The calculated detection limit for methanol was 30 ppm and more than 50 % of the algal activity remained after 10 days. Tetrachloroethylene was used as an example of a compound in aerosol form. The aerosols were produced with an atomiser, and a fan in the sampling chamber distributed the aerosols. The limit of detection for tetrachloroethylene was calculated to be 10 ppm and a linear calibration curve was achieved in the range of 0-250 ppm. The device was said to be suitable as an early warning system to protect workers from harmful chemicals, particularly where the chemicals had not yet been identified.

A construction similar to one of the ethanol biosensors described earlier (Mitsubayashi *et al.*, 1994), was also developed to monitor trimethylamine (TMA) in gas- and liquid-phase (Mitsubayashi and Hashimoto, 2000). The enzyme system was changed to flavin-containing monooxygenase 3 and the measurements were performed by measuring the steady state current when (β -Nicotinamide adenine dinucleotide phosphate) NADP^+ was reduced to NADPH in a substrate recycling system. The response time, 90 % of steady state current, was approximately 2 minutes after the start of exposure. The TMA gas was supplied by a gas generator and the concentrations were measured from 10 to 98 ppm. When investigating interfering compounds it was found that triethylamine gave a minor response.

1.4.3.7 Odours

When odours are detected the sensor is usually referred to as an electronic nose. A device using coated piezoelectric crystals was developed by Okahata and Shimizu to detect odours and perfumes in gas-phase (Okahata and Shimizu, 1987). Different coatings were tested and it was found that a lipid bilayer had the best characteristics for the odours represented by β -ionone. The response time for the device was 5 minutes when exposed to a saturated atmosphere of β -ionone.

Piezoelectric crystals coated with 4 different lipid films used for the detection of odours represented by 8 organic compounds (e.g. amyl acetate, β -ionone, methanol etc.) were described by Muramatsu and co-workers (Muramatsu *et al.*, 1989). The crystals were fixed in a vessel and the samples were injected into the vessel as liquids. The patterns for the different odours were then normalised and compared.

Mixtures of asolectin and cholesterol were used by Muramatsu and co-workers in a device developed for odour recognition (Muramatsu *et al.*, 1991). The lipids were coated on piezoelectric crystals and the frequency shift was measured when the odours, represented by 8 organic compounds, adsorbed to the coated crystal. The odours were vaporised by injecting liquid sample into the vessel in which the crystal was positioned and the resonant frequency and the resonant resistance was measured both before and after injection. The patterns for the different compounds were then compared with the aim of recognising the odours.

Wu described a device for odour detection using olfactory receptors coated on a piezoelectric crystal (Wu, 1999). Olfactory receptor proteins (ORP) were used in an attempt to mimic the human sense of smell. Crude ORPs and ORPs fractionated into five groups were coated on the crystals to establish the patterns from 6 organic compounds (e.g. n-caproic acid, isoamyl acetate, linalool etc.) used as odours. According to the authors the sensor did not lose sensitivity after storage for 5 months and it could be used continuously for 10 weeks without loss of sensitivity.

Synthesised peptides have also been used as coating materials for a piezoelectric crystal biosensor (Lin *et al.*, 2000). Five groups of different peptides were used on a commercial system to monitor odours represented by ammonia, methyl amine, 2-mercaptoethanol, acetic acid and chlorobenzene. This array of sensors generates a fingerprint of each compound depending on the interactions between the odours and each sensor. For this type of sensor array it is evident that an efficient method

to handle all data is needed, such as a neural network or a multivariate statistical method.

1.4.3.8 Other sensors

Okada and co-workers developed a biosensor for determination of NO₂ in air (Okada *et al.*, 1983). Nitrite oxidising bacteria were immobilised on an acetylcellulose membrane. The membrane was then attached to an oxygen electrode and covered with a gas-permeable Teflon membrane. The sample was prepared in a gas bag, pumped into the system and dissolved in a buffer, which was pumped through the sample cell of the biosensor. The decrease in oxygen, caused by an increased activity of the micro-organisms when NO₂ was present, was measured. The minimum concentration of determination was calculated to 0.51 ppm and the calibration curve was linear below 255 ppm. The sensor was said to be reusable for 400 assays or 24 days and only respond to NO₂. No experiments were performed with other inorganic gases (e.g. NO, SO₂, NH₃).

A biosensor for nitrogen monoxide was described by Aylott and co-workers (Aylott *et al.*, 1997). It consisted of a sol-gel containing cytochrome c spin-coated onto a glass substrate. A gas flow-through cell covered the sol-gel for the gaseous sample to get in contact with the enzyme. When the NO attached to cytochrome c a shift of the absorption wavelength occurred, which was measured spectrophotometrically. Since the bond between NO and cytochrome c was reversible the sensor could be used for repeated exposures of NO. The standard deviation was calculated to 1 % of the response when 5 repeated exposures to 10 ppm NO were made. The limit of detection was calculated to 1 ppm and the range of detecting NO was calculated to 1-25 ppm. The authors found no evidence of interference from oxygen, nitrogen or carbon monoxide. However, NO₂ was found to bind to cytochrome c thereby giving rise to interference. The authors therefore concluded that cytochrome c only could be used for detecting NO_x and not NO selectively.

A sensor for monitoring sulphur dioxide in air has been described by Matuszewski and Meyerhoff (Matuszewski and Meyerhoff, 1991). It was also used to monitor alcohol as mentioned earlier. A gas-phase biosensor for the direct determination of gaseous sulphur dioxide (SO₂) in the atmosphere was also developed by O'Sullivan (O'Sullivan, 1996). A mixture of agarose and carboxymethylcellulose was chosen from a range of matrices as the media for immobilisation of SOD. Agarose (1 % w/v) and carboxymethylcellulose (1 % w/v) retained a relatively high proportion of water over a three hour period, thus preventing enzyme dehydration and allowing efficient dissolution of SO₂. The sensor method was compared to the standard method for SO₂ determination and a correlation coefficient of 0.999 obtained indicating efficient dissolution of SO₂, in the matrix as well as accurate production of air/sulphur mixtures by the gas rig and efficient functioning of the biosensor. The reproducibility of the biosensor was noted to be good with a coefficient of variation (CV) of 0.96 % obtained for n=10. The linear range was 0-13.5 ppm and the limit of detection was 73.9 ppb.

Hart and co-workers described another type of biosensor for measuring SO₂ in air (Hart *et al.*, 2002). The paper described two similar types (s- and b-type) of screen-printed amperimetric biosensors. They consisted of a carbon working electrode and a Ag/AgCl electrode deposited onto poly(vinylchloride) sheets. The s-type was constructed by mixing the bio-components (SOD and cytochrome c) into the supporting electrolyte and deposited on top of a polycarbonate membrane, which was fixed on the screen-printed surface. The b-type was constructed by mixing the bio-components into the carbon ink and spread over the working electrode. The cell potential was set at +0.3 V versus Ag/AgCl. The linear range was 4-50 ppm and the limit of detection was 4 ppm. Both types were found to be operational for 24 hours at room temperature and the b-type biosensor was found to be operational after storage in dry state for 3 months in a refrigerator. The current reached 90 % of the maximum value within 110 and 220 s for the s- and b-type biosensors respectively.

There is not only a need to monitor chemical compounds in air, but also a need to monitor micro-organisms in air. In some industrial environments workers can be exposed to high levels of micro-organisms. Biological warfare is another application that is in need of fast sampling methods for micro-organisms in air. For this purpose Ligler and co-workers developed a lightweight biosensor that was fixed in a remotely piloted aeroplane (Ligler *et al.*, 1998). Aerosolised bacteria were sampled using a plastic cyclon air sampler with a constant addition of buffer solution. A portion of the liquid sample was pumped over an optic fibre coated with polyclonal antibodies against the bacteria in question. The micro-organisms attached to the antibodies and a reagent solution containing fluorescent-labelled antibodies against the same bacteria was pumped over the optic fibre. The fluorescent signal from the labelled antibodies was measured with a laser fluorimeter. All this equipment was mounted in the aeroplane to collect, identify and transmit continuous information to an operator on the ground. The system was tested by releasing harmless bacteria in various amounts in the air while sampling with the aeroplane. The detection limit of the fibre optic probe was calculated to 3000 colony forming units (cfu)/ml when liquid samples were used and the dried probes could be rehydrated after several months without significant loss of activity. With some modifications to the system it could probably be developed into a sensor for monitoring micro-organisms in air for occupational and environmental purposes.

Much attention has also been given to the field of electroantennography (EAG). Although, EAG devices are rarely described in the literature as biosensors they are constructed by connecting insects, or parts of insects, to electronic devices. Odours that penetrate the insect antennae interact with specific proteins, which then yield an electrical signal. The use of insects, connected to field-effect transistors, has been well described by Schöning and co-workers (Schöning *et al.*, 2000).

1.4.4 Biosensors for formic acid

Biosensors have previously been used to monitor formate or formic acid in liquid phase but no scientific material was found concerning monitoring of formic acid in gas-phase. In 1980 a whole-cell biosensor for formic acid was published (Matsunaga *et al.*, 1980). It contained a micro-organism which produces hydrogen from organic compounds. In order to discriminate some organic compounds a Teflon membrane was used to cover the immobilised micro-organisms. Organic compounds that could penetrate the membrane, e.g. other organic acids, were found not to be metabolised by the micro-organisms. Hence, no interfering compounds were found.

Formate dehydrogenase (FDH) based enzymatic electrochemical biosensors have also been investigated for liquid samples. Kulys and co-workers described a biosensor with the enzyme and cofactor trapped behind a dialysis membrane on a graphite electrode modified with N-methylphenazium and tetracyanoquinodimethane (Kulys *et al.*, 1991). The sensor had a response time of 2 to 3 minutes (90 % of steady state) and the concentration of formic acid was investigated up to 5 mmol/l. However, the biosensor could only be used for one day due to stability problems and a new biosensor had to be calibrated before use. A voltammetric biosensor for determination of formate was presented by Tzang and co-workers (Tzang *et al.*, 2001). It was constructed by attaching a nylon mesh, with immobilised FDH, on a glassy carbon electrode modified with 3,4-dihydroxybenzaldehyd. It had a linear detection range for formate of 0.07 to 1.1 mM and the limit of detection was calculated at 0.05 mM.

A fibre-optic biosensor for automated detection of formate and other compounds was based on fluorimetric detection of NAD(P)H consumed or generated during enzymatic reactions (Schelp *et al.*, 1991). Different enzymes were used to determine different compounds and the resulting change in NAD(P)H concentration was measured with a fluorimetric detector. The NAD(P)H generated or consumed was regenerated by a second enzymatic reaction with

another enzyme. Formate was measured using FDH as the initial enzyme and lactate dehydrogenase to generate the NADH to NAD⁺. The biosensor was connected to an automated analysis system and the linear concentration range for formate was 1-10 mg/l.

2 Materials and methods

2.1 Materials

The respective sources of chemicals and materials used were as follows:

Sigma (St. Louis, MO, USA): MELDOLA'S BLUE: hemi salt, 90 % dye; GLYCEROL: ~99 %; NICOTINAMIDE ADENINE DINUCLEOTIDE oxidised form (NAD⁺): ~98 %; NICOTINAMIDE ADENINE DINUCLEOTIDE reduced form (NADH): disodium salt, ~98 %; FORMALDEHYDE DEHYDROGENASE (FoDH): [EC 1.2.1.46] from *Pseudomonas putida*, 5.7 U/mg solid; ALCOHOL DEHYDROGENASE (AcDH): [EC 1.1.1.1] from Bakers Yeast, 92 %, 428 U/mg protein; ALDEHYDE DEHYDROGENASE (AdDH): [EC 1.2.1.5] from Bakers Yeast, 55 %, 6.9 U/mg protein

Fluka (Buchs, Switzerland): DIMETHYLFORMAMIDE (DMF): ≥99.8 %; 2,4-DINITROPHENYLHYDRAZINE (DNP): ~99 %; FORMATE DEHYDROGENASE (FDHs): [EC 1.2.1.2] from *Candida boidinii*, ~0.4 U/mg powder

Riedel-de Haën (Seelze, Germany): FORMIC ACID: ≥98 %

Merck (Darmstadt, Germany): METHANOL: >99.8 %; ACETIC ACID: glacial, 100 %; PARAFORMALDEHYDE: extra pure; SODIUM HYDROXIDE (NaOH): p.a., >99.98 %; PHOSPHORIC ACID (H₃PO₄): p.a., 85 %; POTASSIUM CHLORIDE (KCl): p.a., 99.5 %; POTASSIUM DIHYDROGEN PHOSPHATE (KH₂PO₄): p.a., 99.5 %

J. T. Baker (Phillipsburg, NJ, USA): METHANOL: ACETONITRILE: HPLC gradient grade, >99.8 %; CARBON DISULFIDE (CS₂): GC grade, 99.9 %

Kemetyl (Haninge, Sweden): ETHANOL: S-sprit, 99.5 %

MCA (Cambridgeshire, UK): SCREEN-PRINTING INKS: carbon paste, I45 and Ag/AgCl paste, C20R15

ESL Europe (Reading, UK): INSULATING INK: 242-SB

Cadillac Plastic Limited (Swindon, UK): POLYESTER SHEETS: Melinex MST5725

Millipore (Bedford, MA, USA): MEMBRANE FILTERS: AA08, FG02, FH05, FA1, FS3 and LS5

Maplin (Milton Keynes, UK): IDC EDGE CONNECTOR

Loctite Sweden AB (Gothenburg, Sweden): CYANOACRYLATE GLUE: Loctite 420

3M (Cergy-Pontoise, France): OFFICE TAPE: Scotch Magic Tape

Recombinant formate dehydrogenase (FDHr) from *Pseudomonas sp.101*, 28 U/ml (25°C), total protein 3.6 mg/ml, was supplied by Prof. Vladimir Tishkov, Department of Chemical Enzymology, Chemistry Faculty, M.V. Lomonosov Moscow State University, Moscow, Russian Federation.

2.2 Equipment

The respective suppliers of the equipment used were as follows:

Carnegie Medicin (Stockholm, Sweden): MICRO-INJECTION PUMP: CMA/100

Hamilton Company (Reno, NV, USA): SYRINGES: 1 ml, model 1001: 5 ml, model 1005 and 10 ml, model 1010

J E Meinhard Associates Inc. (Santa Ana, CA, USA): NEBULISER: Meinhard nebulizer TR-30-A3

Vaisala (Helsinki, Finland): RELATIVE HUMIDITY AND TEMPERATURE METER: HMI 14a R.H. & T indicator

Eco Chemie (Utrecht, the Netherlands): ELECTROCHEMICAL ANALYSER: μ Autolab type II; SOFTWARE: General Purpose Electrochemical Software (GPES)

Bioanalytical Systems, Inc. (West Lafayette, IN, USA): BATTERY-POWERED POTENTIOSTAT: LC-3E "Petit Ampère"

Intab (Stenkullen, Sweden): PC-LOGGER: AAC-2

DEK (Weymouth, UK): SCREEN PRINTER: DEK 248

Supelco (Bellefonte, PA, USA): ADSORPTION TUBES: ORBO 53, silica tubes

Cetac (Omaha, NE, USA): IC COLUMN: IC-Sep AN1

Dionex (Sunnyvale, CA, USA): IC COLUMN: AS11; GUARD COLUMN: AG11

SeQuant (Umeå, Sweden): ANION SUPPRESSOR COLUMN: 10 cm

Coricon AB (Knivsta, Sweden): ION CHROMATOGRAPH: Triathlon autosampler, Series III pump, IC-21 series II column oven, JD-21 series II conductivity detector

Agilent (Palo Alto, CA, USA): GAS CHROMATOGRAPH: HP 5890; CAPILLARY COLUMN: Carbowax 20M, 25 m, 0.20 mm id

SKC Inc. (Eighty Four, PA, USA): ADSORPTION TUBES: Anasorb 747; GLASS FIBRE FILTERS: type AE, 13 mm, 0.3 μ m pore size; PORTABLE PUMPS: Pocket pumps

Waters (Milford, MA, USA): HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: 616 pump, 600s controller, 717 plus autosampler, 2487 dual λ absorbance detector; MILLENNIUM³² CHROMATOGRAPHY MANAGER: Version 3.0

Jones Chromatography (Hengoed, UK): HPLC COLUMN: Genesis 120A, 4 μ , C18

The experimental design and the multiple linear regression (MLR) were performed using Modde 4.0. Principal component analysis (PCA) and partial least square (PLS) were performed using Simca 8.0. Both software packages were supplied by Umetrics (Umeå, Sweden). S-Plus 2000 (MathSoft, Cambridge, MA, USA) was used to carry out the linear and the robust regressions on the data from the storage experiments. SPSS 10.0 (SPSS Inc., Chicago, IL, USA) was used to perform the analysis of variance (ANOVA). A result was considered statistically significant when the significance probability was less than 5 %. Calculations of the responses and the response rates were facilitated by fitting parts of the achieved amperometric curves to linear functions using WinCurveFit (Kevin Raner Software, Mt Waverley, Australia).

2.3 Generation of vapours

2.3.1 General description

There are many ways of generating atmospheres with known concentrations of a certain compound. Different compounds require different methods of generation depending on the desired concentration, the chemical and the physical properties. Examples of commonly used methods are mixing of gas-streams, direct injection of gas or liquid into a gas-stream, generation of gases by use of diffusion methods (where gases and vapours diffuse through tubes or membranes at a constant rate), and evaporation techniques (where gas is passed through a liquid causing the liquid to evaporate) (Nelson, 1982).

2.3.2 Formic acid

Unless otherwise stated the following method was used to generate the formic acid atmospheres. The atmospheres were generated using a method that continuously injects a liquid into a gas stream (see Figure 2.1). The method is based on the evaporation of a liquid using a nebuliser. The nebuliser is constructed so that a rapid stream of gas flows past the nozzle of the nebuliser where the liquid is slowly injected into the system. This creates small aerosols that are quickly vaporised in the mixing chamber. The injection of the liquid was controlled by a micro-injection pump. A gas tight syringe was connected to the pump and the formic acid concentrations were controlled by changing the concentration of formic acid in the injected liquid and by changing the speed of the micro-injection pump.

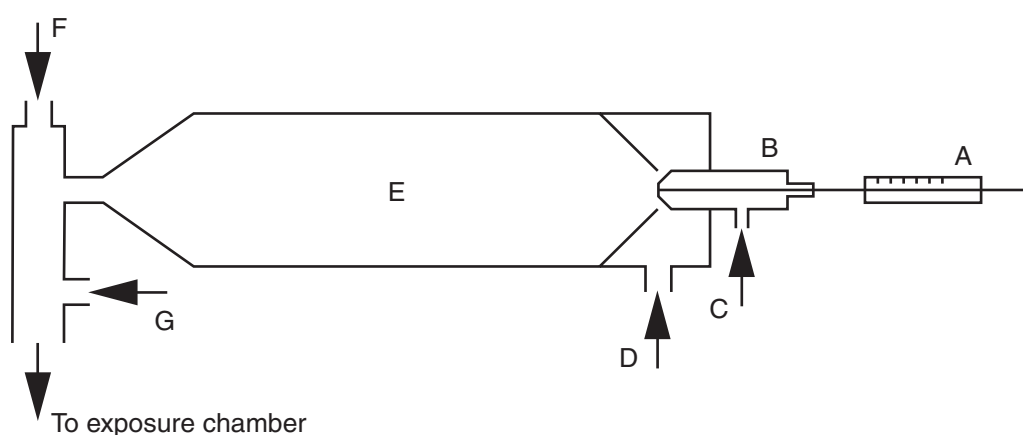


Figure 2.1: The evaporation chamber consisted of a syringe controlled by a micro-injection pump (A), a nebuliser (B), air inlet to the nebuliser, 0.7 l/min (C), compressed air, 4.5 l/min (D), an evaporation chamber (E), compressed humidified air for dilution, 60 l/min (F) and an inlet for formaldehyde vapour (G). The flow rates included are those used for the experiments.

In the evaporation chamber the gas stream was diluted with compressed air to facilitate evaporation. The gas stream was then diluted further with compressed, humidified air to achieve an atmosphere with controlled relative humidity. The

temperature and relative humidity was measured in the exposure chamber. The exposure chamber was made of Teflon and constructed as described in Figure 2.2.

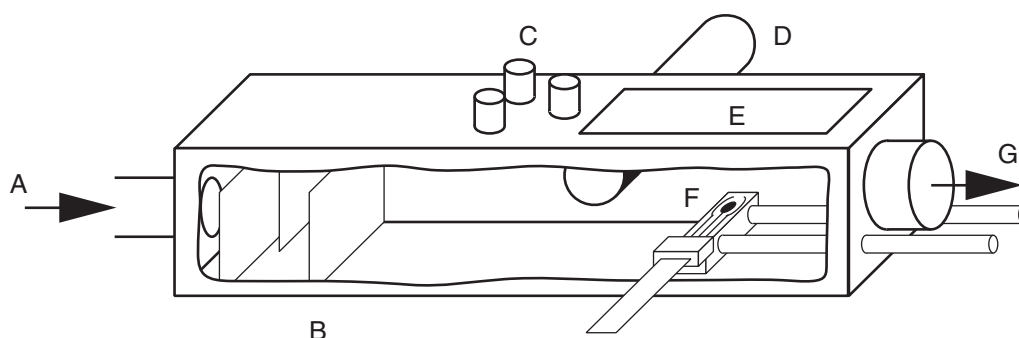


Figure 2.2: The exposure chamber consisted of an inlet connected to the generation system (A), three obstruction plates to give thorough mixing of the air stream (B), three¹ outlets for sampling with adsorption tubes (C), a meter for relative humidity and temperature (D), an opening (E) in the exposure chamber for sampling with the biosensor including an aluminium block to ensure the temperature of the biosensor was controlled (F)² and an outlet (G).

Similar generation systems have been used successfully to generate other compounds by the Programme for Chemical Exposure Assessment at the National Institute for Working Life in Umeå (Sunesson *et al.*, 1995; Lindahl *et al.*, 1996; Sunesson *et al.*, 1999). The relative humidity was regulated by adjusting the airflow through a moisturising system consisting of three water-filled dispersion bottles.

2.3.3 Acetic acid

Since formic and acetic acid are easily mixed, the generation of acetic acid vapour was performed in the same manner as the formic acid. That is, by continuous injection of acetic acid into the air stream using a micro-injection pump and a

¹ The number of connections was increased to six in Chapter 5.

² The aluminium block was used for temperature control during the multivariate evaluation (Chapter 4). Otherwise the biosensor was at room temperature.

nebuliser. When generating formic and acetic acid vapours together the two liquids were mixed in an aqueous solution and injected into the system. A mixture of 79 mg/ml formic acid and 206 mg/ml acetic acid was used for the generation with a 1 ml gas tight syringe injecting at 4.1 $\mu\text{l}/\text{min}$. When generating acetic acid alone a 206 mg/ml acetic acid solution was injected at 4.1 $\mu\text{l}/\text{min}$.

2.3.4 Methanol

The risk of esterification of formic acid to methyl formate was evident when methanol and formic acid were mixed. Therefore, there was a need for separate injections to simultaneously generate vapours of the two liquids. Injections of methanol and formic acid were controlled using the micro-injection pump with two gas tight syringes. However, only one nebuliser was connected to the system and the syringes therefore had to be connected together. Peak tubing was used to connect the syringes to a Y-connector. To ensure as short a mixing time as possible, the Y-connector was placed as close as possible to the nebuliser. In addition, a narrow bore capillary was used to connect to the nebuliser.

The formic acid solution was a 79 mg/ml water solution in a 1 ml gas tight syringe. It was injected at 4.1 $\mu\text{l}/\text{min}$. The methanol, injected at 41 $\mu\text{l}/\text{min}$, was used undiluted with a 10 ml gas tight syringe. When generating only methanol the formic acid in the 1 ml syringe was replaced by water.

2.3.5 Formaldehyde

Formaldehyde was generated using a permeation tube containing paraformaldehyde placed in a GC-oven. By changing the temperature of the permeation tube different amounts of formaldehyde will diffuse through a permeation membrane and into a gas stream. The equipment had been calibrated “in house” for formaldehyde vapour concentrations up to 0.7 mg/m^3 . The gas stream over the permeation tube was approximately 270 ml/min and the temperature was set at 82°C to give a concentration of approximately 0.6 mg/m^3 in the exposure chamber. Achieving the exact concentrations of the vapours was

not important since they were measured using the reference methods, as described later.

The formaldehyde generation system was connected to the formic acid generation system as described in Figure 2.1. The formic acid vapour was generated as described earlier.

2.4 Analytical reference methods

2.4.1 General description

To determine what concentrations were achieved in the exposure chamber it was of utmost importance to have a reliable alternative method for comparison. There are a large number of sampling and analysing methods validated by NIOSH and OSHA. These reference methods can, therefore, be used to ensure well-validated results.

2.4.2 Formic acid

To verify the formic acid concentration in Chapter 3, a method for determination of formic acid in air using sampling on silica adsorption tubes (ORBO 53) and suppressed ion chromatographic analysis developed by NIOSH was used (NIOSH, 1994). The method was used as described by NIOSH, with the exception that 10 mM NaOH was used as eluent pumped at 1 ml/min through an ICSep AN1 column. The suppressor solution pumped through the suppressor column was 5 mM H₂SO₄ at 4 ml/min.

To determine the concentrations of formic acid in Chapter 4 and 5, the analysis was performed using an AS11 analytical column and an AG11 guard column connected to the ion chromatographic system. The eluent in this case was 0.5 mM NaOH.

When decreasing the eluent concentration to 0.5 mM chromatographic separation of formic acid and acetic acid was possible. Hence the same method was used to sample and analyse these two compounds.

The sampling rates for the determination of formic acid were set to approximately 190 ml/min and the sampling time was one hour, unless otherwise stated.

2.4.3 Acetic acid

In Chapter 5 the acetic acid concentrations were sampled and analysed using the same procedure as for formic acid. Acetic acid had a shorter retention time compared to formic acid. The reason for using rather low concentration of NaOH as eluent was mainly to ensure sufficient separation between formic and acetic acid.

2.4.4 Formaldehyde

The sampling and analysis of formaldehyde was performed using an “in house” method that has been accredited according to EN 45001 by the Swedish Board of Accreditation and Conformity Assessment (SWEDAC). This method is based on active sampling of formaldehyde using 2,4-dinitrophenylhydrazine (DNP) coated 13-mm glass fibre filters and analysis on HPLC. The method has been published earlier (Levin *et al.*, 1985) but a few alterations have been made. The glass fibre filters were impregnated with a solution of 300 mg recrystallised DNP, 0.15 ml 85 % H_3PO_4 , 1.5 ml 20 % glycerol in ethanol and 9 ml acetonitrile that was free from formaldehyde. The filters were then dried in a stream of air at room temperature and then stored in a freezer until use. Before use, the filters were placed in the filter holders and used the same day. The sampling was performed for one hour with sampling rates of approximately 190 ml/min.

The formaldehyde 2,4-dinitrophenylhydrazone, formed on the filter by the reaction between formaldehyde and DNP, was eluted from the filters by shaking for 5 min with 3 ml of acetonitrile. Aliquots of 10 μl were injected into the HPLC

system. The column was a 150 mm C18 column, the eluent solution contained 60 % acetonitrile and 40 % water and the flow rate was set at 1 ml/min.

2.4.5 Methanol

The determination of methanol was performed using active sampling and analysis with GC according to a method developed by OSHA (OSHA, 1991). Sampling was performed using Anasorb 747 adsorption tubes at a flow rate of approximately 50 ml/min. The methanol was desorbed by transferring the contents of the tubes to vials and adding a 50/50 mixture of carbon disulphide (CS₂) and dimethylformamide (DMF). The samples were desorbed for one hour and were vigorously shaken several times during this procedure. A small portion of the sample (1 µl) was injected into the GC system, which consisted of a HP 5890 GC with flame ionisation detection and a Carbowax 20M column.

2.5 Calculations

2.5.1 Multivariate methods

A two-level Plackett-Burman design was used in the evaluation of the biosensor in Chapter 4. This design (*Design I*) is a balanced orthogonal *Resolution III* design and all estimated main effects will be confounded with interaction effects. However, this would also have been the case if a fractional factorial design of *Resolution III* had been used in the first screening (Carlson, 1992). It was assumed that a linear first order model was sufficient for identifying the most important factors and for this a *Resolution III* design was adequate. A more elaborate study was then undertaken in *Design II* using a 2⁶⁻¹ fractional factorial design with the most important factors for which their possible interaction effects were also estimated. This approach is labour-saving since strong interaction effects are most likely associated with factors, which by themselves have significant main effects.

2.5.1.1 Mathematical method

A straight forward method for determining which factors have a significant influence on the responses is to fit individual response surface models to each

response by MLR, and then to assess whether or not the terms associated with each factor exert an influence on the response above the noise level. When responses have large random error variance, such a procedure is not very efficient since only highly significant factors can be detected. To evaluate the experiments PCA can be used to factorise the response matrices. This procedure will model the systematic variance of the responses and leave the noise unmodeled. The principal component (PC) score vectors can then be used as responses in separate MLR models. The PCA removes a large part of the random noise variation and this tool reduces the standard error of the estimated coefficients and makes it possible to clearly discern significant factors, either from cumulative normal probability plots of the coefficients or by a t-test when the coefficients are compared with their standard errors. Since the score vectors are mutually orthogonal, this procedure will model independent properties of the biosensor. A thorough account of this combined PCA-MLR procedure has been presented by Carlson and co-workers (Carlson *et al.*, 1992).

It was assumed that the variations of each response, y_i , see equation (2.1), could be modelled by a truncated Taylor expansion of the settings, x_j , of the experimental factors i.e.

$$y_i = \beta_{i0} + \sum_{j=1}^k \beta_{ij} \times x_j + \varepsilon \quad (2.1)$$

where ε is the random error term.

2.5.2 Statistical methods

2.5.2.1 ANOVA

ANOVA is frequently used when comparing more than two independent sample means. The null hypothesis to be tested is that the means are the same, and the alternative hypothesis is that two of the means are not the same. When using ANOVA a few important assumptions have to be made. Normal distributions of the outcome variable values are assumed but the F-test in ANOVA is quite robust

with respect to violation to this assumption. However, more critical is the assumption that the variances in the groups are the same (Dawson and Trapp, 2001).

2.5.2.2 *Regression*

Simple linear regression is widely used to determine the equation that describes the linear relationship between two variables according to:

$$y = a + bx \tag{2.2}$$

The significance test of regression yields one P -value associated with the slope (b) and one P -value associated with the intercept (a) of equation (2.2). If $P < \alpha$ (the level of significance) the slope is considered not statistically separated from zero and, hence, y is not affected by x . Although linear regression is considered a relatively robust procedure there are a few assumptions that have to be addressed. The error of y has to be normally distributed and the values of y must have equal variance for each value of x . It is also assumed that each value is independent of the other.

Robust regression is another procedure in which a linear regression can be performed. This procedure has the advantage of being minimally affected by any extreme values in the independent, dependent or both variables (Anon., 1999).

2.5.2.3 *Student's t-test and significance correction*

Since ANOVA assumes that the variations in all investigated groups are equal, it is not always applicable. When the variance differs, other methods need to be used, for example, multiple, regular Student's t-test combined with a sequentially rejective method for significance correction as described by Holm (Holm, 1979). Sequentially rejective means that the hypotheses are rejected one by one until no further rejections can be made. This is performed using the P -value that is

calculated from each t-test. The hypotheses are then placed in the order of increasing P . Starting with the P for the hypothesis with the lowest P -value, the P -value is compared with α divided with the number of tests (n) that have been performed. Hence, if $P_1 \leq \frac{\alpha}{n}$ then the first hypothesis is rejected and the next P -value is compared with $\frac{\alpha}{n-1}$, and so on. This is performed until no further rejections can be done. If a P_i -value is larger than $\frac{\alpha}{(n+1)-i}$, then that hypothesis and the following hypotheses are not rejected and the procedure is stopped.

3 Development of a formic acid biosensor

3.1 Introduction

A formic acid-selective enzyme was found to be a suitable biological component for the development of a gas-phase biosensor for formic acid. The enzyme, FDH (Popov and Lamzin, 1994), is a NAD^+ -dependent dehydrogenase and therefore requires the presence of NAD^+ to function properly. Since this is a redox reaction (NAD^+ is reduced to NADH) it can easily be incorporated into an electrochemical biosensor (Lobo *et al.*, 1997). The reoxidation of NADH to NAD^+ can then be performed at an electrode surface, producing an electrical signal. However, this oxidation is a slow process and requires a high potential at the electrodes, which in turn can cause increased background current and increased risk of interfering compounds reacting at the electrode (Gorton, 1986). One way to solve this problem is the introduction of a mediator, which has the ability of reacting quickly with NADH and is easily reoxidised at a substantially lower potential. One such mediator is Meldola's blue (see Figure 3.1), which is a phenoxazine that has been used in a number of electrochemical biosensors (Wedge *et al.*, 1999).

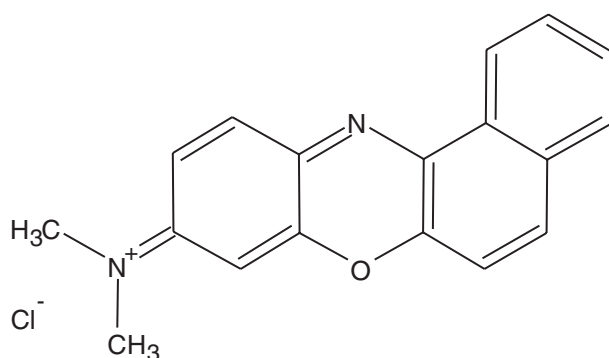


Figure 3.1: Chemical structure of Meldola's blue

Formic acid was measured by adding it to an electrochemical cell which contained the enzyme. Due to the neutral pH the salt of formic acid (formate) is produced. Formate reacts with FDH in presence of the co-factor, NAD^+ , which is reduced in

the process to NADH (Höpner and Knappe, 1974). NADH is then reoxidised to NAD^+ when MB^+ is reduced to MBH. The reoxidation of MBH to MB^+ was then performed at the electrode surface. The reaction scheme is shown in Figure 3.2.

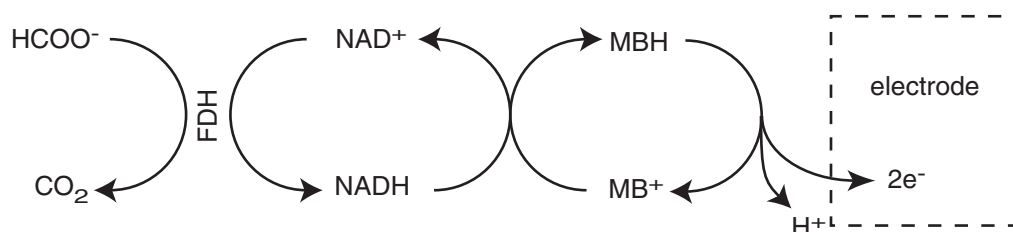


Figure 3.2: *Electron flow diagram of the formic acid biosensor where MB^+ and MBH are the oxidised and reduced forms of Meldola's blue, respectively.*

In this chapter, initial studies for the construction of a diffusive sampling device, based on biosensor technology, for monitoring personal exposure to formic acid is described. The aim of this work was to ascertain how the biological system could be attached to the screen-printed electrodes to produce a biosensor that was functional in air.

3.2 Experimental

3.2.1 Variability of the exposure chamber

An investigation of the homogeneity and reproducibility of the atmosphere generated in the exposure chamber was carried out by generating formic acid (2.9 mg/m^3) in the chamber and measuring the concentrations using three adsorption tubes for each generation. The results were then analysed using a mixed-design ANOVA using tube positions (3 levels) with repeated measurements on the number of generations of the formic acid (6 levels).

3.2.2 Biosensor

3.2.2.1 Electrodes

Inexpensive, disposable electrodes were manufactured using screen-printing to deposit carbon paste, Ag/AgCl paste and an insulating ink on polyester sheets. The design is such that only the electrode areas and contact pads are left exposed (Figure 3.3). The sensors were then cut to their final size, which was approximately 15 x 50 mm (Figure 3.3d).

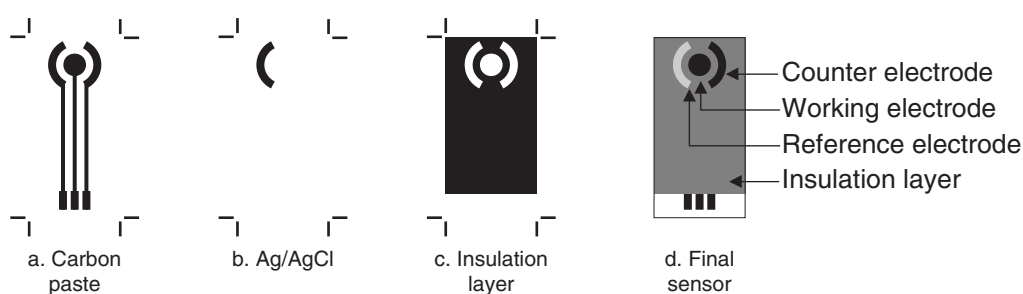


Figure 3.3: *The disposable sensors were constructed by screen-printing pastes in different layers on polyester sheets. Black areas in a, b and c shows the layer printed in each step.*

The screen-printed sensors were connected to an electrochemical analyser (μ Autolab) by a connector manufactured from an IDC edge connector and standard IDC cables. The μ Autolab was controlled by a personal computer with the General Purpose Electrochemical Software (GPES) supplied by the manufacturer.

Seventeen electrodes were used to investigate the variability of the electrochemical response of the electrodes. The variability was measured in a stirred solution containing phosphate buffer (0.1 M, pH 7.0 with 0.1 M KCl) and 5.3×10^{-5} M Meldola's blue. A potential of -50 mV versus the internally printed Ag/AgCl reference electrode was applied. After 500 seconds, a 10 μ l aliquot of a 2 μ g/ μ l solution of NADH was added and the peak heights measured.

3.2.2.2 Enzyme system and immobilisation

Initial enzyme immobilisation experiments were performed using sol-gel, alginate gel and glycerol solution, respectively. The sol-gel was constructed according to an earlier described method (Ellerby *et al.*, 1992). The protein solution, added to the sol-gel, consisted of 39 mg/ml FDHs, 4 mg/ml NAD⁺ and 1 mg/ml Meldola's blue in phosphate buffer. An aliquot (50 µl) of the mixture was placed on the electrodes and stored at 4°C over night. The alginate gel was prepared by mixing approximately 20 µl of a 4 % sodium alginate solution, dissolved in a phosphate buffer (0.05 M, pH 7.0 with 0.1 M KCl), with 7 µl of a 20 U/ml solution of FDHs, 2 µl of a 40 mg/ml solution of NAD⁺ and 1 µl of a 20 mg/ml solution of Meldola's blue. Approximately 10 µl of the gel was placed on the electrodes and the electrodes were then dipped in 0.1 M CaCl₂ to complete the gel. The glycerol solution used to immobilise the enzyme, cofactor and mediator on the screen-printed electrodes consisted of a mixture of glycerol and phosphate buffer. Glycerol has been used previously in a biosensor for analysing phenol in air and proved to be very efficient in this application, mainly because of its ability to retain water and its ability to concentrate phenol in the gel (Dennison *et al.*, 1995). The glycerol solution consisted of 80 % (w/w) glycerol and 20 % (w/w) phosphate buffer (0.1 M, pH 7.2 with 0.1 M KCl). An aliquot (20 µl) of the solution was mixed with 7 µl of a 20 U/ml solution of FDHs, 2 µl of a 40 mg/ml solution of NAD⁺ and 1 µl of a 20 mg/ml solution of Meldola's Blue. An aliquot (10 µl) of this mixture was applied to the screen-printed sensors to cover all three electrodes. The comparison was performed by measuring the gaseous formic acid above an equilibrated 0.012 M solution of formic acid using the two different types of immobilisation.

3.2.2.3 Membranes.

Gas-permeable membranes were placed on top of the glycerol solution to ensure that the glycerol solution was secured on the electrodes. Nine types of membrane were investigated (Table 3.1).

Table 3.1: *Membranes investigated.*

Membrane code	Pore size (μm)	Wettability	Material
AA08	0.8	Hydrophilic	Mixed cellulose esters (nitrate and acetate)
FG02	0.2	Hydrophobic	PTFE with PE backing
FH05	0.5	Hydrophobic	PTFE with PE backing
FA1	1.0	Hydrophobic	PTFE with PE backing
FS3	3.0	Hydrophobic	PTFE with PE backing
LS5	5.0	Hydrophobic	PTFE unlaminated
PC01	0.1	Hydrophilic	Polycarbonate
PC08	0.8	Hydrophilic	Polycarbonate
PC8	8.0	Hydrophilic	Polycarbonate

The membranes were cut into squares of approximately 14 x 14 mm. Aliquots of 10 μl of the glycerol solution were placed on the electrodes and the membranes were attached to the electrodes by applying small amounts of cyanoacrylate glue to the edges of the membrane squares. The electrodes were then connected to the electrochemical analyser and exposed to formic acid in the exposure chamber. The potential was -50 mV versus the internally printed Ag/AgCl reference electrode and the current at steady state was measured.

3.2.2.4 Testing the biosensor

The biosensors described above, were tested in an exposure chamber with formic acid concentrations set at 1.9, 3.7 and 5.6 mg/m³, which is near the Swedish threshold limit value of 5 mg/m³ for formic acid, set by the SWEA (Arbetskyddsstyrelsen, 2000). The biosensors were equilibrated in the chamber before the formic acid was added. A potential of -50 mV (versus the internally printed Ag/AgCl reference electrode) was applied and when the signal had reached steady state (approximately 3 minutes) the micro-injection pump was started and a formic acid atmosphere of 1.9 mg/m³ was generated in the exposure chamber. After 20 minutes, the concentration of formic acid was increased to 3.7 mg/m³ and after another 20 minutes the concentration was increased to 5.6 mg/m³.

3.2.2.5 Storage

In an initial investigation of storage stability, 42 electrodes, using the LS5 membrane, were prepared as above. The storage experiment was performed by maintaining batches of 18 electrodes at 4°C and -15°C, respectively. After storing the electrodes for 1, 2 and 3 days, 6 electrodes from each storage condition were analysed each day. The analyses were performed by exposing the electrodes to 6 mg/m³ of formic acid in air and measuring the current at steady state.

As seen in the Results section (Figure 3.8), the storage stability was poor. Before performing the experiments described in the following sections of this thesis, one significant change in the design was made. The filters were attached to the sensors using regular office tape instead of the cyanoacrylate glue. This did not facilitate the fabrication of the biosensors but it avoided the contact between the glycerol solution and the glue. Other types of tapes could therefore also be considered.

Storage stability experiments were also performed using the modified sensor design. After storing biosensors for 0, 3, 6, 10 and 20 days at -15°C steady state currents were measured at two concentrations of formic acid (1.4 and 2.9 mg/m³). A total of 15 biosensors were used, giving three biosensors to use for measurement on each occasion. Each disposable biosensor was used to measure both concentrations of formic acid. Since the main problem with the storage stability was suspected to be that the enzyme was not stable enough, a recombinant and more stable FDH (FDHr) was used (Tishkov *et al.*, 1999). There was also a long period of investigation of the storage problems. Hence, the experimental conditions between the first and the second set of storage experiments were not identical – the glycerol solution contained 2.7 U/ml of FDHs and 7.5 U/ml of FDHr in the initial and second storage experiments, respectively. Also, the formic acid vapour concentrations were 6 mg/m³ for the initial storage experiment and, 1.4 and 2.9 mg/m³ for the second.

3.3 Results and discussion

3.3.1 Variability of the exposure chamber

To ensure reliable concentrations in the exposure chamber six generations were performed and analysed using the reference method for formic acid. The average concentration of formic acid in the 18 adsorption tubes (3 tubes x 6 generations) was 2.81 mg/m³ and the CV was 5 %. The CV for the three tubes in each generation varied between 1 and 9 % and the average concentration varied from 2.69 to 2.92 mg/m³. However, no significant difference was found between the generations or between the tube positions according to the ANOVA.

3.3.2 Variability of the electrode response

The average peak height measured after the addition of NADH to the Meldola's blue/buffer solution was 0.064 μ A and the CV of the electrochemical response of the screen-printed electrodes was 13 % (n=17). The experiments were performed over four days and on each day new solutions were made. No difference was observed in the size of the signal with time, either between or within groups.

3.3.3 System for immobilisation of the enzyme

The sol-gel method was discarded because the transparent silica gel did not adhere properly to the surface of the screen-printed electrodes and hence cracked. When the enzyme system was immobilised in glycerol the response was larger and quicker compared to immobilisation in alginate gel (Figure 3.4). The alginate gel has a less permeable structure and this probably caused the difference in response. For this reason, glycerol was chosen as the immobilisation media.

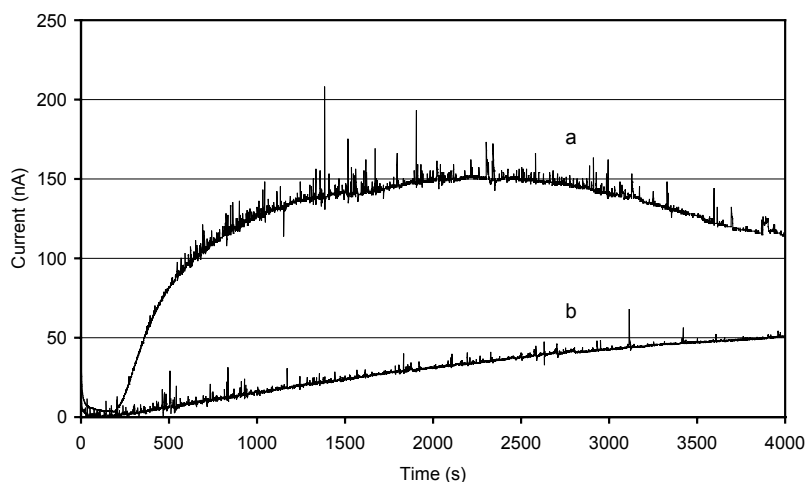


Figure 3.4: *Response of screen-printed electrodes with FDHs, NAD^+ and Meldola's blue immobilised in glycerol (a) and alginate gel (b) and exposed to formic acid in air.*

3.3.4 Choice of membrane

Of the nine membranes that were investigated, the LS5 membrane gave a stable response for over 60 minutes and the CV of steady state current between six electrodes was 15 %. The AA08 membrane was not suitable for this application and produced no signal, probably because of the ability of the membrane to absorb the glycerol solution into the membrane. The polycarbonate membranes (PC01, PC08 and PC8) responded with far more variation than the LS5 membranes. This may be attributed to the fact that these membranes were less rigid in the construction and were therefore more difficult to attach to the sensors without causing damage to the biosensor system. Electrodes with the fluoropore membrane (FG02, FH05, FA1 and FS3) all produced larger responses than electrodes with the LS5 membrane, but they also exhibited larger variation in the signal within each group of electrodes. Figure 3.5a shows the experiments performed with the LS5 membrane and Figure 3.5b shows the experiments performed with the FA1 membrane as an example of the fluoropore membrane type. All fluoropore membranes gave a similar response as the FA1 membrane, with larger response but much greater variation in the signal. Some of the

electrodes with fluoropore membrane did not maintain a steady response for 60 minutes as can be seen in Figure 3.5b. Although the response was lower for the electrodes with the LS5 membrane, this membrane was chosen for further investigation since the stability within the group of electrodes was better. Steady-state currents were reached after 4-15 minutes using this membrane on the biosensor.

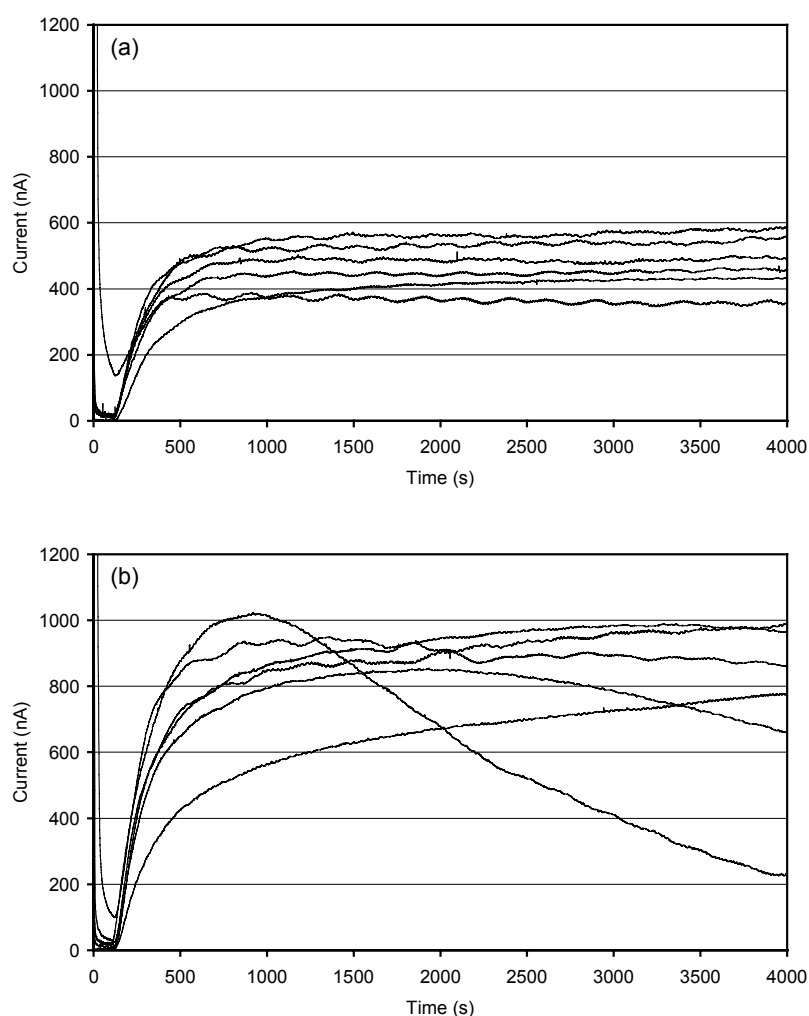


Figure 3.5: Comparison of the membranes used to secure the glycerol solution on the electrodes performed by exposing them to formic acid in air and measuring the amperometric response at steady state. a) LS5 membrane and b) FA1 membrane.

3.3.5 Linearity of sensor response

When the biosensors were exposed to formic acid concentrations between 1.9 and 5.6 mg/m³ they responded well to the increase in concentration in the test atmosphere, as can be seen in Figure 3.6. A fast response time was achieved, which indicates that the device could be used as a real-time monitor. Six experiments were performed using the same conditions and all the responses showed the same pattern. One of the biosensors, however, did have approximately 17 % lower response. This is illustrated in Figure 3.7 by the three points well below the calibration curve. It also shows a linear correlation between the formic acid concentrations and the responses.

Unless otherwise stated all formic acid concentrations presented in this chapter are calculated values. However, the verification of the formic acid concentrations using the NIOSH method showed that the concentrations were only between 60 and 75 % of the calculated values, at the three different concentration levels. This could be caused by adsorption of formic acid to the surfaces of the generation system. However, it shows that a separate reference sampling is needed for each formic acid concentration.

3.3.6 Storage stability

Figure 3.8 shows that the performance of the biosensors was not maintained after storage. The amperometric response decreased by 50 % after only one day's storage at -15°C. Since storage stability is an important feature, this had to be improved. When the biosensors were stored at 4°C the response decreased by 80 % after one day's storage.

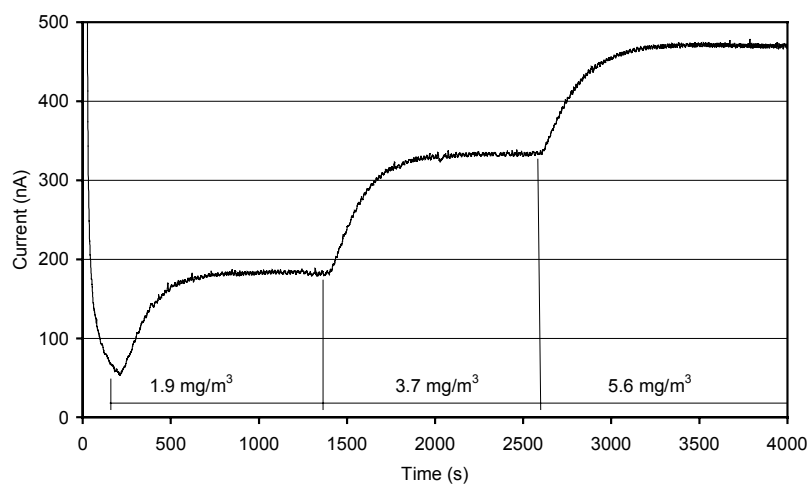


Figure 3.6: Amperometric response of a biosensor, with FDHs, NAD^+ and Meldola's blue immobilised in glycerol and covered with LS5 membrane, when exposed to 1.9, 3.7 and 5.6 mg/m^3 of formic acid in air.

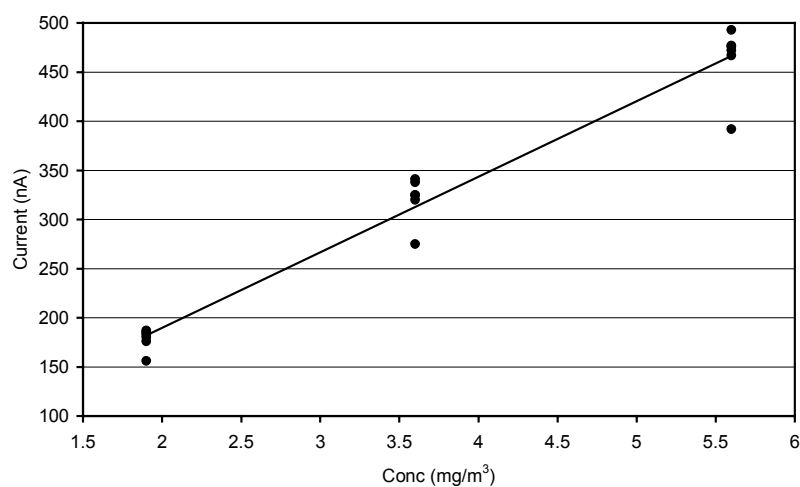


Figure 3.7: The correlation between the formic acid concentrations and the amperometric response for six experiments. Conditions were the same as for Figure 3.6.

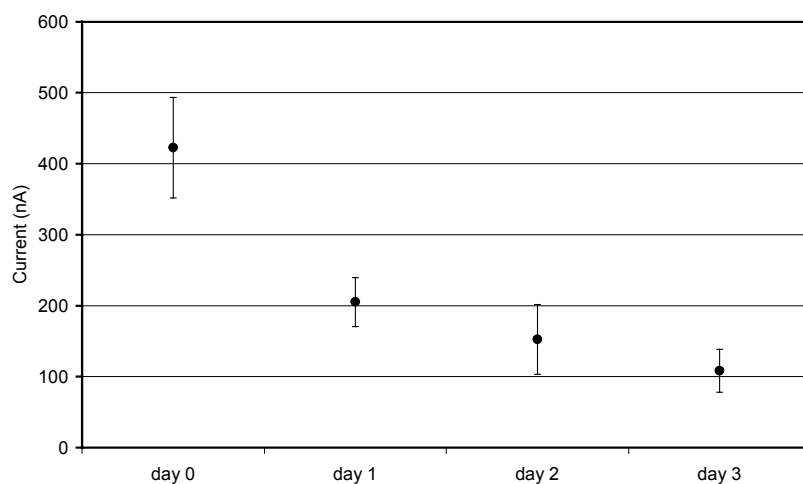


Figure 3.8: *Determination of the storage stability of biosensors with FDHs, NAD^+ and Meldola's blue immobilised in glycerol and covered with LS5 membranes attached with glue, at -15°C . Analyses were performed after 0, 1, 2 and 3 days storage. The error bars represent the standard deviation for each experiment.*

By using regular office tape to fix the membrane filter to the sensor, the storage stability of the biosensor was considerably improved. The results (Figure 3.9) showed that the slopes of the steady state currents versus the storage time for this period were not significant according to linear or robust regression. This was a major improvement of the storage stability compared to the earlier construction that had a 50 % reduction of the response after only one day of storage. It can therefore be concluded that a more thorough investigation of how the biological system in the biosensor was affected by the glue would have been appropriate at an earlier stage. However, the effect of the glue was only seen on biosensors that had been stored and does not affect other conclusions drawn from the studies performed.

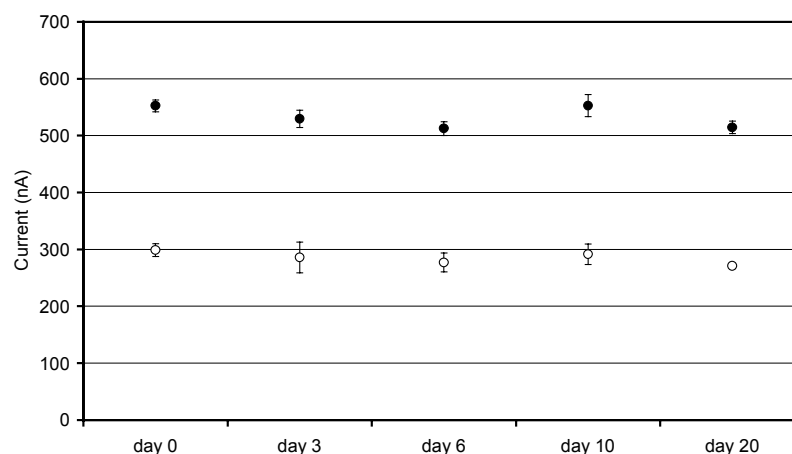


Figure 3.9: *Determination of storage stability using biosensors with membranes attached with office tape when the biosensors were exposed to formic acid concentrations of 1.4 mg/m^3 (○) and 4.9 mg/m^3 (●). The error bars represent the standard deviation for each experiment.*

3.4 Conclusions

This study demonstrated the potential for a simple, inexpensive and specific personal passive "real-time" sampler based on biosensor technology, for measurement of formic acid in air. The enzyme system can be immobilised in glycerol and kept in place using a gas-permeable membrane producing electrodes, which are operationally stable and give a linear response. By making a small change in the original design, that is using regular office tape instead of glue to attach the membrane to the electrode, sensors with good storage stability could be produced. The use of glycerol has the advantage of preventing loss of water during operation of the biosensor. The system described shows promise in meeting the considerable demand for a simple, specific and inexpensive method for personal exposure monitoring.

4 Multivariate evaluation

4.1 Introduction

Although multivariate optimisation using experimental designs is an important tool during the development of new methods and technologies there are only very few examples of multivariate evaluation aiming to optimise the performance of biosensors in the literature (Danzon and Schwedt, 1996a; Danzon and Schwedt, 1996b; Situmorang *et al.*, 2000). However, pattern recognition and characterisation of different environments using PLS and PCA are frequently used with other types of sensors, i.e. electronic noses (Eklöv *et al.*, 1999) and tongues (Krantz-Rülcker *et al.*, 2001).

In this chapter a multivariate study of different factors that may influence the performance of the formic acid biosensor is presented. The objective of this study was to elucidate which factors are important and to determine the optimum operational conditions of the biosensor. Many experimental factors may exert an influence on the measurable responses from the biosensor. Since these factors also may interact with each other, it is necessary to study their influence by statistically well designed experiments (Carlson, 1992). It is also desirable to show that experimental design, PLS and the combination of PCA and MLR provide important information during development of biosensors.

4.2 Experimental

4.2.1 Materials

The biosensor design was described in Chapter 3. For the experiments described in this chapter, the LS5 membranes were attached to the sensors using the regular office tape and the enzyme used was the FDHr. The temperature of the biosensors was controlled by placing them on top of an aluminium block with temperature controlled water running through it, see Figure 2.1. During the experiments the

biosensors were connected to the electrochemical analyser and the responses were measured as described below.

4.2.2 *Factors and responses*

In order to determine which experimental factors are most important for the performance of the biosensor a series of sensors were constructed where 11 factors could be varied (see Table 4.1).

Table 4.1: *Variables included in the multivariate experiments*

	Abbr.	Unit
the concentration of glycerol	<i>Gly</i>	mg/g
the concentration of Meldola's blue	<i>MB</i>	mg/g
the concentration of NAD ⁺	<i>NAD</i>	mg/g
the concentration of FDHr	<i>FDH</i>	mg/g
the concentration of KCl	<i>KCl</i>	M
the concentration of phosphate buffer	<i>PhC</i>	M
pH of the phosphate buffer	<i>pH</i>	
the amount of the solution placed on the electrode	<i>v</i>	μl
the electrochemical potential	<i>E</i>	V
the temperature of the biosensor	<i>T</i>	°C
the relative humidity in the sampled environment	<i>Rh</i>	%

Relative humidity was included because it was suspected that the biosensor could loose water due to evaporation at low levels of relative humidity and thereby affect the response. By including it as a factor in the multivariate evaluation, it can be compensated for or limits for possible use of the biosensor can be set if it has a significant impact on the response.

The ideal performance of the biosensor would be a rapid change in the measurable response value when the formic acid concentration is increased and a high and stable current at steady state for a continuous formic acid concentration. It was assumed that the rate of the response change to variation in the formic acid concentration could be different depending on the initial formic acid concentration and on how long the biosensor had been used. The following six measurable responses were therefore chosen for the evaluation and optimisation:

- $y_1 = C1$: Current at steady state when the biosensors were exposed to a formic acid vapour concentration of 1.4 mg/m^3 .
- $y_2 = C2$: Current at steady state when the biosensors were exposed to a formic acid vapour concentration of 2.9 mg/m^3 .
- $y_3 = C3$: Current at steady state when the biosensors were exposed to a formic acid vapour concentration of 4.8 mg/m^3 .
- $y_4 = R1$: Initial response rate when the formic acid vapour concentration was increased from 0 to 1.4 mg/m^3 after approximately 8 minutes equilibration.
- $y_5 = R2$: Initial response rate when the formic acid vapour concentration was increased from 1.4 to 2.9 mg/m^3 at approximately 25 minutes.
- $y_6 = R3$: Initial response rate when the formic acid vapour concentration was increased from 2.9 to 4.8 mg/m^3 at approximately 40 minutes.

$C1$ - $C3$ were used to ensure as high response as possible and to determine whether the responses to different concentrations of formic acid were linear. $R1$ - $R3$ were used to determine any difference in the rate depending on the time of use and the initial formic acid concentration when the concentration were changed.

4.2.3 Experimental design

4.2.3.1 Design I

The initial experiments, incorporating the eleven factors considered, were laid out according to a twelve-run Plackett-Burman design (Plackett and Burman, 1946). The design was augmented with five centre-point experiments to obtain an estimate of the experimental error variance and to make it possible to detect non-linear response variation (Table 4.2).

Table 4.2: Design I: Plackett-Burman design of the eleven experimental factors included in the investigation.

Exp.	<i>Gly</i>	<i>MB</i>	<i>FDH</i>	<i>NAD</i>	<i>PhC</i>	<i>pH</i>	<i>KCl</i>	<i>v</i>	<i>E</i>	<i>Rh</i> ^a	<i>T</i>
1	495	0.6	0.792	2.4	0.096	7	0.144	10	0.2	40	35
2	495	1.2	0.576	3.6	0.096	7	0.096	10	0.2	80	25
3	297	1.2	0.792	2.4	0.144	7	0.096	6	0.2	80	35
4	495	0.6	0.792	3.6	0.096	8	0.096	6	0	80	35
5	495	1.2	0.576	3.6	0.144	7	0.144	6	0	40	35
6	495	1.2	0.792	2.4	0.144	8	0.096	10	0	40	25
7	297	1.2	0.792	3.6	0.096	8	0.144	6	0.2	40	25
8	297	0.6	0.792	3.6	0.144	7	0.144	10	0	80	25
9	297	0.6	0.576	3.6	0.144	8	0.096	10	0.2	40	35
10	495	0.6	0.576	2.4	0.144	8	0.144	6	0.2	80	25
11	297	1.2	0.576	2.4	0.096	8	0.144	10	0	80	35
12	297	0.6	0.576	2.4	0.096	7	0.096	6	0	40	25
13	396	0.9	0.684	3	0.12	7.5	0.12	8	0.1	60	30
14	396	0.9	0.684	3	0.12	7.5	0.12	8	0.1	60	30
15	396	0.9	0.684	3	0.12	7.5	0.12	8	0.1	60	30
16	396	0.9	0.684	3	0.12	7.5	0.12	8	0.1	60	30
17	396	0.9	0.684	3	0.12	7.5	0.12	8	0.1	60	30

^a) During initial experiments, it was found that low relative humidity in the sampled atmosphere fouled the biosensor too quick to receive a reliable response. The range of relative humidity was therefore restricted to 40-80 % instead of the 20-80 % that was initially intended.

4.2.3.2 Design II

Evaluation of the results obtained in Design I showed that six factors were important. With these factors, an extended study was undertaken using a Resolution VI 2^{6-1} fractional factorial design. The design allows independent estimates of possible interacting effects. The design was augmented with five centre-point experiments. The design, with the chosen factors, is shown in Table 4.3 and the non-included factors were set as follows: *Gly*, 396 mg/g; *pH*, 7.5; *KCl*, 0.096 M; *Rh*, 50 % and *T*, 25 °C. Since these factors did not significantly influence the performance of the biosensor within normal ranges they could be set to any value within the model. A common setting for variables not included in this type of design is the media value and *Gly* and *pH* were set to these values. However, *KCl* was set to the lower level because 0.1 M *KCl* is often used to ensure reliable electrochemical cells. *Rh* was set to 50 % since this value is often

used when evaluating biosensors. Even though T did not have a significant effect on the performance of the biosensor, the combination of $T = 35^{\circ}\text{C}$ and $Rh = 40\%$ caused the biosensor to foul after less than two minutes causing missing values in the response matrix. Therefore, T was set to the lower level. All experiments were performed in random order as determined by the built-in random generator in the Modde package.

It was assumed that the errors of the observed responses had a normal distribution. For arguments for this assumption see (Carlson, 1992), pages 45-49. The residuals from the PCA modelling were checked as indicators of possible outliers. No such outlier indicators were detected. Normal probability plots of residuals did not show deviation from normality. Therefore, the combination of PCA-MLR was considered as acceptable for the present study.

4.3 Results and discussion

4.3.1 Multivariate experiments - Design I

4.3.1.1 Principal component analysis of the responses

PCA of the data from the six responses in Table 4.4 afforded three significant PCs ($77 + 14 + 6\%$ explained variance) according to cross-validation (Wold, 1978). The scores from these PCs were used as response matrix in the MLR to receive an estimate of the most important factors in the model. Some combinations of settings of the changed factors caused the biosensor to foul at an early stage. This can be seen as missing data in Table 4.4. The missing data did not cause any problem when the data was evaluated since the NIPALS (nonlinear iterative partial least squares) algorithm used for calculating the PCs tolerates missing data; for instance see (Wold, 1978).

Table 4.3: *Design II: 2^{6-1} fractional factorial design of the six experimental factors chosen for further investigation.*

Exp.	<i>MB</i>	<i>FDH</i>	<i>NAD</i>	<i>PhC</i>	ν	<i>E</i>
1	0.3	0.36	1.2	0.048	8	0
2	1.5	0.36	1.2	0.048	8	0.2
3	0.3	0.792	1.2	0.048	8	0.2
4	1.5	0.792	1.2	0.048	8	0
5	0.3	0.36	4.8	0.048	8	0.2
6	1.5	0.36	4.8	0.048	8	0
7	0.3	0.792	4.8	0.048	8	0
8	1.5	0.792	4.8	0.048	8	0.2
9	0.3	0.36	1.2	0.144	8	0.2
10	1.5	0.36	1.2	0.144	8	0
11	0.3	0.792	1.2	0.144	8	0
12	1.5	0.792	1.2	0.144	8	0.2
13	0.3	0.36	4.8	0.144	8	0
14	1.5	0.36	4.8	0.144	8	0.2
15	0.3	0.792	4.8	0.144	8	0.2
16	1.5	0.792	4.8	0.144	8	0
17	0.3	0.36	1.2	0.048	12	0.2
18	1.5	0.36	1.2	0.048	12	0
19	0.3	0.792	1.2	0.048	12	0
20	1.5	0.792	1.2	0.048	12	0.2
21	0.3	0.36	4.8	0.048	12	0
22	1.5	0.36	4.8	0.048	12	0.2
23	0.3	0.792	4.8	0.048	12	0.2
24	1.5	0.792	4.8	0.048	12	0
25	0.3	0.36	1.2	0.144	12	0
26	1.5	0.36	1.2	0.144	12	0.2
27	0.3	0.792	1.2	0.144	12	0.2
28	1.5	0.792	1.2	0.144	12	0
29	0.3	0.36	4.8	0.144	12	0.2
30	1.5	0.36	4.8	0.144	12	0
31	0.3	0.792	4.8	0.144	12	0
32	1.5	0.792	4.8	0.144	12	0.2
33	0.9	0.576	3	0.096	10	0.1
34	0.9	0.576	3	0.096	10	0.1
35	0.9	0.576	3	0.096	10	0.1
36	0.9	0.576	3	0.096	10	0.1
37	0.9	0.576	3	0.096	10	0.1

Table 4.4: Measured responses and calculated scores (*t*) and loadings (*p*) from the first experimental design.

Exp.	C1(nA)	C2(nA)	C3(nA)	R1(nA/s)	R2(nA/s)	R3(nA/s)	t[1]	t[2]	t[3]
1	-	-	-	-	-	-	-	-	-
2	293	491	806	0.664	0.625	0.706	0.517	-0.152	0.198
3	322	462	447	0.746	0.412	0.022	-1.504	2.120	0.074
4	260	517	644	0.866	0.868	0.644	0.859	0.432	1.434
5	-	-	-	-	-	-	-	-	-
6	228	368	477	0.481	0.240	0.117	-2.972	0.338	0.163
7	279	495	743	0.427	0.345	0.303	-1.157	0.087	-1.014
8	278	582	1145	0.884	1.043	0.939	3.048	-0.787	0.712
9	81	-	-	-	-	-	-5.426	-2.048	-0.026
10	326	632	1158	0.665	0.830	0.875	2.734	-0.578	-0.795
11	317	530	616	0.704	0.582	0.216	-0.301	1.444	-0.156
12	184	317	547	0.428	0.221	0.160	-3.369	-0.494	0.416
13	317	601	933	0.781	0.692	0.485	1.413	0.632	-0.474
14	274	557	894	0.679	0.750	0.606	1.049	-0.176	0.021
15	275	515	787	0.667	0.549	0.495	0.058	0.139	-0.073
16	307	595	948	0.813	0.827	0.631	1.891	0.312	0.025
17	240	490	848	0.562	0.509	0.433	-0.458	-0.461	-0.221
p[1]	0.310	0.454	0.426	0.337	0.462	0.434			
p[2]	0.583	0.183	-0.464	0.434	-0.016	-0.471			
p[3]	-0.383	-0.416	-0.402	0.514	0.455	0.218			

4.3.1.2 Regression of experimental factors on response scores

The estimated coefficients were small. A cumulative normal probability plot showed that six variables had coefficients that deviated from the normally distributed random error. These factors were *MB*, *FDH*, *NAD*, *PhC*, *v* and *E* and they were investigated further in the second experimental design.

4.3.2 Multivariate experiments - Design II

4.3.2.1 Principal component analysis of the responses

PCA of the data from the six responses in Table 4.5 afforded three significant principal components according to cross-validation (76 + 14 + 7 % explained variance). The loading plot of the first two PCs (Figure 4.1) showed that all responses had approximately the same importance for the model determined by

the distance to origin. It also showed that the three response rates are very close together on the loading plot. Current response *C3* is quite close to the cluster of response rates but the other two current responses are well separated from one another and the other responses. The *C3* column in Table 4.5 does also contain missing data. Under certain conditions, it was impossible to obtain data from the last steady-state level and the data were therefore excluded.

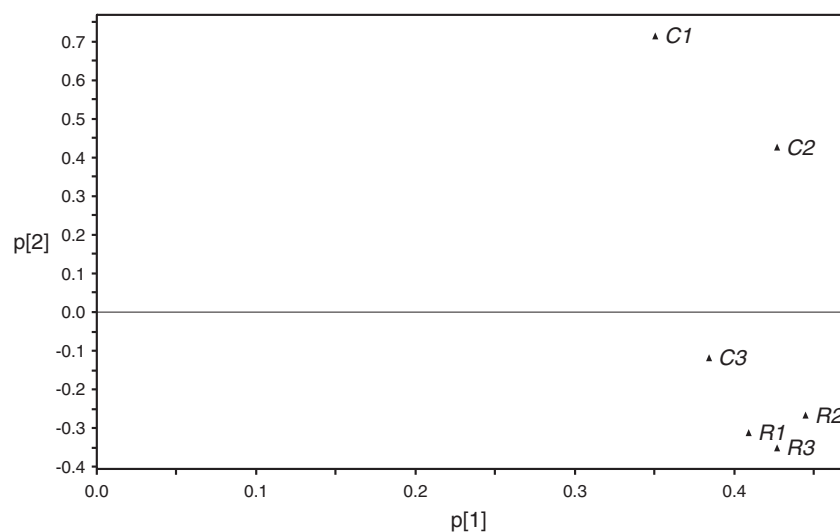


Figure 4.1: Loading plot of the first two PCs (76 + 14 % explained variance) from PCA of the response matrix in experimental design II.

4.3.2.2 Regression of experimental factors on response scores

The scores and loadings from the PCA of the responses are shown in Table 4.5 and an MLR, of the experimental factors with interaction terms to the three score responses, was performed. Cumulative normal probability plots of the estimated coefficients (Figure 4.2) showed one significant factor, *FDH*, for the first score response, four significant factors, *MB*, *FDH*, *E* and the interaction term between *FDH* and *NAD* (*FDH*NAD*), for the second score response and five significant factors, *PhC*, *v*, *E*, *NAD*PhC* and *NAD*E*, for the third score response. Since the first PC explained most of the variance in the model, the first response score was also the most interesting (Bratchell, 1989). The significance of *FDH* for the first

score response and thereby for the rate and current responses was obvious and the concentration of the enzyme always plays an important role in enzymatic biosensors. *MB* was found to be significant in the second PC and the interpretation of the normal probability plot (Figure 4.2b) and the loading plot from the PCA (Figure 4.1) was that *MB* has a positive effect on *C1* and *C2* but a smaller negative effect on *C3*, *R1*, *R2* and *R3*. This finding means that the determination of the optimum settings is difficult since *C1* and *C2* respond differently than *C3* to the settings of *MB*.

4.3.2.3 *Separate PLS models for the current and rate responses*

As some responses were strongly associated, separate PLS models were established for the rate responses, where the Y block consisted of *R1*, *R2* and *R3*, and for the current responses, where the Y block consisted of *C1*, *C2* and *C3*. The X block consisted of all factors and their cross-product terms. The PLS loading plots for these models are shown in Figure 4.3. As mentioned above *FDH* was the most important factor when evaluating the response scores and this can also be seen in these plots. *FDH* had a strong influence on all the responses and this can be seen in both PLS plots. The PLS analysis confirmed the conclusions from the PCA-MLR analysis.

Table 4.5: *Measured responses and calculated scores (t) and loadings (p) from the second experimental design.*

Exp.	C1(nA)	C2(nA)	C3(nA)	R1(nA/s)	R2(nA/s)	R3(nA/s)	t[1]	t[2]	t[3]
1	213	377	568	0.519	0.263	0.192	-3.769	-0.736	-0.187
2	296	512	677	0.577	0.322	0.240	-1.591	1.116	-0.045
3	217	441	727	0.671	0.499	0.392	-1.447	-1.526	0.116
4	266	534	675	0.603	0.463	0.372	-0.953	0.130	-0.064
5	285	528	744	0.564	0.411	0.310	-1.021	0.688	0.367
6	257	445	514	0.535	0.322	0.149	-3.091	0.455	-0.766
7	302	589	742	0.985	0.771	0.586	2.118	-0.584	-1.205
8	375	697	926	0.959	0.801	0.672	4.105	0.986	-0.304
9	238	425	-	0.487	0.324	0.186	-3.078	-0.092	0.175
10	250	476	663	0.527	0.305	0.215	-2.460	0.269	0.196
11	263	540	997	0.827	0.662	0.417	1.088	-0.937	0.811
12	314	570	793	0.640	0.507	0.390	0.241	0.944	0.260
13	264	518	-	0.606	0.440	0.322	-1.096	0.121	0.500
14	291	485	685	0.399	0.313	0.188	-2.348	1.309	0.529
15	244	544	1036	0.740	0.622	0.627	1.320	-1.562	1.595
16	310	579	796	0.838	0.683	0.432	1.288	0.201	-0.504
17	258	491	752	0.775	0.434	0.303	-0.912	-0.405	-0.199
18	278	455	641	0.595	0.299	0.207	-2.264	0.579	-0.263
19	285	570	846	1.076	0.765	0.555	2.256	-1.164	-0.879
20	362	662	776	0.906	0.677	0.397	2.186	1.578	-1.001
21	259	443	695	0.665	0.391	0.316	-1.654	-0.372	-0.147
22	298	521	668	0.629	0.368	0.240	-1.309	1.046	-0.315
23	302	590	891	1.021	0.890	0.760	3.396	-1.341	-0.497
24	289	542	646	0.984	0.674	0.346	0.577	-0.299	-1.780
25	213	384	-	0.582	0.356	0.211	-3.169	-1.058	-0.295
26	277	465	598	0.530	0.328	0.164	-2.535	0.820	-0.357
27	296	553	-	0.942	0.666	0.490	1.591	-0.556	-0.615
28	320	590	868	0.989	0.634	0.488	2.011	0.076	-0.516
29	264	497	-	0.615	0.457	0.327	-1.141	-0.048	0.357
30	302	548	812	0.657	0.470	0.281	-0.300	0.851	0.300
31	214	391	-	0.776	0.550	0.429	-1.293	-2.231	-0.284
32	307	610	975	0.868	0.734	0.592	2.566	-0.313	0.487
33	312	596	914	0.787	0.605	0.473	1.514	0.358	0.435
34	343	651	1015	0.920	0.711	0.515	3.051	0.635	0.390
35	340	641	991	0.733	0.613	0.493	2.165	1.087	0.950
36	288	545	870	0.791	0.609	0.401	0.722	-0.182	0.185
37	324	611	940	0.816	0.667	0.483	2.027	0.467	0.383
p[1]	0.350	0.427	0.384	0.409	0.445	0.427			
p[2]	0.716	0.426	-0.119	-0.312	-0.266	-0.352			
p[3]	-0.159	0.058	0.785	-0.548	-0.193	0.130			

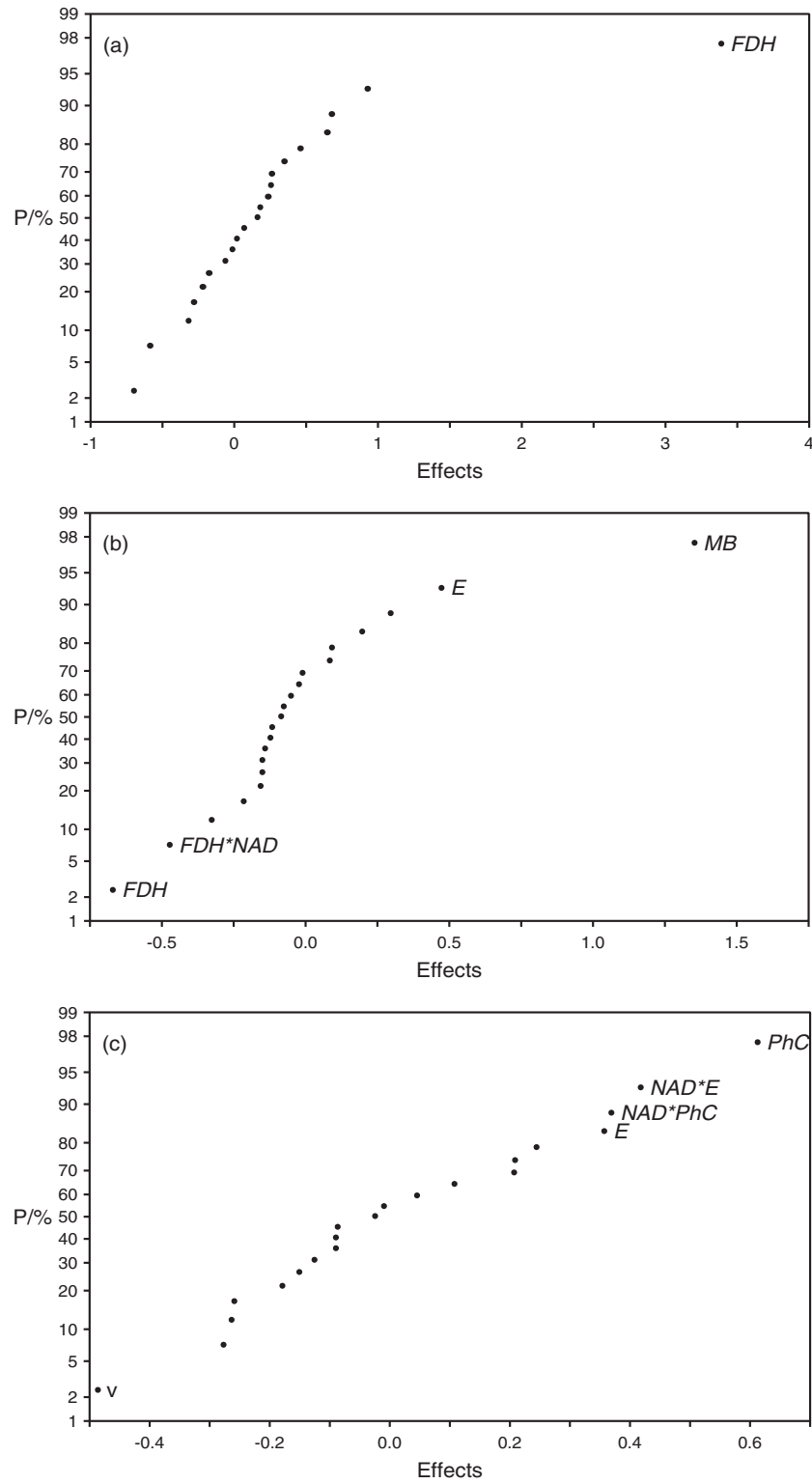


Figure 4.2: Cumulative normal probability plots of effects from PCA-MLR in experimental design II: (a) PC 1; (b) PC 2; (c) PC 3. The points deviating from a straight line have a significant effect on the model.

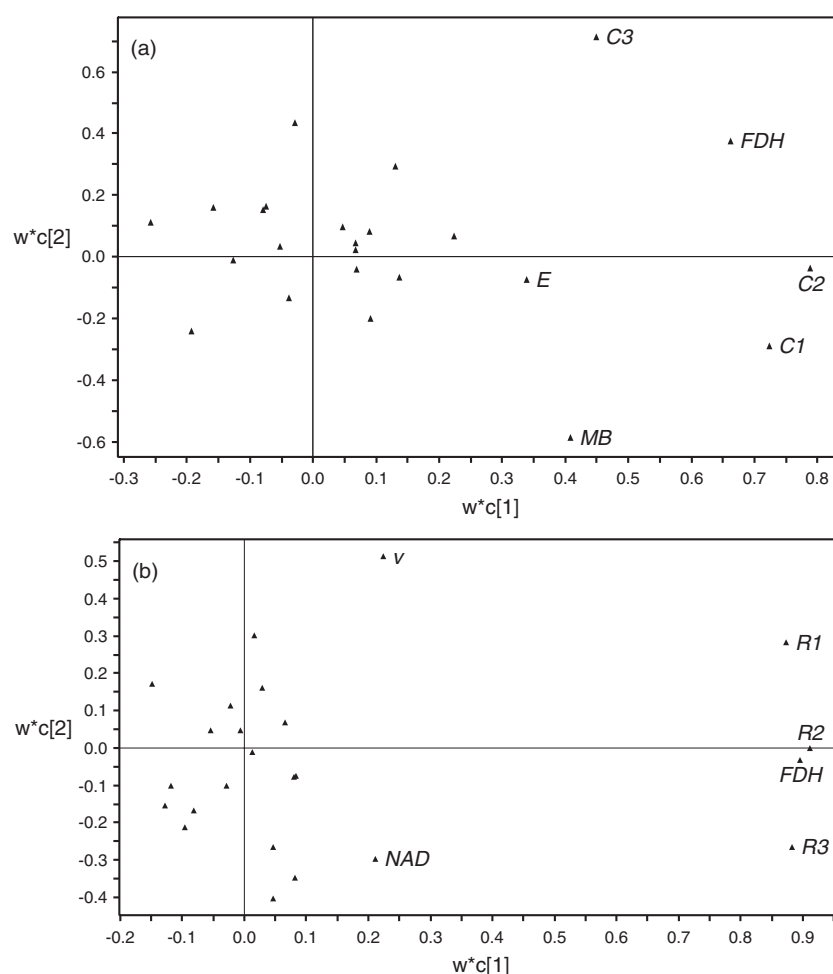


Figure 4.3: PLS loading plots from experimental design II. The plots show the first two PLS loadings for: (a) the model for the current responses, $C1$, $C2$ and $C3$; (b) the model for the rate responses, $R1$, $R2$ and $R3$.

4.3.3 Optimisation of the biosensor

When optimising the construction of the biosensor there were of course some practical limitations that had to be considered. Since the amount of enzyme had a major impact on the performance, this was the most important factor to maximise within these practical limitations. There was also a limit to how much glycerol the solution could contain. Too large amount of glycerol would complicate the addition of the other components in the solution. Therefore, the 80 % glycerol level that would have been favourable in regard to water retention (Dennison, 1995) was not reached. However, this did not cause any problem since glycerol

did not have a significant effect on the performance of the formic acid biosensor, as concluded from Design I.

To obtain large response and fast response rate the settings of the experimental factors were set using the coefficients of the MLR of the first PC of the response matrix. Of the eleven factors initially considered, six were included and five were excluded from the second design. The values of the included factors were set as described in the second experimental design.

The results from the second design yielded that *MB* and *PhC* had coefficients close to zero. Therefore, these factors were set to their media levels (0.9 mg/g and 0.096 M respectively) in the experiments performed to compare optimised and non-optimised biosensors. The coefficients of the remaining factors (*FDH*, *NAD*, *v* and *E*) were calculated to 1.7, 0.47, 0.34 and 0.33, respectively. Hence, the values used in four additional experiments were set to 0.97 mg/g, 3.85 mg/g, 11 μ l and 0.13 V, respectively. The additional experiments were, therefore, performed in the direction where the factors contributed to larger responses and response rates to confirm the results from the model interpretations.

The results from four optimised biosensors were compared with results achieved from experiments using four biosensors, which did not have optimised parameters, but had the parameters set to the values described in Chapter 3. They were compared using Student's t-test followed by a sequentially rejective method for significance correction. As can be seen in Table 4.6 there was a general improvement in the biosensor performance following optimisation. Although *C1* was found to be of borderline significance, it did produce an increase 21 % when comparing before and after optimisation. The steady state currents *C2* and *C3* increased by 30 % and 18 % respectively and the response rates (*R1* and *R2*) increased by 47 % and 89 % respectively. The third response rate, *R3*, was not found to be significantly increased. This was mainly due to the large variation in the *R3* data from the biosensors that were not optimised. The variation was caused

by a disturbance of the amperometric curves that occurred at the time when $R3$ was measured.

Table 4.6: Comparison of the three responses and the three response rates for the eight biosensors. The test compared biosensors that did not have optimised parameters (Before Optimisation) with biosensors that had been optimised (After Optimisation).

Before optimisation						
Sensor no	$C1(\text{nA})$	$C2(\text{nA})$	$C3(\text{nA})$	$R1(\text{nA/s})$	$R2(\text{nA/s})$	$R3(\text{nA/s})$
1	236	442	762	0.76	0.63	0.43
2	246	426	760	0.60	0.37	0.73
3	219	426	751	0.92	0.50	1.10
4	238	431	720	0.64	0.38	0.27
Average	235	431	748	0.73	0.47	0.63
SD	11	7	20	0.14	0.12	0.37
After optimisation						
Sensor no	$C1(\text{nA})$	$C2(\text{nA})$	$C3(\text{nA})$	$R1(\text{nA/s})$	$R2(\text{nA/s})$	$R3(\text{nA/s})$
5	264	535	876	1.01	0.90	0.79
6	258	525	852	1.13	0.82	0.77
7	310	593	908	1.11	0.92	0.85
8	305	588	895	1.06	0.94	0.80
Average	284	560	883	1.08	0.89	0.80
SD	27	35	24	0.05	0.06	0.04
Increase	21 %	30 %	18 %	47 %	89 %	27 %
P^a	0.027	0.004	0.000	0.012	0.003	0.423
α^b	0.025	0.012	0.008	0.017	0.01	0.05

^a) Student's t -test followed by a sequentially rejective method for significance correction.

^b) Corrected level of significance, which the P -values are compared with.

4.4 Conclusion

This work shows how a particular combination of multivariate methods can provide a useful approach to the improvement of the performance of a biosensor and helpful information about the biosensor system. The approach adopted here has revealed factors that appear to influence performance and possible interactions between some of those factors, thus enabling an approach to optimising sensor

performance. The combination of PCA and MLR offers a very useful way of visually interpreting sets of multiple responses.

5 Selectivity and field test

5.1 Introduction

It is of utmost importance to develop inexpensive, fast and easy-to-use samplers for occupational and ambient environments. Although diffusive sampling has reduced cost and increased simplicity, there are still needs for methods that give fast responses, which can quickly be related to a procedure, a process or an event.

To ensure that the biosensor was selective for formic acid, and not affected by other compounds, three additional volatile chemicals were used for the investigation. These were methanol, formaldehyde and acetic acid. They were chosen because they can be closely related to formic acid. Therefore, it can be assumed that these chemicals would interact with the enzyme, FDH. It is also crucially important to test the biosensors in an environment for which it is supposed to be used. The formic acid biosensor was developed for exposure measurements in occupational environments. Therefore, the test site chosen was a work-place setting in a glutam production unit where a problem of formic acid exposure had already been established.

The objective of this study was to evaluate the formic acid biosensor in terms of selectivity and on-site use. Investigations into exchanging the formate selective enzyme for other enzymes, in order to expand the utility of the method, are also presented.

5.2 Experimental

5.2.1 *Selectivity test*

The selectivity of the formic acid biosensor was investigated by exposing the sensor to three possible interfering compounds in the gas phase. The gases were generated both with and without the addition of gaseous formic acid so that the

extent that the interfering agents reacted with FDHr could be determined. The compounds used, besides formic acid, were acetic acid, formaldehyde and methanol. The gases were generated and determined as described in Chapter 2 and set at approximately 4 mg/m³ for formic acid, 10 mg/m³ for acetic acid, 0.5 mg/m³ for formaldehyde and 450 mg/m³ for methanol.

The biosensors were constructed according to the outcome of the optimisation described in Chapter 4. For each combination of gas atmospheres five biosensors were tested. At the same time the atmospheres were sampled using the reference methods described in Chapter 2 to determine the actual concentrations of the gases. The relative humidity was set at 50 % in all experiments.

The biosensors were connected to an electrochemical analyser and the steady state currents were measured when 0.13 V was applied. The currents were then compensated for (divided by) the determined concentrations of formic acid, which yielded a unit of A/(mg/m³). One-way ANOVA comparisons with post-hoc Bonferroni test (Dawson and Trapp, 2001) were performed for the experiments containing formic acid and for the experiments not containing formic acid, separately. The hypotheses, in both cases, were that there were no differences between the groups.

5.2.2 Field measurements

5.2.2.1 Method comparison

The biosensors were tested on-site in a factory using glue containing between 10 and 30 % formic acid to produce glulam products. The glue is hardened by heating and during this process formic acid is evaporated into the factory environment. The atmosphere was monitored using both the silica tube reference method and biosensors at a stationary position close to the equipment used for hardening the glue. The silica tubes were connected to a vacuum pump ensuring flow rates at approximately 190 ml/min. The sampling times for the four different

sampling periods were between 10 and 90 minutes, and three tubes were connected in parallel at each occasion.

The biosensors were constructed according to the outcome of the optimisation described in Chapter 4 and stored at below -15°C until use (16 days). The biosensors were used as single-use samplers and the steady state currents were read after 5 minutes. A calibration was performed by exposing 10 biosensors to different formic acid vapour concentrations in the exposure chamber. The concentrations were approximately 0, 0.1, 0.6, 1.1 and 2.4 mg/m^3 , and two biosensors were used at each concentration. The vapour concentrations were determined using three silica tubes at each occasion according to the reference method described in Chapter 2. After sampling, the tubes were capped and stored in room temperature until the analysis was performed. The biosensor calibration curve that was achieved was then used to evaluate the biosensors used in the field tests.

The biosensors used in the field test were transported in a Styrofoam box containing ice-blocks so that the temperature of the biosensors was kept below 0°C during transportation. To ensure that the biosensors were not affected by the transportation to the sampling site, and thereby did not deviate from the achieved calibration curve, four biosensors were left unused in the transportation box. These were tested in the exposure chamber after transportation back to the laboratory. They were then exposed to 0.1 and 0.6 mg/m^3 of formic acid vapour with two biosensors used on each occasion. Since the relative humidity was measured at 25 % during the experiments in the glulam factory, the post field test controls were also performed at this humidity. The calibration and the field test of the biosensors were performed using a portable battery-powered potentiostat connected to a PC-logger.

5.2.2.2 Exposure measurements

To estimate the levels of formic acid vapours that the workers at the glutam production unit are exposed to, personal exposure measurements were performed on four workers with slightly different work tasks. The measurements were performed by active sampling using three silica adsorption tubes connected in parallel to a portable pump on each worker. The airflow through the tubes was set at approximately 60 ml/min and the sampling was performed for approximately 1.5 hours. After sampling the silica tubes were stored and analysed in the same way as the tubes from the stationary sampling (see Chapter 2).

5.2.3 Investigation of other enzymes

The design of the biosensor makes it easy to exchange one enzyme for another. This was performed by modifying the glycerol solution by adding other NAD^+ -dependent dehydrogenases. The amount of the investigated enzymes and the concentrations of the vapours used are presented in Table 5.1. The rest of the constituents were the same as described earlier in this chapter.

Table 5.1: NAD^+ -dependent dehydrogenases used instead of FDHr in the biosensor.

Enzyme	U/biosensor	reacts with ^a	vapour used	conc ^b (mg/m ³)
formaldehyde dehydrogenase (FoDH)	0.47	formaldehyde	formaldehyde	0.6
aldehyde dehydrogenase (AdDH)	0.28	aldehydes	formaldehyde	0.6
alcohol dehydrogenase (AcDH)	0.89	prim. sec. alcohols and hemiacetals	methanol	250

^a) according to (Webb, 1992), ^b) no reference methods were used during these experiments. The concentrations were determined theoretically.

5.3 Results and discussion

5.3.1 Analyte selectivity

The selectivity of the biosensors was investigated by exposing the biosensors to acetic acid, formaldehyde and methanol vapours. Active sampling was used to determine the concentrations of the vapours for the different experiments. The concentrations determined according to the reference methods are presented in Table 5.2. The concentrations of formic acid were used to adjust for the differences in formic acid concentration when the biosensors were used.

Table 5.2: Average concentrations according to reference methods described in Chapter 2. FA=formic acid, AA=acetic acid, Me=methanol, Fo=formaldehyde.

Vapour(s)	FA ^a	AA ^a	Me ^a	Fo ^a
No vapour	n.m. ^c	-	-	-
AA	n.m. ^c	9.59 ^c	-	-
Me	n.m. ^b	-	468 ^b	-
Fo	n.m. ^b	-	-	0.508 ^b
FA	3.88 ^c	-	-	-
FA + AA	3.11 ^c	9.61 ^c	-	-
FA + Me	3.53 ^b	-	438 ^b	-
FA + Fo	3.67 ^b	-	-	0.511 ^b

n.m. not measurable (limit of detection was calculated at 0.1 mg/m³ when sampling 60 minutes at approximately 200 ml/min)

^a) Vapours measured in mg/m³, ^b) n=3, ^c) n=6

The results from the biosensors exposed to “clean air” were compared with the results from the biosensors exposed to acetic acid, methanol and formaldehyde vapours using a one-way ANOVA with post hoc Bonferroni test. The result from this analysis showed that there was a significant difference between the “clean air” and acetic acid vapour exposed biosensors. This is clearly shown in Figure 5.1. The current measured after 5 minutes was on average 14 nA higher for the acetic acid-exposed biosensors compared with the blank exposed biosensors. As can be seen in the figure, one of the values for the methanol exposed biosensors was an extreme value; this has not yet been explained.

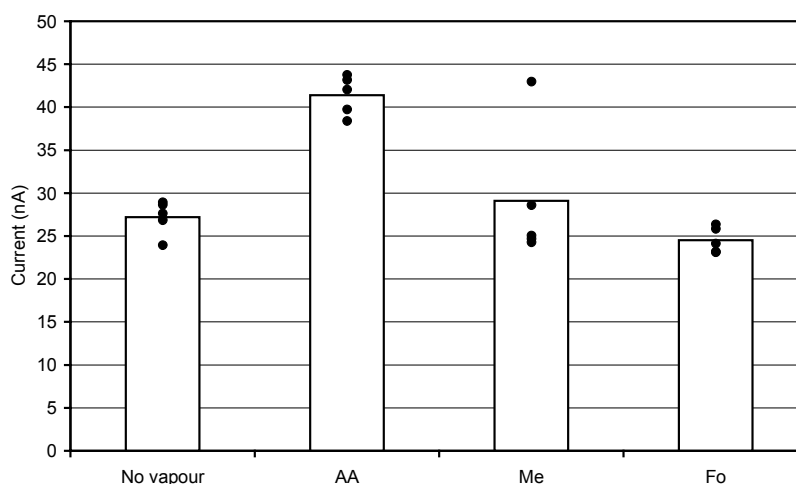


Figure 5.1: Exposure of biosensors to possible interfering compounds. The bars represent the average steady state currents when the formic acid biosensors were exposed to different gases without exposure to formic acid. Five experiments were performed for each compound (●). AA=acetic acid, Me=methanol, Fo=formaldehyde.

Figure 5.2 shows how the response of the biosensor was influenced by acetic acid, methanol and formaldehyde vapours in combination with formic acid vapour. An ANOVA with post hoc Bonferroni test showed that the addition of acetic acid vapour significantly increased the response. The adjusted current increased by 24 % when the biosensors were exposed to 9.6 mg/m^3 of acetic acid and 3.1 mg/m^3 of formic acid as compared with 3.9 mg/m^3 of formic acid alone. There were also increases in the responses when the biosensors were exposed to 440 mg/m^3 of methanol in combination with 3.5 mg/m^3 of formic acid, and 0.5 mg/m^3 of formaldehyde in combination with 3.7 mg/m^3 of formic acid. However, these increases were not statistically significant. Hence, it is quite clear that acetic acid vapour affects the biosensor and thus it is important to be aware of any acetic acid sources at a sampling site where the biosensor is used. However, the sensitivity of the biosensor is much larger for formic acid than for acetic acid; that is, it would require high concentrations of acetic acid to generate a

measurable response. Another issue to take into account is that the adjusted current incorporates an additional source of error, i.e. the determination of the formic acid concentrations using the reference method. As can be seen in Table 5.2, the concentration of formic acid differs between the experiments, although the generations of formic acid vapour were performed in the same way.

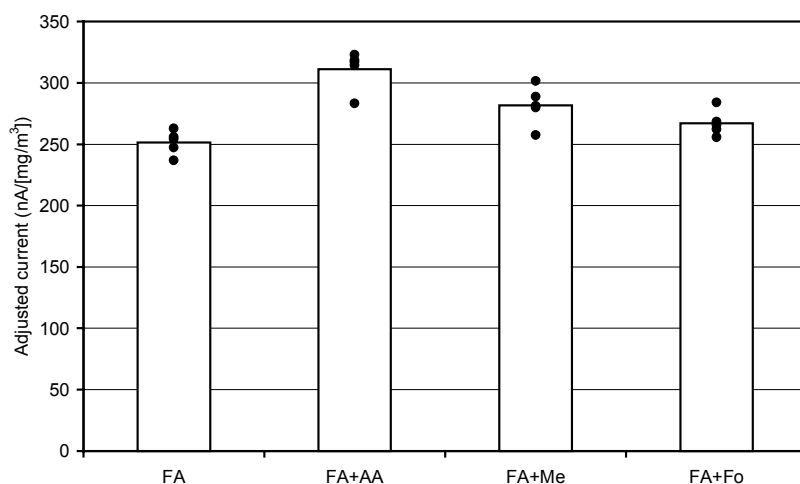


Figure 5.2: Exposure to possible interfering compounds. The bars represent the average adjusted steady state currents when the formic acid biosensor was exposed to different gases with simultaneous exposure to formic acid. Five experiments were performed for each compound (●). FA=formic acid, AA=acetic acid, Me=methanol, Fo=formaldehyde.

5.3.2 Field measurements

5.3.2.1 Method comparison

Prior to the field test 10 biosensors were used for calibration in the range of 0.02 mg/m³ to 2.4 mg/m³ of formic acid vapour (see Figure 5.3). The calibration curve was linear within this range and the limit of detection was calculated to 0.03 mg/m³ (3 x standard error of the calibration curve). In Figure 5.3 the post field test control values, marked as unfilled circles, can also be seen. These

showed that the biosensors had not been affected by the transportation to and from the sampling site.

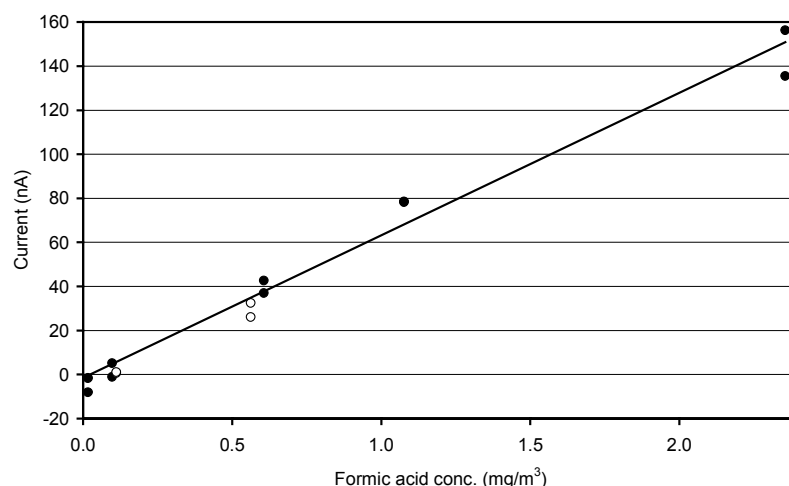


Figure 5.3: Calibration curve of the formic acid biosensor. Steady state currents when the formic acid biosensor was exposed to formic acid of concentrations determined by the reference method for formic acid. Two experiments were performed for each concentration (●). The calibration curve is described by $y = 64.7x - 1.5$ ($R^2 = 0.98$). Post field test controls were performed at two concentrations (○).

In the field test experiments 23 biosensors were used: 17 biosensors at a suspected high formic acid vapour concentration (close to the heater for hardening the glue), and 6 biosensors at a suspected low formic acid vapour concentration (at another part of the factory). The temperature and the relative humidity were measured at 25°C and 25 %, respectively. It was suspected that the low relative humidity was stretching the limits of the biosensor since it has been investigated at 40-80 % during the multivariate optimisation described in Chapter 4. However, no evidence was found for this during the experiments. Contrary to what had been suspected so far, the biosensor could be used at relative humidity below 40 %, at least when it is used as a single-use device over a short period of time. This increases the usability of the biosensor in field applications. Although it would

probably be possible to compensate for low relative humidity, it is of course an advantage if the biosensor is usable over as wide of a range as possible.

Since the actual concentration of the formic acid vapour was unknown the sampling time for the reference method was varied from 10 to 90 minutes. In Figure 5.4 the determined concentrations of formic acid on the four different occasions are represented by the horizontal lines. The lines also indicate how many biosensors were used during each sampling occasion. It would have been preferable, although not practically possible, to perform one reference sampling for each biosensor measurement. However, this would have required 5 minute sampling times, which was suspected not to be long enough to collect enough formic acid in the sample for analysis. Therefore, the reference method was not completely comparable with the biosensor measurements. Only one biosensor was investigated in this way (Experiment no. 6 in Figure 5.4) and it was in agreement with the reference samples. There was large variation in the biosensor data, which was not observed for the reference method. This may be caused by a variation between the biosensors. However, this is less likely since it has not been seen in earlier experiments. Therefore, the reason may be due to a variation in the formic acid vapour concentration on the site. Such a variation would not have been picked up by the reference method since the sampling time was too long. Hence, this demonstrates the usability of direct-reading instruments in occupational environments. Short peaks of high chemical exposures will not be detected by traditional two-step measurement procedures. The potential to produce biosensors of this type at a low cost makes it possible to assess the exposure during a workday by frequently changing the biosensor strip. Although this procedure would be inconvenient, it would make it possible to find suspected exposure peaks. Optimally, it would be possible to use the biosensor as a direct-reading instrument over a period of a workday. However, this has yet to be achieved. The main restriction is that the biological system in the biosensor is not yet stable enough. However, there are a number of techniques under development, which may produce biological or biomimetic systems with increased stability.

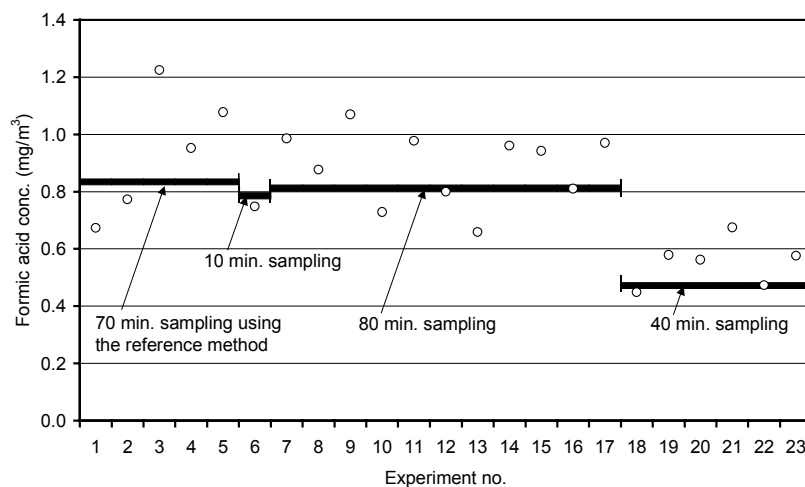


Figure 5.4: Field test of biosensors presented in chronological order. (○) represents each biosensor. Formic acid concentration was calculated using the calibration curve (Figure 5.3). (—) represents the concentrations of formic acid determined using the reference method. Each line represents one reference-sampling occasion.

5.3.2.2 Personal exposure measurements

The personal exposure measurements of formic acid were performed to determine if the exposure levels were in the concentration range that the biosensor could be used. Sampling equipment was worn by four workers working with glulam production in different ways. There was found to be some variation in the exposure levels of the different work tasks. The exposure levels were 0.6, 0.7, 1.1 and 1.9 mg/m³ of formic acid. As a reference, the Swedish threshold limit value (TLV) for formic acid has been set at 5 mg/m³ (Arbetskyddsstyrelsen, 2000). The glulam production unit also has a policy of changing the workers' tasks at least once per day, which ensures that no one is constantly exposed to a higher level of formic acid.

5.3.3 Investigation of other enzymes

To test if the biosensor design was suitable for measurement of other compounds, the FDHr enzyme was exchanged with three other NAD⁺-dependent dehydrogenases (FoDH, AcDH and AdDH). The altered biosensors were then tested by exposing them to the corresponding analytes in gas-phase. However, none of the biosensors responded to the exposure. A FoDH based biosensor for formaldehyde measurements had been investigated earlier, and the results were negative (Dennison, 1995). These biosensors contained a glycerol solution and the reason for the lack of response was said to be the poor ability of glycerol solutions to dissolve formaldehyde. Hence, it was no surprise that the FoDH biosensor did not respond to the formaldehyde exposure. The lack of response from the AcDH and AdDH biosensors may be due to the same reason, but the enzymes may also have a poor functionality caused by the glycerol solution itself. Therefore, it may be possible to construct a functioning biosensor that does not include the glycerol solution. However, this would require another method of retaining water in the biosensor. Hence, that would work against the purpose of a biosensor design that can easily be changed to measure different gaseous compounds. The FoDH, AcDH and AdDH biosensors would most likely have benefited from a thorough optimisation procedure, as was performed for the FDHr biosensor. The conditions for the experiments were performed with parameters optimised for the FDHr-formic acid system, but this does not mean that the parameters are suitable for other enzymes.

5.4 Conclusions

The selectivity and field tests showed that the range of the biosensor was suitable for use at occupational formic acid concentrations, and only minor effects from suspected interfering compounds were found. Only acetic acid had a significant effect on the biosensor response. However, the response from formic acid was far greater than from acetic acid. The exchange of the enzymes was far from simple and the investigation shows that the glycerol solution, under the conditions tested, was not a suitable matrix for the alternative enzymes explored.

6 General discussion

The aim of this thesis was to evaluate the possibility of developing a gas-phase biosensor which can be used for chemical exposure measurements. Focus was put on the construction of a formic acid biosensor – from development to field evaluation.

6.1 User friendliness

When measuring personal exposures to chemicals it is of utmost importance to have measurement devices that do not interfere with the person's activities. This usually means that the smaller the devices are the better. Although the biosensor presented in this thesis is not a finished product, it can already be used in a way that traditional two-step sampling/analysis methods cannot. It can produce a result within minutes of starting a measurement, which is an important feature in devices intended for chemical exposure assessment. In this thesis it is shown that a very simple and easy-to-produce device is suitable for measuring formic acid directly in air.

6.2 Environmental requirements

The biological component in a biosensor requires a certain amount of humidity in order to work properly. When using biosensors to monitor directly in air there is no consistent supply of water as is the case when sampling in liquids. Therefore, it is important to solve or to work around this problem. When looking at the scientific literature the issue of water loss in the biosensors due to low relative humidity in the sampled atmosphere has been ignored in some publications and in other publications the problem has been solved in a number of different ways.

Relative humidity is, for instance, not an important issue when the sampled gas is dissolved in a flow of liquid, which is then passed over a liquid-phase biosensor (Okada *et al.*, 1983; Matuszewski and Meyerhoff, 1991; Vianello *et al.*, 1996; Ligler *et al.*, 1998). This also applies when the gas is passed through a liquid reactor and the produced gas is monitored with a gas sensor (Okada *et al.*, 1981;

Karube *et al.*, 1982), when gas is dissolved into liquids in other ways or when there is a constant addition of liquid to the system (Goodson and Jacobs, 1974; Rindt and Scholtissek, 1989; Mitsubayashi *et al.*, 1994; Naessens and Tran-Minh, 1998a; Mitsubayashi *et al.*, 2000; Mitsubayashi and Hashimoto, 2000). Biosensors can also contain different amounts of liquid, with the ones containing larger amounts of liquid being less prone to the effects of low relative humidity (Okahata and Shimizu, 1987; Hämmerle *et al.*, 1996). Non-physiological media for enzymes have been used to retain water. Glycerol is hygroscopic and retains water well. Although biosensors constructed with this material are probably more sensitive to low relative humidity, it does open the field for smaller biosensors that can be used as direct reading instruments for a longer period of time (Dennison *et al.*, 1995). Immobilisation in reversed micelli has also been used to prevent water loss as it acts as a barrier to evaporation (Dennison *et al.*, 1996). Some areas of interest are not concerned with the problems of water retention, e.g. breath alcohol detection. Firstly, the human breath contains large amounts of water, and secondly, the measurements are usually performed within a minute, which is short enough not to be affected significantly by water loss. This is applicable for most biosensors that have a short time for detection and they are usually activated by wetting the active part (Park *et al.*, 1995; Yee *et al.*, 1996; Park *et al.*, 1999; Smyth *et al.*, 1999).

The issue of relative humidity is very important when the above favourable circumstances do not apply and therefore investigations of gas-phase biosensors should be performed over as large a range of relative humidity as possible. However, it is not always possible to investigate a large range and this was the case in Chapter 4. Initial experiments showed that the biosensors fouled at relative humidities below 30 % and the multivariate evaluation was therefore performed at humidities between 40 and 80 %. However, the field test showed that they could be used at lower humidities, at least for short sampling periods. It was also evident that the sampling period could be extended if the humidity of the sampled atmosphere was over 40 %.

6.3 Immobilisation

Different types of immobilisation media (alginate gel, sol-gel, glycerol solution) for the biological system were investigated in this thesis. It was found that a glycerol solution was the most promising of the investigated media. It gave a faster response than alginate gel when exposed to formic acid vapour. Although sol-gel is an interesting technique it is usually used in optical biosensors and was found not to be suitable for the present application. The surface of the screen-printed electrodes was most likely the cause of the failure with the sol-gel. It appeared that the transparent silica gel did not adhere properly to the surface.

Since a glycerol solution was used as immobilisation media the viscosity was higher than for an aqueous solution. Hence, the mass transfer of the compounds in the glycerol solution can be assumed to be slower, causing a slower response time. However, this is the price that has to be paid for the glycerol's ability to retain water.

6.4 Membranes

The biosensor was constructed with a membrane covering the glycerol solution. This was mainly to keep the solution in place, since the liquid has a tendency to move, but the membrane also helped to decrease the transport of water vapour to and from the glycerol solution.

The membrane found to give most stability and least variation in the response was a PTFE membrane with 5 μm pore size. The membrane also works as a diffusion barrier, restricting the diffusion of formic acid into the glycerol solution. This diffusion can be described by the laws of diffusion, mentioned in Chapter 1.3.1.2. Hence, the biosensor has a constant flow of formic acid through the membrane dependent on the vapour concentration. The formation of formate in the glycerol solution can be assumed to be quick and the back diffusion of formic acid from the biosensor could therefore be assumed to be slow. It is important to have this type of diffusion barrier included in the system since it makes the sensor less

sensitive to airflow of the surrounding atmosphere. That is the amount of formic acid diffusing through the membrane would not be dependent on the velocity of the air passing the biosensor, within reasonable limits.

6.5 Stability

Another important issue for biosensor development is the stability. Since biosensors incorporate biological systems they are particularly sensitive not only to humidity, but also to temperature, pH and destructive chemicals. Both operational and storage stability have to be investigated when developing biosensors (Nistor *et al.*, 1999).

The operational stability of the formic acid biosensor was satisfactory for the initial investigations. However, it was dependent on the formic acid concentration for which it was exposed to. It can therefore be suspected that the recirculation of the components in the biosensor was not functioning properly, and that one of the components was degraded in some way. The instability of the biosensor can probably be attributed to FDH, NAD⁺ or Meldolas' blue, since these components are the most sensitive in the system. However, the cause of instability has not been investigated in detail in this thesis.

Despite the loss of operational functionality after a certain time the biosensor can still be used with satisfactory performance within defined limits. For instance, it can be used as a direct reading instrument when measuring lower levels of formic acid vapour or as a single-use biosensor when only short sampling periods are needed.

During the investigation of how to solve the stability problems found in Chapter 3, the enzyme system was initially considered the most likely cause of the problem. A number of polymers were examined for use with or instead of the glycerol solution, in order to stabilise the enzyme system, e.g. Nafion, poly

vinylpyridine and Eastman AQ. However, all presented other problems, such as excessive water loss from the biosensor.

The early problem of storage stability was addressed in Chapter 3 and it was discovered that this was caused by the glue that was initially used to attach the membrane to the sensor. The solution to this problem was to attach the membrane in another way and the use of a simple tape was found to be satisfactory for this purpose. There are obviously one or several components in the glue that poison the biosensor system. However, the problem of decrease in response was only found in stored biosensors; in biosensors used directly following construction (see Chapter 3) the glue did not cause any observable problem. Cyanoacrylate glues are used as adhesives in medical and dental surgery, and are considered biologically degradable (Causton, 1992). They are also used in various industrial applications and in homes as all-purpose glues. The adhesive properties are developed by polymerisation triggered by hydroxyl groups on the surfaces to be glued, and since proteins contain large numbers of these groups the cyanoacrylates are very adhesive to biological tissues. This effect is used in some surgical applications, instead of sutures. This biological interaction is also most likely the cause of the rapid degradation of the biosensors with glued membranes. When attaching the membrane using cyanoacrylate glue it probably reacts with weak base groups in the enzymes and causes a loss of enzyme activity.

6.6 Performance optimisation

Few papers have previously been published which use a multivariate approach to the development and evaluation of biosensors. Multivariate techniques have the advantage of considering all variables at the same time. Interaction effects are also taken into account with this approach. Therefore, this type of evaluation appears to be preferable over methods which evaluate one parameter at the time, since these inevitably fail to find possible interaction effects. It is perhaps surprising that multivariate methods are not more widely employed in reports of biosensor development.

The multivariate evaluation and optimisation performed in Chapter 4 showed that the most important variable for the responses was the amount of enzyme in the glycerol solution. This result was not surprising since the enzyme usually has a key roll in biosensors. However, more surprising was that no other variables were clearly significant in these investigations. Although Meldola's blue was significant in the second PC it did give contradictory results since Meldola's blue had a positive effect on two of the response variables and a smaller negative effect on the rest. However, the first PC explained 76 % of the variation and was therefore considered of greater importance. Although PLS was also used it was mainly utilised to verify the results from the combined PCA-MLR. In order to increase the response and the response rate, optimisation of the biosensor was performed based on the results from the multivariate evaluation. This procedure increased the values of the response variables from between 18 and 89 %. It is difficult to compare the results from the multivariate evaluation and optimisation with a traditional one-variable-at-the-time (OVAT) approach since the latter has not been performed in this thesis. However, the importance of a multivariate approach has been explained by Box and co-workers, who show that it has the ability to extract reliable results from a smaller number of experiments and, more importantly, that it also has the ability to detect interacting variables (Box *et al.*, 1978). Since the OVAT approach does not consider interactions there is always a risk of not reaching optimum conditions. As has been mentioned in Chapter 1, multivariate methods are rarely used in biosensor development and the use of OVAT approach is frequent. For example, a paper published by Park and co-workers describes the development of a differential-type biosensor for determination of breath alcohol (Park *et al.*, 1999). They describe a step-wise investigation of the effects of hydration time, pH of the hydration buffer and temperature. With this approach the authors assume that there are no interacting effects between the variables. In this example it would not have been unreasonable to assume that some of the variables might interact with each other. Other variables would also be appropriate to investigate for possible interactions,

e.g. construction variables. To include these possibilities the experiments have to be planned so that all variables can be varied at the same time, and this can be achieved using experimental design followed by a multivariate analysis to generate the result. Hence, the multivariate approach described in Chapter 4 was used. There are, of course, many ways to incorporate a multivariate approach as a tool in the development of biosensors and one way has been described in Chapter 4.

6.7 Sampler validation

When reviewing the literature on biosensors for air monitoring there are a number of articles that do not have well-described systems for generating test atmospheres. Also in many cases there are a lack of reliable reference methods to determine the gaseous concentrations that are used for the tests. In addition, most of the sensors have not been sufficiently validated, which is a requirement from the EU when it comes to developing devices for air monitoring (CEN, 1995; CEN, 1996; CEN, 2001a; CEN, 2001b). These standards describe the requirements placed on methods for the monitoring of workplace and ambient atmospheres, with a focus on the use of diffusive samplers. Requirements include tests on how the samplers are influenced by variables such as air velocity, air temperature, air humidity, exposure concentrations, sampling time and storage etc. They also state what is required when generating the test atmospheres and when verifying the exposure concentrations. Although these requirements apply to diffusive samplers they may also be suitable for gas-phase biosensors. There is also a standard for direct reading instruments used in workplace atmospheres for detecting toxic gases, which is also applicable (CEN, 1999a; CEN, 1999b). It covers a large range of aspects that are interesting when using a direct reading electrical instrument to monitor gaseous chemicals. However, many of these requirements are only interesting for devices that will actually be used in practical applications. Examples of validations of diffusive samplers can be found for sampling NO₂ using the Willems badge (Hagenbjörk-Gustafsson *et al.*, 1999) and for sampling acetaldehyde using the GMD sampler (Lindahl *et al.*, 1996). No

standard protocol has been written for gas-phase biosensors specifically. However, in the gas-phase biosensor literature there are examples of a number of different techniques for generating and validating test atmospheres, e.g. sampling of headspace and verification with GC-MS (Smyth *et al.*, 1999), generation of ethanol using a breath alcohol simulator and verifying with GC (Park *et al.*, 1999), using commercially available gas tubes and verification using a colourimetric method (Okada *et al.*, 1983), and generation using a permeation tube and verifying the concentrations gravimetrically or with Draeger tubes (Dennison *et al.*, 1996). However, papers on biosensor evaluation seldom discuss the need for proper standardised validation.

6.8 Field tests

To ensure that the biosensor can be used for its intended purpose, selectivity and field investigations were performed. The selectivity of the biosensor was tested by exposing the biosensor to acetic acid, methanol and formaldehyde. It was found that high levels of acetic acid exposures gave a significant response from the biosensor. It has previously been reported that the nearest structural homologues of formic acid, e.g. acetate, propionate, oxalate, pyruvate and methanol, do not have affinity to FDH (Quayle, 1966), or at least only a very weak one (Popov and Lamzin, 1994).

However, the response from formic acid was far greater and the interference from acetic acid should not cause any misleading results in practice if the problem is understood. Methanol and formaldehyde did not interfere detectably with the biosensor. Depending on the precise application of the biosensor, it will probably be necessary to test for other possible interfering compounds to ensure accurate measurements are achieved in the field.

It is also very important to validate sampling devices in real environments. This was performed in a glutam factory where the workers are exposed to formic acid. Although the biosensor is not yet suitable for being carried by workers, it did

prove suitable for measuring the formic acid vapour concentration at a work station. Since the final target of this research programme is a device for personal exposure measurement, further work is required to minimise the size of the complete system to ensure that it will not be intrusive for the workers.

6.9 General conclusions

In general, gas-phase biosensors can offer exquisite detection limits and selectivity, but the instability of isolated biological systems is aggravated by the need to operate in air. Nevertheless, the literature shows a number of innovative approaches to engineering solutions to those problems and niche applications of biosensors for air monitoring can be expected to materialise as a commercial reality. The field of biosensor research and development is rapidly expanding. The use of biosensors in air monitoring is mainly targeted on real-time devices for monitoring atmospheric pollution or research applications, but there is also a need for fast and simple devices for personal exposure measurements in occupational environments; biosensor technology could make an important contribution to this objective. Another major advantage with biosensors is that the manufacturing process can be inexpensive due to mass-production technology that is now widely available. Increased demand for more frequent and more varied analyses in the work place can be expected to catalyse biosensor developments in this area and we can expect to see commercially available devices in due course.

7 Future work

When looking at biosensors for air monitoring the conclusion can be drawn that this application has not received the same attention as other areas. This could be attributed to the fact that the major commercial area for biosensors has, up to now, been in the field of medicine. While biosensors are being developed for air monitoring in several laboratories, there are still some requirements that have yet to be fulfilled for them to be accepted generally for this application.

The field of personal exposure monitoring using biosensors has not previously been addressed. Although this thesis shows the potential of using biosensors in this area there is still work to be done. Stability, which is often mentioned as a problem in biosensor development, has to be improved. Recently, there have been a number of reports of advances in areas such as molecular imprinted polymers (Piletsky *et al.*, 2001) and engineered proteins (O'Connell and Guilbault, 2001) and these techniques could have a tremendous effect on the development of future biosensors or biomimetic sensors. For instance, it may be possible to develop biosensors with synthetic enzymes or receptors that can be optimised for a specific purpose and will offer significantly increased operational and storage stability.

In the case of the formic acid biosensor presented in this thesis, future work should include investigations on how to increase the operational and possibly the storage stability of the device. To make this biosensor into a practical and useful tool for personal exposure measurements the electronic instrumentation also has to be further developed for this purpose, e.g. small, portable and easy to use. However, the methods that exist today in the electronics and diagnostics industry should make this a relatively inexpensive and simple process.

8 References

- Anon. (1999). *S-Plus 2000. Guide to statistics*. Seattle, WA, USA: MathSoft.
- ACGIH (2001). *Threshold limit values for chemical substances and physical agents and biological exposure indices*. Cincinnati, OH, USA: American Conference of Governmental Industrial Hygienists.
- Albery, W. J., Cass, A. E. G., Mangold, B. P. & Shu, Z. X. (1990a). Inhibited enzyme electrodes. Part 3: A sensor for low levels of H₂S and HCN. *Biosens. Bioelectron.* **5**: 397-413.
- Albery, W. J., Cass, A. E. G. & Shu, Z. X. (1990b). Inhibited enzyme electrodes. Part 1: Theoretical model. *Biosens. Bioelectron.* **5**: 367-378.
- Albery, W. J., Cass, A. E. G. & Shu, Z. X. (1990c). Inhibited enzyme electrodes. Part 2: The kinetics of the cytochrome oxidase system. *Biosens. Bioelectron.* **5**: 379-395.
- Arbetskyddsstyrelsen (2000). *Hygieniska gränsvärden och åtgärder mot luftföroreningar, AFS 2000:3 (in Swedish)*. Solna, Sweden: Publikationsservice.
- Aylott, J. W., Richardson, D. J. & Russell, D. A. (1997). Optical biosensing of gaseous nitric oxide using spin-coated sol-gel thin films. *Chem. Mater.* **9**: 2261-2263.
- Barzana, E., Klibanov, A. M. & Karel, M. (1989). A colometric method for the enzymatic analysis of gases: the determination of ethanol and formaldehyde vapours using solid alcohol oxidase. *Anal. Biochem.* **182**: 109-115.
- Berlin, A., Brown, R. H. & Saunders, K. J. (1987). *Diffusive sampling. An alternative approach to workplace air monitoring*. London, UK: the Royal Society of Chemistry.

Box, G. E. P., Hunter, W. G. & Hunter, J. S. (1978). *Statistics for experimenters* 510-526. New York, USA: John Wiley & Sons, Inc.

Bratchell, N. (1989). Multivariate response surface modelling by principal components analysis. *J Chemom* **3**: 579-588.

Carlson, R. (1992). *Design and optimization in organic synthesis*. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Carlson, R., Nordahl, Å., Barth, T. & Myklebust, R. (1992). An approach to evaluating screening experiments when several responses are measured. *Chemom. Intell. Lab. Syst.* **12**: 237-255.

Case, G. D. & Crivello, J. F. (1990). A film badge biosensor for hazardous environmental chemical agents. *Proc. Natl. Conf. Hazard. Wastes Hazard. Mater.* **7**: 229-233.

Causton, B. E. (1992). Medical and dental adhesives. In *Materials science and technology*. Cahn, R. W., Haasen, P. & Kramer, E. J. (Eds.) **14**: 291-292. Weinheim, Germany: VCH.

CEN (1995). *Workplace atmospheres - General requirements for the performance of procedures for the measurement of chemical agents*. (Standard No. EN482). Brussels, Belgium: Comité Européen de normalisation.

CEN (1996). *Workplace atmospheres - Diffusive sampling for the determination of gases and vapours - Requirements and test methods*. (Standard No. EN838). Brussels, Belgium: Comité Européen de normalisation.

CEN (1999a). *Workplace atmospheres - Electrical apparatus used for the direct detection and direct concentration measurement of toxic gases and vapours - Part 1: General requirements and test methods*. (Standard No. EN45544-1). Brussels, Belgium: Comité Européen de normalisation.

CEN (1999b). *Workplace atmospheres - Electrical apparatus used for the direct detection and direct concentration measurement of toxic gases and vapours - Part 2: Performance requirements for apparatus used for measuring concentrations in the region of limit values.* (Standard No. EN45544-2). Brussels, Belgium: Comité Européen de normalisation.

CEN (2001a). *Ambient air quality - Diffusive samplers for the determination of concentrations of gases and vapours - Part 1: General requirements.* (Standard No. EN13528-1). Brussels, Belgium: Comité Européen de normalisation.

CEN (2001b). *Ambient air quality - Diffusive samplers for the determination of concentrations of gases and vapours - Part 2: Specific requirements and test methods.* (Standard No. EN13528-2). Brussels, Belgium: Comité Européen de normalisation.

Clark Jr., L. C. & Lyons, C. (1962). Electrode systems for continuous monitoring in cardiovascular surgery. *Ann N Y Acad Sci* **102**: 29-45.

Dabek-Zlotorzynska, E. & McGrath, M. (2000). Determination of low-molecular-weight carboxylic acids in the ambient air and vehicle emissions: a review. *Fresenius. J. Anal. Chem.* **367**: 507-518.

Danzer, T. & Schwedt, G. (1996a). Chemometric methods for the development of a biosensor system and the evaluation of inhibition studies with solutions and mixtures of pesticides and heavy metals. 1. Development of an enzyme electrodes system for pesticide and heavy metal screening using selected chemometric methods. *Anal. Chim. Acta* **318**: 275-286.

Danzer, T. & Schwedt, G. (1996b). Multivariate evaluation of inhibition studies on an enzyme electrodes system with pesticides and mixtures of mercury and pesticides. *Anal. Chim. Acta* **325**: 1-10.

Dawson, B. & Trapp, R. G. (2001). *Basic and clinical biostatistics* (3rd ed.). USA: Lange Medical Books/McGraw-Hill.

Dennison, M. J., Hall, J. M. & Turner, A. P. F. (1995). Gas-phase microbiosensor for monitoring phenol vapor at ppb levels. *Anal. Chem.* **67**: 3922-3927.

Dennison, M. J., Hall, J. M. & Turner, A. P. F. (1996). Direct monitoring of formaldehyde vapour and detection of ethanol vapour using dehydrogenase-based biosensors. *Analyst* **121**: 1769-1773.

Dennison, M. (1995). Gas-phase enzyme biosensors. PhD Thesis, Cranfield University, Cranfield, England.

Eklöv, T., Mårtensson, P. & Lundström, I. (1999). Selection of variables for interpreting multivariate gas sensor data. *Anal. Chim. Acta* **381**: 221-232.

Ellerby, L. M., Nishida, C. R., Nishida, F., Yamanaka, S. A., Dunn, B., Valentine, J. S. & Zink, J. I. (1992). Encapsulation of proteins in transparent porous silicate glasses prepared by the Sol-Gel method. *Science* **255**: 1113-1115.

Engelmark Cederborg, S. (2001). Miljövänlig myrsyra (in Swedish). *Kemivärlden med Kemisk Tidskrift* 28-30.

Gaughlitz G. (2000). Optical sensors. In *Biosensors for environmental monitoring*. Bilitewski, U. & Turner, A. P. F. (Eds.) 28-51. Amsterdam, The Netherlands: Harwood Academic Publishers.

Gibson, L. T., Cooksey, B. G., Littlejohn, D. & Tennent, N. H. (1996). Pollution from showcases. *Museum practise* **1**: 46-47.

Gibson, L. T., Cooksey, B. G., Littlejohn, D. & Tennent, N. H. (1997a). Determination of acetic acid and formic acid vapour concentrations in the museum environment by passive sampling. In *European Cultural Heritage Newsletter on Research (ECHNLR)*. The European Commission DG XII **10**: 108-112.

Gibson, L. T., Cooksey, B. G., Littlejohn, D. & Tennent, N. H. (1997b). Determination of experimental diffusion coefficients of acetic acid and formic acid vapours in air using a passive sampler. *Anal. Chim. Acta* **341**: 1-10.

Gibson, L. T., Cooksey, B. G., Littlejohn, D. & Tennent, N. H. (1997c). A diffusion tube sampler for the determination of acetic acid and formic acid vapours in museum cabinets. *Anal. Chim. Acta* **341**: 11-19.

Gil, G. C., Mitchell, R. J., Chang, S. T. & Gu, M. B. (2000). A biosensor for the detection of gas toxicity using a recombinant bioluminescent bacterium. *Biosens. Bioelectron.* **15**: 23-30.

Goodson, L. H. & Jacobs, W. B. (1974). Application of immobilised enzymes to detection and monitoring. In *Enzyme engineering*. Pye, E. K. & Wingard, Jr. L. B. (Ed.) **2**: 393-400. New York, USA: Plenum Press.

Gorton, L. (1986). Chemically modified electrodes for the electrocatalytic oxidation of nicotinamide coenzymes. *J. Chem. Soc., Faraday Trans.* **82**: 1245-1258.

Guilbault, G. G. (1983). Determination of formaldehyde with an enzyme-coated piezoelectric crystal detector. *Anal. Chem.* **55**: 1682-1684.

Hagenbjörk-Gustafsson, A., Lindahl, R., Levin, J.-O. & Karlsson, D. (1999). Validation of a diffusive sampler for NO₂. *J. Environ. Monit.* **1**: 349-352.

Hämmerle, M., Hall, E. A. H., Cade, N. & Hodgins, D. (1996). Electrochemical enzyme sensor for formaldehyde operating in the gas phase. *Biosens. Bioelectron.* **11**: 239-246.

Hart, J. P., Abass, A. K. & Cowell, D. (2002). Development of disposable amperometric sulfur dioxide biosensors based on screen printed electrodes. *Biosens. Bioelectron.* **17**: 389-394.

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scand J Statist* **6**: 65-70.

Höpner, T. & Knappe, J. (1974). Formate - Determination with formate dehydrogenase. In *Methods of enzymatic analysis*. Bergeyer, H. U. (Ed.) (2 ed.) 1551-1555. New York, USA: Academic Press Inc.

HSE (2001). *EH40/2001 Occupational exposure limits 2001*. UK: Health and Safety Executive.

International conference. *Measuring air pollutants by diffusive sampling*; 26-28 Sept 2001, Montpellier, France. (in press).

Iqbal, S. S., Mayo, M. W., Bruno, J. G., Bronk, B. V., Batt, C. A. & Chambers, J. P. (2000). A review of molecular recognition technologies for detection of biological threat agents. *Biosens. Bioelectron.* **15**: 549-578.

Kaisheva, A., Iliev, I., Christov, S. & Kazareva, R. (1997). Electrochemical gas biosensor for phenol. *Sens. Actuators, B* **44**: 571-577.

Kaisheva, A., Iliev, I., Kazareva, R., Christov, S., Wollenberger, U. & Scheller, F. (1996). Enzyme/gas-diffusion electrodes for determination of phenol. *Sens. Actuators, B* **33**: 39-43.

Karube, I., Okada, T. & Suzuki, S. (1982). A methane gas sensor based on oxidizing bacteria. *Anal. Chim. Acta* **135**: 61-67.

Kauppinen, T. (1996). Exposure assessment - a challenge for occupational epidemiology. *Scand J Work Environ Health* **22**: 401-403.

Kawamura, K., Ng, L.-L. & Kaplan, I. R. (1985). Determination of organic acids (C1-C10) in atmosphere, motor exhausts, and engine oils. *Environ. Sci. Technol.* **19**: 1082-1086.

- Krantz-Rülcker, C., Stenberg, M., Winqvist, F. & Lundström, I. (2001).** Electronic tongues for environmental monitoring based on sensor arrays and pattern recognition: a review. *Anal. Chim. Acta* **426**: 217-226.
- Kulys, J. J., Bilitewski, U. & Schmid, R. D. (1991).** Reagentless biosensors for substrates of dehydrogenases. *Anal. Lett.* **24**: 181-189.
- Lautenberger, W. J., Kring, E. V. & Morello, J. A. (1980).** A new personal badge monitor for organic vapors. *Am Ind Hyg Assoc J* **41**: 737-747.
- Levin, J.-O., Andersson, K., Lindahl, R. & Nilsson, C.-A. (1985).** Determination of sub-part-per-million levels of formaldehyde in air using active or passive sampling on 2,4-dinitrophenylhydrazine-coated glass fiber filters and high-performance liquid chromatography. *Anal. Chem.* **57**: 1023-1035.
- Levin, J.-O. & Lindahl, R. (1994).** Diffusive sampling of reactive compounds - A review. *Analyst* **119**: 79-83.
- Liesivuori, J. & Kettunen, A. (1983).** Farmers' exposure to formic acid vapor in silage making. *Ann. Occup. Hyg.* **27**: 327-329.
- Liesivuori, J., Laitinen, J. & Savolainen, H. (1992).** Kinetics and renal effects of formic acid in occupationally exposed farmers. *Arch. Toxicol* **66**: 522-524.
- Liesivuori, J. & Savolainen, H. (1991).** Methanol and formic acid toxicity: biochemical mechanisms. *Pharmacol. Toxicol* **69**: 157-163.
- Ligler, F. S., Anderson, G. P., Davidson, P. T., Foch, R. J., Ives, J. T., King, K. D., Page, G., Stenger, D. A. & Whelan, J. P. (1998).** Remote sensing using an airborne biosensor. *Environ. Sci. Technol.* **32**: 2461-2466.
- Lin, C. F., Wu, T. Z., Hao, O. J., Lin, Y. C. & Rau, Y. R. (2000).** Biosensor for detecting odorous compounds. *J. Environ. Eng.* **126**: 446-450.

- Lindahl, R., Levin, J.-O. & Mårtensson, M. (1996).** Validation of a diffusive sampler for the determination of acetaldehyde in air. *Analyst* **121**: 1177-1181.
- Lobo, M. J., Miranda, A. J. & Tuñón, P. (1997).** Amperometric biosensors based on NAD(P)-dependent dehydrogenase enzymes. *Electroanal* **9**: 191-202.
- Matsunaga, T., Karube, I. & Suzuki, S. (1980).** A specific microbial sensor for formic acid. *European J. Appl. Microbiol. Biotechnol.* **10**: 235-243.
- Matuszewski, W. & Meyerhoff, M. E. (1991).** Continuous monitoring of gas-phase species at trace levels with electrochemical detectors. Part 1. Direct amperometric measurement of hydrogen peroxide and enzyme-based detection of alcohols and sulfur dioxide. *Anal. Chim. Acta* **248**: 379-389.
- Mitsubayashi, K. & Hashimoto, Y. (2000).** Development of a gas-phase biosensor for trimethylamine using a flavin-containing monooxygenase 3. *Electrochemistry* **68**: 901-903.
- Mitsubayashi, K., Hashimoto, Y., Nakayama, Y., Montesinos, T., Noguer, T. & Marty, J.-L. (2000).** Gas-sensors with high selectivity using a biological catalytic system. *J. Adv. Sci.* **12**: 211-216.
- Mitsubayashi, K., Yokoyama, K., Takeuchi, T. & Karube, I. (1994).** Gas-phase biosensor for ethanol. *Anal. Chem.* **66**: 3297-3302.
- Muramatsu, H., Tamiya, E. & Karube, I. (1989).** Detection of odorants using lipid-coated piezoelectric crystal resonators. *Anal. Chim. Acta* **225**: 399-408.
- Muramatsu, H., Tamiya, E. & Karube, I. (1991).** Odorant recognition using quartz resonators coated with a mixed film of asolectin and cholesterol and monitoring the viscoelastic change of the film. *Anal. Chim. Acta* **251**: 135-141.
- Naessens, M. & Tran-Minh, C. (1998a).** Whole-cell biosensor for determination of volatile organic compounds in form of aerosols. *Anal. Chim. Acta* **364**: 153-158.

- Naessens, M. & Tran-Minh, C. (1998b).** Whole-cell biosensor for direct determination of solvent vapors. *Biosens. Bioelectron.* **13**: 341-346.
- Nelson, G. O. (1982).** *Controlled test atmospheres. Principles and techniques.* Ann Arbor, MI, USA: Ann Arbor Science Publishers, Inc.
- Ngeh-Ngwainbi, J., Foley, P. H., Kuan, S. S. & Guilbault, G. G. (1986).** Parathion antibodies on piezoelectric crystals. *J. Am. Chem. Soc.* **108**: 5444-5447.
- Nielsen, G. D., Hansen, L. F., Nexø, B. A. & Poulsen, O. M. (1998).** Indoor air guideline levels for formic, acetic, propionic and butyric acid. *Indoor Air Suppl.* **5**: 8-24.
- NIOSH. (1994).** Formic acid: Method 2011. In *NIOSH Manual of Analytical Methods.* Eller, P. M. & Cassinelli, M. E. (Eds.) (4th ed.). Cincinnati, OH, USA: National Institute for Occupational Safety and Health.
- Nistor, C., Emnéus, J., Gorton, L. & Ciucu, A. (1999).** Improved stability and altered selectivity of tyrosinase based graphite electrodes for detection of phenolic compounds. *Anal. Chim. Acta* **387**: 309-326.
- O'Connell, P. J. & Guilbault, G. G. (2001).** Future Trends in Biosensor Research. *Anal. Lett.* **34**: 1063-1078.
- O'Sullivan, C. K. (1996).** Microbiosensor for the detection of gaseous sulphur dioxide in the atmosphere. PhD Thesis, Cranfield University, Cranfield, England.
- Okada, T., Karube, I. & Suzuki, S. (1981).** Microbial sensor system which uses *Methylobacterium* sp. for the determination of methane. *European J. Appl. Microbiol. Biotechnol.* **12**: 102-106.
- Okada, T., Karube, I. & Suzuki, S. (1983).** NO₂ sensor which uses immobilized nitrite oxidizing bacteria. *Biotechnol. Bioeng.* 1641-1651.

Okahata, Y. & Shimizu, O. (1987). Olfactory reception on a multilayer-coated piezoelectric crystal in a gas phase. *Langmuir* **3**: 1171-1172.

OSHA. (1991). Methyl alcohol: Method 91. In *OSHA Analytical methods*. Salt Lake City, UT, USA: US Department of Labor, Occupational Safety and health Administration, OSHA Analytical Laboratory.

OSHA. (1993). Acetic and formic acid in workplace atmosphere: Method 186. In *OSHA Analytical methods*. Salt Lake City, UT, USA: US Department of Labor, Occupational Safety and health Administration, OSHA Analytical Laboratory.

Park, J.-K., Yee, H.-J., Lee, K. S., Lee, W.-Y., Shin, M.-C., Kim, T.-H. & Kim, S.-R. (1999). Determination of breath alcohol using a differential-type amperometric biosensor based on alcohol dehydrogenase. *Anal. Chim. Acta* **390**: 83-91.

Park, J.-K., Yee, H.-J. & Kim, S.-T. (1995). Amperometric biosensor for determination of ethanol vapor. *Biosens. Bioelectron.* **10**: 587-594.

Piletsky, S. A., Subrahmanyam, S. & Turner, A. P. F. (2001). Application of molecularly imprinted polymers in sensors for the environment and biotechnology. *Sens Rev* **4**: 292-296.

Plackett, R. L. & Burman, J. P. (1946). The design of optimum multifactorial experiments. *Biometrika* **33**: 305-325.

Popov, V. O. & Lamzin, V. S. (1994). NAD⁺-dependent formate dehydrogenase. *Biochem J* **301**: 625-643.

Quayle, J. R. (1966). Formate dehydrogenase. *Methods Enzymol* **9**: 360-364.

Rajakovic, L., Ghaemmaghami, V. & Thompson, M. (1989). Adsorption on film-free and antibody-coated piezoelectric sensors. *Anal. Chim. Acta* **217**: 111-121.

Reshetilov, A. N., Donova, M. V., Dovbnya, D. V., Il'yasov, P. V., Boronin, A. M., Leasers, T. & Green, R. (1998). Membrane-bound dehydrogenases of gluconobacter oxydans: sensors for measuring sugars, alcohols, and polyoles. *Bull Exp Biol Med* **126**: 702-704.

Rindt, K.-P., Flauß, A., Feldbrügge, R., Prieß, U. & Kock, K. (1992). *Enzyme diffusion badges as biochemical devices for the dosimetric measurement of toxic compounds from air*. Lübeck, Germany: Drägerwerk.

Rindt, K.-P. & Scholtissek, S. (1989). Quantitative detection of gaseous substances at low ppm and sub-ppm levels by means of enzyme diffusion badges. In *Biosensors. Applications in medicine, environmental protection and process control*. Schmid, R. D. & Scheller, F. (Eds.) **13**: 405-415. GBF Monographs. Weinheim, Germany: VCH.

Saini, S., Surareungchai, W., Turner, A. P. F. & Downs, M. E. A. (1995). Preliminary investigation of a bioelectrochemical sensor for the detection of phenol vapours. *Biosens. Bioelectron.* **10**: 945-957.

Schelp, C., Scheper, T., Bückmann, F. & Reardon, K. F. (1991). 2 Fiberoptic Sensors With Confined Enzymes and Coenzymes - Development and Application. *Anal. Chim. Acta* **255**: 223-229.

Schöning, M. J., Schroth, P. & Schütz, S. (2000). The use of insect chemoreceptors for the assembly of biosensors based on semiconductor field-effect transistors. *Electroanal* **12**: 645-652.

Siekmann, H. & Kleine, H. (1992). Direct-reading instruments. In *Clean air at work. New trends in assessment and measurement for the 1990s*. Brown, R. H., Curtis, M., Saunders, K. J. & Vandendriessche, S. (Eds.) 156-161. Cambridge, UK: the Royal Society of Chemistry.

Situmorang, M., Hibbert, D. B. & Gooding, J. J. (2000). An experimental design study of interferences of clinical relevance of a polytyramine immobilized-enzyme biosensor. *Electroanalysis* **12**: 111-119.

Smyth, A. B., Talasila, P. C. & Cameron, A. C. (1999). An ethanol biosensor can detect low-oxygen injury in modified atmosphere packages of fresh-cut produce. *Postharvest Biol Technol.* **15**: 127-134.

Spicer, C. W., Nishioka, M. G., Trayser, D. A., Burkholder, H. M., Hupp, D. E., Kenny, D. V., Vermillion, W. D. & Ward, G. F. (1991). *Sampling and analysis of formic acid in methanol vehicle exhaust (Final report)*. Coordinating Research Council.

Sunesson, A.-L., Nilsson, C.-A. & Andersson, B. (1995). Evaluation of adsorbents for sampling and quantitative analysis of microbial volatiles using thermal desorption-gas chromatography. *J. Chromatogr. A* **699**: 203-214.

Sunesson, A.-L., Sundgren, M., Levin, J.-O., Eriksson, K. & Carlson, R. (1999). Evaluation of two adsorbents for diffusive sampling and thermal desorption-gas chromatographic analysis of monoterpenes in air. *J. Environ. Monit.* **1**: 45-50.

Thévenot, D. R., Toth, K., Durst, R. A. & Wilson, G. S. (1999). Electrochemical biosensors: Recommended definitions and classification (Technical Report). *Pure Appl. Chem.* **71**: 2333-2348.

Tishkov, V. I., Galkin, A. G., Fedorchuk, V. V., Savitsky, P. A., Rojkova, A. M., Gieren, H. & Kula, M.-R. (1999). Pilot scale production and isolation of recombinant NAD⁺- and NADP⁺-specific formate dehydrogenases. *Biotechnol. Bioeng.* **64**: 187-193.

Turner, A. P. F., Aston, W. J., Higgins, I. J., Bell, J. M., Colby, J., Davis, G. & Hill, H. A. O. (1984). Carbon monoxide:acceptor oxidoreductase from

Pseudomonas thermocarboxydovorans strain C2 and its use in a carbon monoxide sensor. *Anal. Chim. Acta* **163**: 161-174.

Turner, A. P. F. & Bilitewski, U. (2000). Conclusion. In *Biosensors for environmental monitoring*. Bilitewski, U. & Turner, A. P. F. (Eds.) 405-408. Amsterdam, The Netherlands: Harwood Academic Publishers.

Turner, A. P. F., Aston, W. J., Davis, G., Higgins, I. J., Hill, H. A. O. & Colby, J. (1985). Enzyme based carbon monoxide sensor. In *Microbial gas metabolism*. Poole, R. K. & Dow, C. S. (Ed.) 161-170. London, UK: Academic Press.

Tzang, C. H., Yuan, R. & Yang, M. S. (2001). Voltammetric Biosensors for the Determination of Formate and Glucose-6-Phosphate Based on the Measurement of Dehydrogenase- Generated Nadh and Nadph. *Biosens. Bioelectron.* **16**: 211-219.

Ulfvarson, U. (1995). Why should measurements be done? In *First Nordic workshop on quality assurance in occupational hygiene*. Levin, J.-O. (Ed.) *Scand J Work Environ Health* **21**: 312-314.

Vianello, F., Stefani, A., Di Paolo, M. L., Rigo, A., Lui, A., Margesin, B., Zen, M., Scarpa, M. & Soncini, G. (1996). Potentiometric detection of formaldehyde in air by an aldehyde dehydrogenase FET. *Sens. Actuators, B* **37**: 49-54.

Webb, E. C. (1992). *Enzyme nomenclature*. San Diego, CA, USA: Academic Press.

Wedge, R., Pemberton, R. M., Hart, J. P. & Luxton, R. (1999). Recent developments towards disposable screen-printed biosensors incorporating a carbon ink modified with the redox mediator, Meldola's Blue. *Analusis* **27**: 570-577.

White, S. F. & Turner, A. P. F. (1997). Mediated amperometric biosensors. In *Handbook of biosensors and electronic noses: medicine, food and the environment*. Kress-Rogers, E. (Ed.) 227-244. Boca Raton, FL, USA: CRC Press.

Williams, A. K. & Hupp, J. T. (1998). Sol-gel-encapsulated alcohol dehydrogenase as a versatile, environmentally stabilized sensor for alcohols and aldehydes. *J. Am. Chem. Soc.* **120**: 4366-4371.

Wold, S. (1978). Cross-validatory estimation of the number of components in factor and principal components models. *Technometrics* **20**: 397-405.

Wu, T.-Z. (1999). A piezoelectric biosensor as an olfactory receptor for odour detection: electronic nose. *Biosens. Bioelectron.* **14**: 9-18.

Yee, H.-J., Park, J.-K. & Kim, S.-T. (1996). Disposable thick-film amperometric biosensor with multiple working electrodes fabricated on a single substrate. *Sens. Actuators, B* **34**: 490-492.

Zhang, S., Wright, G. & Yang, Y. (2000). Materials and techniques for electrochemical biosensor design and construction. *Biosens. Bioelectron.* **15**: 273-282.