Biosensors for Food Analysis

Biosensors for Food Analysis

Edited by

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Preface

This Symposium was organised by the Royal Society of Chemistry Food Chemistry Group to stimulate discussion and future research in the development of biosensors for food analysis. The need to bring researchers from a variety of scientific disciplines together with the food industry is a constant requirement to ensure that insights and understanding in modern developments are shared. Therefore, the proceedings of the Symposium are being published to widen the scientific debate with the hope that questions which appear to be hindering progress in developing biosensor instruments for food analysis can be resolved thus enabling their commercial availability and use.

The success of the Symposium was greatly enhanced by the support received from the following people and organisations. Hillsdown Holdings plc and CWS Limited both gave generous financial support. Much assistance was received from my advisors to whom I am indebted for their encouragement whilst organising the Symposium: Dr R. Fenwick (Institute of Food Research, Norwich), Professor B.L. Wedzicha (University of Leeds) and in particular Dr R.L.S. Patterson (Queens University, Belfast), Professor M. Spiro (Imperial College, London) and Professor A.P.F. Turner (Cranfield University, Bedford). In addition, a number of students at the University of Leeds helped with running the Symposium. They were Miss J. Dimarkou, Miss D. Galani, Mr A. Griffin, Mr J. Guimar and Mr J. Zabetakis. The Tetley Group Limited were also generous in allowing me time and providing administrative support for the organisation of the Symposium; in particular I would like to thank Mrs K. Irvine and Mrs D. Martin for their secretarial assistance.

The completion of the proceedings has been due to the encouragement and support given by Mrs J. Freshwater and Miss K. Ferguson at The Royal Society of Chemistry, Cambridge. I would like to acknowledge the editorial advice received from Professor M. Spiro (Imperial College, London), Dr N. Ward (University of Surrey, Guildford) and Dr K. Kerrola and Dr K.N. Wood (The Tetley Group Limited, London); however, I retained responsibility for the editorial decisions.

The veracity of the contents of the papers is the responsibility of the authors. Through editing I have attempted to ensure that the chapters fitted into a coherent structure and are easily understood. Faults and omissions in the content are regrettable. However,

symposia proceedings can only present the available material and rely upon other publications to fill the gaps. It is my hope that further research will be stimulated to the benefit of both the food industry and of its customers.

Finally, I would like to thank my family, Androulla, Simone and Maria without whose tolerance and support this book would not have been published.

Andrew Scott
November, 1997

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Glossary

$h\nu$	light
λ	wavelength
$\lambda_{(max)}$	optimum wavelength for measuring absorbance
ANN	artificial neural network
ASP	advanced processing board
ATP	adenosine triphosphate
D-LDH	lactate dehydrogenase which metabolises the D form of lactate
L-LDH	lactate dehydrogenase which metabolises the L form of lactate
DNA	deoxyribonucleic acid
Epitope	part of the antibody which binds to its antigen
Fc	section of the antibody which contains the epitope
FIA	flow injection analysis
FMN	flavin mononucleotide, oxidised form
$FMNH_2$	flavin mononucleotide, reduced form
GOD	glucose oxidase
HCG	human chorionic gonadotropic hormone
IMS	ion mobility spectrometry
ISE	ion selective electrode
Mab	monoclonal antibody
MOSFET	metal oxide semiconductor field effect transistor
NAD^+	nicotinamide dinucleotide, oxidised form
$NAD(P)^+$	nicotinamide adenine dinucleotide phosphate, oxidised form
NAD(P)H	nicotinamide adenine dinucleotide phosphate, reduced form
NADH	nicotinamide dinucleotide, reduced form
NMP-TCNQ	*n*-methylphenazinium tetracyanoquinonedimethane
ONP	*ortho*-nitrophenol, 2-nitrophenol
OPEE	organic phase enzyme electrode
PC	personal computer
PVC	polyvinyl chloride
SAW	surface accoustic wave
SCE	saturated calomel electrode
TIRF	total internal reflection fluorescence
YSI	Yellow Springs Instrument Company

1
Introduction

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1 INTRODUCTION

The potential advantage of using biosensors for food analysis is a rapid, specific quantification without the need for extensive sample preparation. Rapidity is accomplished by coupling the biological component to a transducer to obtain an immediate result. Specificity is achieved through the reaction of an analyte with a particular biological component selected from a range which occur naturally; examples of these biological components are enzymes, antibodies, lectins and DNA. The transducer transforms the specific recognition of the analyte into an electronic, optical or similar signal which can be readily quantified. The specificity of biological components for particular analytes reduces the need for extensive sample extraction and analyte isolation before measurement can take place. An additional advantage is the possibility of measuring the analyte in its natural micro-environment thus avoiding analyte modification during sample preparation. Biosensor achievements have revolutionised the ability medical practitioners have to provide rapid and appropriate treatment for their patients in the clinic. In addition, diabetics can now measure blood glucose within minutes and can more effectively manage their own blood glucose level through their diet and their use of insulin. The consequent improvement in quality of life for diabetics is enormous. Another area of application has been the rapid detection of pregnancy which has obvious benefits in relieving stress and enabling women and their partners to make plans for the immediate future and to ensure the health and the care of the mother and baby.

Food analysis is a tool used to satisfy a broad range of requirements for both industry and government which endeavour to provide a healthy and nutritious diet for people around the world. Freshly harvested food can be taken to the kitchen and prepared for immediate consumption or can be packed and transported across the world, undergoing a sophisticated series of manufacturing processes along the way. The basic rationale for food analysis is to verify that food retains the qualities at harvest until it is consumed. The food may simply be tasted before making a purchase or it may be subjected to a sophisticated range of laboratory analyses, such as gas chromatography-mass spectrometry (e.g. detection of olive oil adulteration),[1] to determine the complete range of constituents of some processed product. Analysis can be used to confirm the healthy nature of the food to ensure compliance with legislation. Food products are diverse in nature not only because of the range of plants and animals that occur due to the inherent complexity of their constituents but also because of the sophisticated demands of consumers for conveniently

pre-prepared foods which can be consumed without any further preparation. The diversity of foods, the requirement for a long shelf life, the increasing amount of technology used in farming, the fortification of foods with additional nutrients, adulteration of foods and the consumer's demand for convenience all conspire to make an analyst's job seemingly impossible. The consequent need for a large number of rapid and precise means of estimating a long list of nutrients and contaminants has created a demand for tests which give immediate and accurate results. Consequently, research is constantly searching for indices of food which can be tested to assess food quality and safety.[2] With this in mind many analysts, researchers and food technologists look to biosensor technology as a promising solution.

This introduction provides an illustration of the problems and requirements encountered in food analysis. These issues need to be addressed by instrument researchers and developers in order that they may produce the technology to replace existing methods which often fall short of completely meeting the food industry's requirements.

2 FOOD ANALYSIS

Food analysis satisfies two basic needs: (i) to assess quality and (ii) to ensure compliance with legislation. The food industry uses analysis for quality assessment while compliance with legislation is the primary provenance of government agencies, although the two are inextricably linked. An example of quality assessment is emulsion stability. Milk, infant formulae, mayonnaise and coffee creamer products are processed to form an emulsion, stable over a given life time; whereas ice cream and whipping creams are expected to remain as emulsions for a stated period before being readily de-stabilised.[3] For a good quality food, the emulsion must perform as required and analyses are used to ensure that the processing and formulation are correct to achieve the required emulsion performance.

An example of industry and government inter-linked requirements is the case of vanillin. This flavour is common in foods and can be obtained from three main sources: vanilla beans (natural), lignin (semi-synthetic) and guaiacol (synthetic). The food industry needs to know the source of the vanillin because the natural form is more expensive and the government agencies need to ensure compliance with labelling regulations. Currently the different forms of vanillin can only be distinguished by sophisticated analyses such as isotopic analysis using nuclear magnetic resonance.[4] Government agencies also wish to ensure good nutrition from food and need to prevent misrepresentation through the sale of adulterated foods (such as meat).[5] More recently, legislation has focused on pack labelling, additives and ingredients, irradiation, contaminants, environmental issues,[6] the use of health claims in marketing foods[7] and genetically modified foods.[8] Government agencies are keen to ensure that health claims on labelling are truthful (and not misleading), valid, consistent with recognized medical and nutritional principles and comply with regulations.

The human senses (vision, smell and taste) are often more sensitive than most forms of food analysis. They can detect small differences in colours and low concentrations of flavours and aromas. Indeed, low concentrations of allergens and poisons can affect people before they are easily detected analytically. Sophisticated sensory analysis is holistic in its

approach and compares the similarities and differences of foods but cannot provide absolute quantification and is a destructive form of analysis.[9] In contrast, instumental analysis, despite its limitations, can be used to provide quantitative results on which to base judgements.

Food quality assessment encompasses a large range of measurements during the lifespan of the product. This includes: the selection of suitable raw materials, ensuring appropriate processing during manufacturing, verification of the required shelf life during storage, distribution and retail and ensuring the food is nutritious and safe to consume. For food that undergoes further processing the manufacturing conditions need to be optimised to maintain the expected product quality. One reason for food processing is to stabilise the nutrients in vegetables (eg blanching before freezing) which naturally deteriorate after harvest.[10] The blanching process is optimised to meet the requirements of each batch of vegetables being processed. For recipe dishes correct product formulation is an additional consideration. The resulting processed food is expected to have good nutritional values, sensory characteristics, shelf life attributes, no contamination and health and safety properties. The quality of the food packaging also influences these attributes together with the manufacturing environment which also may affect on the health and safety of their staff. The same concerns form the basis of standards set in legislation by governments.

3 THE PROBLEMS

Food analysis has six basic interests: the diverse nature of foods, form of the analyte, access to the analyte, the stability of the analyte during and after isolation, the stability of the food in its packaging and the appropriate level of sampling for analytical results to provide a true estimate of the qualities encountered in the bulk material.

Food diversity is clearly reflected in the wide-ranging diets throughout the world and the range of food industry sectors. The growth in demand for a wide range of so called ethnic foods is one of the most significant changes in the market place in the last decade. In addition, there are cultural influences on the preparation of and preferences for food. For example, kosher-observant Jews abide by particular regulations: the use of acceptable animals (mammals that chew the cud and have split hooves, domesticated birds that are not birds of prey, and fish with fins and scales); that blood is removed from the animal and properly discarded; that meat and dairy products are kept separate and different equipment is used during preparation and processing.[11] Different cultures have different taste preferences which international food companies recognise so recipes are modified accordingly.[12]

The nature of the food and the heterogeneous distribution and form of the analyte combine to create a complexity of issues to be resolved by the analyst in his endeavour to quantify the amount of an analyte. In the majority of cases, the analyst needs to separate the analyte from the food before detection and measurement can be accomplished. Extraction and isolation procedures normally begin with homogenising the food which changes the local micro-environment surrounding the analyte and this potentially affects its form; sample preparation for the detection of pathogens often includes dilution,

centrifugation, filtration, partition and enrichment.[13] Homogenisation also increases the opportunities for confounding chemical reactions to occur; transformation, chelation or modifications due to pH effects (e.g. natural food colours).[14] The broad range of foods include: meat; fish, dairy; cereal; fruit; vegetables; baked goods; beverages; confectionery; and snacks. The range of food analytes comprises solids, liquids and gases as ionic, radical or neutral species; each presenting a different challenge for the food analyst. Furthermore, most food components undergo changes during digestion to produce more soluble components which may or may not improve their nutrient value. This is illustrated by the case of vitamin A; *trans*-retinol has vitamin A activity.[15] However, foods contain over 600 known carotenoids, precursors of *trans*-retinol, of which only approximately 50 (e.g.β-carotene) can be converted enzymically to vitamin A in the human intestine.[16] How can an analysis for vitamin A in the original food be accurate when there are different forms which could become active once digested? A further consideration with vitamin A and carotenoids is the rapid decomposition on contact with light, oxygen, moisture and high temperatures which are difficult to control during sample preparation to provide sample homogeneity for analysis. The analyte also can be bound (chelated) or freely available. This causes significant problems when determining the amount of water which affects deterioration of foods and has lead to the proliferation of analytical methods to measure water content.[17]

Access to the analyte is a further challenge. Analytes can be nutrients, anutrients, additives or contaminants. Nutrients such as vitamins, proteins, sugars and fats; additives such as preservatives, antioxidants and flavours; contaminants such as bacteria and fungi, toxins, pesticides and hormones. The analyte can be soluble in water or fat and, as if this were not enough, it is rare to find an analyte homogeneously distributed throughout the food. Microbial contamination (e.g. *Listeria*, *Salmonella*, *Escherichia coli*) and the toxins which can be produced (e.g. aflatoxin)[18] commonly occur within food in small pockets often referred to as 'hot spots' - an analytical needle in a haystack. For example, there are seven species of *Listeria* of which *Listeria monocytogenes* is the most significant pathogen; its effects are most important for pregnant women, babies, farm workers, veterinary surgeons and those who are ill because their immune response is less effective. *L. monocytogenes* can only be distinguished from related species by specific biochemical tests when it is isolated.[19] The concentration of this bacteria in foods (commonly occurring in raw vegetables, milk, soft cheese and ready prepared salads) is usually too low to be detected directly by the common plating techniques and it needs to be isolated and allowed to multiply to the detectable level. In foods the doubling time of bacteria numbers for *L. monocytogenes* is approximately 1.5 days at 4°C but less than 1 h at 35°C. If the food is not kept refrigerated, but at room temperature (20°C) or in a car on a hot day, an undetectable level of bacteria can quickly multiply to become a serious problem to human health. The location of the consequent large numbers of bacteria will be in a small, 'bite sized' volume of food and not homogeneously distributed. The seriousness of this problem is illustrated by records showing 374 cases of infection and 118 deaths between 1979 - 1987. Both the food industry and government agencies are keen to prevent any health risks due to pathogens such as *Listeria* and more recently *Escherichia coli* 0157:H7 as shown by the reduction in cases related to *Listeria* recorded after 1985 as a result of industry and government action.[20,21]

Analytes vary in their stability, a major cause of analyte change is due to food spoilage which naturally begins at harvest and continues thereafter; a common example is the development of brown discoloration in fruits and vegetables.[22] Where food does not travel far, e.g. from the field to the farmer's table, it can be eaten quickly and deterioration is not a major problem. However, food is more commonly harvested and prepared for transport to the consumer which may mean travelling thousands of miles through a variety of climates before processing, packaging and distribution to the shops. There are two basic reasons for processing foods, the first is to stabilise the nutrients and the second is to improve convenience for the consumer. A wide range of methods have been developed to stabilise or preserve foods and improve convenience. These including: salting, drying, canning, fermentation, freezing, chilling and more recently modified atmosphere storage and packing. The quality of the food is dependent on: dried food remaining dry (for example, herbs and spices); the cans having a complete seal; the food remaining frozen or chilled; etc. Modified atmosphere packaging has been developed to maintain food in as fresh a state as possible with a significantly reduced rate of deterioration. However, if the pack leaks the food rapidly deteriorates with consequences for both nutritional and health status, the latter resulting from rapid bacterial growth. Analysis is undertaken to ensure that the preservation technique is maintained (e.g. modified atmosphere packaging).[23] In addition, the preservation technique often alters the form, concentration, stability and accessibility of an analyte. Finally, once purchased, the consumer may store the food before consumption. The stability of foods after purchase therefore needs to be assured by correct processing and packaging.

Analysis of appropriate samples of food is used to estimate the total quantity of analyte in the bulk material because total analysis would mean total destruction of the food. The principle of taking a sample is based on the assumption that it is a true representative of the whole material and therefore the measured result will enable estimation of the analyte concentration in the bulk material. Statistics are used to provide the valid basis for this procedure using tables to give the number of samples and sampling points. However, the number of samples often required are larger than can be analysed in a reasonable time and / or the costs of the analyses are higher than is acceptable. The industry usually uses a statistically valid number of samples for analysis during setting up a food manufacturing process to optimise the quality and safety of the product and subsequently uses a much lower number of samples to monitor the manufacturing process.

4 COMMON ANALYSES

The two basic aspects to food analysis are the range of analytes and the analytical techniques. These are illustrated in Tables 1 and 2. The variation of foods containing an analyte alters the ease of analysis; for example, measuring acidity in a beverage is simpler than the same measurement in fruit or the measurement of pesticides in nuts. Similarly the analysis techniques vary in their degree of sophistication; for example, measuring water by the oven drying method requires less sophisticated equipment and skill than measuring minerals by inductively coupled plasma-mass spectrometry. It is clear, however, that the range of analyses, the need for skilled analysts, the expense of equipment and reagents and the time consuming nature of the analyses mean that it is not always possible to provide the

Table 1 *Typical analytes measured in foods*

Analyte	Foods
Acidity	beverages, fruit, vegetables
Alcohol	beverages
Antibiotics	dairy, meat
Colour	beverages, fish, fruit, meat, vegetables
Fat type	dairy, fish, meat, nuts
Fatty acids	dairy, fish, meat, nuts, vegetables
Fibre	cereal
Flavours	beverages, cereal, dairy, fish, fruit, meat, vegetables,
Gluten	cereal
Hormones	dairy, meat
Microbial species	beverages, cereal, dairy, fish, fruit, meat, nuts, vegetables
Minerals	beverages, cereal, dairy, fish, fruit, meat, nuts, vegetables
Pesticides	beverages, cereal, dairy, fish, fruit, meat, nuts, vegetables
Phytate	cereal
Preservatives	beverages, cereal, dairy, fish, fruit, meat, nuts, vegetables
Protein	cereal, dairy, fish, fruit, meat, nuts, vegetables
Starch	cereal, nuts, vegetables
Sugars	beverages, cereal, dairy, fruit, vegetables
Sweeteners	beverages, dairy
Toxins	beverages, cereal, dairy, fish, fruit, meat, nuts, vegetables
Vitamins	beverages, cereal, dairy, fish, fruit, meat, nuts, vegetables
Water / Moisture	beverages, cereal, dairy, fish, fruit, meat, nuts, vegetables

Table 2 *Typical analytical techniques used in food analysis*

Analyte	Technique
Acidity	titration, pH meter
Antibiotics	high pressure liquid chromatography, microbial plate technique, immunoassay
Bacteria and fungi	plate techniques, immunoassay
Colour	spectroscopy, tristimulus colorimetry
Fats	extraction and gravimetric techniques
Fatty acids	gas chromatography and high pressure liquid chromatography
Hormones	high pressure liquid chromatography, immunoassay
Minerals	combustion, atomic absorption spectroscopy, inductively coupled plasma mass spectrometry, neutron activation analysis, atomic emission spectroscopy and x-ray fluorescence
Pesticides	gas chromatography, gas chromatography-mass spectrometry and high pressure liquid chromatography
Preservatives	spectroscopy, high pressure liquid chromatography
Protein	kjehldal digestion
Sugars & sweeteners	spectroscopy, refractometry, high pressure liquid chromatography, gas chromatography
Toxins	high pressure liquid chromatography, immunoassay
Vitamins	spectroscopy, high pressure liquid chromatography, microbial plate technique, immunoassay
Water	oven drying, Karl Fischer titration, refractometry, nuclear magnetic resonance spectroscopy, near infra red spectroscopy

results of food constituent measurement when and where it would be most appropriate. Often analyses have to be performed for retrospective action if there is a problem and for historical records because it is not always possible to provide analytical results at the

correct time for commercial judgements to be made. This also means that additional levels of safety are included in manufacturing processing conditions and packaging specifications because analytical results cannot be available in time to ensure products are safe. For all these reasons, the promise that biosensors can reduce the time taken for analysis, whilst maintaining accuracy and precision of the results, is an attractive proposition to the food analyst and to those making commercial judgements about food every day.

5 BIOSENSORS AND FOOD ANALYSIS

The proceedings of this symposium on 'Biosensors for Food Analysis' seeks to assist in the evaluation of biosensors to satisfy this need and identify the issues which will allow this technology to be used much more widely. The objective, therefore, was to bring together academics in the field of biosensor research and food industry technologists to review the current state of the art. This review sought to answer three basic questions:

1. What lessons can we learn from current research?

2. What knowledge can be used as the basis for further developing biosensors for food analysis?

3. What are the key issues which need to be overcome for biosensors to be made commercially available for the food industry?

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BIOSENSORS

2
An Introduction to Biosensors

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1 INTRODUCTION

A biosensor may be defined[1] as a compact analytical device containing a biological or biologically derived sensing element (*e.g.* enzymes, antibodies, microorganisms or deoxyribose nucleic acid - DNA) either integrated with or in intimate contact with a physicochemical transducer (*e.g.* electrochemical, optical, thermometric or piezoelectric). The usual aim is to produce a continuous or semi-continuous digital electronic signal which is proportional to a specific chemical or group of chemicals. Devices may be configured as fixed or portable instruments giving qualitative or quantitative information. A number of comprehensive texts and review articles may be of further interest to the reader.[1-4]

As the academic literature on biosensors continues to diversify, a clear trend within industry to critically evaluate and invest in focused areas is emerging. Medicine has provided the main driving force behind biosensor development to date, but the appeal to the food industry of pocket-sized devices capable of on-the-spot measurement of a wide range of analytes is obvious. Equally attractive is the possibility of on-line monitoring of complex biochemical parameters during production or processing of foods. In a process situation biosensors can provide information which is simply not available using conventional analytical techniques. The ability to provide continuous data on a specific analyte can be invaluable for optimisation and / or control. The relevance of biologically-based sensing systems to effects of toxins make biosensors ideal candidates for broad range alarms. The exquisite specificity of some enzymes, on the other hand, can distinguish between stereo isomers of the same compound to give precise information about food content. Increasing concern about chronic exposure to very low levels of residues necessitates the development of ultra-sensitive assays for analytes such as pesticides, herbicides, antibiotics and hormones. A key technology here is the construction of immunosensors, which may also find application in meat speciation and microbial identification. Some of the features and benefits of biosensors for use in the food industry are highlighted in Table 1.

Despite the obvious benefits, biosensor technology has been slow to penetrate the food industry. The authors had believed that the enormous scale of food production and quality control would provide a natural home for technology transfer from high technology advances in the medical field. However, it is now clear that public pressure, legislation

Table 1 *Features and Benefits of Biosensors for the Food Industry*

FEATURE	BENEFIT
Targeted specificity	Versatility (from definitive identification of single compound to broad range)
Electronic integration	Elegant and compact instrument design resulting in ease of use
Operate in complex matrices	Reproducibility and simplicity resulting from lack of need for sample preparation
Fast response time	High throughput in automatic analysers. Decreased user time for spot checks
Continuous signal	More accurate understanding of fluctuations & facility to actuate corrective action
Small size	Portable, inexpensive, multiple simultaneous assays
Mass producible	Inexpensive, disposable (hygienic), can be widely dispersed

and international investment will combine to promote expansion of biosensor technology in environmental monitoring, in advance of major applications in the food industry. The reasons for this are complex, involving the market, the nature of the industry and the limitations of the technology. It is hoped that this introduction will set the scene for more detailed presentations on the technical achievement to date together with an analysis of possible future scenarios exploiting the benefits offered by this fast moving technology.

2 THE BIOSENSOR AS AN ANALYTICAL TOOL

The biosensor field is multidisciplinary, encompassing such diverse areas as electrochemistry, optics, biochemistry, electronic engineering and a host of other science and engineering disciplines. Biological molecules, coupled to transducers, which convert the biological signal into an electronic one, have been used since the early 1960s to improve the specificity of chemical sensors.[5] Miniaturisation, mass production and improvements in computing power have advanced the analytical capabilities of such devices and allowed them access to a wider range of applications.

The transduction element of a biosensor must be capable of converting a specific biological reaction (binding or catalytic) into a response which can be processed into a useable signal. This element must also be suitable for the immobilization of the biological component at, or close to, its surface. The commonly used transducers are outlined in Table 2.

Table 2 *Transducers Commonly used in Biosensors*

Transducer	Examples
Electrochemical	
Amperometric	Clark oxygen electrode, chemically modified electrodes
Potentiometric	Ion-selective electrodes, field effect transistors
Conductimetric	Platinum electrodes
Optical	Optical fibres, evanescent field devices
Acoustic	Piezoelectric crystals, surface acoustic wave devices
Thermal	Thermistor, thermopile

Amperometry was the basis of the first biosensor, described by Clark and Lyons in 1962,[5] in which glucose oxidase was immobilized next to a Clark oxygen electrode. It has continued to be the most popular approach to biosensing largely due to its inherent simplicity, the ease of mass production and the low cost and availability of instrumentation. The technique involves the measurement of current at a fixed potential. The signal is dependent on the rate of mass transfer to the electrode surface and hence it is common to use a diffusion barrier to minimise the variations due to turbulence and to extend the linear range of the sensor. In the simplest mode of operation, the release of an electroactive product or consumption of reactant due to a biocatalytic reaction can be monitored directly at an inert working electrode such as a platinum wire. Modification of the electrode material with electron donors or acceptors is a popular theme at present.[2] The aim is to provide a charge transfer pathway between the biocatalytic component and the electrode surface. The transfer of electrons at the electrode can then be monitored at a lower potential, minimising interference problems. A further benefit is that this approach overcomes the oxygen dependence of certain enzymic reactions, such as those based on oxidases.

Coulometry is similar to amperometry in that it involves the use of a constant applied potential. In this technique, however, the total charge transfer is monitored, rather than the current. It is therefore an absolute method, requiring no calibration, since the total charge passed is independent of kinetics. In order to consume all of an electroactive species generated by an analytical reaction in a reasonable time, it is necessary to carry out the assay in a small and accurately known volume. This has most conveniently been achieved in the capillary-fill-device.[6]

Potentiometric biosensors were originally hampered by the relatively high cost, poor performance and the scarcity of suitable ion-selective electrodes (ISEs). The choice of devices is now much broader, performance has improved (see Table 3), but ISEs can still prove expensive, particularly for single use applications. Potentiometry, however, offers a further level of specificity, since the device itself is often highly selective. There is, understandably, a great deal of interest in lowering the manufacturing cost of ISEs. In

Table 3 *Some Commercially Available Ion-selective Electrodes*

Electrode	Type	Concentration Range
Barium	Liquid Ion-Exchange	10^0 - $5x10^{-5}$ M
Bromide	Solid State	10^0 - $5x10^{-6}$ M
Calcium	Liquid Ion-Exchange	10^0 - $5x10^{-5}$ M
Chloride	Solid State	10^0 - 10^{-5} M
Copper	Solid State	10^0 - 10^{-6} M
Cyanide	Solid State	10^{-2} - 10^{-6} M
Fluoride	Solid State	10^0 - 10^{-6} M
Iodide	Solid State	10^0 - 10^{-5} M
Nitrate	Liquid Ion-Exchange	10^0 - $5x10^{-5}$ M
Potassium	Liquid Ion-Exchange	10^0 - $5x10^{-5}$ M
Silver	Solid State	10^0 - 10^{-6} M
Sodium	Glass	10^0 - 10^{-6} M
Sulfide	Solid State	10^0 - 10^{-5} M

1970, Bergveld realised that it was possible to use a modified field-effect transistor as an ion-selective device.[7] The promise of silicon-based technology, with its inherent low manufacturing cost,[1] was extremely attractive. Despite this promise, it was many years before devices of this type became practical, mainly due to encapsulation problems. Unfortunately, the solutions to these problems have meant that the final device is little cheaper to manufacture than the original ISE. More recently, light addressable potentiometric sensors (LAPS) have been developed.[8] Once more, the device is based on silicon technology. A measurement is made by illuminating the back of a silicon chip, which causes a photocurrent to flow. Modulation of the light results in an alternating current, the magnitude of which depends on the potential across the sensor. A chemical reaction at the surface shifts the surface potential and hence affects the current. Adjustment of the external potential, to maintain constant current, allows the rate of the reaction to be monitored. Interesting as this device is, it is not an inexpensive alternative to the conventional ISE.

The field of optics is currently progressing at a very rapid rate due to the demands of optical computing, telecommunications and many other areas. Optical biosensors are benefitting from this advancement of technology, particularly with regard to the reduction in cost of waveguides and instrumentation. Much of the recent work on optical biosensors has concentrated on the use of evanescent wave technology. When light is reflected at an optical interface where there is a change of refractive index, there is a decay of energy away from the point of reflection into the surrounding medium. This energy field, which

extends into the medium for a distance similar to the wavelength of the light, can be used to probe the vicinity of the waveguide. There are many ways in which this principle can be utilised in biosensor applications, but those currently receiving the most attention in the scientific literature are total internal reflection fluorescence (TIRF) and surface plasmon resonance (SPR). With the former, the evanescent wave is used to excite fluorescent molecules bound to the surface of the waveguide. The latter relies on the excitation of the electron plasma (surface plasmon) of a thin metal layer covering the surface of the waveguide. The angle at which the incident light best couples to the surface plasmon is very sensitive to the refractive index in the vicinity of the metallised surface. Binding of large molecules such as antibodies can thus be monitored as the system goes out of resonance. Recently, SPR has appeared in the Pharmacia BiaCore (Pharmacia, Uppsala, Sweden) instrument. The resonant mirror, a variation of SPR, has also recently featured in the Fisons IAsys (Fisons, Cambridge, UK) device.

Piezoelectric transducers are conventionally used to measure small masses of materials in applications such as the vacuum deposition of metals. Such devices are able to generate and transmit acoustic waves in a frequency dependent manner. Changes of mass or density at the crystal surface change the resonant frequency enabling them to be used to monitor, for example, the binding of biological molecules.[9] Bulk wave devices operate by transmitting a wave from one side of the crystal to the other. Surface acoustic wave (SAW) sensors transmit waves along a single crystal face. The basic concepts of this approach are relatively simple, but the use of piezoelectric crystals in liquids is a relatively recent development.

The heat resulting from biological reactions can be detected as a temperature change using one of a range of thermometers, thermopiles (arrays of thermocouples) or thermistors. Such an approach allows virtually every type of reaction to be followed, since heat is either produced or consumed in nearly all reactions of this type. Progress in this area has been restricted by problems associated with thermal interference. Conventional solutions, such as the use of adiabatic flow-through systems have so far resulted in somewhat cumbersome and expensive instruments. A calorimetric system for use as a biosensor, the TAP 3300 device, is sold by Thermometric AB (Sweden). Research into microsystems capable of significantly reducing the bulk and expense of thermometric devices is promising and new instruments may make this approach considerably more attractive in the future.

The biological component of a biosensor is used to confer specificity on the device. It operates by either producing or consuming a component which can be detected by the transducer (biocatalysis-based sensor) or by binding a molecule which can then be measured (affinity-based sensor). The most widely used biomolecules in biosensors are enzymes, which are large protein molecules, which act as biological catalysts. Over 2500 enzymes have now been identified in nature and many of these are now commercially available. Other biocatalytic components include whole cells, tissue slices and organelles. There are also many biological materials which can be employed in affinity devices. These include antibodies, lectins and DNA.

The combination of biological molecules and transducers yields a seemingly infinite

number of possible devices. It is a combination of technological practicalities and financial restraints which control the development and ultimate realisation of biosensors. Hence research and development have concentrated on a relatively narrow range of approaches. The majority of publications involve the use of oxido-reductase enzymes coupled to electrochemical sensors or antibodies combined with optical transducers.

3 TECHNOLOGICAL DEVELOPMENTS

The use of electron transfer compounds (mediators) to shuttle electrons from the active site of the biological component to the electrode surface has revolutionised the field of amperometric biosensors. The use of immobilised mediators, such as ferrocene, was pioneered by Cranfield Biotechnology Centre and Oxford University in the early 1980s as a result of earlier work on fuel cells.[10] A large number of research groups have since become involved in this area and a number of effective mediator compounds have been identified (Table 4).

Table 4 *A Selection of Commonly used Mediators*

Mediator	Operating Potential (versus SCE[a])	Reference
Benzoquinone	+350 mV	11
1,1'-Dimethyl ferrocene	+100 mV	12
Ferrocene	+180 mV	10
Ferrocene 1,1'-dicarboxylic acid	+400 mV	12
Ferrocene monocarboxylic acid	+290 mV	12
Potassium hexacyanoferrate	+450 mV	13
Ruthenium hexamine	+50 mV	6
Tetracyanoquinodimethane	-45 mV	14
Tetrathiafulvalene	+160 mV	15

[a] SCE = saturated calomel electrode

As a means of retention of electron transfer mediators, a number of groups have looked at coupling the active site of the enzyme to the electrode surface by covalently modifying the enzyme, the electrode or both with mediators.[16] Efficient direct electron transfer between the enzyme and the electrode are still the 'holy grail' of workers in this area. This would offer the most straightforward method of coupling these proteins to electronic circuits. Early success with peroxidase has been followed recently by success with other enzymes, including glucose oxidase, which exhibited direct electron transfer when immobilized on a conducting polymer located within the pores of a track-etch membrane.[17]

As described earlier, the detection of the hydrogen peroxide produced by the action of oxidoreductase enzymes is a common and convenient method of biosensor operation. The major drawback has always been that relatively high potentials are required to oxidise hydrogen peroxide at conventional electrodes, which makes such devices prone to interference problems. Recently, attention has been focussed on reducing the oxidation potential in a selective manner by using metallised graphite electrodes.[18]

The use of biological components in the organic phase offers new possibilities both for the design of biosensors and in the construction of devices incorporating an organic phase for use in aqueous samples. Enzymes are by far the most widely studied class of biological material in organic solvents, although recently, antibodies have been investigated.[19] The bulk of the work in this area has concentrated on the relationship between the enzyme and the solvent. Starting with Organic Phase Enzyme Electrodes (OPEEs), the work has extended into organic phase enzyme thermistors,[20] and optical and piezoelectric devices. Some remarkable changes in the properties of such biocatalysts have been found, including greater thermal stability and changes in substrate specificity. However, it is probably the access to a greater range of analytes by extending operation outside of the aqueous phase that is the most exciting feature of these devices.

One of the problems encountered during electrochemistry in organic solvents is that caused by the low conductivity often encountered. Microelectrodes, which are generally considered to be microscopic in at least one dimension, have a number of advantages which facilitate their use in low conductivity environments. The reduction in the faradaic current leads to a decrease in the ohmic losses in the matrix.[21] Further advantages of such electrodes include a decreased time taken to reach a steady state since mass transfer to and from the electrode is very rapid. Screen-printed microelectrode arrays (Figure 1) offer an inexpensive means of producing reproducible devices in large numbers. A logical progression from the above work in organic phases is to develop gas phase biosensors,[22] where the biological component is incorporated in a support matrix which is cast over the microelectrode array. Biosensors for phenol vapours, sulfur dioxide and methane are under development, but the technology will be applicable to a wide range of analytes.

Grating couplers have been used as integrated-optical sensors responding to either changes in refractive index, where they operate as differential refractometers, or to the adsorption or desorption of molecules, where they behave as gas or chemical sensors.[23] The advantage of such devices is that the input beam can be autocollimated with a high degree of accuracy with relative ease. This greatly simplifies the required instrumentation and circumvents the need for expensive optical benches.

A recent development by Cranfield Biotechnology Centre and Bookham Technology is a silicon-based optical chip technology, known as ASOC, which combines standard silicon electronic circuit manufacturing methods with silicon micromachining and innovative integrated optical designs. This combination eliminates the inefficient manufacturing steps, which previously impeded the production of optical chips. The technology has been used for the inexpensive manufacture of optical evanescent wave and absorption spectroscopy-based chemical and biological sensors.

Molecular recognition forms the basis of most biological processes and considerable efforts are being made to develop synthetic recognition systems for given molecules. This approach is of universal interest, but is particularly relevant to the design and construction of novel sensors. The technique of molecular imprinting is an exciting new method of developing new recognition systems. Functional monomers are polymerised in the presence of a 'print' molecule. The functionalities of the print molecule are used to interact with the functionalities on the monomers, such that they are ordered during polymerisation[24]. A further approach, which is aimed at overcoming one of the major drawbacks of biosensors, the instability of the biological component, is to develop artificial enzymes or 'synzymes'. An example of this is the use of a lipophilic amide of trifluoroacetylaniline as a substitute for alcohol dehydrogenase.[25]

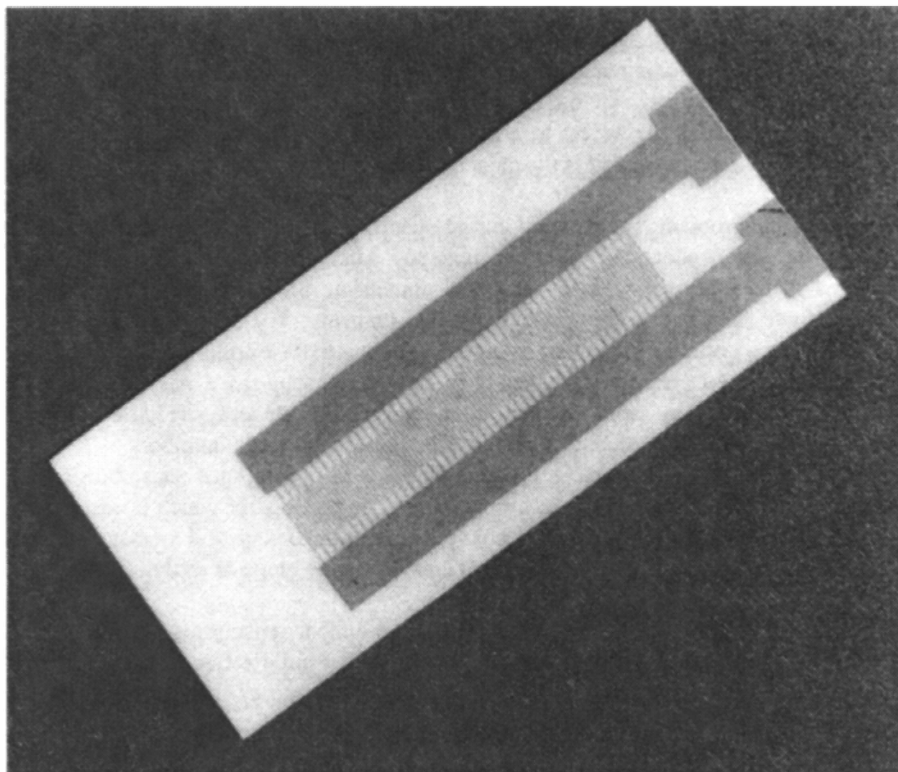

Figure 1 *Screen-printed interdigitated gold microelectrode array*

4 COMMERCIAL DEVELOPMENTS

Despite the surge of interest in biosensors during the 1980s, products were slow to emerge. A failure to appreciate the scale of the problems led to a great deal of hyperbole and spectacular claims for the future. Whereas the authors realise that much work still needs to be done, there has been an encouraging increase in the number of biosensor products

available. A lot of these are at present marketed by small companies, targetting niche applications, but the signs for growth appear good. Led by the Medisense ExacTech blood glucose analyser (Figure 2), approximately 65% of income earned is from mediated amperometric sensors and 80% of the current market for biosensors is for glucose analysis.

A significant recent development is the entry into the marketplace of Boehringer Mannheim as a major player offering biosensor technology with the Acucheck Advantage blood glucose analyser, targetting the $1.3 billion diabetes market. The Yellow Springs Instrument (YSI) company has steadily supplied the food industry with products along with a number of other smaller companies (see Table 5). An interesting recent launch is that of the Orion biosensor for sugar analysis in food, which appears to use very similar chemistry to that used in the ExacTech blood glucose analyser, but in a carbon paste format. At the time of writing, this device has been temporarily withdrawn due to 'technical difficulties'.

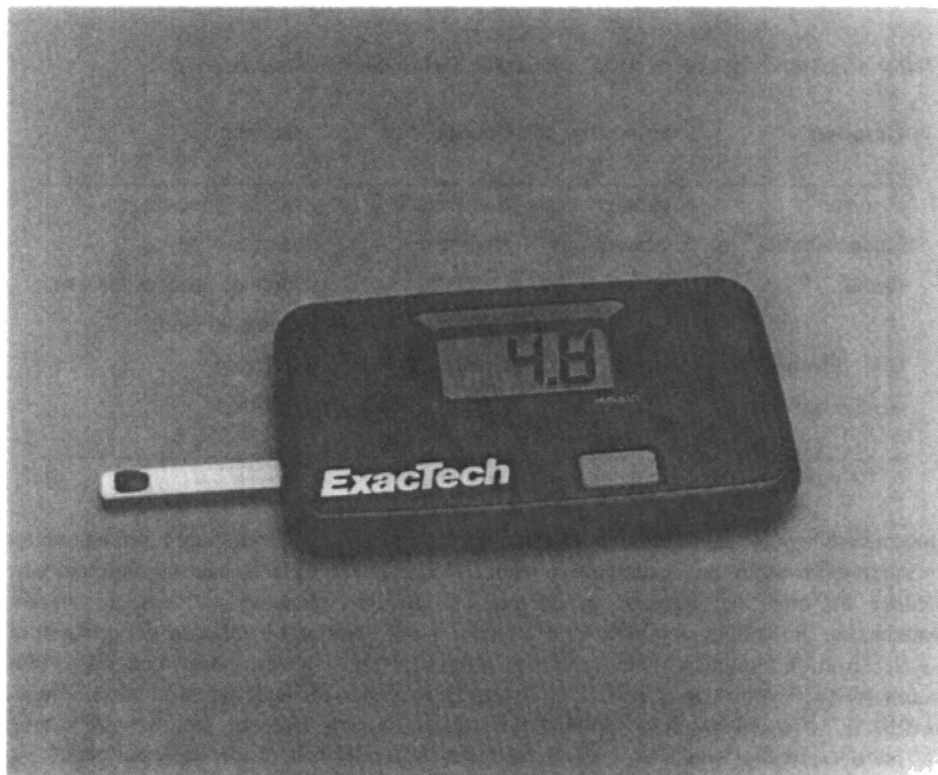

Figure 2 *The Medisense ExacTech blood glucose monitor*

5 BIOSENSORS IN THE FOOD INDUSTRY

A lack of suitable sensors is particularly marked in the food industry, where the chemical and even physical sensors currently used provide only in part the information needed. The remaining information is currently provided from, amongst others, taste panels and sophisticated laboratory procedures. The most important drawbacks of these procedures are:

(i) They are not real time measurements and hence there is a time lag.
(ii) Highly skilled analysts are required.
(iii) The procedures are often time consuming.
(iv) Complex and expensive analytical equipment may be required.

It is important to emphasise that; to be successful, a biosensor needs to offer either an improvement to an existing technology, an existing measurement in a novel environment or an entirely new measurement. Generally, the choice of whether or not to turn to the biosensor approach is governed by financial constraints. Many biosensors have not

Table 5 *Examples of Commercially Available Biosensors for Food analysis*

Company	Nationality	Product	Analyte
Gonotec	Germany	Sensomat B10	Alcohol (brewing)
Kalger GmbH	Germany	Microzym-L	Lactate
Orion*	US		Glucose, lactose, sucrose, lactate, ethanol
TOA Electronics	Japan	Glu-11	Glucose
Yellow Springs	US	1500 Sidekick	Glucose

* - currently unavailable due to technical problems

progressed beyond laboratory demonstration due to the cost of developing a product which is suitable for use in the desired environment. Nevertheless, there is a strong motivation to reduce the time lag between production and quality assurance, at least for certain parameters. Potential candidates for examination are found in all stages of the production cycle: from farming (pesticide residues, fertilisers and ripeness), raw materials (food adulteration, freshness), process control (organoleptic considerations, microbiological safety) and distribution. The enormous breadth of products, test sites and the wide variety of matrices, within which an analysis needs to be performed, make the development of biosensors for use in the food industry particularly challenging.

The food industry has a very different set of constraints compared with, for example, the pharmaceuticals industry or medical diagnostics. It is important to consider both the limitations and benefits when selecting the target analyte and medium.

Some of the drawbacks include:

(i) The range of potential applications is enormous. Each product has its own analytical requirements. However, the market for an individual sensor is small compared to, for example, a glucose sensor for use by diabetics.

(ii) Most biosensor research has been aimed at the medical diagnostics field, where the extreme specificity of a biosensor targetting one selected analyte is extremely advantageous. This is not always applicable to food industry requirements, where, for example, a particular taste or smell may be a control parameter.

(iii) Multisensors would be preferable in many instances due to the complexity of food process control requirements.

(iv) Most existing processes have been fine-tuned over many years. Often, little process control is required due to the experience of operating the process over such a long period. The biosensor must provide an increase in productivity or quality of the product to be viable. This is not always possible.

(v) The technology available must be low cost, simple to use and above all, reliable. Long term stability, drift and calibration are problems which often prevent the introduction of biosensors to industrial processes.

There are also some factors which make the introduction of biosensors in the food industry more feasible:

(i) Extreme miniaturisation is not usually required. This has implications on the fabrication process and on the size of the signal.

(ii) Destructive or by-pass sampling techniques are usually tolerable. This may facilitate the use of techniques such as flow-injection analysis with biosensor detection.

(iii) A great deal of work has been carried out in the medical diagnostics industry. Some of the lessons learned in this field may be transferable to food industry requirements.

(iv) It may not be necessary to provide an instant or continuous measurement. An improvement from days to hours or minutes may be considered sufficiently beneficial.

(v) Accuracy and precision requirements may be time dependent. An instant answer to within ±10% may be preferable to ±0.1% a week later.

(vi) The influence of consumers and regulatory bodies may encourage the use of advanced technologies such as biosensors.

A conclusion which can be drawn from the above points is that although the market is very fragmented, there are possibilities for the introduction of broad-based biosensors, preferably those employing a generic technology which can easily be adapted for use in other branches of the industry or other analytes. Some of the broad-based measurements of the food industry are summarized in Table 6.

Table 6 *Some Measurement Requirements of the Food Industry*

Analyte	Where Measured
Total microbial count	Almost universal, but particularly in dairy products, fish and meat
Specific bacteria	As above
Sugars, alcohol	Drinks
Pesticides, herbicides	Vegetables
Cholesterol, fatty acids	Oils, fats, butter, margarine
Glucose, ethylene	Fruit ripeness
Glucose, lactate	Milk products

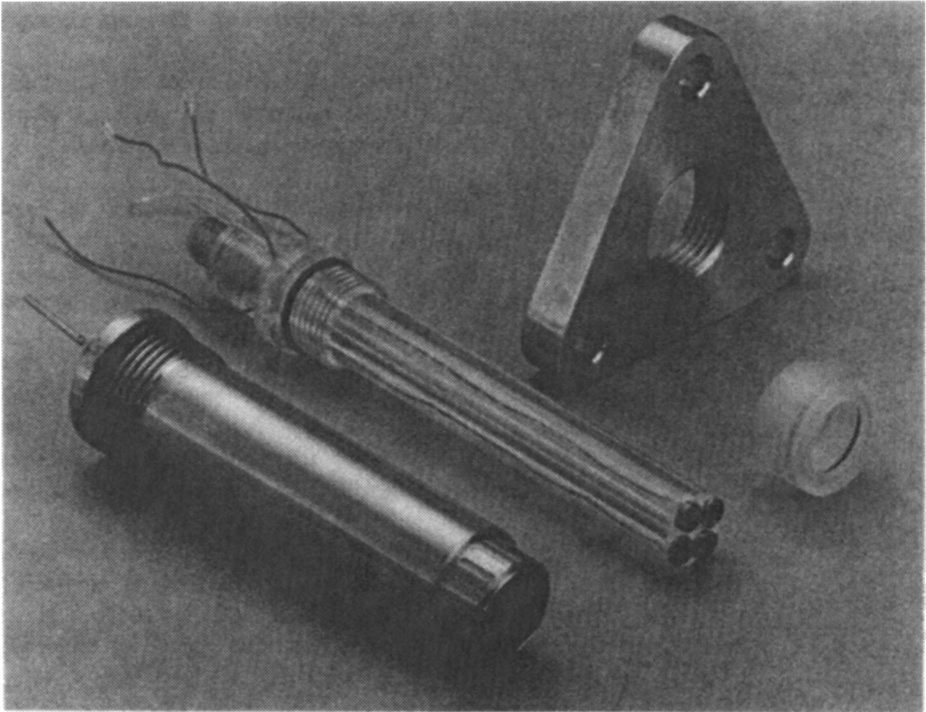

Figure 3 *A steam-sterilisable biosensor for the measurement of glucose during baker's yeast fermentation*

An area of food production which appears more receptive than most to the introduction of new technology, such as biosensors, is fermentation. There are a very wide range of fermented food products and the degree of equipment sophistication is equally broad. Most fermentations, however, operate over relatively long periods and involve comparatively high value products. Early work involved the development of *in situ*

biosensor probes,[26] in which a retractable biosensor was mounted in a steam sterilisable outer sheath (see Figure 3). More recently, the favoured approach has been on-line flow injection analysis (FIA). Such systems have relied on many forms of detection.[27] Biosensors have been widely deployed based on mediated,[28] oxygen detecting[29] and hydrogen peroxide detecting[18] amperometry, potentiometry,[30] optical methods[31] and thermal approaches.[32]

Rapid bacterial detection and quantitation is probably the most sought after analysis in the food industry. It is also one of the most difficult to achieve reliably. A number of biosensor approaches have been adopted by various groups over the last decade or so. An interesting approach which has been developed as far as an advanced prototype instrument involves the assessment of meat freshness using a biosensor array.[33] The sensor measures glucose simultaneously at depths of 2 and 4 mm and relates the depletion of this to microbial deterioration. A photograph of an early prototype can be seen in Figure 4.

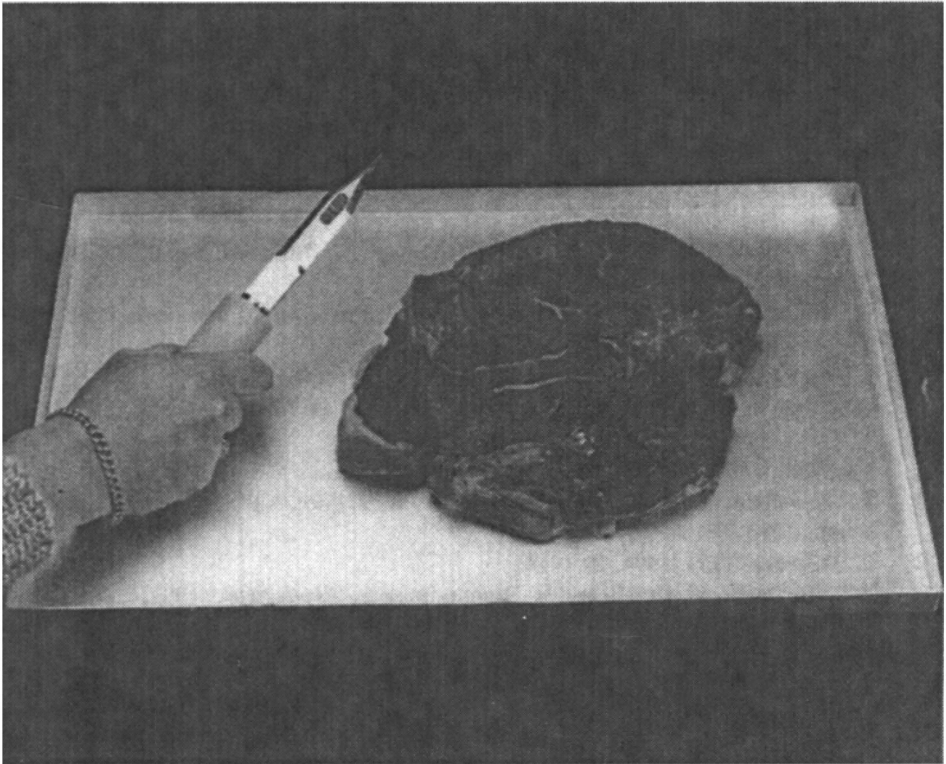

Figure 4 *The depth profile of glucose concentration can be used to determine microbial contamination of meat samples*

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3
Enzyme Biosensors

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1 INTRODUCTION

Over the last decade or so, a variety of biosensors based on elegant concepts has been described in the literature (recent reviews can be found in references 1-3). In most cases, these highly attractive analytical tools result from the intimate combination of a transducer and an immobilized enzyme layer. Electrochemical sensors for oxygen or hydrogen peroxide detection or involving a mediator are the most common transducers used for the design of the so-called enzyme electrodes. Practically, β-D-glucose and L-lactate are the two main analytes for which instruments based on this technology are available on the world market and which are routinely used in bioprocesses. This small number can be explained by the difficulties, generally not encountered when feasibility is explored in the laboratory but which must be overcome for practical and extended use with real samples.

Many parameters have been considered concerning the enzyme itself, its origin, specificity and availability, immobilization procedure and stability both operational and on storage, compatibility with working conditions of the transducer *etc.* In complex media selectivity is probably the key parameter to take into account, a compromise between all the requirements is usually accepted leading to different configurations in the design of analytical devices for food analysis.

In our group, special attention was given to the preparation of the enzyme layer. Our first goal was the fast and cheap preparation of easy to handle and disposable enzymic membranes which could be associated with electrochemical or optical transducers. This could be achieved using pre-activated polyamide membranes enabling enzyme immobilization in a few minutes. The electrochemical based sensor then developed allowing the determination of glucose, lactate, phosphate *etc* with the suitable enzymes.

Assays could be performed in various foodstuffs including wine, honey, dairy products *etc.* More recently, a fibre optic-based enzyme sensor with enzymes producing luminescence from fireflies for adenosine triphosphate (ATP) or from luminescent bacteria for the reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) was designed. Auxilliary enzymes, namely dehydrogenases co-immobilized with the bacterial luminescent system allowing different metabolites to be determined, like sorbitol, ethanol or oxaloacetate. Enzyme activity could also be assayed with lactate dehydrogenase as model enzyme in cell culture processes. Both types of transduction lead to very low

detection limits and wide dynamic ranges. The fact that generally no tedious pre-treatment of the sample is required makes such systems particularly attractive for food analysis.

2 ENZYME IMMOBILIZATION

The properties and characteristics of the biosensing layer are extremely important in the design of biosensors, especially for the reliability versus time since they are repeatedly in contact with the sample. Various immobilization methods can be chosen for preparing the sensing layer. Two main categories of layers can be distinguished: pre-formed artificial membranes bearing functional groups, which can be activated and further coupled to selected enzyme or polymer matrices in which the enzyme molecules are trapped and which can be used for the direct coating of the transducer. In our group chemically activated collagen membranes were first used as pre-formed membranes for enzyme immobilization.[4] The activation was based on acyl-azide formation through a three-step treatment of the carboxyl group of lateral chains of glutamate and aspartate residues available on collagen. After washing, to remove all the reagents which could be damaging for the enzyme, spontaneous coupling was achieved by simple immersion of the activated membrane in a freshly prepared enzyme solution. A variety of enzymes including oxidases, transferases, hydrolases or lyases were immobilized on such membranes. Despite good stability of the bound enzymes, the main drawback was the time required, more than three days, for the activation process which should be kept mild with regard to the proteinic nature of the support itself. Immunodyne™ membranes from Pall, Glencove, USA provided in a preactivated form were selected for their handiness and good reproducibility of the immobilization procedure.[5] These polyamide membranes are provided in a dry state in moisture proof bags. Briefly, enzyme immobilization can be obtained as follows by simple membrane wetting: for example with glucose oxidase, a solution at 10 mg ml^{-1} in 0.1M phosphate buffer, pH 7 is prepared and 10 μl applied to both faces of the membrane. The reaction is completed within one minute. The enzymic membrane is rinsed in buffer and bioactive discs of a suitable size adapted to the electrode tip are cut out of the membrane. The main advantage of these membranes is the extremely simple and fast procedure which enables enzymes to be immobilized in a few minutes without tedious and hazardous steps. The bio-active membranes can be stored either in buffer or in a dry form.

3 ENZYME ELECTRODES

We mainly focused on oxidase-based electrodes or on oxidase-containing multienzyme systems when such sequences were needed if no oxidase could be found and used directly for the target analyte to be assayed. Two approaches can be considered: either oxygen consumption is followed with a Clark-type electrode, or hydrogen peroxide production is monitored with a platinum electrode, both poised at the adequate potential. Among the two possibilities, monitoring the appearance of hydrogen peroxide was chosen (Figure 1).

The glucose electrode was first designed with glucose oxidase covalently bound onto collagen membrane.[5,6] After injection of the glucose-containing sample in the reaction cell

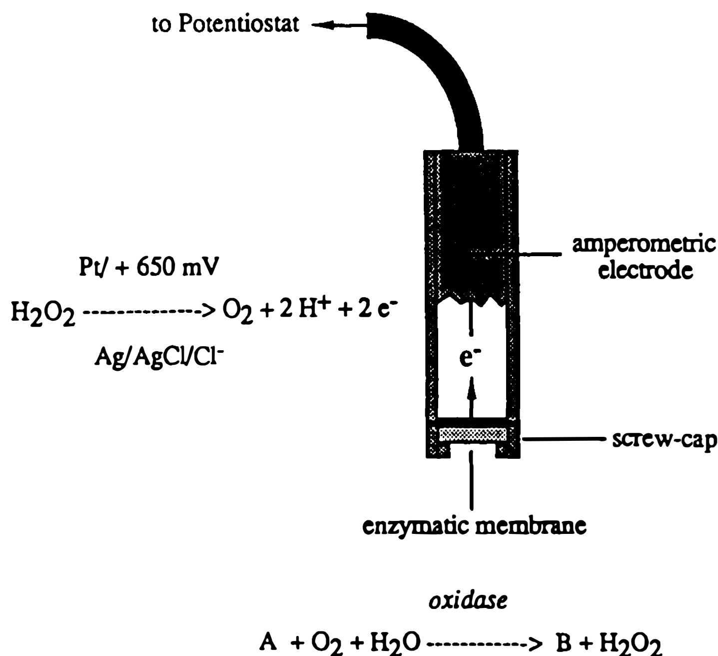


Figure 1 *Principle of amperometric enzyme membrane electrode*

3.1 Glucose Electrode

Scheme 1

where the biosensor tip is immersed, glucose oxidation leads to the production of gluconic acid and hydrogen peroxide which is detected on a platinum anode with a potential poised at +650 mV versus a silver/silver chloride reference. The current output due to hydrogen peroxide oxidation increases and reaches a plateau, the variation being proportional to glucose concentration in a wide range. Performances of this enzyme sensor were the following:

- detection limit as low as 10^{-8} M
- dynamic range extending from 10^{-7} M to $2x10^{-3}$ M
- sensitivity between 1 to 3 mA M^{-1} depending on the activity of the membrane for a 8 mm diameter platinum tip
- operational stability good over a 6 month period

Based on this work, a self-contained enzyme electrode Gluc 1 was designed and commercialized by Solea-Tacussel, France.

3.2 Selectivity

Selectivity is one key parameter to consider when thinking of the practical use of biosensors. Indeed, a high selectivity is needed for direct analysis. The selectivity of the sensor depends on both the specificity of the enzyme and on the presence of electroactive species in the sample. With glucose oxidase, the specificity of the enzyme is good and the β -D-glucose anomer is the main substrate to be oxidized at a high rate. This is not the case for other enzymes, especially galactose oxidase and alcohol oxidase which accept several substrates or with D-amino acid oxidases active on various amino acids.

In complex media various contaminants which are electroactive at the poised potential may exist in the sample; this is not only restricted to food analysis but also affects biomedical analysis or environmental control. An example of this is ascorbic acid which can be readily oxidized together with hydrogen peroxide at the above mentioned potential. Several ways have been considered by different authors to avoid such a drawback. In the approach developed by Clark and his group,[7] the bioactive membranes used were obtained by sandwiching a glutaraldehyde-treated enzyme layer between a cellulose acetate membrane and a polycarbonate Nucleopore membrane. The role of the cellulose acetate membrane in contact with the platinum elelctrode is to act as a barrier for small molecules and to prevent oxidation of interfering species with hydrogen peroxide leading to an overestimated measurement.

One possibility to avoid interferences is to be able to work at another potential (for instance +160 mV versus a saturated calomel electrode - SCE). This can be achieved with oxidase reactions by replacing oxygen with electrode mediators like ferrocene or its derivatives.[8] The system further developed and now thoroughly used in diabetes as a pen-size biosensor for glucose analysis in whole blood becomes insensitive to oxygen content and is not subject to ascorbic or uric acid interferences. Undoubtedly, this is a very promising approach which could be extended to food analysis.

A high selectivity can also be obtained with a differential set-up involving a non-enzymic electrode allowing the continuous subtraction of the interfering current due to electroactive species.[9] This is the way we chose and a prototype of the semi-automatic analyser 'Glucoprocesseur®' based on this principle was then developed in France by Solea-Tacussel. Injections of the crude glucose-containing sample into a specially designed thermostatted cell, equipped with the two electrodes, are performed with a glass capillary micropipette. Filling and emptying the cell are automatic.

3.3 Response time

For the self-contained enzyme electrode Gluc 1, the steady-state response time varies between 3 and 5 minutes. This time was considered too long for practical applications of an analyzer and in this instrument the derivative of the current was monitored instead of the direct current. The result was then obtained on a digital display only 30 seconds after manual injection of the glucose-containing sample, but a 90 second interval between assays was necessary to allow washing and filling of the measurement cell.

3.4 Use In Food Analysis

In the laboratory, tests were performed with either the self-contained electrode Gluc 1 or the semi-automatic analyzer Glucoprocesseur® equipped with collagen or polyamide membranes. Either single or, when necessary, multi-enzyme systems were tested. Results obtained with different target analytes are summarized below.

3.4.1 Glucose. Determination of glucose in honey and jam could be performed with the enzyme electrode analyzer. With a series of 10 assays, excellent values of the coefficient of variation ranging from 0.7-1.7% were obtained.[5]

Using the same electrode design, enzyme sensors for other analytes have been also developed with single or multi-enzyme systems.

3.4.2 Galactose. A galactose electrode[10] could be made with galactose oxidase but the enzyme is not specific for galactose and oxidizes other galactosides like lactose or raffinose. This restricts, in principle, the use of a galactose electrode to mediums where such interferent sugars are not present.

3.4.3 Lactose. β-galactosidase hydrolyses lactose to produce glucose and galactose. The performances of this sensor were poor with a rather high detection limit and a narrow linear range.[10]

3.4.4 Sucrose. Three enzymes were involved: invertase which hydrolyses sucrose into fructose and α-D-glucose, mutarotase converting α-D-glucose into β-D-glucose, and glucose oxidase. The linear range was narrow compared to glucose and the sensitivity low.[10]

3.4.5 Maltose. The related two enzyme biosensor involving glucoamylase and glucose oxidase was used for maltose determination.[11] An asymmetrical immobilization could be performed on collagen membranes with each enzyme bound on only one face of the membrane.

3.4.6 L-Lactate. A very sensitive assay of L-lactate could be achieved with lactate oxidase.[12] Accurate measurements of L-lactate could be performed in several types of dairy products including cream cheese, yogurt, whey, sauerkraut broth and wine.

3.4.7 Oxalate. The self-contained enzyme electrode involving oxalate oxidase or the related analyzer enabled the determination of oxalate with minimal pre-treatment in various samples including soft drinks, chocolate, spinach or sorrel.[13]

4 LUMINESCENCE PHOTOBIOSENSOR

Based on our experience in the development of enzyme electrodes, a fibre optic sensor was designed,[14] based on luminescence enzymes from either the firefly for ATP or from marine bacteria for NAD(P)H detection. The enzymes were bound to pre-activated polyamide

membranes associated through a screw-cap to one end of a fibre optic bundle connected to a luminometer (Figure 2). The bioactive tip was inserted in a light-proof measurement cell in which samples can be injected through a septum. The same set-up can be used whatever the light emitting system on the membrane.

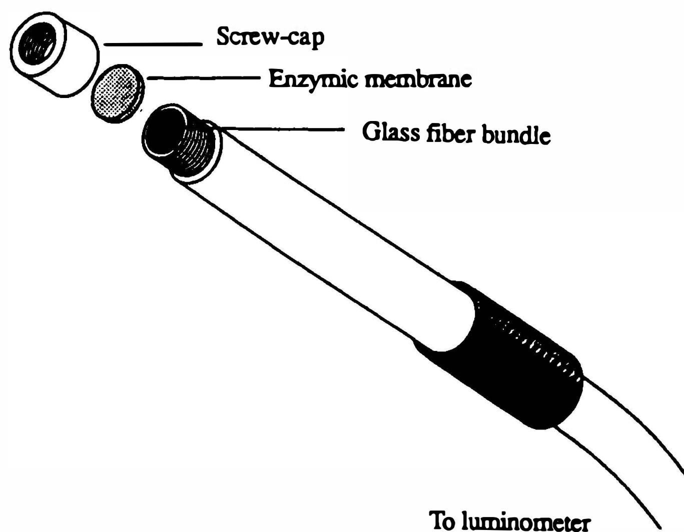


Figure 2 *Fibre optic biosensor tip*

4.1 Detection System

4.1.1 ATP determination. In the presence of its substrates luciferin (LH_2), Mg^{2+}, molecular oxygen and ATP, the firefly luciferase (from *Photinus pyralis*, EC 1.13.12.7) provides a light emission according to the reaction:

$$ATP + LH_2 + O_2 \xrightarrow{\textit{firefly luciferase}} AMP + PPi + Oxyluciferin + CO_2 + h\nu$$

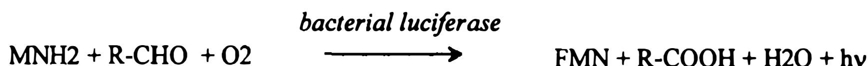
Scheme 2

Under appropriate conditions, the intensity of the light (λ_{max} = 560 nm) is proportional to the ATP concentration over a wide dynamic linear range.

4.1.2 NAD(P)H determination. Two consecutive enzymic reactions are necessary, using the luminescent marine bacteria systems (from *Vibrio fischeri* or *Vibrio harveyi*; λ_{max} = 490 nm)

$$NAD(P)H + FMN \xrightarrow{\textit{oxidoreductase}} NAD(P)^+ + FMNH_2$$

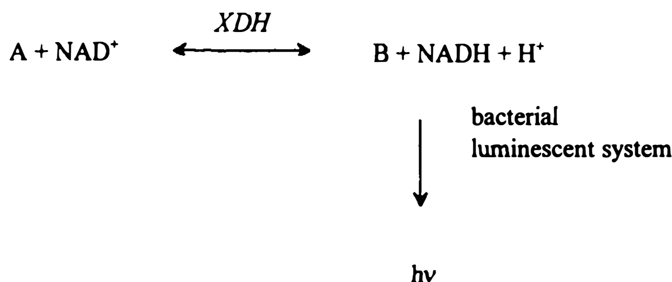
Scheme 3



Scheme 4

4.2 Determination of Analytes in Food Analysis

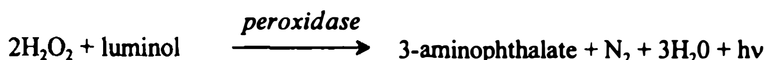
4.2.1 Sorbitol and Ethanol. The microdetermination of various target analytes (A) also could be achieved providing suitable auxilliary enzymes like dehydrogenases (XDH) converting A into B can be co-immobilized in the sensing layer, with the luminescent system.



Scheme 5

This was the case with sorbitol dehydrogenase for D-sorbitol and with alcohol dehydrogenase for ethanol, which could be measured from $2x10^{-8}$ M to $2x10^{-5}$ M and from $4x10^{-7}$ M to $7x10^{-5}$ M, respectively.[15]

4.2.2 Detection of Hydrogen Peroxide. Chemiluminescence with the luminol-hydrogen peroxide-peroxidase system was also exploited using the same set-up. The reaction is the following (λ_{max} = 425 nm):



Scheme 6

4.2.3 Glucose in Drinks. The feasibility of chemiluminescent flow analysis of glucose with the fibre optic sensor involving co-immobilized glucose oxidase and peroxidase was established recently.[16] Hydrogen peroxide generated by the glucose oxidase reaction in the presence of glucose as previously indicated, was detected using the chemiluminescence reaction of luminol catalyzed by peroxidase. The detection limit was 0.25 nmol with a coefficient of variation lower than 4% for repeated measurements of 2.5 nmol glucose. The method was applied to glucose analysis in soft drinks such as Schweppes Indian Tonic Water and Coca-Cola on diluted degassed samples. The results were in good agreement with those obtained with a standard spectrophotometric method,

using a reagent kit based method measuring the end point of the hexokinase / glucose-6-phosphate dehydrogenase reaction by detecting the reduced form of nicotinamide adenine dinucleotide (NADH) at 340 nm.

5 CONCLUSION : GENERAL REQUIREMENTS FOR THE PRACTICAL USE OF BIOSENSORS IN FOOD ANALYSIS

Even though a large number of papers dealing with biosensors have been published in the last decade, reliable devices are still scarce. Attempts were made in our group to overcome identified bottlenecks impairing their use for practical applications. Enzyme electrodes and luminescence photobiosensors were developed and several analytes important in food analysis could be reliably determined with such sensors in real samples.

From our experience, the constraints existing in the different domains where biosensors could be used as valuable tools explain the difficulties encountered for their acceptance. The main problem may be the selectivity of the system and thus the confidence the user has in the values obtained. A second point concerns the lifetime of the bio-active membrane in complex media. Other points have to be carefully addressed when the design of a new instrument is planned. For instance, will the system be operated by technicians or unskilled persons? Is a long stand-by possible without recalibration or could periodic calibrations be performed automatically? Can an error detector be incorporated in the signal processing system? *etc.* Obviously the architecture of the instrument and its cost will depend on the answers to these and many other questions. My hope is that these problems could be solved soon by the different groups active in the domain so that systems having a suitable performance may be widely available in the near future.

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4
Optical Affinity Biosensors

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1 OPTICAL AFFINITY BIOSENSORS - A DEFINITION

A biosensor is characterised by a biological sensing element intimately connected or integrated within a transducer which converts the biological event into a response that can be further processed.[1] The biological sensing element can be either catalytic (*e.g.* enzyme, microorganism) or non-catalytic, also denoted 'affinity sensing element' (*e.g.* antibody, receptor). The transduction technologies described so far fall into the following categories: electrochemical, calorimetric, acoustic and optical. An optical affinity biosensor is therefore a biosensor based on a non-catalytic biological sensing element intimately connected to an optical transducer. The transducer measures the change in optical properties when the sensing element interacts with another molecule (normally the analyte). Depending on the technology chosen for the transducer these changes can be proportional to mass surface concentration changes on the surface of the transducer.[2]

Another approach is to detect absorption, fluorescence or luminescence. In this case it normally means that some sort of label (possessing the absorptive, fluorescent or luminescent properties) has to be introduced in the assay configuration. Label-dependent biosensor assays are very close in function to the already existing and comercially available immunoassay systems based on the well known enzyme linked immunosorbent assay, fluoroimmunoassay, *etc.* configurations and will not be considered here. For a review of label-dependent optical biosensors, see Reference 3. Non-label affinity biosensors are characterized by real-time measurement of the interaction between the sensing element and the analyte (Figure 1).

The baseline of the sensorgram in Figure 1 is the transducer with a pre-immobilized antibody as the sensing element. When the antigen is injected, the progress of the binding event is monitored as a change in the optical signal (in this case expressed as an arbitrary unit: RU) which is proportional to mass surface concentration. Following the interaction the transducer with its sensing element can be regenerated by, for example, lowering the pH and thus dissociating the antibody-antigen binding. In this way the biosensor can be reused and is ready for another sample.

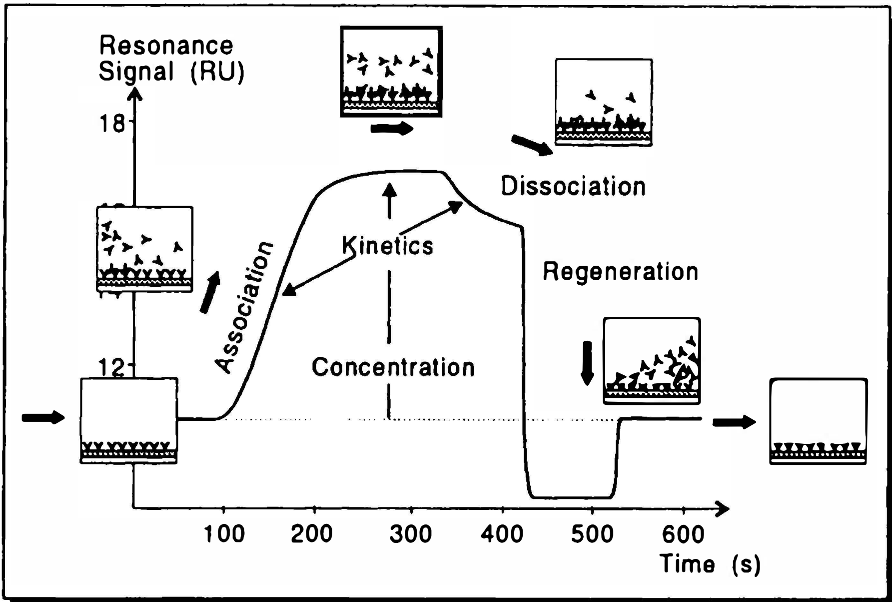

Figure 1 *A typical sensorgram of an antibody-antigen interaction*

2 SYSTEM CONFIGURATION AND AVAILABILITY

A biospecific interaction is a dynamic series of events described in terms of association and dissociation rates, the affinity of the participating molecules for each other, and the stoichiometry of the molecular complex formed. The real time measurement of a biospecific interaction by an optical affinity biosensor allows the following analytical information to be obtained: specificity / identity, concentration / activity, affinity, kinetic rate constants, stoichiometric relationship, relative binding pattern and co-operativity of the binding.

There are now four systems on the market for label-free real-time measurements of biomolecular interactions. Of these Pharmacia Biosensor's BIAcore has collected nearly all of the rapidly growing applications as illustrated by the reference list (Figure 2).

The applications with BIAcore from Pharmacia has tripled in less than one year as a result of more than 200 BIAcore users worldwide. More than half of the references are in the fields of receptor-ligand / signal transduction and antibody characterization / engineering.

Pharmacia Biosensor recently launched BIAlite which is a cut-down version of BIAcore. The other commercial system manufacturers are Fisons Applied Sensor Technology (UK) with its IASYS and Artificial Sensing Instruments (Switzerland) with its BIOS-1. Published work on these systems are few because the systems have been available only relatively recently. A comprehensive survey of the commercially available systems is found in Reference 4. Except for the optical affinity biosensor these systems also incorporate means for liquid handling (Figure 3).

	1993 January	1994 January
Antibody characterisation / Engineering	6	26
Receptor-ligand and Signal transduction	3	25
Nucleic acids	0	3
Vaccine, Virus and Infectious disease	3	3
Other fields	0	3
Review articles	3	6
Methodology	2	8
Surface chemistry	3	3
System description	7	12
Total	27	89

Figure 2 *The number of references for applications with BIAcore.*

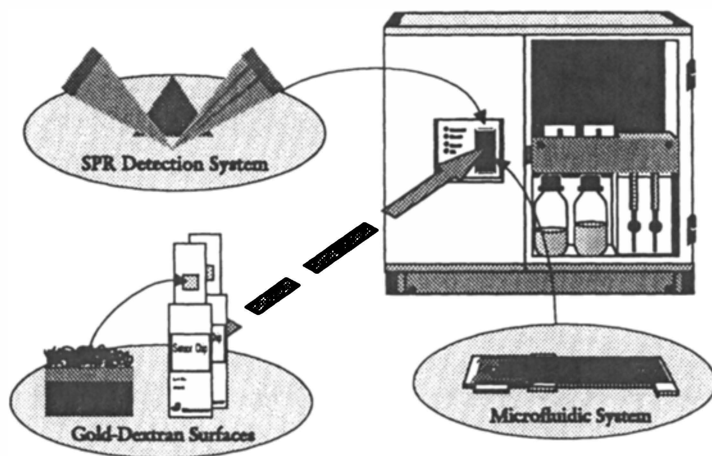

Figure 3 *The basic components of an analytical system for biospecific interaction analysis. The figure shows the transduction technique based on surface plasmon resonance used by Pharmacia Biosensor.*

All the presently available systems are of an open architecture. This means that the sensing element and the application are user defined. To support the immobilization of the sensing element a number of coupling chemistries are available (Figure 4). The immobilization is performed either manually or as a predetermined automatic procedure (in BIAcore).

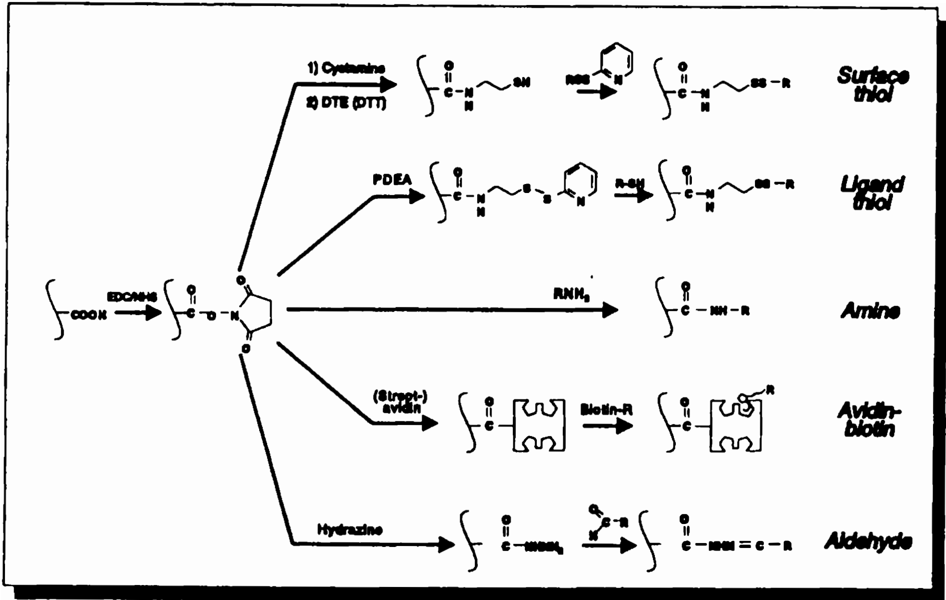

Figure 4 *Coupling Chemistries*

The most common coupling procedure is amine coupling which couples molecules through their primary amines. In other cases where coupling through primary amines is unsuitable other alternatives are available:

1 Two approaches for disulfide exchange:
 (a) Thiol groups are introduced on the surface by immobilizing cystamine which in reduced forms can react with proteins modified with reactive disulfides.
 (b) Reactive disulfides are immobilized on the sensor surface which then react with thiol-containing molecules.
2 Biotinylated reagents (peptides, DNA) can be immobilized with high affinity through amine coupled avidin surface.
3 Molecules oxidized to aldehydes can couple to surfaces activated by hydrazine or carbohydrazide. The method is suitable for coupling of glycoconjugates and polysaccharides.

3 APPLICATION AREAS

The versatility of non-label optical affinity biosensors will now be illustrated by examples from screening of reagents for immunoassay test development.

To determine isotype is of importance when considering stability and storage. Monoclonal antibodies (MAb) of isotype M and G3 are often more unstable than MAbs of isotype G1, G2 and G2b. Figure 5 shows a typical sensogram for isotype determination. The unknown MAb (αβ-human chorionic gonadotropic hormone - HCG) binds to a pre-immobilized antibody directly towards the Fc part of the MAb (αFc). The isotype reagents are injected in sequence. The first four reagents do not bind. The fifth reagent (α -G1) binds and hence indicates isotype. The reagents can be injected in any sequence and still generate the same result.

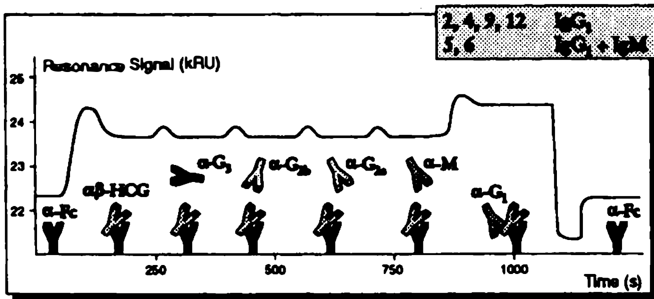

Figure 5 *Antibody characterization : Isotope determination*

When constructing a sandwich assay it is important to know the relative binding pattern between MAbs. The first and the second antibody need to have different epitope specificity. With affinity biosensors it is possible to do the mapping on non-purified MAbs in culture supernatant. Figure 6 shows a typical sensorgram for pairwise epitope mapping.

1. An antibody (αFc) directed towards the Fc part of the MAb is immobilized to the surface.
2. The first MAb is injected and binds to the αFc. The increase in response as the sample passes over the surface is partly due to other proteins in the culture supernatant, but these will not bind to αFc.
3. An irrelevant antibody is injected in order to block all remaining sites on the αFc surface.
4. The antigen is injected and binds to the first MAb.
5. The second MAb is injected and if it has a different epitope specificity it will bind to the antigen. If it has the same epitope specificity as the first MAb the site is occupied and no binding occurs.
6. After regeneration with HCl the surface is ready for the next pair.

Using affinity biosensors, each step can be monitored; the amount of the first MAb, the efficiency of the blocking, the ability of the antigen to bind to the first MAb and a Yes/No answer for the second MAb. This is unique for non-label affinity biosensors where conventional techniques only allow control over the final labelled step. Testing a panel of MAbs against each other can result in a binding matrix as shown.

When an antibody in solution binds to a surface immobilized ligand the observed binding under certain conditions will be limited by mass transport of the antibody to the surface. Under such conditions binding rates are proportional to the active antibody concentration and independent of the antibody-antigen affinity. The upper left of Figure 7 shows the binding of an antibody at different concentrations to its antigen. The time

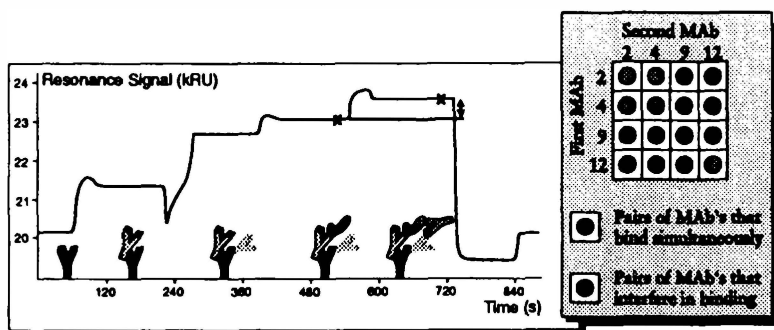

Figure 6 *Antibody characterization : Epitope mapping*

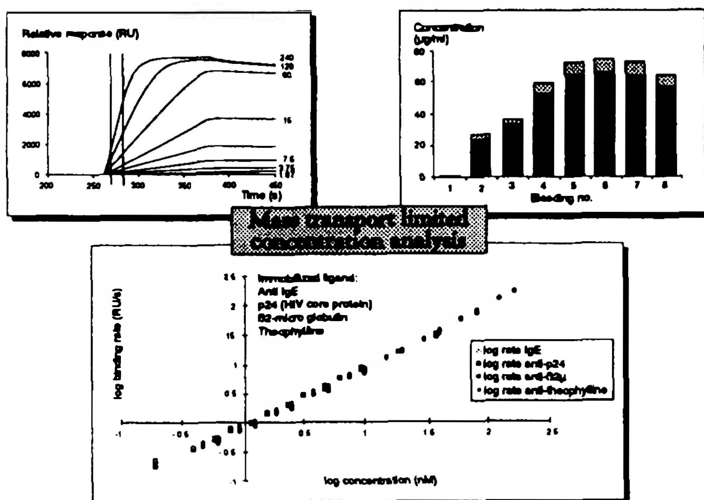

Figure 7 *Antibody characterization : Determination of active antibody concentration*

window where the binding is limited by mass transport is marked. Using this approach one 'universal' standard curve can be generated and used to evaluate the concentration of different antibodies. This type of analysis is illustrated in the lower part of Figure 7 using the interaction of different antibodies with their antigen (anti-IgE/IgE, anti-p24/P24, anti-b2microglobulin/ b2microglobulin, anti-theophylline/theophylline). The upper right of Figure 7 shows an example from an immunization where the active antibody concentration is monitored over time.

The affinity of MAbs is important to consider when high sensitivity and short test times are needed. The MAb is immobilized to the sensor surface. The antigen is injected and the binding to the MAb is analysed. Figure 8 shows an overlay plot of MAb 2, 4, 9, 12 when binding the antigen at the same concentration. The overlay plot gives immediate information on differences in association and dissociation rates which makes an affinity ranking possible. MAb 4 and 2, binding to the same epitope have highest affinities. MAb 9 and 12 have similar dissociation rates to MAb 2 and 4, but since the association rates are lower, the affinities will be lower.

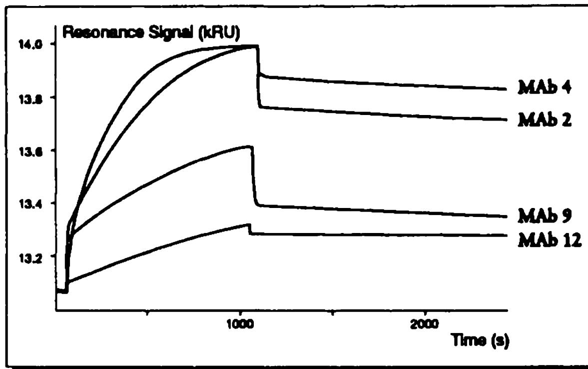

Figure 8 *Antibody characterization : Affinity ranking*

The main advantage of using non-label techniques is the time saving (Figure 9). Real time BIA analysis is a useful tool in the characterization of MAbs with no need for purification or labelling. The use of hybridoma culture supernatants allows the start of characterization at a very early stage, saving unnecessary cloning work, and thereby time, in development of MAbs. An example from Nycomed where BIAcore is compared with conventional methods shows impressive time saving.

The most appreciated feature of optical affinity biosensors in the reference list (Figure 2) is the quantitative kinetic analysis. This is important in the areas listed in Table 1.

System performance is illustrated in Figure 10 where three different concentration assays in human serum are performed in BIAcore and compared with other commercially available assays. Concentration analysis in BIAcore are correlated to three commercial

assays. The sensitivity of BIA concentration analysis will depend on the size of the analytes, design of the assay and quality of the reagents. In BIAcore, using a sandwich assay, it is possible with good reagents to determine a concentration of 40 pM with an acceptable coefficient of variation.

BIAcore™

- No purification
- No labelling
- Earlier characterisation
- Kinetic information

	BIAcore™	Conventional	
	Time	Method	Time
Isotyping	Day 1	ELISA	One day
Affinity	Day 1 & 2	RIA	Weeks + labelling
Kinetics	Day 1 & 2	NA	NA
Epitope map	Over night	ELISA	Weeks + labelling
Assay	Day 2	Various EIA	Days - Weeks
Extended map	Day 3	ELISA	One day + labelling
SUM	2 - 3 days		Weeks - Months

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Figure 9 *Antibody characterization : Comparison between BIAcore™ and conventional methods*

Table 1 *Areas where Kinetic Information is Needed*

- **Quantification of effects of structural changes on interactions**
  - Understanding of structure-function relations
  - Design of affinity pairs
- **Characterization of Biopharmaceutical products**
  - Recombinant proteins
  - Characterization of the immune response in vaccine development
- **Development of assays based on affinity**
  - Selection of reagents
- **Development of purification schemes**
  - Selection of affinity ligands and conditions for use
  - Study the effect on function of conditions used

4 OPTICAL AFFINITY BIOSENSORS IN THE FOOD INDUSTRY

Biosensor use in the food industry may be divided into research applications at major food research centres and routine analysis in, for example, quality control of raw materials and monitoring / control of manufacturing processes. The commercially available affinity biosensors are designed for research purposes and find applications in the research centres,

e.g. for test development. Although no routine analysis with affinity biosensors in the food industry has yet been described it is expected that the response from consumer and legislative pressures will enhance the development of such applications. The main features for affinity biosensors in this arena are the time saving (few assay steps and complete automization) and the reliability (built in quality control from the real time signal).

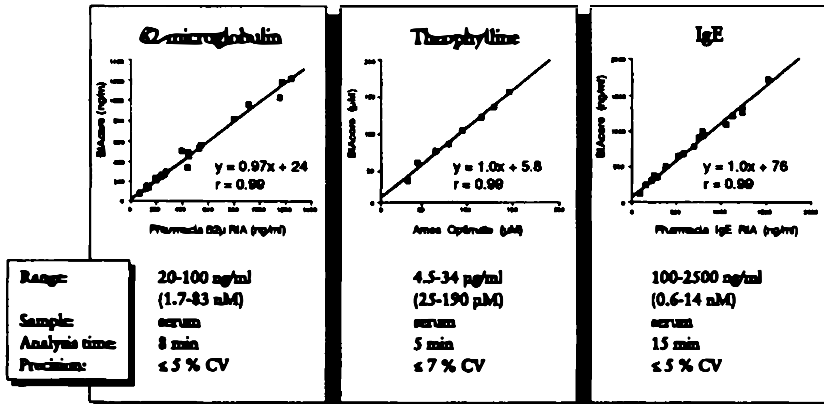

Figure 1 0 *System performance : Concentration determination*

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5
Stabilisation of the Biological Component of Biosensors

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1 SUMMARY

The storage stability of amperometric enzyme electrodes has been enhanced using a proprietary stabilising combination consisting of polycation D5106 and a sugar alcohol, lactitol. Different types of *N*-methylphenazinium tetracyanoquinonedimethane (NMP-TCNQ) mediated biosensors have been produced using the enzymes L-lactate oxidase, L-malate dehydrogenase and L-glutamate oxidase. The addition of the stabilising combination promoted an increase in the activity of the enzyme component during desiccation and conferred elevated storage stability on the enzyme, indicated by an increase in the shelf life of the desiccated biosensors under conditions of thermal stress at 37 °C. The results obtained from the biosensor activities on storage correlate approximately with the activity profiles observed for the enzymes in solution after desiccation in the presence of the stabilising combinations.

2 INTRODUCTION

Biosensors provide an extremely useful methodology for the electrochemical measurement of a large range of substances in foods and beverages by utilizing the specificity of biological components and electrochemical transducers. The biological components of such systems include enzymes, receptor proteins and antibodies, most of which exhibit inherent instability when isolated as pure preparations.

The useful lifetime of biosensors usually depends on the retention of the biological activity of the bio-component of the sensor. This may vary from days to months depending on the method of manufacture, the storage conditions and the stability of the biomolecule used. Most biosensors reported to date have been prepared and used under laboratory conditions which demonstrate the feasibility and characteristics of the biosensor very well. Studies regarding the long term storage of biosensors, which is necessary for the production of commercial products having an acceptable shelf life, have rarely been carried out.

Glucose oxidase which is used in many commercially available biosensors for glucose determination[1,2] is fairly resistant to denaturation. Using this enzyme it is possible to produce very stable, long lived, dry glucose electrodes by incorporating an excess of

enzyme into the matrix during fabrication in the absence of any additives.[3] Few other enzymes have been used successfully to produce biosensors which retain an acceptable shelf life on long term storage. Some examples from the literature include a biosensor for salicylate[4] prepared using the enzyme salicylate hydroxylase which retained acceptable enzyme activity over 3 months storage at room temperature. Uricase entrapped and air dried exhibited 14 months dry stability in a biosensor for uric acid.[1] Similarly, air dried, covalently linked enzyme membranes formed from a collagen film with immobilized glucoamylase and glucose oxidase gave a storage stability of 2 months at room temperature when measuring maltose as substrate and 5.5 months when measuring glucose.[5] It can be seen from these examples that the consistent production of long lived, stable biosensors remains problematical due to the labile nature of the enzymes or other biomolecules that are used.

We have previously stabilised a number of different enzymes using a combination of a positively charged polymer and a sugar alcohol, lactitol.[6-9] In most cases the activity of the enzymes was estimated using colorimetry, however, we have previously reported enhanced activity of the enzymes, alcohol oxidase, peroxidase and L-glutamate oxidase in alcohol and glutamate biosensors using the stabilising combination.[8] We report here the extension of this work to include malate dehydrogenase and lactate oxidase based biosensors for malate and lactate estimation and longer term stability of L-glutamate biosensors using polyelectrolyte-lactitol combinations.

3 EXPERIMENTAL

3.1 Materials

Malate dehydrogenase (MDH) from bovine heart (type M-9004), DL-malate, lactic acid hemicalcium salt and NADH were obtained from Sigma, L-glutamate oxidase was supplied by Yamasu Shoya Co. Ltd., Choshi, Chiba 288, Japan and lactate oxidase (LOx) was obtained from Genzyme diagnostics. Lactitol was a generous gift from Cortecs Ltd., Deeside, North Wales. Sodium phosphate buffer salts were obtained from BDH Merck Ltd. All other chemicals and reagents were obtained from Aldrich Ltd. Graphite sheet and silver foil were purchased from Johnson Matthey Ltd. Silver loaded epoxy resin was obtained from RS Components Ltd. NMP-TCNQ was prepared by the method of Melby (1965).[10] Silver/silver chloride electrodes were prepared using silver foil by the method of Sawyer and Roberts jnr. (1974).[11]

Polycation D5106 stabilising solution was prepared using proprietary methods developed by Leeds Biochemicals, 175 Woodhouse Lane, Leeds, LS2 3AR and pre-mixed with lactitol solutions to produce the polyelectrolyte stabilising combination.

3.2 Preparation of Mediated Enzyme Biosensors

Graphite sheet (1 mm thick) was cut into 6 mm x 2 mm strips and bonded to short lengths of insulated copper wire using silver loaded epoxy resin. The conductive surfaces were then covered with rapid set Araldite (Ciba-Geigy) to produce insulated electrodes

having one conductive face of graphite. The electrodes so formed were baked at 80 - 100 °C for 1 hour and then cooled to room temperature. A saturated solution of NMP-TCNQ in acetonitrile was layered onto the graphite surface in four 5 µl aliquots; the electrodes were allowed to air dry between each addition.

The NMP-TCNQ modified graphite electrodes were then immersed in a solution of 20 mg ml^{-1} *N*-cyclohexyl-*N*-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate in 200 mM acetate buffer, pH 4.5, for 90 minutes then the electrodes were then rinsed with 200 mM carbonate buffer, pH 9.0. Immediately 5 µl aliquots of enzyme solution (MDH 50 units and LOx 4 units) in the same carbonate buffer were added to the conducting faces of the activated electrodes and the electrodes were incubated for 120 minutes in a humid atmosphere to prevent drying out. Stabilisation of the enzyme electrodes was carried out by rinsing with 20 mM phosphate buffer pH 7.0 to remove excess enzyme solution then 5 µl of a solution of polycation D5106 and lactitol (5% w/v) in 20 mM phosphate buffer pH 7.0 was pipetted onto the conducting surface where the enzyme was immobilized and the electrodes were air dried in a vacuum desiccator in the presence of silica gel. Unstabilized electrodes were treated in an identical fashion but the stabilising combination was replaced with 5 µl of 20 mM phosphate buffer pH 7.0.

The dry electrodes were then stored for various periods of time over silica gel at a temperature of 37 °C until use. L-glutamate biosensors were constructed in a similar fashion as reported in Gibson *et al.* 1992.[8]

3.3 Storage and Activity Measurement

Dried biosensors were stored at 37 °C over silica gel as desiccant for various periods of time. For long term comparative studies with the NMP-TCNQ glutamate biosensors a number of electrodes were also stabilised with trehalose and stored desiccated at 37 °C.

Residual activity measurement for malate electrodes was carried out using 100 mM diethanolamine buffer pH 9.2 containing 50 mM potassium chloride, this buffer and pH has been reported to be optimal for the malate to oxaloacetate reaction (biochimica information, Boehringer Mannheim).[12] Lactate electrodes were measured using 100 mM sodium phosphate buffer containing 50 mM potassium chloride. In both cases the electrodes were immersed in 5 ml of the respective buffer and poised at +300 mV with respect to a saturated calomel (SCE) reference electrode using a model 362 EG & G potentiostat. The current was recorded using a 'Datatrace' flat bed chart recorder set at 50 mV full scale, which was equivalent to a maximum deflection of 500 nA. The baseline was allowed to stabilise with the solution being stirred constantly to promote equal mixing. The response of lactate electrodes was recorded by adding 10 µl of 400 mM lactic acid (hemicalcium salt) to give a final concentration of 0.8 mM. Malate was measured by adding 100 µl of a 50 mM solution of NAD (final concentration 1.0 mM) followed by 20 µl of a 400 mM solution of malic acid giving a final concentration of 1.6 mM. In most cases duplicate responses were recorded for these initial studies.

To measure residual activity for the mediated glutamate electrodes, five replicates were used for each assay point. The electrodes were immersed in 20 ml of 40 mM

phosphate buffer, pH 7.4, containing 100 mM potassium chloride. The electrodes were poised at a constant potential of +100 mV with respect to a silver/silver chloride electrode using the same equipment described above. When a stable baseline was obained, 200 μl of a 1 M solution of glutamic acid was added to give a final concentration of 10 mM and the responses recorded.

3.4 Measurement of Lactate Biosensor Responses in Deoxygenated Buffer

The phosphate buffer was deoxygenated by: (i) degassing under vacuum at room temperature followed by saturation of the buffer with nitrogen for 10 minutes, this process was then repeated; and (ii) by bringing 50 - 100 ml of buffer to the boil then cooling the solution whilst continuously gassing with nitrogen. Lactate sensor responses from the same sensor were then measured as before in the deoxygenated buffers using 0.8 mM lactate as substrate and compared to the response obtained in untreated buffer.

4 RESULTS AND DISCUSSION

4.1 Malate Biosensor

Energetically the reaction from oxaloacetate to malate is favoured under physiological conditions, however at alkaline pH malate is oxidized utilizing NAD as cofactor, which in this case was added to the reaction buffer before electrochemical measurements were taken. The sensor responses were due to the reoxidation of the NADH produced from this reaction at the electrode at +300 mV versus SCE which is mediated by NMP-TCNQ adsorbed on the graphite surface.[12] The reoxidation of the NADH back to NAD also assists in driving the reaction towards malate oxidation. Usually dehydrogenase enzymes incorporated into such NAD / NADH responsive electrodes are entrapped by membranes, however, in this case direct covalent immobilization of the enzyme has been successfully achieved. The reaction sequence is depicted diagramatically in Figure 1.

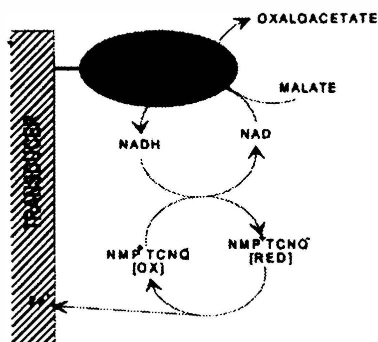

Figure 1 *Schematic reaction sequence of malate biosensor. NADH generated from the oxidation of malate by carbodiimide immobilized malate dehydrogenase is electrochemically reoxidised at the sensor surface by adsorbed NMP-TCNQ.*

The response characteristics of the sensor in this format are quite low (between 0.83 nA to 5.83 nA mm^2) and the dynamic range is limited (0.4 - 7.2 mM malate), however enough enzyme activity was observed to give stability measurements.

4.2 L-Lactate Biosensor

Lactate oxidase is a flavoprotein which uses molecular oxygen to oxidise lactate to pyruvate producing hydrogen peroxide as the second reaction product. The production of peroxide or the consumption of oxygen have both been used for the detection of lactate using this enzyme and form the basis of commercially available sensor systems for lactate (Yellow Springs Instruments Inc. and Analox Ltd.). Conducting salts such as NMP-TCNQ have previously been demonstrated to act as mediators for a variety of flavoenzymes including L- and D-amino acid oxidases, choline oxidase and xanthine oxidase.[14] In the case of lactate oxidase, direct electron transfer between the NMP-TCNQ adsorbed on the electrode surface and flavin group of the the enzyme is indicated, the amperometric responses being similar in magnitude in the presence and absence of molecular oxygen, Table 1. Based on this evidence the schemetic reaction sequence for the lactate sensor is depicted in Figure 2.

Table 1 *Lactate Biosensor Response in the Absence of Oxygen*

Oxygenated Buffer (nA)	Deoxygenated Buffer (nA)	
	Gassing at Room Temperature	Gassing Boiled Buffer
250	245	238

4.3 Stabilisation of the enzyme activity

The responses of the malate and lactate biosensors have been determined after incubation at 37 °C for various time periods in the presence and absence of the stabilising compounds, Figures 3 and 4. It can be clearly seen that the sensors dried with stabilisers exhibit much greater responses with respect to the length of storage at elevated temperature when compared to the sensors dried in buffer alone. Also the initial responses of the sensors immediately after drying are higher in the presence of the stabilisers indicating less activity is lost during the drying step. These results for immobilized enzymes in the biosensor format are consistent with results obtained for enzymes in solution when dried with the stabilisers.[8]

4.3 L-Glutamate Biosensor

The production and stabilisation of an NMP-TCNQ mediated L-glutamate biosensor based on immobilized L-glutamate oxidase has been demonstrated using a combination of polycation D5106 and lactitol as the stabilisation agents. Unstabilised biosensors were fairly unstable on storage at 37 °C, losing over 50% activity within the first week.

Stabilised biosensors incubated at temperatures of 4, 25 and 37 °C retained between 65% and 88% initial activity over an incubation period of 12 weeks, Figure 5. Direct electron transfer between the flavin group of the enzyme and the NMP-TCNQ mediator has been indicated in initial experiments using nitrogen saturated buffer giving a proposed reaction scheme of the same type as lactate oxidase shown in Figure 2.

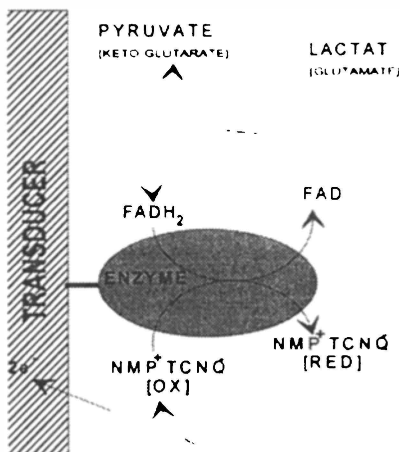

Figure 2 *Schematic reaction sequence of lactate biosensor. The bound FAD cofactor of the carbodiimide immobilized lactate oxidase is reduced to $FADH_2$ as lactate is oxidised to pyruvate. The reduced cofactor is then directly reoxidised at the sensor surface by the adsorbed NMP-TCNQ.*

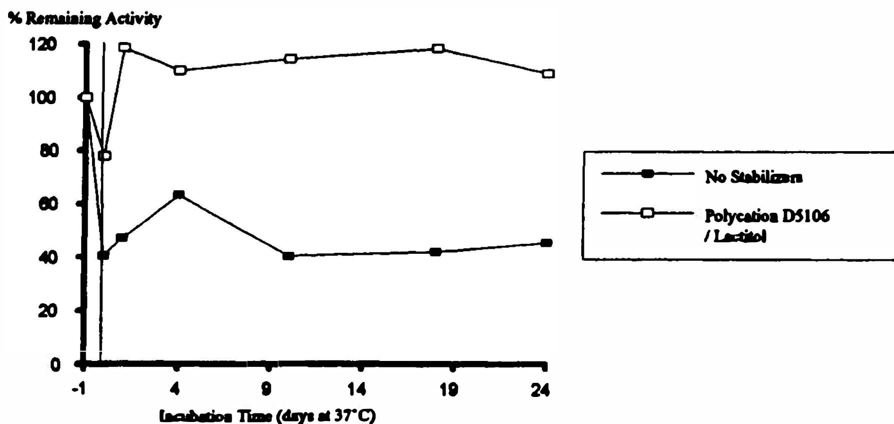

Figure 3 *NMP-TCNQ mediated malate biosensor. The initial response of the malate biosensor as a percentage of the enzyme activity of biosensors measured before the drying step is shown at the zero time point indicated by the vertical line. The subsequent points on the graph are average biosensor responses to 1.6 mM malate as a percentage of the initial activity after incubation at 37 °C.*

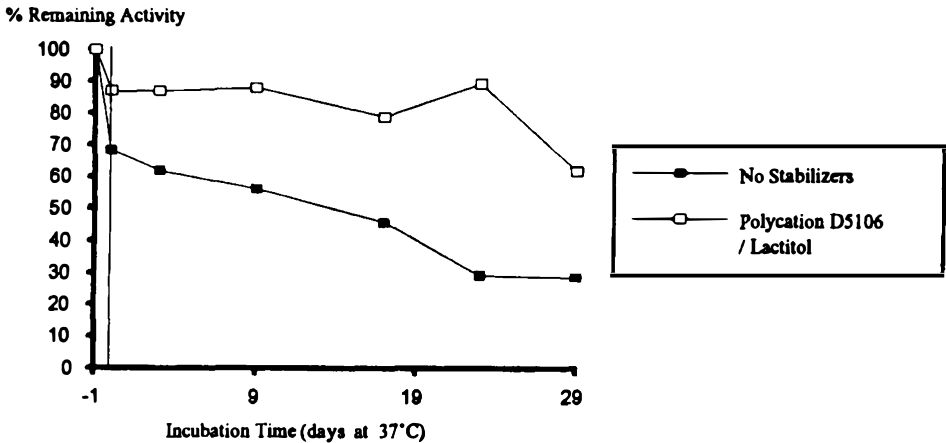

Figure 4 *NMP-TCNQ mediated lactate biosensor. The initial response of the lactate biosensor as a percentage of the average enzyme activity of biosensors measured before the drying step is shown at the zero time point indicated by the vertical line. The subsequent points on the graph are average biosensor responses to 0.8 mM lactate as a percentage of the initial activity after incubation at 37 °C.*

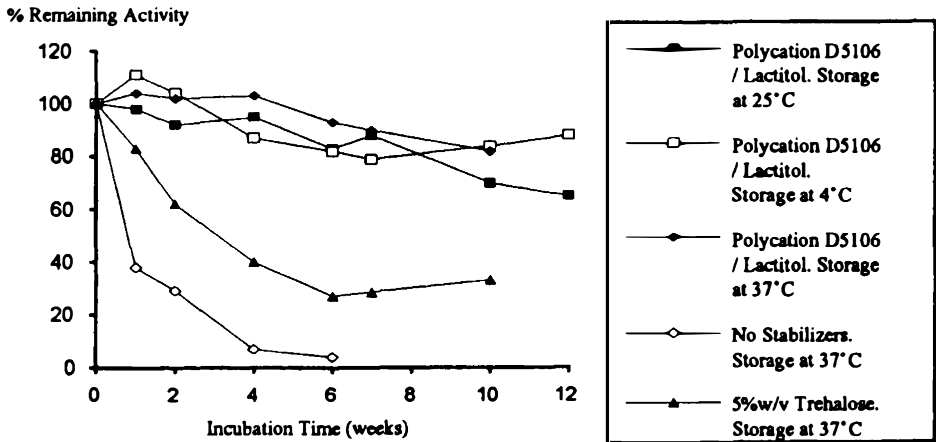

Figure 5 *NMP-TCNQ mediated L-glutamate biosensor. The stability of L-glutamate biosensors was estimated using elevated temperature degradation at 37 °C. Initial values of enzyme activity were taken from freshly dried biosensors and the subsequent time points were percentages relative to the initial activity. Five replicates were used for each time point.*

5 CONCLUSIONS

The application of the stabilisation system described allows enzyme based biosensors, previously reported as being low in stability, to be dry stabilised and to confer a useful working shelf life for commercial applications. The incorporation of the stabilisers in the production of biosensors for lactate and malate prevents the loss of enzyme activity during the drying step and therefore would be beneficial in the production of biosensors based on these enzymes.

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A Preliminary Model and Evidence for the Mechanism of Stabilisation of Analytical Enzymes in Aqueous Solution by Polyelectrolytes and Sugar Derivatives

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1 SUMMARY

We have demonstrated that it is possible to enhance the activity retention of enzymes in aqueous solution when tested under conditions of thermal stress by using a proprietary stabilising mixture consisting of soluble polyelectrolytes and sugar derivatives. A prelimary theoretical model for the mechanism of the stabilisation effect observed has been postulated.

2 INTRODUCTION

Enzymes have been used in analytical techniques for many years, having the advantages of specificity and sensitivity over conventional chemical methods. A major disadvantage of enzymes is that they are labile in nature and require careful handling to avoid inactivation. Many techniques have been employed to increase the stability of enzymes including immobilisation,[1] entrapment,[2] chemical modification,[3] crosslinking,[4] protein engineering[5] and admixtures with various types of chemicals.[6]

We have previously reported the use of combinations of polycationic polyelectrolytes and sugar alcohols in the production of stabilised dry enzyme preparations[7,8] and the application of these systems in both a single analytical reagent for alcohol [8] and biosensor stabilisation.[9-11] A theoretical model for the mechanism of dry enzyme stabilisation by combinations of polyelectrolytes and sugar derivatives has been postulated.[12] In this paper we provide evidence that combinations of polyelectrolytes and sugar derivatives can also be used to stabilise enzymes in aqueous solution and a theoretical model explaining the stabilising effects observed is suggested. The application of this fundamental investigation into enzyme interactions to create stable molecules will enable the production of commercially viable analytical biosensors and related analytical methods.

3 MATERIALS AND METHODS

Alcohol oxidase (MOx) from *Hansenula polymorpha* was obtained from Leeds Biochemicals, 175 Woodhouse Lane, Leeds, LS2 9JT. Glucose oxidase (GOx) from *Aspergillus niger* and horseradish peroxidase type 4 (HRP) were obtained from Biozyme. Yeast alcohol dehydrogenase (YADH) was obtained from Sigma. Polycation (D5105, D1104, G7106 and HS106) and polyanion D1104 stabilising solutions were prepared using proprietary methods developed by Leeds Biochemicals. All other chemical reagents were obtained from Sigma and BDH and were of analytical grade.

3.1 Enzyme Stability Testing

An Eppendorf tube containing the buffer/stabiliser was equilibrated to the desired temperature in a Techne Dri heating block. Concentrated enzyme solution was then added. Aliquots were taken periodically and were analysed immediately for residual enzyme activity, using the enzyme assay methods described below. The time taken for enzyme preparations to loose 50% of their initial activity (denoted as $T_{50\%}$) when incubated at elevated temperatures was chosen as a measure of enzyme stability. The concentrations of enzymes used in the stability measurement experiments were 3.2 mg ml^{-1} for MOx, 0.1 mg ml^{-1} for GOx, 0.04 mg ml^{-1} for HRP and 0.03 mg ml^{-1} for YADH.

3.2 Enzyme Assay Methods

Alcohol oxidase activity was measured using the standard assay procedure described in Gibson *et al.* [8] Horseradish peroxidase activity was measured using the assay procedure described in Gibson *et al.*[12] Glucose oxidase and yeast alcohol dehydrogenase were assayed by the standard methods described in Bergmeyer.[13]

3.3 Native Agarose Gel Electrophoresis

Various GOx (0.76 mg ml^{-1})/polycation (0.5% w/v) mixtures were loaded into a 2% w/v agarose gel. The gels were run in 89 mM tris/phosphate buffer pH 8.0 at constant voltage (75 V) for 3 hours. After electrophoresis GOx was visualised by soaking the gel in an activity stain comprised of 18.2 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), 1.5 units ml^{-1} peroxidase, 0.11M glucose and 100 mM sodium phosphate buffer pH 6.0.

4 RESULTS AND DISCUSSION

The effect of adding polyelectrolytes to solutions of enzymes is shown in Table 1. The presence of polyelectrolytes enhanced the activity retention of all enzymes tested, indicating a positive effect on enzyme stability. The uncharged control polymers when tested did not give this stabilising effect, which suggests that an electrostatic interaction between the polyelectrolytes and the enzymes is important in conferring the enhanced stability observed.

Table 1 *Effect of Polyelectrolytes on the Thermal Stability of Enzymes*

Enzyme	Buffer	Temperature (°C)	Stabiliser*	$T_{50\%(mins)}$
MOx	100 mM Sodium phosphate pH 8.0	56	None	11.3
			UCP D5015 (2.0% w/v)	10.1
			PC D5105 (2.0% w/v)	14.2
			UCP G7106 (2.0% w/v)	8.3
			PC G7106 (2.0% w/v)	20.0
	100 mM Sodium phosphate pH 6.0	59	None	2.5
			UCP D1104 (1% w/v)	3.1
			PA D1104 (1% w/v)	27.4
GOx	Sodium acetate pH 4.8 (ionic strength=0.072)	66	None	3.6
			PC D5105 (0.5% w/v)	16.2
			PC G7106 (0.5% w/v)	9.0
			PC HS106 (0.5% w/v)	5.1
YADH	10 mM Sodium phosphate pH 7.0	48	None	4.3
			PA D1104 (1% w/v)	59.0
HRP	25 mM Sodium phosphate pH 8.0	69	None	16.0
			PC D1104 (1.0% w/v)	24.0
			PC D5105 (1.0% w/v)	21.0

* UCP=Uncharged polymer, PC=Polycation, PA=Polyanion

Figure 1 *Native agarose gel electrophoresis of GOx/polycation mixtures. Lane 1=native GOx, Lane 2=GOx/polycation D5105, Lane 3=GOx/polycation G7106, Lane 4=GOx/polycation HS1106.*

The formation of soluble glucose oxidase/polycation complexes is demonstrated by native gel electrophoresis of GOx/poylcation mixtures in Figure 1. Native GOx (isoelectric point=4.2) migrates towards the anode because the enzyme is negatively charged at pH 8.0 (lane 1). GOx in the presence of polycations D5105 and HS1106 (lanes 2 and 4 respectively) migrate towards the cathode. This demonstrates that GOx binds to the polycations forming a soluble complex containing an excess of cationic charges. The polycation D5105/GOx complex migrates further than the polycation HS106/GOx complex. This observation suggests that the polycation D5105/GOx complex is either smaller in size or has a larger net positive charge than the polycation HS1106/GOx complex.

Interestingly, the polycation G7106/GOx mixture (lane 3) appears to form two types of soluble complex, evidenced by two migrating bands on the gel. The first type of complex migrates towards the cathode, and must therefore contain an excess of polycationic charges. The second type of complex, however, migrates towards the anode, but is less mobile than the negatively charged native enzyme. This result suggests that this complex contains an excess of GOx molecules making the overall charge on the complex negative. The formation of different types of soluble protein/polyelectrolyte complexes has also been demonstrated by Tolstoguzov [14] and Gekko *et al.*[15]

The effect of adding sugars to solutions of enzymes and polyelectrolytes is shown in Table 2. In each case, the sugar enhanced the stabilising effect of the polyelectrolyte on the enzyme. The stabilisation effect observed when both sugars and polyelectrolytes are used as soluble additives to enzyme solutions appears to be greater than the individual effects of

Table 2 *Effect of Combinations of Polyelectrolytes and Sugar Derivatives on the Thermal Stability of Enzymes*

Enzyme	Buffer	Temperature (°C)	Stabiliser*	$T_{50\% (mins)}$
MOx	100 mM Sodium phosphate pH 8.0	59	None	2.0
			PC D1104 (1% w/v)	29.6
			Lactose (10% w/v)	2.1
			PCD1104 (1% w/v) + Lactose (10% w/v)	36.1
YADH	10 mM Sodium phosphate pH 7.0	48	None	4.3
			PA D1104 (1% w/v)	59.0
			Glycerol (10% w/v)	55.0
			PA D1104 (1% w/v) + Glycerol (10% w/v)	78.3
HRP	25 mM Sodium phosphate pH 8.0	69	None	16.0
			PC D1104 (1% w/v)	24.0
			Glycerol (10% w/v)	25.5
			PC D1104 (1% w/v) + Glycerol (10% w/v)	34.5
			Mannitol (10% w/v)	26.5
			PC D1104 (1% w/v) + Mannitol (10% w/v)	36.0

* PC=Polycation, PA=Polyanion

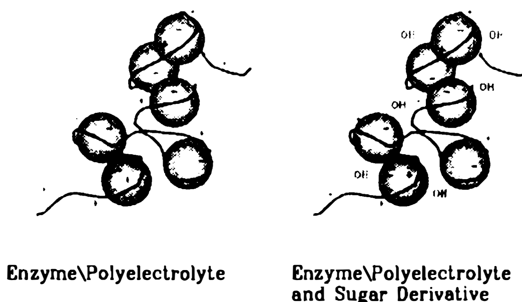

Figure 2 *Theoretical model of mechanism of enzyme stabilisation*

either of the additives alone. This is very clearly demonstrated by reference to the stabilisation of MOx by a combination of polycation D1104 and lactose as additives as shown in Table 2. A prelimary model for the mechanism of enzyme stabilisation is shown in Figure 2.

Enzyme molecules at pHs above their isoelectric point will be negatively charged overall and as such will tend to attract and interact electrostatically with polycations. Conversely, at pHs below their isoelectric points enzyme molecules will have a net positive charge and will tend to interact with polyanions. However, in practice it is found that polyelectrolytes will interact to varying degrees with certain enzymes at pHs above and below the isoelectric point (unpublished results).

The theoretical model assumes that in enzyme solutions of appropriate ionic composition association between polyelectrolytes and enzyme molecules will occur, forming a soluble enzyme/polyelectrolyte complex where polyelectrolyte molecules surround the enzyme molecules and hold them in a sort of 'electrostatic cage'. It would be expected that the soluble enzyme/polyelectrolyte complex would require greater amounts of energy to disrupt the molecular structure when compared to free enzymes in solution. Experimentally, we have demonstrated that enzymes exhibit enhanced stability in the presence of soluble polyelectrolytes. We have also shown that glucose oxidase forms soluble electrostatic complexes with polycations.

The second part of the theoretical model comes into play when, in addition to polyelectrolytes, polar cosolutes such as sugars and their derivatives are also present in enzyme solutions. In this case, the presence of the polar cosolute decreases the dielectric constant of the aqueous medium. This has the effect of increasing the strength of the electrostatic interactions between the enzyme molecules and the polyelectrolytes, resulting in a soluble enzyme/polyelectrolyte complex with enhanced thermal stability in aqueous solution.

Further experimental work in our laboratory is aimed at elucidating the details of the molecular interactions between stabilisers and several model enzymes.

5 CONCLUSIONS

Enzymes can be stabilised in aqueous solution by using a combination of soluble polyelectrolytes and sugar derivatives. The proposed model for the mechanism of enzyme stabilisation was, in part, substantiated by the evidence obtained from enzyme stability studies in solution. Four different enzymes exhibited enhanced stability in the presence of polyelectrolytes of differing chemical structures. When the polyelectrolytes were replaced with uncharged control polymers of analogous molecular structure little stability was detected, suggesting that electrostatic interactions between polyelectrolytes and enzymes are responsible for the stabilisation effect observed. Native agarose gel electrophoresis of various GOx/polycation mixtures demonstrated that electrostatic interactions between GOx and polycations resulted in the formation of soluble GOx/polycation complexes. Inclusion of sugars into aqueous solutions of enzyme/polyelectrolyte mixtures resulted in enhanced stability of the enzyme.

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7

The Application of Computer-aided Experiment Design and of Formulation Optimization to Biosensor Assembly

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1 INTRODUCTION

Recent years have seen a large growth in biosensor research both in the field of medical diagnostics and in the field of biotechnology related monitoring including rapid food analysis.[1] As a highly sophisticated discipline, food analysis has attracted much attention with respect to biosensor research and development.[2] More sophisticated detection, including the assessment of the freshness and the taste of fish and meat, is now assisted by the recently reported freshness and taste sensors.[3,4] However, there has been a relatively limited number of commercialized biosensor products either for medical diagnosis or for food analysis. This is probably due to the fact that there has been insufficient effort in the optimization of the performance of the prototype biosensors, resulting from the laboratory research, enabling successful adaptation into commercially feasible sensor products. This paper stresses the importance of the optimization of the performance of the biosensors in designing prototype biosensor system and of the modification of prototype biosensors to produce commercially feasible sensor products.

2 FORMULATION OPTIMIZATION AND BIOSENSOR DEVELOPMENT

The fundamental working principles of biosensors have not changed greatly since Clark and Lyon[5] invented the first biosensor, an enzyme electrode as it was originally named. A biosensor is, in fact, a combination of several devices which have stand alone functions. These functions include the bio-recognition element, the signal transducer and the result display device. The most fundamental of the functions is the bio-recognition device where the major biochemical reactions take place.

In modern biosensor systems, there is usually more than one material included in the bio-recognition device. Each of these materials in turn has specific functions in the bio-recognition process. The materials may be the enzymes, the electron mediators, the polymeric binders or other additives. Although a workable bio-recognition device requires only one or two of these materials, it is not rare to find the bio-recognition device in a commercialized biosensor containing more than ten components. Thus, it is important to optimize the composition of the bio-recognition device for the performance of the biosensor system and of the manufacturing cost of the commercial products.

Since biosensors are often working in the environment with complex compositions, a bio-recognition device, in many cases, has to involve a selective permeation device in order to filter interfering substances from the targeted substances. Such a selective permeation device is often in a membranous format. In the Clark and Lyon enzyme electrode, this selective permeation device is the glass membrane which allows the diffusion of only the H^+ ions through the glass membrane to reach the electrode surface. The Reflotron Cholesterol sensor system, manufactured by Boehringer Mannheim GmbH, is another example of biosensor system incorporating a selective permeation device. This biosensor system has been designed to measure the cholesterol content in a liquid sample, particularly a blood sample. A fabric filtration layer, incorporated in this sensor system, permits the selective diffusion of plasma through the layer while holding back the erythrocytes.

Membranes used in the bio-recognition device have to give satisfactory rates of transport of the permeants and satisfactory rates of separation of the interferents. Since in many cases, biosensors for food analysis are desired to work under atmospheric or near atmospheric pressure, the transport of the permeants through the membrane can only be the result of the structural functionalities of the membranes rather than of the pressure. The small size of a biosensor adds significant difficulties to the development of membranes for biosensors. Thus, membranes used in biosensor systems are required to have sophisticated structure and functions. It is well-known that the structural functionalities of membranes are heavily dependent on their composition. Therefore, the optimization of the composition of membranes becomes one of the important steps in the development of biosensor systems in order to achieve the desired separation and desired transport.

A typical example of a complex bio-recognition device is the Medisense glucose biosensor systems which are used by nearly 50% of the diabetics in the U.S. Though originally designed for the detection of glucose in human blood, these biosensors can be successfully adapted to use in the detection of glucose contained in food and other media. A typical Medisense glucose electrode has its configuration displayed as Figure 1.

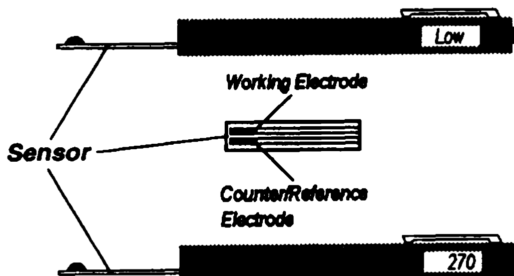

Figure 1 *The Medisense biosensor system*

The Medisense glucose system is composed of two major parts; the pen-sized electronic transducer and display unit integration, and the bio-recognition device which is the working enzyme electrode and the reference electrode. Both the reference electrode and the working electrode are screen-printed onto a polyvinyl chloride (PVC) substrate.[6,7] The reference electrode is a simple Ag/AgCl formulation while the working electrode consists of a mixture of more than ten relevant enzymes, polymeric modifiers and other additives. Each component involved in the working electrode plays an important role in the function of this sensor system. Therefore, the composition of each component in the working electrode must be optimized in order to achieve the best performance of this system. In fact, the final commercialized Medisense glucose sensor system is the result of successful optimization of the formulation of the working electrode, taking into account the fine-tuning of the parameters relevant to screen-printing technology.

3 STRATEGIES FOR THE OPTIMIZATION OF BIOSENSOR SYSTEMS

Assuming that the working principles of a biosensor system have been established, the next step is the formulation of the bio-recognition device. First of all, the materials involved must be evaluated for their importance in the formulation. This usually can be achieved using multi-factor factorial experiments. Formulation of the bio-recognition device containing various proportions of the materials of interest, whose compositions are determined by the principles of factorial design, are tested and results recorded. The results can be easily processed using the basic principles associated with factorial analysis to give an indication of the contribution of each component to the performance of the biosensor system being investigated. As a result, the necessary ingredients of a biosensor system can be determined.

Following this is the optimization of the proportions of the necessary ingredients in the biosensor system or the optimization of the biosensor formulation. The task of formulation optimization is, in fact, the optimization of the response/performance space illustrating the relationship between the biosensor performance and the biosensor composition/formulation. The fundamental idea is to assume that there exists a certain relationship between the performance of the biosensor and the composition of the materials contained in the biosensor system, which can be expressed mathematically as:

$$Y = f(X_1, X_2, \ldots, X_n)$$

Scheme 1

where, Y denotes the performance of the biosensor system and X_1, X_2,..., X_n denote the proportion of Component *1*, Component *2* ..., Component *n* included in the biosensor system. Then, the task of the formulation optimization becomes the optimization of the expression (Scheme 1) to obtain the optimal value of performance *Y*. The values of X_1, X_2, ...X_n corresponding to the optimal *Y*, give the composition of the optimal biosensor system.

In practice, the modelling of the performance-composition relationship of a biosensor system involves two steps. Firstly, the type of the model should be specified. We propose that the following model could be used for most biosensor systems.

$$Y \cap Y_{i(1)} \times Y_{i(2)} \times \ldots \times Y_{i(n)}$$

Scheme 2

while

$$Y_{i(1)} = a_{i(1)} + b_{i(1)}(X_1 - X_{i(1)}) + c_{i(1)}(X_1 - X_{i(1)})^2 + d_{i(1)}(X_1 - X_{i(1)})^3$$

Scheme 3

and

$$Y_{i(2)} = a_{i(2)} + b_{i(2)}(X_2 - X_{i(2)}) + c_{i(2)}(X_2 - X_{i(2)})^2 + d_{i(2)}(X_2 - X_{i(2)})^3$$

Scheme 4

and so on to

$$Y_{i(n-1)} = a_{i(n-1)} + b_{i(n-1)}(X_{n-1} - X_{i(n-1)}) + c_{i(n-1)}(X_{n-1} - X_{i(n-1)})^2 + d_{i(n-1)}(X_{1n-1} - X_{i(n-1)})^3$$

Scheme 5

where, $i(1) = 1, 2, \ldots, N_1$; $i(2) = 1, 2, \ldots, N_2$; ...; $i(n-1) = 1, 2, \ldots, N_{n-1}$; $N_1, N_2, \ldots, N_{n-1}$ represent the number of biosensor formulations containing different fractions of components *1, 2, ..., n-1*. The terms in *Y* and *X* have the same meanings as defined previously.

This model, the spline-interpolation model, has high versatility especially in the case of complex multi-component systems.[8,9] The following discussion of the design of experiments for the optimization of the performance of a biosensor system and of the optimization of the performance of the biosensor is concentrated on a biosensor system containing three interested components. Full details of this approach have been given elsewhere.[8,9] First of all, a set of six experiments has to be performed. These experiments involve the evaluation of the differences in performance of the biosensor systems containing various amounts of the three components of interest. The compositions of these components in the biosensor system are given in Table 1.

Table 1 *The Compositions of the Biosensor System for the Initial Investigation*

Experiment Number	Composition (%)		
	Component		
	1	2	3
1	0	0	0
2	50	0	50
3	100	0	0
4	0	50	50
5	0	100	0
6	50	50	0

It should be emphasized that the compositions given in Table 1 are relative ones. In other words, a composition of 0% of Component *i* does not have to mean that the biosensor system contains none of Component *i* but means that the amount of Component *i* in the biosensor system is at the low end of the investigated composition range of this component in the biosensor system. The data points, *i.e.* the measured performances of the biosensor systems with their compositions shown as Table 1, would allow the construction of the performance surface, *i.e.* the modelling of the relationship between the performance of the biosensor and the composition of the biosensor. The principles of the modelling are schematically shown in Figure 2. That is, the value of the biosensor performance is the product of the values of the two splines representing the contribution of the Component 1 and of the contribution of the Component 2 to the biosensor performance.

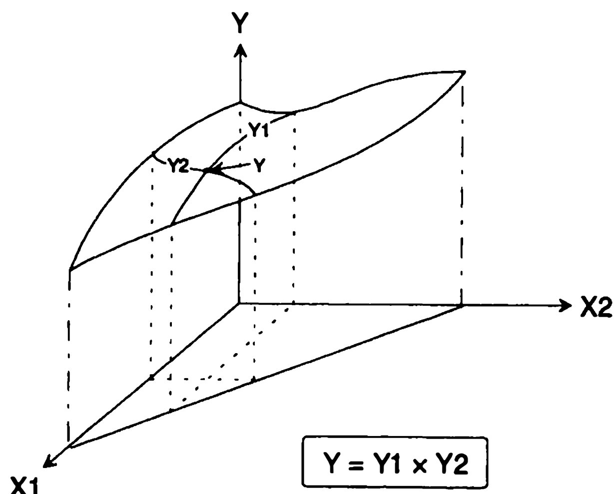

Figure 2 *The principles involved in the modelling of performance surfaces based on the spline-interpolation. The symbols have the same significance as those previously defined.*

The biosensor system performance value with any component composition can be predicted using this model. Thus, the optimal performance of the biosensor system can be obtained through the numerical optimization of the model. The performance surface for the individual biosensor performance can be built using the measured biosensor performances of the six biosensor formulations listed in Table 1. Therefore, multi-objective optimization can be conducted to deduce the biosensor composition which gives the overall optimal performance. The composition of the biosensor system giving the optimal biosensor performance is that of the optimal biosensor formulation.

The simulation error of the performance-composition model is one of the important criteria in evaluating the reliability of the model obtained. In some cases, a model acquired from six initial biosensor formulations may not be reliable enough. Therefore, more data

points will be required for the modelling. It is suggested that the values of the performance of biosensors, whose compositions are given in Table 2, would enable reliable modelling of even very complicated biosensor systems.

Table 2 *The Composition of the Biosensor Systems for Further Investigation*

Experiment Number	Composition (%)		
	Component		
	1	2	3
1	1	0	0
2	0	1	0
3	0	0	1
4	1/3	1/3	1/3
5	0.8536	0.1464	0
6	0	0.8536	0.1464
7	0.1464	0	0.8536
8	0.1464	0.8536	0
9	0	0.1464	0.8536
10	0.8536	0	0.1464

4 THE 'EXPERT' COMPUTER PROGRAMME

The performance optimization task outlined above is difficult to calculate without the aid of a computer. Several complicated mathematical operations are required to perform the multi-variable simulation and multi-variable and multi-objective optimizations. Consequently, a computer program has been written to calculate the results from the

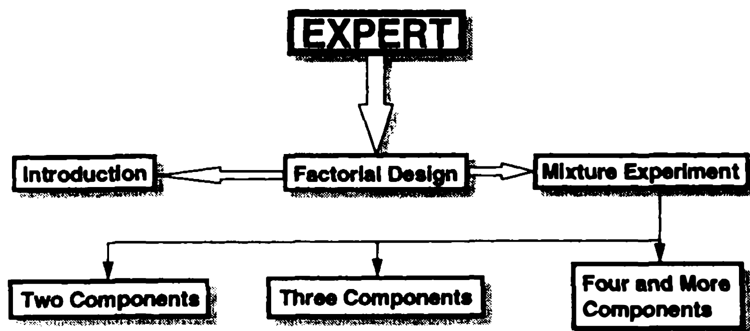

Figure 3 *Structure of the Expert software package*

formulae. This program consists of analysis of variation (ANOVA) for the designed factorial experiments and the multi-variable optimization required by the designed mixture experiments. The program is divided into three parts. The structure of the program is schematically illustrated as Figure 3.

Detailed background information is provided in the 'Introduction' part of the program explaining the factorial experimental design, the design and analysis of mixture experiments and the use of the computation package . The 'Factorial Design' part allows the input of the data from the designed factorial experiments, provides the ANOVA table and determines the contribution of each component of the biosensor system to the performance parameter being investigated.

The 'Mixture Experiment' has been dedicated to the modelling of the performance of the biosensor system using the data obtained from design experiments. The program also provides: (i) a curve illustrating the relationship between the performance of a biosensor system containing two components of interest and the proportions of these components in the biosensor system; (ii) a surface visualizing the relationship between the performance of a biosensor system containing three major components and the proportions of these components in the biosensor system; (iii) a tabulated data set to show the relationship between the performance of a biosensor containing four or more components and the proportions of these components in the biosensor system.

Multi-objective and multi-variable optimizations are automatically performed by the program and information is presented concerning the overall optimal performance and the relevant proportions of each component. Typical displays of the program, for an optimization case involving a biosensor system containing three components of interest are given in Figures 4 and 5. Figure 5 shows contours corresponding to the performance surface given in Figure 4.

The Expert package can be run under the MS-DOS with a minimum requirement of an 8086 microprocessor and 640KB of RAM (Random Access Memory). The menu-driven operation provides ease of use of the Expert package.

5 FORMULATION OPTIMIZATION AND BIOSENSOR DEVELOPMENT - A CASE STUDY

The following case study is provided to demonstrate the usefulness of the Expert package in formulation optimization during biosensor development . A polymeric membrane for plasma separation was required to be incorporated into the Medisense glucose sensor system.

The target was to achieve the optimal rate of plasma diffusion through the membrane and the optimal rate of erythrocyte separation. Three polymeric components were used in the membrane formulation of which the compositions were to be optimized.

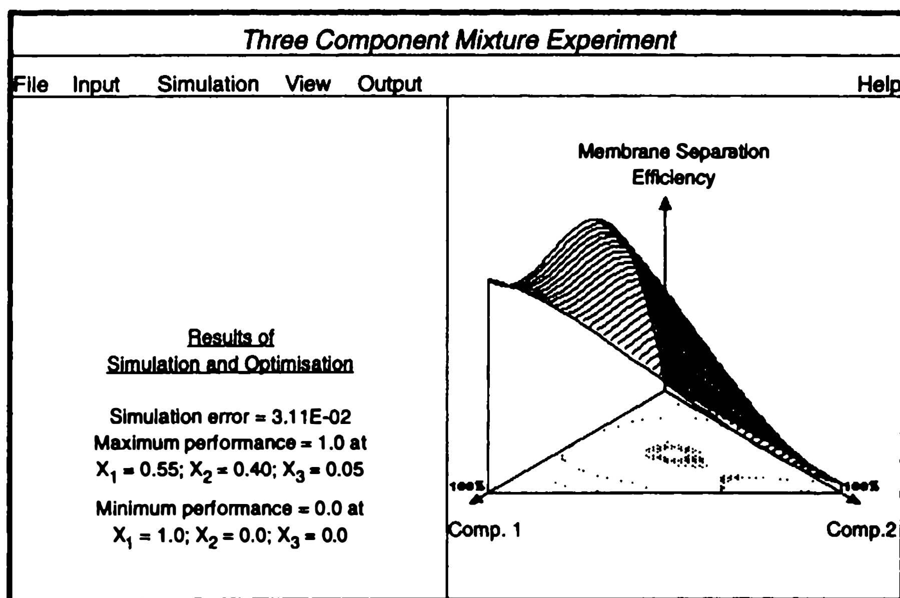

Figure 4 *Typical display of the Expert package. This shows the relationship between the performance of a biosensor and the proportions of the interested components - The Response Surface.*

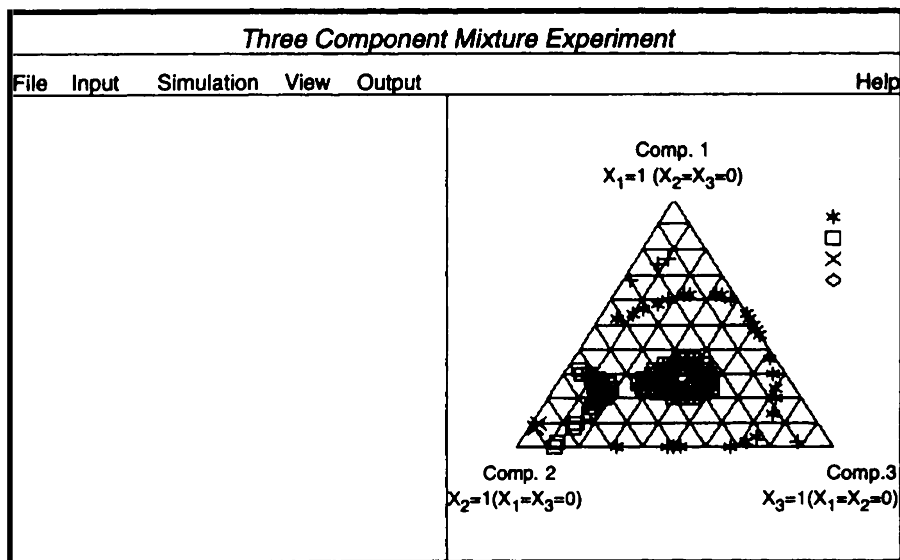

Figure 5 *Typical display of the Expert package. This shows the relationship between the performance of a biosensor and the proportions of the interested components - The Contours corresponding to the Response Surface.*

Membranes containing the three chosen polymeric components, with their compositions given as Table 2, were prepared and incorporated within the Medisense glucose sensor systems. The rates of erythrocyte separation by the membranes and the rates of plasma diffusion through the membranes were measured. Using the Expert program, the relationships between the rate of erythrocyte separation and the membrane composition and between the rate of plasma diffusion and the membrane composition were generated, as shown in Figure 6 and Figure 7.

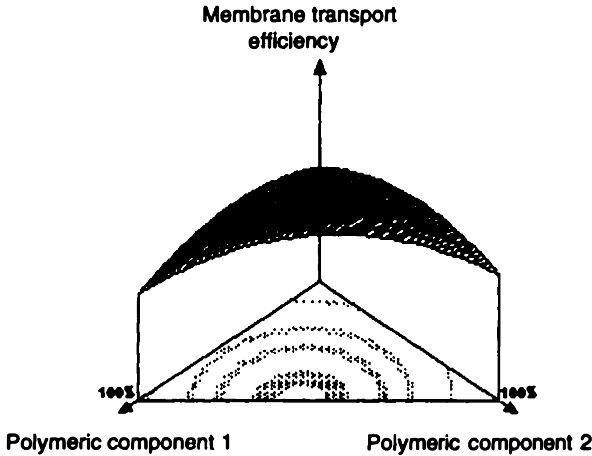

Figure 6 *Performance surface illustrating the relationship between the membrane transport efficiency and the membrane composition*

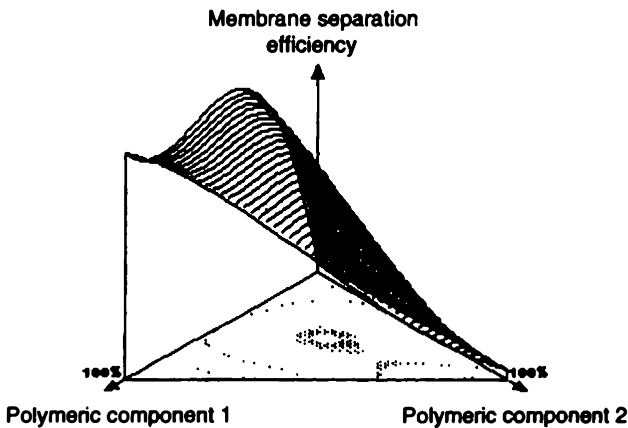

Figure 7 *Performance surface illustrating the relationship between the membrane separation efficiency and the membrane compositions*

The Expert software package also yields the optimal membrane formulation which gives both the optimal plasma transport rate and the optimal erythrocyte separation rate. This optimal membrane formulation contains 38% of polymeric component 1, 42% of polymeric component 2 and 20% of polymeric component 3.

6 CONCLUSIONS

Formulation optimization and the design of experiments, focussed on the provision of data for modelling the performance-composition relationship, are important steps in the efficient development of biosensor systems. The Expert software package, described here, can significantly facilitate the optimization of the performance of biosensor systems; the programme design being based on the proposed experimental design and performance-composition modelling approach,.

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Practical Construction and Function of Biosensor Systems for Quality Control in the Food and Beverage Industry

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1 INTRODUCTION

Analytical chemistry seeks to develop methods which allow the measurement of specific species with the minimum of steps in the reaction sequence. It would be highly preferable to have a system which did not require any reagents, specifically recognised the species to be analysed, and produced a signal which was easily converted into a meaningful numeric result. Wet chemistry analysis as it was developed during the mid 20th century was far from this ideal. Although automated analysers were developed during the 1960's, these still used large quantities of reagents, and were, in general, using colorimetric assays to detect the analytes being tested. This presented many problems when measuring clinical samples such as blood. Samples required pre-treatment to remove the red blood cells and could still foul the system with serum protein. These systems were also slow to produce results as in most cases they appeared as a peak on a chart recorder and a technician had to read the result from a chart calculated from the response to a series of standards. Clearly there was room for improvement in this system especially when clinicians required results quickly, particularly in the case of traumatised patients.

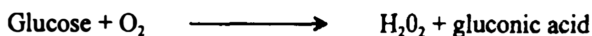
2 BIOSENSORS

2.1 Development of the first Biosensor

During the 1950's Dr. Leland (Lee) Clark Jr. was working on the development of a heart lung machine to allow total by-pass of the heart during open heart surgery. As part of this work he developed the first polarographic oxygen probe to measure the oxygen content of the blood in his apparatus. The use of electrochemistry to measure an element was not new but Dr. Clark's adaption of the electrode to measure oxygen through a gas permeable membrane was to revolutionise the measurement of oxygen and a whole series of other analytes.

During the mid 20th century biochemists were also hard at work discovering a vaste number of enzymes which were uniquely specific for a very narrow range of substrates. Thus during the 1950's Lee Clark obtained a sample of a newly purified enzyme, glucose oxidase. He used the enzyme to remove the oxygen from a solution and in the process he

noted that when the glucose oxidase reacted with glucose he could register the removal of oxygen from the solutions using an oxygen electrode (Scheme 1).



Scheme 1

This reaction produced hydrogen peroxide and Dr. Clark knew that if he reversed the polarity on the oxygen electrode the electrode would become a peroxide sensor.[1] Thus one evening, sitting at his kitchen table Lee Clark tried rubbing some glucose oxidase onto a platinum anode wire. He placed the wire together with a silver/silver chloride electrode into a saline solution and using a couple of resistors and a battery he poised the platinum electrode at +0.6 volt. Using a rubicon string galvanometer he was able to observe a current flow when there was glucose present in the solution. This was the first crude biosensor, but it would not function in the presence of blood because the catalase present in the blood destroyed the peroxide. Lee Clark hit upon the idea of using a cellophane membrane to exclude the blood cells and catalase from the electrode, having only a cigarette packet wrapper available he tied a piece over the platinum electrode and was able to demonstrate that he could detect glucose even in the presence of blood. The glucose oxidase adhered tenaciously to the platinum and in the end he was only able to remove it by scrubbing with toothpaste! Here then was the basic membrane electrode from which most commercial biosensors were to develop. This design, however, merely physically trapped the enzyme behind a dialysis membrane. This meant that the enzyme was mobile and somewhat unstable. Furthermore, the membrane not only allowed glucose access to the electrode surface it also allowed other electrochemically active compounds access to the electrode. Thus compounds such as ascorbate, urate and acetomophen (Tylenol/paracetamol) could diffuse into the electrode and were capable of generating a signal which then gave a false glucose value.

It was at this point that the Yellow Springs Instrument company (YSI) began to try to commercialize the membrane technology. Early sensors used glucose oxidase bound in a resin attached to the back of a polycarbonate membrane, such that the enzyme was between the electrode and the membrane. The electrode consisted of a platinum anode and a silver cathode. The electrode was poised at +0.6 volt and thus suffered from the same problem as Clark's first system. In order to overcome this a second electrode covered with the polycarbonate membrane alone, was included in the measurement chamber (Figure 1) in the hope that the interfering substances in the whole blood sample would give a signal at the second electrode and that the glucose content of the blood could be obtained by the difference between the two readings.[2] Unfortunately, it is not possible to obtain two electrodes which give identical current response to the same electroactive compound, even if they have the same surface area. Therefore this approach failed and it became obvious that some method to protect the electrode from these interferents must be found. This led to the invention of an extremely thin cellulose acetate polymer film which was constructed to have a molecular sieve property which only allowed the passage of molecules lower than Mr 300. Hydrogen peroxide could pass through the membrane but larger molecules such as ascorbate could not. A membrane sandwich was developed which consisted of an inner (nearest to the electrode) cellulose acetate membrane, an enzyme layer, and an outer

polycarbonate membrane (Figure 2 and Plate 1). The polycarbonate served two purposes, firstly it provided a barrier to the solids and higher molecular weight proteins in the sample solution. Secondly it acted as a diffusion limiting membrane slowing the rate of glucose transport to the membrane. The enzyme layer was now immobilized between the outer and inner membranes by crosslinking with gluteraldehyde, this also helped glue the two membranes together. The cellulose acetate membrane excluded both interferents and small molecular weight proteins which might otherwise have fouled and passivated the electrode surface.

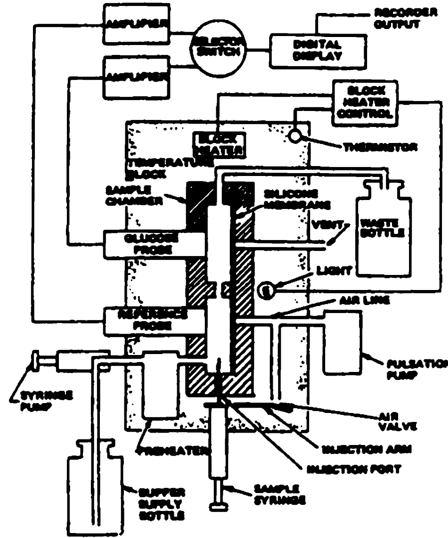


Figure 1 *Dual electrode analyser*

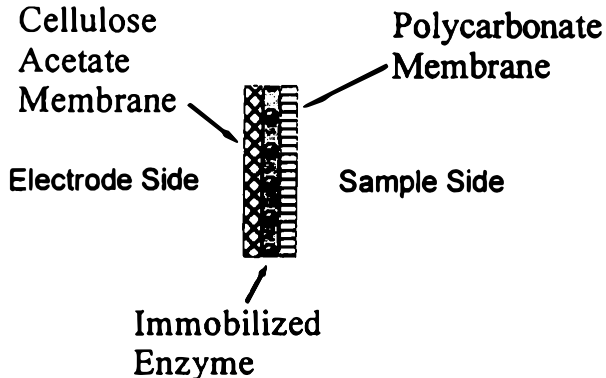


Figure 2 *Trilayer membrane sandwich*

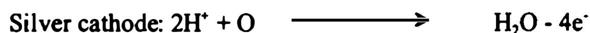
***Plate 1** Electron micrograph of a section through a membrane sandwich. Upper layer: polycarbonate; Middle layer: immobilized enzyme; Lower layer: cellulose acetate.*

2.2 Mechanism of Enzyme Membrane Operation

Figure 3 shows an exploded diagram of the enzyme membrane. The glucose molecules on the outside of the membrane pass through the polycarbonate membrane at a controlled rate and are converted to hydrogen peroxide (Scheme 1). Interferent molecules also pass through this layer but remain unchanged. The peroxide passes through the cellulose acetate membrane to the platinum, silver / silver chloride electrode where it is oxidised to water and oxygen (Schemes 2 and 3). The electron flow causes a current to pass through the electrode and this current is directly proportional to the rate of conversion

Platinum anode: $H_2O_2 \longrightarrow 2H^+ + O + 2e^-$

Scheme 2



Silver cathode: $2H^+ + O \longrightarrow H_2O - 4e^-$

Scheme 3

of the glucose to peroxide in the enzyme layer. Figure 4 shows a typical curve for the current versus time using such a glucose electrode. When the glucose is first injected into the sample chamber there is a rapid increase in current as the hydrogen peroxide builds up at the anode. However, after a few seconds the current reaches a plateau as the rate of peroxide formation in the enzyme layer equals the rate of peroxide destruction at the

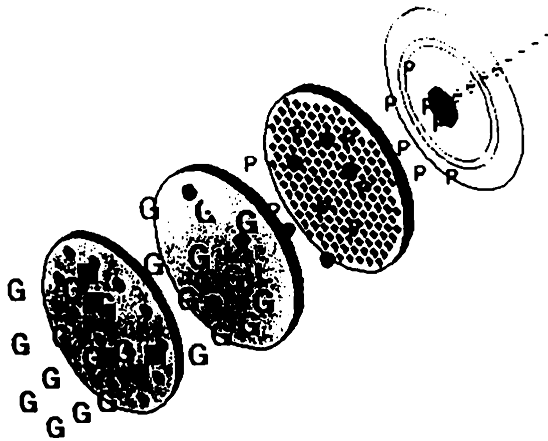


Figure 3 *Mechanism of glucose measurement in a biosensor. G = glucose molecule; P = peroxide, • = interferent molecules.*

electrode. The steady state current is therefore a direct measurement of the amount of glucose in the sample under investigation. Since the rate of glucose conversion to peroxide is governed by the enzyme turnover rate and kinetics, the peroxide electrode is never saturated; therefore these membrane biosensors are controlled by the characteristics of the enzyme layer and the outer diffusion limited membrane.

The use of oxidase enzymes in the membrane biosensors introduces two extra controlling factors to their operation. Firstly, they consume oxygen from the solution containing the analyte. Thus under circumstances where oxygen is limiting in the sample solution, this could lead to erroneous results. In the YSI analyser this is prevented by diluting the sample 1:10 to 1:20 into an oxygen saturated buffer. The second potential controlling factor is the formation of peroxide which is an inhibitor of the oxidase enzyme. There is not a way of completely eradicating this problem, but washing of the membrane after each measurement removes the excess peroxide and restores the enzyme layer to its original activity.

The enzyme membrane biosensor therefore fulfils many of the criteria mentioned above for an ideal analytical process. The sensor is reagentless merely requiring a buffer to allow for dilution of the sample. It requires no sample preparation, specifically recognises one or only a very small number of compounds and produces a direct electrical signal which is easily converted to a concentration of analyte by comparison with a standard solution. This technology forms the basis for all of the measurements made in the YSI analyser line. Table 1 lists the common analytes and the membranes used to measure them together with the enzymes immobilized in the membrane. Of course these are peroxide based systems which therefore confine measurements to an end product which can be measured with an oxidase. This type of biosensor must be modified to allow the use of other enzyme systems in order to broaden the analytes which can be measured using membranes.

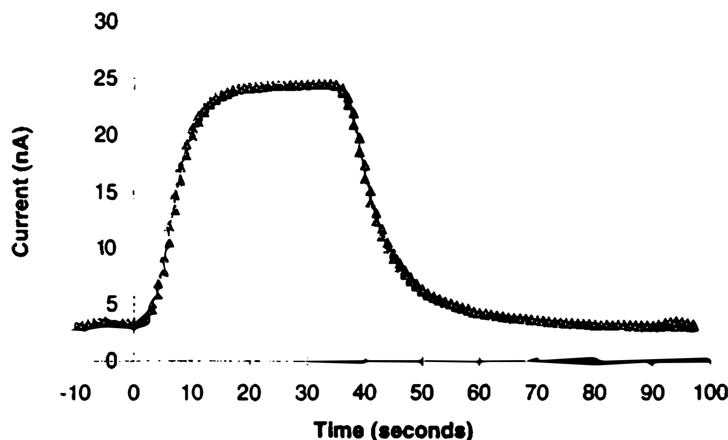


Figure 4 *Current response of the electrode to glucose injection*

Table 1 *Analytes measured by YSI membrane biosensors*

Membrane	Analyte	Sample Type
Glucose oxidase	Glucose	Blood, serum, plasma, dextrose in vegetables, ice- cream, cereals, peanut butter, *etc.*
Glucose oxidase Mutarotase invertase	Sucrose	Vegetables, ice-cream, peanut butter, baked goods, cereal. Effluent monitoring.
Lactate oxidase	Lactate	Blood, plasma, serum, spinal fluid. Lunch meats, cooked foods, *etc.*
Galactose oxidase	Lactose	Cheese, whey.
Alcohol oxidase	Ethanol	Alcohol in beers and wines
Alcohol oxidase	Methanol	Aspartame (pre-treatment with chymotrypsin).
Glutamate oxidase	Glutamate	Monosodium glutamate in food.
Choline oxidase	Choline	Infant formula.

2.3 Practical Construction of Membranes

The construction of a membrane biosensor element is shown in Figure 5. The trilaminate membrane is glued to an 'O' ring which fits into a collar on the platinum silver / silver chloride electrode. The electrode is slightly domed to stretch the membrane when the probe is screwed into its holder. This gives a tight contact between the cellulose acetate backing of the membrane and the surface of the electrode, thus ensuring a fast response to the presence of the analyte. The 'O' ring not only serves as support for the membrane, it also acts as a seal between the probe and the probe holder preventing leakage from the chamber. The contents of the probe chamber are stirred continuously during measurements to ensure its correct mixing and dilution. This creates a biosensor element which is easily replaced and inexpensive to produce, yet long lasting (glucose lasted 14-56 days, lactate 14-28 days, ethanol 7-56 days in use).

The above system is essentially similar for all the membranes produced by YSI. However, significant difficulties can arise when producing some of these membranes. These difficulties lie in the stability of the enzymes used in the membranes. Glucose oxidase is a very stable glycoprotein. It is stable up to 70 °C, and can be crosslinked and thus it can be immobilized with an agressive chemical such as glutaraldehyde. Many other enzymes are less stable and subject to severe inhibition or structural damage during immobilization with glutaraldehyde. In order to make the membrane element of the biosensor a practicable commercial entity, it must be stored and shipped dry. This means that the enzymes must be able to be dried in the membrane sandwich without loss of activity, which is the most difficult part of designing a new element. Unfortunately many enzymes are very unstable and either lose activity during drying or storage over extended periods (months). The stabilisation of enzymes has been attempted using a large number of different chemicals including mono, di, and tris-saccharides, polysaccharides and other polymers such as polyethylene glycol.[3,4,5,6] Few of these compounds have been effective in stabilising enzymes, especially when they are immobilized in thin films. We have studied the stabilisation of a notoriously unstable enzyme, alcohol oxidase. This enzyme has eight subunits and requires all these to be in the correct orientation and active in order to carry out the catalysis of alcohol oxidation. Initial studies indicated that the monosaccharides were poor stabilisers,[7] but disaccharides and polyalcohols were moderately good at stabilising the free enzyme (Figure 6). In the process of purifying the enzyme, however, we also noted that when bound to DEAE-Sepharose, the enzyme could be dried and then reconstituted without loss of activity. Soluble DEAE-dextran was, however, harmful causing complete inactivation of the enzyme (Figure 7).

Further research revealed that a combination of a charged polymer and disaccharide such as lactitol completely stabilised the enzyme (Figure 7). The lactitol/DEAE-dextran stabiliser system effectively stabilised the alcohol oxidase membrane sandwiches, and they could be stored for months in the dry state without loss of activity. Such electrodes have excellent linearity (Figure 8), and can be used for hundreds of assays over periods of 14 to 56 days at room temperature depending on use and sample concentration. By manipulating

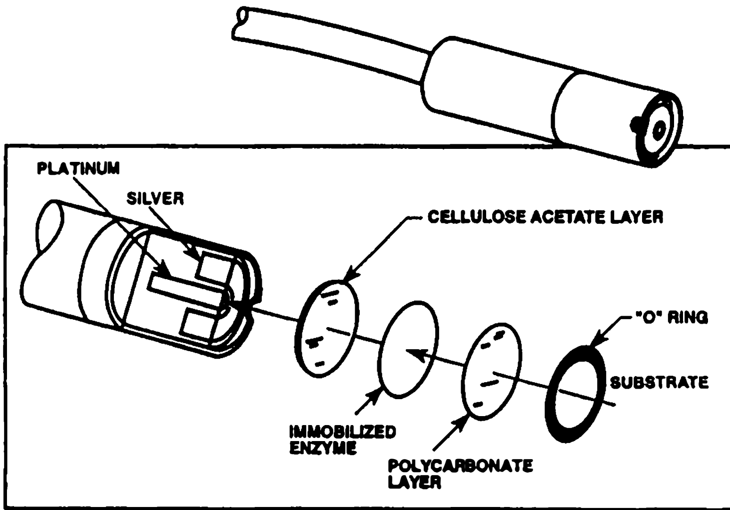


Figure 5 *Exploded diagram of a YSI membrane and electrode*

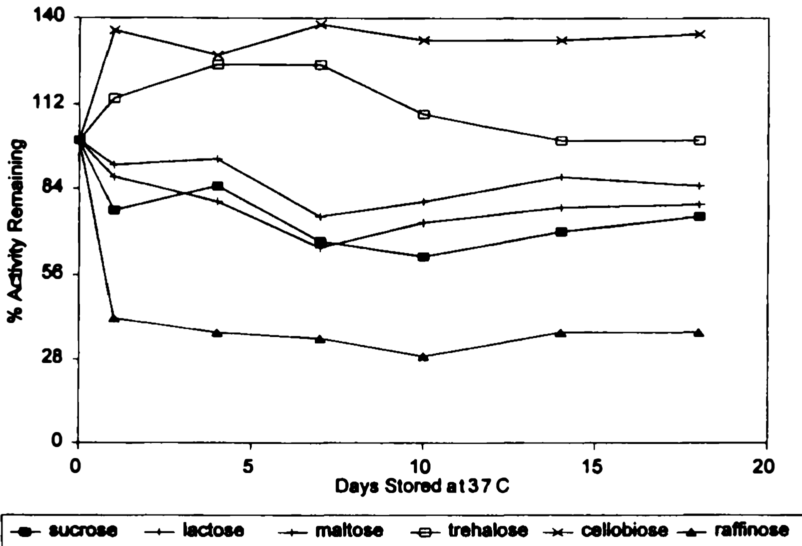


Figure 6 *Stabilisation of alcohol with various disaccharides*

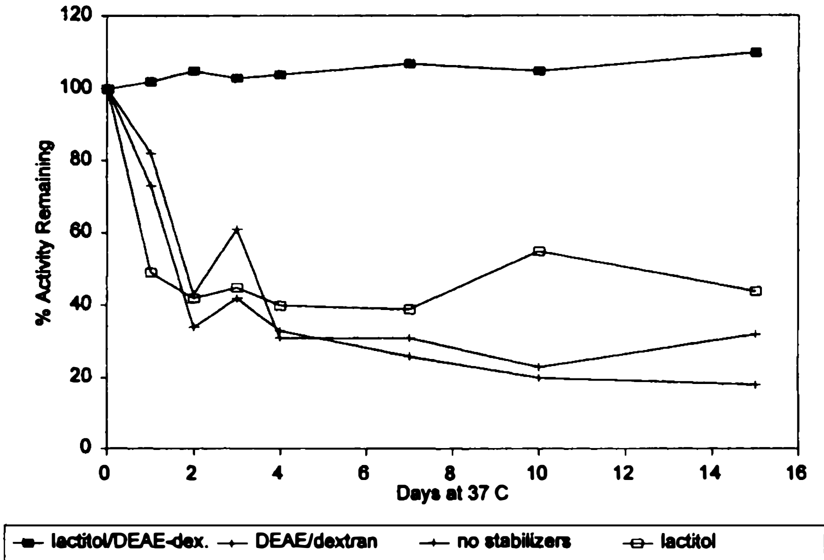


Figure 7 *Stabilisation of alcohol oxidase by various poly- and di- saccharides*

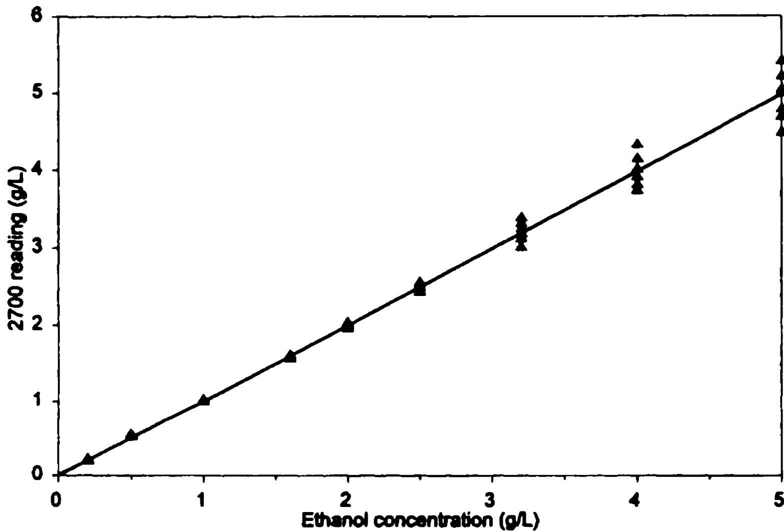


Figure 8 *Linearity of a standard YSI ethanol membrane. A 2 g L^{-1} standard was used to calibrate the system.*

the diffusion limiting characteristics of the external membrane, it is possible to extend the normal range from 0-5 g L^{-1} to 0-60 g L^{-1}, well within the range of alcohol concentration in most beers (Figure 9). Table 2 shows the concentration of various beers analysed using a YSI 2700 analyser equipped with two alcohol membranes. Commercial beers were diluted and injected into a model 2700 analyser equipped with ethanol membranes, and the alcohol concentration was checked using a Sigma Chemical Company kit (322-BT). It can be seen that the results compared well with the spectrophotometric assay.

2.4 Mediated Enzyme Electrodes

The membrane sandwich electrodes described above have some disadvantages, especially due to their dependence on oxygen to form hydrogen peroxide. In this oxygen limited state these electrodes can give inaccurate results. Thus many research groups have sought to develop electrodes which would couple electron flow to or from the active enzyme without using oxygen as a mediator. This technology uses an electron carrier such as ferrocene[8] which shuttles between the enzyme reaction and the electrode. Perhaps the most successful example of this technology is the Medisense glucose electrode which uses glucose oxidase and a 1,1'-dimethylferrocene mediator.[9] This is a disposable electrode aimed at the diabetic home glucose test market. However, not all enzymes can be mediated via an electron carrier. We were unable to find a compound which would mediate electron transfer from alcohol oxidase to an electrode.

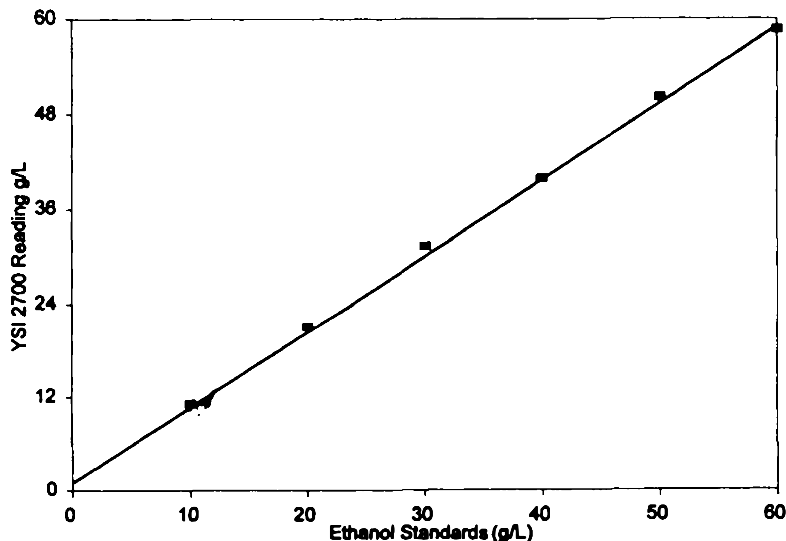


Figure 9 *Linearity of a highly diffusion limited alcohol oxidase membrane. A 50 g L^{-1} standard was used to calibrate the system.*

Table 2 *Alcohol concentration in various beers*

Beer	Alcohol Concentration (% w/v)	
	Enzyme Assay	Ethanol biosensor
1	4.39	4.40
2	3.67	3.55
3	3.57	3.75
4	3.76	3.75
5	3.87	3.89
6	3.79	3.74

In 1982 Kulys[10] described an amperometric method of detection for peroxide based on peroxidase immobilized at a *N*-methylphenazinium-tetracyanoquinodimethane ($NMP^+/TCNQ^-$) coated graphite electrode. We developed a sensor involving the co-immobilization of the highly specific methanol oxidase from *Hansenula polymorpha*, together with horseradish peroxidase. Electrodes consisting of base graphite were layered with 20 µl of a saturated solution of $NMP^+/TCNQ^-$ (prepared by the method of Melby[11]) in acetonitrile. When the solvent had evaporated, these electrodes were placed in a solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate in 0.2 M acetate buffer pH 4.5 for 90 min. They were then washed in carbonate buffer (pH 7.0) containing 250 units ml^{-1} of both peroxidase and methanol oxidase for 60 minutes. The silver / silver chloride counter / reference electrode was prepared using the method of Sawyer and Roberts.[12] Figure 10 is a diagram of the graphite enzyme electrode. These electrodes were placed in a flow injection analysis apparatus and used to test various solutions containing alcohol, the electrode was poised at +100 mV. These electrodes were linear to a maximum concentration of about 16 mM ethanol, although the electrode did not saturate until 120 mM, only the first part of the curve was linear. The optimum temperature for activity of the electrode was 39 °C and optimum pH was 7.3.

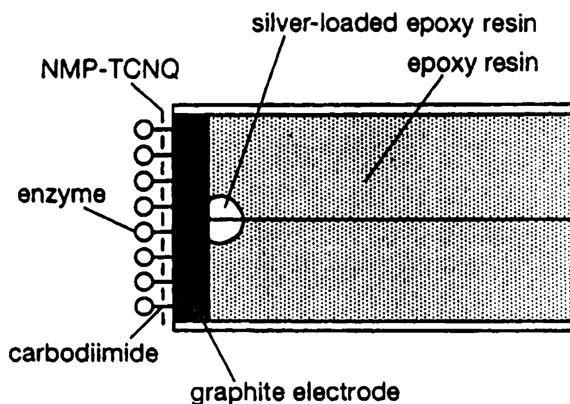


Figure 10 *Construction of a mediated graphite electrode biosensor*

2.5 Electrode Stability

The storage of electrodes prior to use is always a problem, especially when dealing with labile enzymes such as methanol oxidase. Although it was possible to use the electrodes in FIA equipment for 30-50 assays, a more likely scenario would be that these would be disposable electrodes which would be calibrated, used for up to 5 readings and then discarded. Under any circumstances, for electrodes to be commercially useful, they must be capable of storage at ambient temperatures for months or years.

Single electrodes were prepared, then dipped in various solutions of potential stabilisers. The electrodes were then dried under vacuum and stored at 37, 25 or 4 °C over silica gel. Electrodes were taken at intervals and their response to 4 mM ethanol tested as described previously. Electrodes which received no treatment lost sensitivity rapidly, losing 70% of initial activity in three days. Surprisingly, trehalose, a potential stabiliser of proteins, did not effect stability, neither did cellobiose. However, polyhydroxyl alcohols such as inositol and lactilol substantially improved the stability on storage at 37 °C (Figure 11). The best results were obtained using DEAE-dextran/lactilol stabilisers. Initially, a solution containing 1% DEAE-dextran and 5% lactilol was used giving 67% of initial response to alcohol after 35 days storage at 37 °C. 80% response was obtained after the same time for electrodes dipped in a mixture of 0.25% DEAE-dextran and 25% lactilol. A ratio of 10:1 lactilol to DEAE-dextran seemed to give the best results and a mixture of 1% DEAE-dextran/10% lactilol was used in a series of experiments to determine the effect of pH on storage of the electrodes. The best results were obtained at pH 6.0, well below the pH for optimum activity of both enzymes on the electrode.

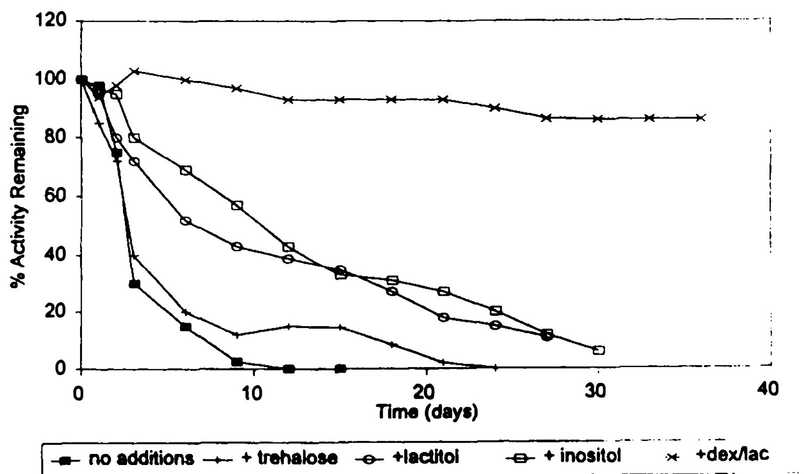


Figure 11 *Mediated alcohol / peroxidase electrode stability. Electrodes were stored dry at 37 °C and then tested with 4 mM ethanol.*

2.6 Stability of Electrodes in the Flow Injection Analysis System

Electrodes stored in the dry state remained active for many months. However, once installed in the flow injection analysis (FIA) flow cell they did lose activity. Multiple assays led to a significant loss in catalytic current, the rate at which this occurred was initially around 1.1% per assay, but after 15 to 20 assays this slowed to around 0.7% per assay. Stripping of the enzyme and or $NMP^+/TCNQ^-$ from the electrode is almost certainly a contributory factor to this loss. During electrode preparation it is likely that much of the enzyme is merely adsorbed to the electrode surface and would be easily displaced by liquid flow. It is interesting to note that at a buffer flow rate of 7 ml min^{-1}, initial loss increased to 1.5% per assay, thus supporting the above theory. Enzyme inactivation probably plays a part in the loss of current, as even when left at room temperature in the carrier buffer, as much as 3% of total activity is lost per hour.

3 BIOSENSORS FOR FOOD ANALYSIS

3.1 Analysis of Commercial Beers and Fermentation Broth

The FIA system was used to assay various commercial beer and larger samples and the results compared with those obtained using gas chromatography. In most instances, the results obtained using the FIA system were between 0 and 10% lower than those observed by gas chromatography (GC). This was almost certainly due to interference from substances other than alcohol in the beer. This was especially noticeable in the low alcohol beer (Table 3).

Table 3 *Ethanol analysis of commerically available beers and lagers*

Beer	Alcohol Concentration (mM)		Standard Deviation
	GC Analysis	FIA Analysis [n=6]	
A	148.37	111.13	3.48
	149.22	96.98	5.29
B	575.28	586.95	13.03
	569.45	563.72	4.99
C	813.01	748.59	12.90
	820.05	738.49	8.10
D	1010.61	937.71	20.49
	1060.35	970.84	9.96
E	723.99	693.03	15.71
F	1 517.8	1 450.71	16.39

When the Brewing Research Foundation (BRF, UK) followed a pilot plant fermentation process using the FIA equipment, a similar negative interference in the reading was noted 20 hours after innoculation of the brew (Table 4). However, subsequent measurements were very close to those obtained using a proprietary instrument developed at the BRF. This illustrates the major problems encountered with mediated electrodes.

Mediation of electron transfer is used to allow lower voltages to be applied to the electrodes. This in turn means that electroactive species such as ascorbic acid will not be oxidised at the electrode surface and therefore the problems seen with interference at +600 mV should be drastically reduced. This is generally the case, however, as can be seen above, the major problem is the fact that even at +100 or +50 mV, interfering compounds can still get to the electrode surface and be oxidised at these low voltages, thus making this form of electrode unsuitable for the measurement of commercial samples of beer (Table 4). Workers such as Heller[13,14] who have developed more sophisticated electron relays from the active site of the enzyme to the electrode, have used anti-interference layers of peroxide to overcome this problem on glucose electrodes. Nevertheless, the use of a bare electrode to allow electrode transfer via mediators presents other problems. Many workers observe that serum will passivate such electrode surfaces in a few minutes and we were unable to use directly mediated glutamate electrodes with anything other than buffered glutamate solutions.

Table 4 *Alcohol formation during a pilot beer fermentation*

Time (hrs)	Specific Gravity	Alcohol Concentration (%v/v)	
		BRF Alcohol meter	FIA System mediated electrode
0	1.04	-	-
20	1.03	1.53	0.06
26	1.02	2.04	2.15
41	1.01	3.03	3.11
49	1.01	3.53	3.24
71	1.01	4.21	4.77
89	1.01	4.17	4.83

3.2 Glutamate Biosensors

Two types of glutamate biosensors were constructed, one using conducting salt NMP-TCNQ mediated graphite electrode (as described above) and the other using the membrane sandwich technique. The enzyme used in both cases was glutamate oxidase. The mediated probe was linear to 10 mM and the membrane electrode was linear to nearly 100 mM glutamate.

The mediated graphite electrode was used mainly for academic studies as it was liable to passivation in commercial samples. The membrane sandwich could be used to measure glutamate in salad dressing, barbeque sauce, and chicken noodle soup (Table 5) and in addition was also applied to the measurement of glutamate in blood and at the blood / brain barrier. Figure 12 shows the removal of glutamate from blood in rabbit immediately after the injection of L-glutamate. We later constructed an electrode (Figure 13) which could be applied to the surface of a rabbit's brain in order to follow the level of glutamate at the brain barrier after injection of glutamate into the rabbit's blood stream (Figure 14).

4 CONCLUSION

The great advantage of membrane sandwich electrodes is that they can be used to measure compounds in very hostile environments which could otherwise coat the electrode surface with polymeric compounds which passivate its response or cause interference. The exclusion of higher molecular weight molecules from the enzyme layer by the polycarbonate membrane, and the exclusion of small molecular weight molecules from the electrode surface by the cellulose acetate layer achieves a practical working electrode. These electrodes are thus protected from adverse conditions by their construction and the use of the membrane makes it simple and practical to replace them once the biological element has degraded. Their limitation is seen in the fact that it is necessary to have an oxidase which recognises the substrate being measured. There are only a limited number of oxidases and this presents a challenge to expand the available analyses. However, this is not impossible; sucrose is measured by co-immobilizing invertase, mutarotase and glucose oxidase. Glutamine can be measured by co-immobilizing glutaminase and glutamate oxidase. We have even co-immobilized chymotrypsin (a proteolytic enzyme) with alcohol oxidase to create an electrode which can quantify the artificial sweetener, aspartame. It is therefore possible to expand the use of this technology to many other analytes. It has the advantage of being simple to use and producing a result in under a minute in most cases. It also requires no sample preparation and is therefore ideal for use in the Food and Beverage Industry where samples often contain solids and need to be measured at the production line or continuously on line. Automation of sample handling and the addition of monitoring stations to these instruments has increased their flexibility thus allowing fermentation monitoring. The use of biosensors to give specific measurement in a production process can reduce process time and increase throughput by quantifying products at the production line. Cost savings can be dramatic as was illustrated when one company changed from measuring choline via a biological method which took 5 days, yielded an answer which was often ±20% of the true value and cost $85 per test. A YSI 2700 equipped with a choline oxidase membrane reduced the assay time to 2 minutes and the cost to around $1-2 per test. Dramatic savings can be made using this kind of technology. The challenge now facing biosensor technologists is to produce electrodes which can be used for 'dipstick-type' analyses. Direct sensing of processes is the next major hurdle to the success of this technology in the food and beverage industry.

Table 5 *Glutamate content of common commercial products*

Type of Food	Monosodium glutamate concentration[a]	
	mM	% w/w
Lite Ranch Dressing	2.53	0.47
Hamburger Seasoning	6.58	5.13
Chicken Noodle Soup	2.79	0.26

[a] Samples were injected directly into a YSI 2700 analyser equipped with glutamate sensors

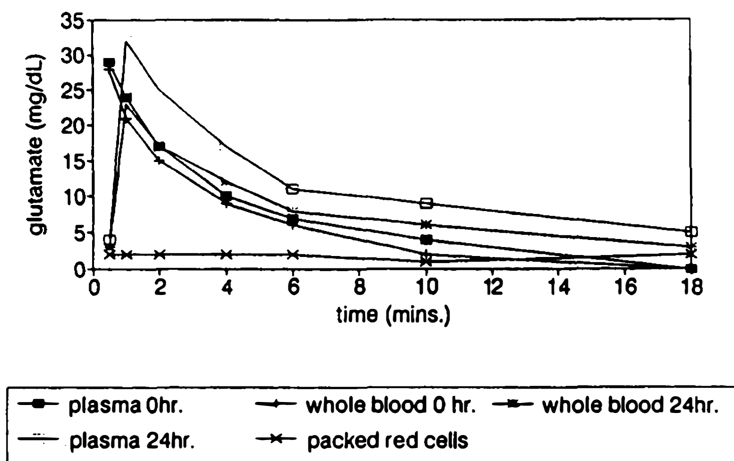


Figure 12 *Glutamate content of blood from a rabbit after intravenous injection. Blood was withdrawn at intervals and injected into a model 27 YSI analyser fitted with a glutamate membrane.*

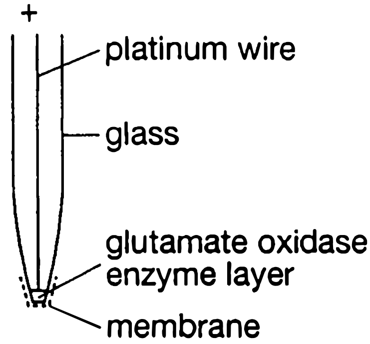


Figure 13 *Construction of a glutamate electrode for use in brain barrier transport studies*

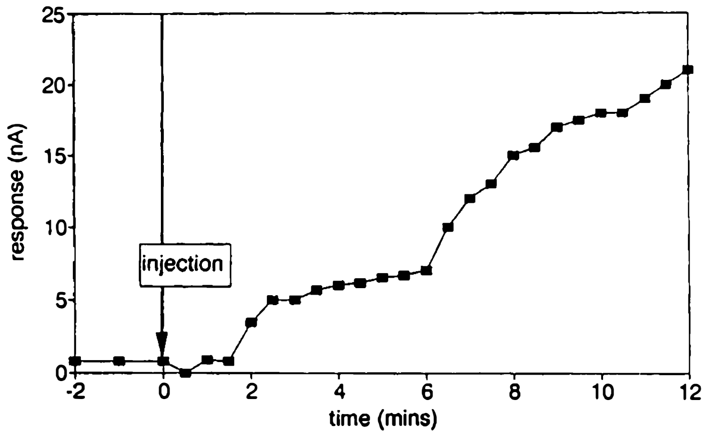


Figure 14 *Electrode response to intravenous injection of glutamate*

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APPLICATION OF BIOSENSORS TO FOOD INDUSTRY REQUIREMENTS

9
An Italian Approach

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1 SUMMARY

Long life milk treated at ultra high temperature (UHT procedure) has been one of the subjects of our research and we developed procedures to control L-lactate in milk for monitoring bacterial fermentation. The possibility of measuring L-lactate in a few seconds directly in whole milk without any conventional preliminary treatment (deproteinization) was demonstrated. However, with some bacteria D-lactate is formed which is not detected by the specific L-lactate biosensor. The research was then extended to develop biosensors for D-lactate exploiting a different pathway of lactate oxidation through D-lactate dehydrogenase instead of the oxidase which was unavailable commercially in this case. This research provides new applications of biosensors whilst eliminating interference through microdialysis and pre-electrolysis procedures.

Another group of metabolites of special interest for food industries are pesticides. Acetylcholinesterase activity is inhibited by phosphoric and carbamic pesticides and the term Total Anticholinesterase Activity (TAA) is being considered for adoption in Italy as a general index for the presence of pesticide in drinking water. New technologies based on Piezoelectric devices for following antigen-antibody reactions directly are considered; this technique can have application by the food industry for measuring low concentrations of specific metabolites such as Atrazine which was measured at ppb level.

2 INTRODUCTION

Food analysis is considered one of the major fields where biosensor technology can be exploited to obtain analytical information in order to evaluate quality, composition and freshness of food. Industries, distributing goods or transforming food using more sophisticated technology, will be more and more aware of the opportunities to exploit biosensors to control food transformation in order to improve product quality. In this paper, we review several lines of research carried out in our laboratory in recent years.

3 DETECTION OF BACTERIAL CONTAMINATION IN STERILE UHT MILK WITH AN L-LACTATE BIOSENSOR.

Monitoring bacterial contamination in milk is especially important in the control of long life milk treated at ultra high temperature (UHT procedure). The current procedure is to incubate milk at an elevated temperature to accelerate fermentation by any active bacteria to check that they have all been inactivated. The progress of fermentation is generally followed by measuring pH. If the pH decreases this indicates bacterial contamination. Measurement of pH is made complicated by the buffering capacity of milk, so very low levels of bacteria may be difficult to detect. An alternative measurement, the determination of L-lactate in milk products, was conveniently carried out using biosensors based on lactate oxidase (EC 1.1.3.2.) from *Pediococcus* species.[1] A flow-injection analysis (FIA) system with an L-lactate biosensor detector was used to monitor the milk fermentation of selected bacteria. This approach was also compared with the commonly used procedure for the measurement of pH.

3.1 Results obtained when monitoring L-lactate during fermentation of milk

Homolactic fermentation was observed when milk was inoculated with *Streptococcus faecalis* (Figure 1A). Milk coagulation began in less than two days after the start of incubation. Similar results were obtained with *Bacillus coagulans* (Figure 1B); however, the fermentation was much slower and the yield of L-lactate was also lower. A rapid milk fermentation occurred with *Enterobacter sakazakii*; this was accompanied by a considerable decrease in pH and the production of gaseous products (CO_2, Figure 1C) which made the identification of this bacteria easy. When the final coagulated fermentation media was analyzed for D-lactate (Table 1), a high concentration was detected and no D-lactate was found in the control experiment milk.

An interesting result was observed in samples inoculated with *Staphylococcus aureus* (Figure 1D). Initially, the pH slowly decreased together with the production of L-lactate which was subsequently metabolized and the pH increased to above the initial value. At the end of the experiment 0.72 mM D-lactate was found, which is very similar to the maximum concentration of L-lactate (0.71 mM) obtained during the course of fermentation. It seems that this bacterial species produces both D- and L-lactate, but only the latter can be further metabolized.

The production of D- and L-lactate depends on the stereospecificity of bacterial lactate dehydrogenases. It was found that L-lactate dehydrogenase is usually dependent on fructose-1,6-diphosphate, which is the key metabolite of the Embden-Meyerhof pathway.[2] However, lactate racemase has been found in some of the *Lactobacillus* species. L-lactate measurement could be a very practical and useful method for the identification of *S. aureus* because the pH changes may not be significant enough for detection using the traditional protocol.

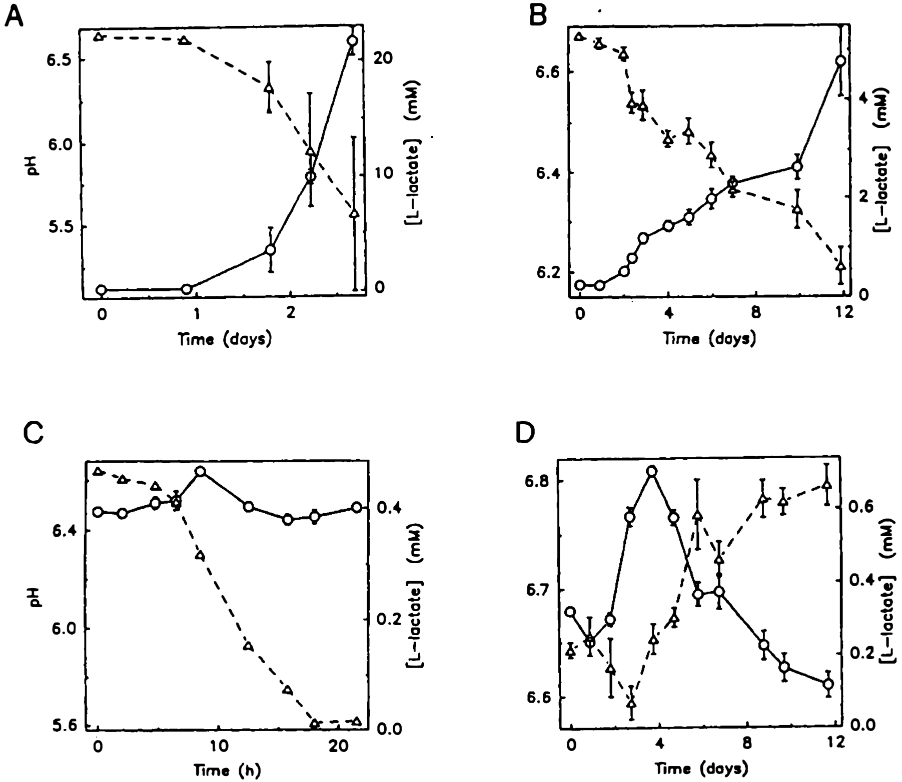

Figure1 *Measurement of pH and L-lactate in UHT milk inoculated with different bacterial strains. (A) Streptococcus faecalis; (B) Bacillus coagulans; (C) Enterobacter sakazakii; (D) Staphylococcus aureus. pH (O); L-lactate (Δ).*

Table 1 *pH, D- and L-Lactate Concentrations Measured after Fermentation*

Innoculum	**Measurement**		
	pH	**L-lactate (mM)**	**D-lactate (mM)**
Control	6.60	0.32	0
Streptococcus faecalis	5.56	21.60	-
Bacillus coagulans	6.21	4.76	-
Enterobacter Sakazakii	5.62	0.40	10.70
Staphylococcus aureus	6.79	0.12	0.72
Bacillus sphericus	6.56	0.31	0

4 DETECTION OF BACTERIAL CONTAMINATION WITH A D- AND L-LACTATE BIOSENSOR

From previous research, summarized above, it was clear that measuring L-lactate could be used for early detection of contamination, but the concomitant determination of L- and D-lactate could prove interesting. D-lactate oxidase is unavailable commercially and therefore D-lactate can only be measured through the use of D-lactate dehydrogenase. Therefore, we developed a suitable procedure to detect L- and D-lactate directly in milk by measuring the reduced form of nicotinamide adenine dinucleotide (NADH) formed at a graphite electrode held at +500 mV with a silver / silver chloride reference electrode. The main problem encountered was due to electrochemical interferences and a suitable procedure for their elimination was devised. Reactions catalyzed by the dehydrogenase enzymes depend on the presence of the NAD^+ coenzyme to produce NADH which is the electrochemically active substance. In addition, direct injection of milk causes rapid fouling of the carbon electrode with high current blank values; we find that these interferences are completely eliminated using microdialysis fiber and a pre-electrolysis cell. Microdialysis is a sampling procedure to filter out all molecules with high molecular weight (proteins, *etc.*) and the pre-electrolysis cell was designed and optimized to eliminate all residual electroactive compounds. This method of L- and D-lactate determination was then used for continuous monitoring of bacterial fermentation in UHT (sterile) milk incubated with *Staphylococcus aureus*. This produces a slight pH variation, however, an early bacterial contamination can be detected using the reported approach measuring L- and D-lactate.

4.1 Flow Injection Analysis System

The scheme of the apparatus is shown in Figure 2. The carrier (0.1 M Tris pH 9 with 0.7 M NaCl) was pumped at constant flow (15 $\mu l\ min^{-1}$) by a peristaltic pump through a microdialysis fiber immersed in the sample. The microdialysis fiber consisted of a hollow fiber (6 cm length) with a tungsten wire (diameter: 0.1 mm) inserted for easy handling. Small molecules such as D- and L-lactate pass through the fiber into the carrier solution which passed into a pre-oxidizing cell where the interference molecules were eliminated. The pre-oxidizing cell consisted of a platinum wire (0.5 mm diameter, 70 mm length) anode inserted in a teflon tube (0.5 mm nominal internal diameter) separated by a dialysis membrane from the cathode (stainless steel) to avoid introducing gas bubbles into the flow system. A 3 V potential difference was applied between the two electrodes through an alkaline battery. The carrier solution was then mixed with a 2 mM NAD^+ tusing a 'T' tube. A three-way valve controls the flow through the L- or D-lactate reactor. Then the carrier solution flows through the wall jet cell equipped with a carbon graphite working electrode to detect NADH produced in the enzyme eactor.

4.2 Results of Bacterial Fermentation of Milk

The inoculum was *Staphylococcus aureus* ATCC 6538p. All fermentations were carried out in half-liter UHT milk boxes. The pH of samples was measured. D- and L-lactate concentration was monitored simultaneously using bioreactors in the flow-system. Figure 3 shows the pH, L-lactate and D-lactate concentrations monitored

during a 6 hour period when UHT sterilized milk (whole, skim, diet) was inoculated with *Staphylococcus aureus*. It can be seen that the pH is almost unaffected by this fermentation, while L- and, particularly, D- lactate shows an impressive and easily detected increase which can be used for quality control.

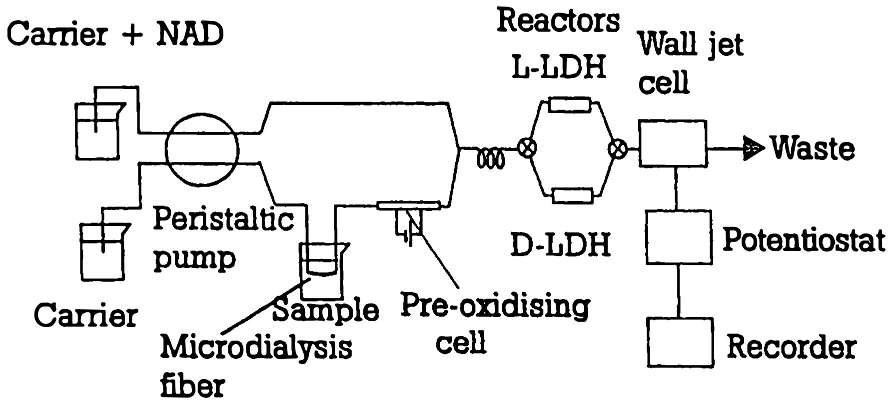

Figure 2 *Diagram of the flow system for the simultaneous measurement of D- and L-lactate. Carrier solution: 0.1 M Tris pH 9, 0.7 M NaCl; NAD^+: 2 mM NAD^+ in carrier; Flow rate: 15 µl min^{-1}.*

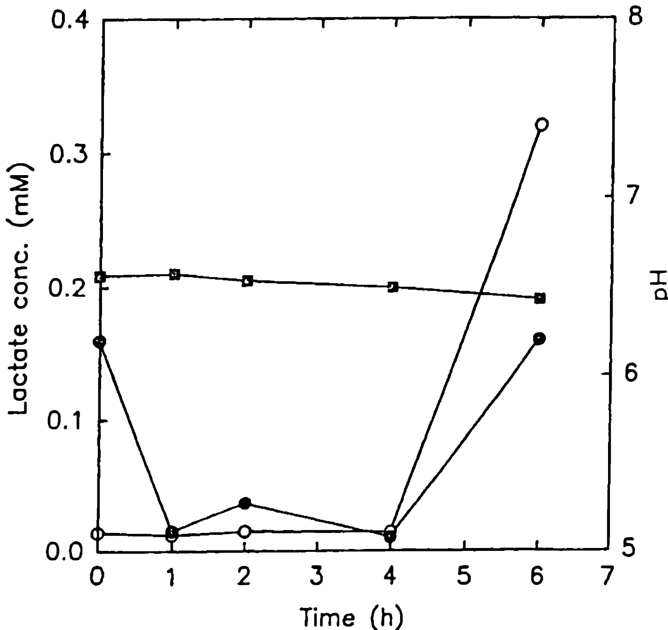

Figure 3 *Recording of pH (■) L- (●) and D- (O)lactate in skim milk (UHT) inoculated with Staphylococcus aureus*

5 TOTAL ANTICHOLINESTERASE ACTIVITY (TAA) AS INDEX OF ENVIRONMENTAL POLLUTION

The measurement of the anticholinesterase activity can be used as a screening test to evaluate the pollution caused by compounds such as organophosphorus pesticides, carbamate derivatives and other compounds which display similar toxicological behaviour. These classes of insecticides have gradually replaced the organochlorines and, although they are characterized by a low environmental persistence, generally they show high acute toxicity. This toxicity has a direct impact on human health because it generates an irreversible inhibition of Cholinesterase (ChE's) which is an enzyme involved in nerve-impulse transmission.

A choline biosensor probe was used to measure the anticholinesterase activity in water samples in order to correlate this parameter with the presence of anticholinesterase compounds such as organophosphorus and carbamate insecticides.[4,5] This measurement is currently used as a 'quality index' and contains biological and chemical information relating to the quality of the sample analyzed. The results from this probe, based upon amperometric measurements, are more sensitive than those based upon potentiometric sensors or modified electrodes.

5.1 Biosensor Assembly

The probe used for the measurement of TAA is constructed using a platinum electrode and poised at +650 mV versus a built in silver-silver chloride reference electrode. It was assembled on an inverted electrode jacket with two membranes: the cellulose acetate membrane and the enzyme membrane. The first membrane is used to protect the platinum electrode from electrochemical interference, mainly from phenols which are widely diffused contaminants present in water. The membranes were secured with an o-ring, the jacket was filled with a 0.1 mol L^{-1} potassium chloride solution, and the electrode was inserted into the jacket to be screwed down until the tip of the platinum was firmly in contact with the membranes.

5.2 Method of Measurement

The reactions are:

$$\text{choline esters} \longrightarrow \text{choline + organic acid}$$

Scheme 1

$$\text{choline} + 2\,O_2 + H_2O \longrightarrow \text{betaine} + 2\,H_2O_2$$

Scheme 2

Reaction 1 is catalyzed by cholinesterase enzymes; reaction 2 is catalyzed by the enzyme choline oxidase. The current output is due to the oxidation of the hydrogen peroxide at the platinum electrode which is correlated to the concentration of choline esters present in

solution.

Pesticide determination was as follows:

Method (1) A solution containing 1 ml distilled water, 1 ml phosphate buffer pH 7.5, 0.2 mol L^{-1} and butyryl cholinesterase (BuChE) 100 mU mL^{-1} was incubated for a fixed time. Then the choline probe was immersed in the solution and allowed to equilibrate until a current baseline was reached; this took less than two minutes. The butyrylcholine was then added to obtain a final concentration of 0.3 mmol L^{-1} and the choline released was measured by the probe for five minutes.

Method (2) In the second step 1 ml sample, 1 ml phosphate buffer and enzyme (both as described above) were incubated for the same time, then the procedure in Method 1 was followed.

Measuring choline production for five minutes was sufficient to obtain a significant current signal. Measurement periods longer than five minutes resulted in a non-linear relation between current and enzyme activity. The calculation of percent of inhibition was calculated according to the formula:

$$I\% = \frac{I_1 - I_2 \times 100}{I_1}$$

where I% is the degree of inhibition. I_1 is the current output measured by method 1 (blank) and I_2 is the current output measured by method 2 (sample). Table 2 shows some results obtained using the biosensor for measurements made on fresh water samples, from several locations in Central Italy, compared with those made using gas chromatography (GC) or high pressure liquid chromatography (HPLC) analysis. Organophosphate pesticides were not detected in any of these samples. TAA was measured in some samples, two of which contained unidentified peaks in the HPLC chromatograms after silica gel fractionation of the water extract.

6 ATRAZINE MEASUREMENT IN WATER WITH A PIEZOELECTRIC QUARTZ CRYSTAL

It is well-known that the resonant frequency of an oscillating piezoelectric crystal can be altered by a change in mass at the crystal surface. The change f in the resonant frequency, f_o, of the crystal may be directly related to the deposited mass m on the surface area A by means of the Sauerbrey equation:[6,7]

$$\Delta f = -2.26 \times 10^{-6} f_o^2 \quad \Delta m/A$$

The piezoelectric crystal consists of a quartz disc with two metal electrodes deposited on both sides. When placed in an electronic oscillator circuit, an oscillating electric field is applied to the crystal and an oscillating mechanical vibration is generated. The rate of this

thickness-shear vibration is a function of the natural resonant frequency of the quartz, which also depends on the mass changes resulting from deposition of substances on the surface of the electrodes. Piezoelectric quartz crystals, therefore, can be used for mass measurements and many gas sensors have been reported. Such crystals have been used for detecting water in gases, organic pollutants, toluene, *etc*. In our research[8] piezoelectric crystals were adapted for the analysis of atrazine by chemically modifying their surface with protein A and a silane derivative with a glutaraldehyde bridge to bind the capturing agent. Competitive assays can be carried out without the need for any complicated separation step.

Table 2 *Analysis of Environmental Water Samples*

Sample	Type of Water Sample	Biosensor Analysis TAA[3] as Paraoxon equivalent (ppb)	GC[1] and HPLC[2] Analysis
Arrone (springs)	small stream	<0.5	no peaks
Arrone (urban area)	small stream	<0.5	no peaks
Arrone (agrigultural area)	small stream	<0.5	no peaks
Arrone (agricultural area)	small stream	1	no peaks
Nemi	lake	1.5	1 un.[4] peak[2]
Bracciano	lake	<0.5	no peaks
Albanolake	lake	<0.5	no peaks
Martignano	lake	<0.5	no peaks
Bolsena	lake	<0.5	no peaks
Aterno	small stream	<0.5	no peaks
S. Gregorio	spring	<0.5	no peaks
Monterosi	lake	<0.5	no peaks
Capodimonte	small stream	<0.5	no peaks
Castiglione	sea	<0.5	no peaks
Tiber (internal harbour)	river	<0.5	2 un. peaks[1]
Tiber (mouth)	river	1	3 un. peaks[1]
Tiber (internal harbour)	river	<0.5	2 un. peaks[1]
Tiber (mouth)	river	1	3 un. peaks[1]

[1] Gas chromatography; [2] High Pressure Liquid Chromatography;
[3] TAA = Total Anticholinesterase Activity; [4] un. = unidentified

6.1 Indirect Assay (competitive)

The Atrazine derivative immobilized on the crystal generated a resonance frequency decrease of 2370±450 Hz (n=10). The binding capacity of the crystal was investigated by immobilizing 1 µg of IgG to the crystal surface which resulted in a resonance frequency decrease of 350±170 Hz (n=8) after 30 minutes of incubation. Figure 4 shows the results obtained with the indirect assay using two different antibody clones. For each side of the crystal 6 µl of atrazine and 4 µl of IgG solution (corresponding to 1 µg IgG per side) were mixed and incubated for 30 minutes. A significant trend was found beginning at 0.1 parts per billion (ppb) to 100 ppb using clone 2; however better results were obtained with clone 1.

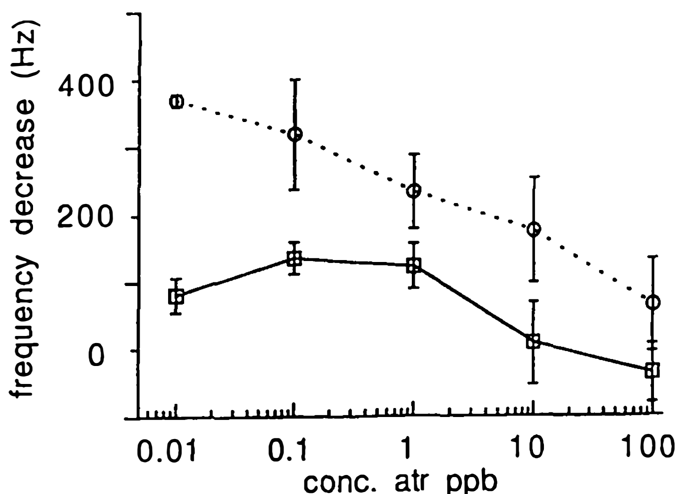

Figure 4 *Response to atrazine solutions in water with the indirect (competitive) assay. The crystals were incubated in the presence of anti-atrazineantibodies (2 µg per side) atrazine (sample). The competition of free and bound atrazine for a limited amount of IgG binding sites occurs, the resulting frequency decrease is indirectly proportional to the concentration of free atrazine; (O) clone 1 and (□) clone 2; the average of frequency of two crystals decreases for each measurement reported.*

The application of the piezoelectric crystal technology for environmental analysis appears to be a promising approach for detection of pesticides in water. Atrazine was investigated in this study as a model system. Direct and indirect (competitive) assays can be used to measure Atrazine, with antibodies and an Atrazine derivative, respectively, immobilized on the surface of the crystal.

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10
A German Approach

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1 INTRODUCTION

Food and fodder products are complex mixtures of chemically diverse compounds therefore highly specific and reliable methods are needed for analysis. In addition to conventional methods such as HPLC and GC, enzymatic assays have found widespread application in food analysis.

Biosensors offer attractive alternatives to existing methods so that monitoring and control of manufacturing processes becomes possible due to improvements in analysis speed and their application on-line. Furthermore, portable biosensors can be used for monitoring products during manufacturing, distribution, and retail.

2 ENZYME SENSORS FOR FOOD ANALYSIS

Most of the enzyme sensors developed for food constituents (for example sugars, amino acids and organic acids) are based on immobilized oxidases in close proximity to Clark-type or modified Clark-type electrodes. The detection is achieved by measuring the oxygen consumed or the hydrogen peroxide produced, respectively (Table 1). Recently, enzyme sensors have been developed for the determination of rapeseed glucosinolates and citrate.

2.1 Biosensor Measurement of Rapeseed Glucosinolates

Glucosinolates, a group of the sulfur-containing heteroglycosides of the plant families Brassicaceae, Resedacea, Caparidaceae and Tropeolaceae, are undesirable anutrients producing goitrogenic and toxic effects in animals. Therefore the control of glucosinolates in food and fodder products which originate from these plants is of great importance. Conventional methods (*e.g.* GC) are time consuming, expensive and require highly skilled analysts.

This biosensor determination of glucosinolates was performed using amperometric enzyme electrodes. After enzymic digestion of the glucosinolates by myrosinase, glucose was determined by glucose oxidase immobilized in close contact to a Clark-type oxygen electrode.

Table 1 *Enzyme Sensors for Application in Food Analysis*

Analyte	Enzyme[1]	Detection Principle[2]	Lifetime (days)	Linear Range (mM)	Application	Ref.
glucose/ sucrose	IN/GOD	amp. oxygen	20	0.2-2.8 0.5 -7.0	instant cocoa	1
glutamate	GLOD	amp. hydrogen peroxide	10	0.001 -1.0	seasonings	2
ethanol	AOD	amp. hydrogen peroxide		0.001 -0.8	beer, wine	3
ethanol	ADH	opt. FIA NADH	28	1.0 - 100.0	beer, baker's yeast culture	4
isocitrate	ICDH/POD	amp. oxygen	1	0.1 -2.0	fermentation broth	5
lactose	β-GAL/POD	amp. hydrogen peroxide	50	0.002 - 3.0	milk	6
putrescine, spermidine, cadaverine	Putrescine -OD	amp. hydrogen peroxide	8	0.03 - 3.0 (μM)	fish freshness determination	7
gluconolactone	GDH/ GOD	amp. oxygen	5	0.02 - 1.0	fermentation broth	8
essential fatty acids	LOX	amp. oxygen	10	0.1 - 1.2	oils	9
acetic acid	AK/ PK/ LDH	opt. FIA NADH	2	10 - 80	vinegar process control	10
butyric acid	*Arthrobacter nicotiniana*	amp. oxygen	7	0.11 - 1.7	milk	11

[1]IN, invertase; LDH, lactate dehydrogenase; GOD, glucose oxidase; GLOD, glutamate oxidase; AOD, alcohol oxidase; ADH, alcohol dehydrogenase; ICDH, isocitrate dehydrogenase; POD, peroxidase; β-GAL, β-Galactosidase; Putrescine-OD, putrescine oxidase; GDH, glucose dehydrogenase; LOX lipoxygenase; AK, acetate kinase; PK, pyruvate kinase.

[2]amp.= amperometric detection; opt.= optical detection; FIA = flow injection analysis; NADH = the reduced form of nictinamide adenine dinucleotide

The isothiocyanates released during digestion are chemically converted into the corresponding thioureas, which are strong inhibitors of the tyrosinase whose activity was measured using catechol as substrate for the enzyme. The oxygen consumption during tyrosinase oxidation of catechol is then measured with a Clark-type oxygen electrode.

However, after enzymic digestion of the glucosinolate progoitrin, the corresponding isothiocyanate spontaneously cyclizes and cannot be converted into the related thiourea. Therefore the content of the progoitrin is not measured by the tyrosinase sensor, but can be estimated from the difference between the glucosinolate concentration measured by the glucose electrode and the thiourea concentration measured by the tyrosinase electrode.

The total glucosinolate concentration found in processed samples of rapeseed meal agreed with those obtained using the reference gas chromatography method.

2.2 Enzyme Sensor for Citrate Measurement

A three enzyme sensor was developed for the determination of citrate. The three enzymes were co-immobilized in gelatin; the enzyme layer being sandwiched between two dialysis membranes and fixed to a modified Clark type electrode.

In the first step citrate lyase converts citrate to oxaloacetate which is then converted into pyruvate and carbon dioxide by the second enzyme, oxaloacetate decarboxylase. The last reaction is to convert pyruvate into acetylphosphate and hydrogen peroxide. The associated oxygen consumption or the production of hydrogen peroxide is measured amperometrically. Preliminary attempts have shown that it is possible to measure citrate in a concentration range of 0.001 - 1 mM.

Enzyme sensors developed in this way can readily be adapted to biosensor based analyzers, which have been developed and commercialized in collaboration with companies such PGW (Medingen), Eppendorf (Hamburg), BST Bios Sensor Technologie (GmbH (Berlin) and EKF (Magdeburg).

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11
A UK Approach

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1 INTRODUCTION

This paper gives an overview of the food and drinks industry operations and the opportunities for biosensors and other chemical sensor systems. The central characteristics of the market and the degree of segmentation is discussed in relation to the materials used, the processes and the products of this major but diverse industry. Sensor application in the food industry must be reviewed in the context of the driving forces; legislative compliance and the search for commercial advantage. In addition, innovation and commercial advantage are key parameters in the food industry supply chain which influence analytical requirements. The opportunities for generic analytical products therefore related to common analytes across several product groups which may operate in different parts of the supply chain.

2 CHARACTERISTICS OF THE FOOD INDUSTRY

2.1 Context

The food and drinks industry deals with a very wide range of materials and products. The range of characteristics of the raw materials and products include: cereal-based materials; meat and fish; sticky jams and preserves; emulsion-based diary and edible oil products; and water-like products such as soft drinks and alcoholic beverages. The processing procedures are correspondingly diverse. They include mixing, blending, fermenting, batch and continuous operations.

Despite its diversity, there are shared themes across the industry:

- the raw materials are organic and usually obtained from living species
- these materials are subject to biodegradation and bacterial contamination
- seasonal and natural variations in supply can cause manufacturing inconsistencies
- there are large requirements for analytical data

The value of the food and drink industry is, not surprisingly, very large. Table 1 gives a breakdown by market sectors for 1991-1992. Alcoholic beverages are notably high, because of excise duty.

Table 1 *Food and Drink Market Sectors by Value 1991-1992*

Product Category	1991 (£m)	1992 (£m)
Alcoholic Beverages	23,378	24,438
Bakerly Products	3,990	4,063
Canned Foods	1,766	1,849
Cereal Products	1,430	1,510
Confectionary	3,965	4,182
Dairy Products	6,206	6,507
Fish and Fish Products	1,939	1,991
Fresh Fruit and Vegetables	5,017	4,843
Frozen Food	2,247	2,234
Hot Beverages	1,313	1,229
Meal Accompaniments	496	856
Meat and Meat Products	9,890	9,898
Oils and Fats	1,060	1,108
Snack Food	1,423	1,427
Soft Drinks	5,844	5,900

Leatherhead FRA: UK Food and Drinks Market 1993

3 TECHNOLOGY APPLICATION STRATEGIES

In this wide area, what is the potential demand for Biosensors? Within the market for analysis in the food industry, three application strategies may be identified:

- substitution of superior services for existing technologies
- generating the means to monitor an existing analyte in a novel environment
- creating an entirely novel measurement

3.1 Substitution of Superior Services for Existing Technologies

From cursory analysis it could be said that biosensor technology could replace many of the measurements now carried out in Quality Control Laboratories. However, there are several forces which make this proposition less likely than may first appear. The principal potential advantages biosensor technology may bring over traditional approaches have been well characterised. They include:

- high specificity conferred by some bio-recognition element, including enzymes.
- reduction in analysis time.
- reduction in skill level of operator.

One of the potential strengths of biosensors over traditional analytical techniques is the cost of obtaining information; the reduction in analysis time and the operator skill level required are of primary importance. However, the cost advantages for replacing traditional techniques in the laboratory may not be as great as a cursory analysis would suggest. This is due to the fact that the analytical approach characterised by biosensors is of a highly specific, narrow nature. Traditionally, biosensor analysis is inflexible but highly specific. This fact has some important consequences for the development of biosensors for the food industry. For example, for any manufacturer of biosensor systems, a large enough potential market must exist to justify development and marketing costs for any single biosensor configuration: any system must be generically applicable. Alternatively a company may seek to combine many specific, inflexible tests in a single system, making the whole package more attractive to potential buyers. In the medical field such an approach has been adopted by I-STAT6, and in the food analysis market by Yellow-Springs Instruments Corp. However, potential buyers would still face the choice between a proven generic system with a specific unproven one.

In all of these cases the new technology will have to compete with existing systems and with alternative approaches. For example, on-line analysis for fat, protein and sugar may be accomplished by FTIR. Several commercial systems of this sort exist and there are precedents for their use commercially by such brand leaders as Coca-Cola Corp.

Biosensor technology will have to offer perceived advantages over existing and competing new technologies in order to win acceptance.

3.2 Generating the Means to Monitor an Existing Analyte in a Novel Environment

One potential area for exploitation is the measurement of analytes in environments where present systems are unable to operate. The limitations imposed on the operating environment of measuring systems are frequently extremes of temperature, pH, viscosity and pressure. The potential for biosensor technology to operate in on-line is frequently severely limited by the process cycle and sometimes by the physical properties of the matrix itself, such as viscosity or hydrophobicity in the case of oils or emulsions. Process cycles involve cleaning regimes consisting of high or low pH conditions, frequently combined with high temperatures. For traditional biosensor systems, containing a heat labile biological element, these conditions represent a considerable challenge. However, there may be opportunities in fermentation industries, where the operating temperatures, pH conditions and viscosity of the matrix are acceptable. Alternatively an in-line configuration may be a more attainable goal than on-line analysis using biosensors; where the sample containing the analyte is brought into contact with the sensor by a sampling system. There may be opportunities for the development of organic phase biosensors for measurements in oils or emulsions, and this field has enjoyed experimental success over recent years.

The development of technically and commercially successful on-line and in-line analytical systems must depend heavily on engineering skills for bringing the sample into contact with the biosensor surface, an area which has perhaps not received as much attention as it merits.

Whilst not constituting a novel environment *per se*, the measurement of analytes in a near line context may be appropriate to consider here. One embodiment of this approach may be a portable, simple to operate, analytical system, using disposable biosensors, and perhaps with data storage facilities. Initially it may be considered that the capability to make measurements in this way would be highly attractive. However, since many of the products of the food industry are solid or semi-solid materials, many of the advantages of biosensor systems in this context may be disregarded because of the need for sample preparation. Clearly the approach needs to be focused on the sample material in addition to the analyte.

This discussion suggests that the development of biosensor systems for the food industry is best focused on drinks and beverages, milk and its products, sauces, fruit and soft vegetables, and perhaps oils. Additionally, there may be opportunities for measurements in process water, effluents and cleaning fluids.

3.3 Creating an Entirely Novel Measurement

The requirement for measuring new analytes may be driven by new legislation, as will be discussed later. This is an incremental function. A completely novel measurement approach, which has generic applications, may open entirely new environments. The BIAcore system, developed by Pharmacia Biosensor AB is an example, and emerging work in machine olfaction may become another.

4 ANALYTES

A classification of biosensor and sensor applications in the food industry is shown in Table 2. The analyte classes are made on the basis of whether the presence or absence of a particular entity is of concern. Generally, as will be shown, in the food industry the absence of certain substances is of great significance. Principally the analytes in this category are associated with the need for legislatory compliance and food safety. It is self evident that the absence of these substances is of commercial importance in an industry where the failure or success of its products is dependent on public perceptions of food safety and quality.

In addition to this classification there is a third category of applications which may be grouped under the heading 'indicators of quality or spoilage'. Here the analytes are principally in the vapour phase, where machine analogues of the human olfactory system may be able to play a valuable role in the future. The indicator analytes may be aldehydes or ketones or complex mixtures of compounds whose presence is associated with malodour by humans.

5 DRIVING FORCES

The principal motivations for chemical testing in the food and drinks industry may be assigned to two influences, the need for legislative compliance and the quest for

commercial advantage. The food and drinks industry is a high volume, low margin industry. At this point we must differentiate between companies whose core business area is the manufacture of food and drink products and those companies who are potential suppliers of analytical instrumentation.

Table 2 *Classification of Biosensor and Sensor Applications in the Food Industry*

Analytes whose presence is important	Analytes whose absence is important
Alcohol	Aflatoxins
Lactic Acid	Herbicides
Vitamins	Ammonia
pH	Higher Alcohols
Sugars	Bacteria
Starch	Nitrite
Flavours	Amines
Total Acidity	Heavy Metals

For food manufacturers the value of obtaining chemical information about a process or a product compared with the perceived or estimated benefits of having that information must show a very favourable cost-benefit ratio. For potential suppliers of analytical systems the questions of absolute market size combined with research, development, manufacturing and marketing costs are paramount. For the application of biosensor systems for the food industry by potential suppliers the inflexibility of a traditional biosensor approach is a barrier to the initiation of development. In the field of home use biosensor products James McCann, former director of Medisense, has estimated that a potential annual market size of at least $40m is required to justify development.[1] This estimate gives some gauge of the cost/market barrier confronting any potential manufacturer of biosensor devices.With regard to legislative compliance, for existing legislation, biosensors of any other new system will only succeed if they offer reduced overheads for information of at least the same quality.

6 COMMERCIAL ADVANTAGE AND LEGISLATIVE COMPLIANCE

The commercial incentive for the use and augmentation of sensing technology within the food industry originates from a number of causes. One of most important is the need for food safety and legislative compliance, the failure to fully control a manufacturing process may result in product withdrawals and damaged brand image.

The principal legislative driving force for the food and drinks industry is associated with the need for microbiological and toxicological food safety. Over a five year period, between 1982 to 1985, five of the most significant food poisoning outbreaks in Canada, the USA and the UK were estimated by the DTI to have cost at least £27m.

The need for rapid microbial testing represents one of the best potential opportunities to apply biosensor technology in the food industry. Besides the defined legislative requirement for testing, there is a recognised need within the industry for quality control and to monitor safety. The technologies under development may be able to combine specificity, conferred by RNA/DNA hybridisation and amplification, with quantification. The associated benefits conferred by rapid detection and enumeration include a reduction of skilled personnel time and therefore an overall potential cost reduction for testing. Besides the reduction of costs associated with food poisoning, an increase in shelf life and reduction in storage overheads may result because of the reduction of the safety certification time from the production plant to point of sale.

Beyond this defensive role played by sensing, a positive contribution may be made by focusing on product quality and the maintenance of profit margins.

In this context the term 'commercial advantage' is intimately associated with a definition of innovation, which is particularly relevant. Innovation may be defined as changes in techniques or procedures which result in the delivery of superior product or service to the consumer. This definition does not use the word technology or mention 'inventions'. This is because inventions in themselves are not innovative, it is their impact which is innovative: an invention may be another way of performing the same function, devised in order to circumvent a competitors' patents. This definition is especially useful when considering the success of an emerging set of technologies, such as biosensor technologies. The new technologies will only succeed in the market place if they can show the potential for demonstrable innovation for the customer.

An examination of the food industry supply chain may be useful here, to focus on the generic opportunities for biosensor technology to contribute to supply chain innovation. A simplified view of the food industry supply chain is shown in Figure 1. Innovation may be sought at any stage in the process. However, the most apparent opportunities for biosensors exist in the first four stages of the supply chain. Process sensing during the manufacture process has already been discussed, and the importance of product quality and assurance is well established, especially with regard to food safety and legislative compliance. The other main potential opportunity may lie in the control of the quality of the natural resources, or raw materials, used by the food industry, and the quality control of the raw materials supplied by the procurer.

From the perspective of the food industry there are issues of defining the quality of the material it buys from a supplier, and of perhaps working with suppliers to optimize the quality of the primary natural resource to the requirements of the industry. These may be two separate entities. Examples may include freshness indicators for fish and meat products, and quality indicators for frozen vegetables, such as vitamin content.

A cursory analysis may indicate opportunities at the point of sale or 'Trade' in the supply chain outline. However, because the relationship between the food industry and the consumer depends heavily on consumer confidence in product quality, any application of freshness indicators at the point of sale is a double-edged sword. One might envisage a packaging system incorporating some indicator of spoilage or freshness for the product.

Such a system may change buying preferences in the consumer for products with short shelf lives, such as dairy products. However, it is unlikely that the consumer would pay a premium for such assurance. Consequently all the development, marketing and manufacturing costs would have to be met by the producer, who would have to be confident that incorporation of a freshness indicator would result in greater market share due to changing consumer preferences. Certainly the economies of scale associated with the volume of the food and drinks industry would favour such a system, especially considering shared development costs through joint-ventures with packaging companies. However, the danger of loss of consumer confidence and product withdrawals for the producer associated with false positives or false negatives makes the incorporation of a freshness indicator a risky proposition.

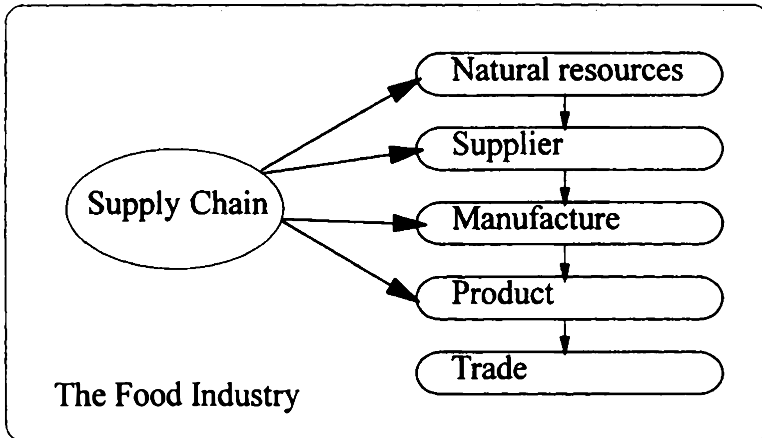

Figure 1 *The Food Industry supply chain*

7 CONCLUSION

This discussion has outlined the major forces shaping the adoption of new testing technology by the food industry. The challenge for any potential manufacturers of biosensor systems for the food industry remain formidable. Potential biosensor producers should select key analytes which are of importance across many points in the supply chain and many product groups in order that the analytical inflexibility of biosensor systems does not impede adoption and to enlarge the accessible market size. Examination of the previous discussion reveals that opportunities may exist for the measurement of sugars, alcohols, rapid microbial detection and perhaps 'aroma' characterisation. The technical and competitive challenges facing the development of biosensor systems with generic application properties should not be underestimated.

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SENSORS BEING DEVELOPED

Enzyme Electrode Sensors for Carbohydrate Analysis

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1 INTRODUCTION

Various modes of electrochemical transducers can be employed in enzyme electrode systems.[1] A convenient way of harnessing oxidase type enzymes is by the amperometric monitoring of the hydrogen peroxide produced in the enzyme-catalyzed reaction with a platinum electrode. For example, for the glucose oxidase catalyzed oxidation of glucose, the platinum electrode is covered with an immobilized enzyme membrane, and the hydrogen peroxide produced is measured by the current output of the cell containing the electrode and linked to glucose concentration. It is best if the enzyme is chemically immobilized; nylon fabric is a particularly useful support matrix for the purpose.[2-6]

The monitoring of the hydrogen peroxide produced by the enzyme-catalyzed reaction, and hence the substrate, is carried out with a potentiostat whereby the indicator amperometric electrode is set at an appropriate voltage with respect to a reference electrode. Setting the electrochemical cell in a flow injection analysis (FIA) system is a convenient arrangement.[2-6] The enzyme electrodes, stored in a refrigerator at 4 °C when not in use, have been found to be still functional after 4 months of intermittent (almost daily) use.[3]

FIA measurements used for measurements with enzyme electrodes allows about 30 measurements to be made in each hour of use. The flow injection approach has the advantage of the carrier stream washing away reaction products after the passage of each injection sample or standard. The linear calibration range of such electrodes is usually <0.001 mM to >1 mM.

2 ENZYME ELECTRODE FOR GLUCOSE

A glucose enzyme electrode has been designed as a model to practicalize the above principles.[3] Thus, in order to prepare the enzyme membrane for covering the indicator platinum electrode, nylon fabric was first activated by treatment with dimethyl sulphate and methanol. Lysine, as a space molecule, was then linked to the activated nylon by immersing the material in a pH 9 solution of the amino acid, and glucose oxidase linked to the lysinated fabric by the agency of glutaraldehyde.[3] The resulting membrane was then

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fitted over the indicator platinum electrode, and assembled along with a silver/silver chloride reference electrode and glassy carbon auxilliary electrode within a three-electrode amperometric modified Stelte micro-cell (Metrohm EA 1102) assembly.[3] Rather than use the commercial Stelte micro-cell version, it is possible to machine a Perspex block to accommodate the electrodes.[7]

The three-electrode amperometric sensing assembly is set up within a FIA system adapted to 500 mm^3 sample sizes (Figure 1), and carbohydrate monitoring (glucose at pH 7 in this case) accords to the electrical current signal given by the hydrogen peroxide produced in the enzyme-catalyzed oxidation of the appropriate sugar. For this purpose, the electrical current produced, when the indicator platinum (enzyme) electrode is set at appropriate voltage with respect to the reference electrode (+600 mV in this case), can be related to the carbohydrate concentration (Figure 2).

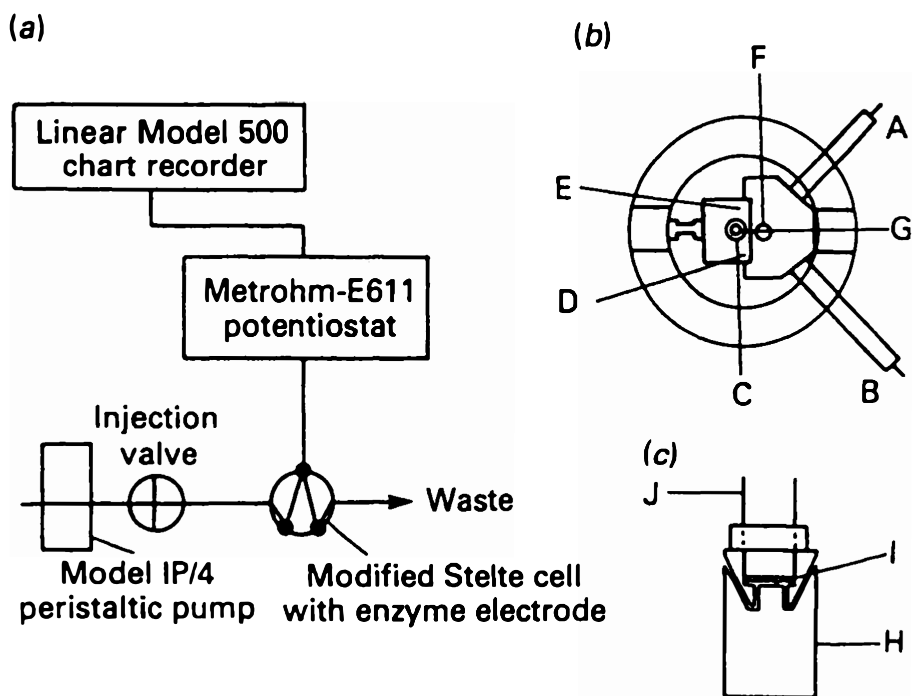


Figure 1 *Amperometric FIA apparatus. FIA apparatus: (a) plan of the modified three-electrode Stelte micro-cell (b) and section through D to E of the micro-cell (c). Key: A, reference silver/silver chloride electrode; B, auxiliary electrode; C, enzyme electrode chamber; D, sample inlet; E, sample outlet; F, reference and auxiliary electrode chamber; G, V-notch on back of Perspex block; H, Perspex block; I, etched channel; and J, enzyme electrode. (from Reference 3).*

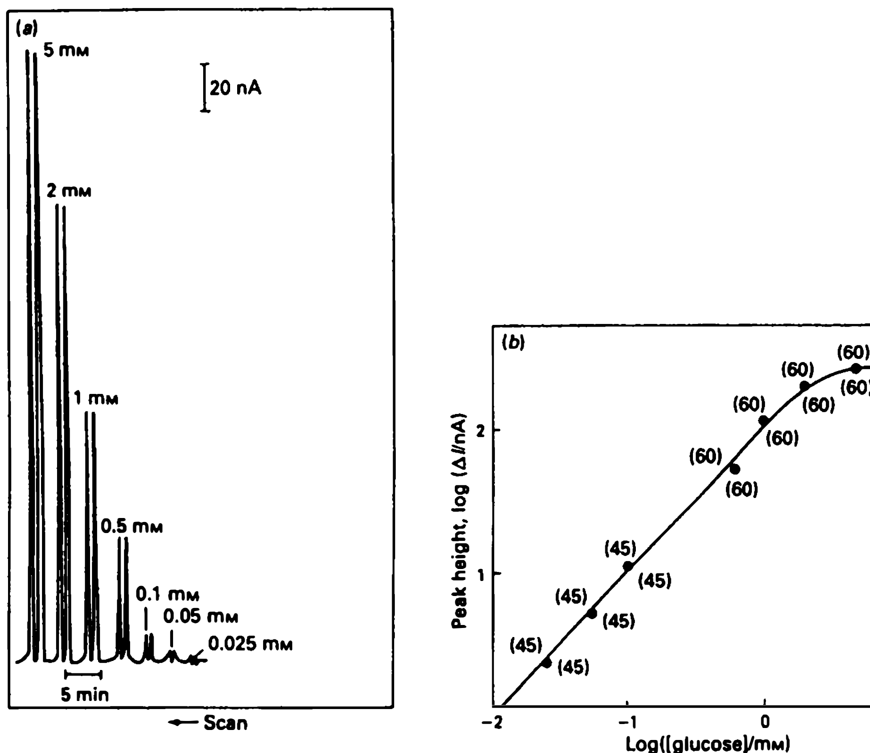


Figure 2 *Typical recorder output. (a) obtained for calibration of glucose with glucose oxidase electrode and calibration plot (b) of data from (a). In (b) numbers above the line indicate response times and those below correspond to wash times. (taken from Reference 3).*

Adding together the response times and wash times corresponding to a peak in Figure 2(a) and a single point in Figure 2(b) shows that it takes just 2 minutes or even less to obtain either a calibration point from a standard or a reading from a sample being analyzed.

The enzyme electrode showed good selectivity towards glucose over galactose, maltose, arabinose, fructose, sucrose, lactose and saccharin.[3] Glucose in foodstuffs was determined by the enzyme electrode,[3] and the results compared favourably with data obtained by other methods (Table 1). For the simplest foodstuffs, sample pre-treatment involved simply leaching with glucose into phosphate buffer by gentle warming (35 °C). Protein-containing samples were treated with 5 cm^3 of potassium hexacyanoferrate(III) (80 mM) and 5 cm^3 of zinc sulphate (250 mM) (Carrez solutions I and II, respectively) in phosphate buffer and diluted to 100 cm^3. After thorough shaking, the filtered solution was analyzed[3] for glucose content (Table 1).

2.1 Overcoming Interference by Ascorbic Acid

In the above case, the hydrogen peroxide product of the enzyme-catalyzed reaction is monitored by the platinum electrode being set at +600 mV versus the silver/silver chloride reference electrode. However, ascorbic acid present in samples causes interference. This can be overcome by lowering the applied potential and using a glucose oxidase/peroxidase bi-enzyme electrode[8] in the presence of hexacyanoferrate(II). This electrode is set at -100 mV versus the silver/silver chloride electrode, since it is the hexacyanoferrate(III) produced by the peroxidase-based catalytic reduction of hydrogen peroxide yielded by the glucose oxidase catalysed reaction that is measured and related to the glucose concentration.[8] This system has been evaluated for glucose in blood serum.[8]

Table 1 *Glucose in Foodstuffs by Soluble Enzyme Kit and Enzyme Electrode Methods*[3]

Sample	Sample pretreatment	Glucose Concentration (%)	
		Kit	Electrode
Strawberry ice cream	Carrez solutions I and II	4.4	4.5
Vanilla ice cream	Carrez solutions I and II	4.7	4.6
Glucose syrup	Dissolve in phosphate buffer (0.1M pH) and warm (35°C)	14.8[a]	13.9
Glucose powder	Dissolve in phosphate buffer (0.1M pH) and warm (35°C)	88.0	88.7
Unrefined syrup	Dissolve in phosphate buffer (0.1M pH) and warm (35°C)	21.4[a]	22.0
Horlicks	Carrez solutions I and II	4.8	3.6
Molasses	Carrez solutions I and II	9.2	9.2
Glucose in flour	Carrez solutions I and II	22.0	21.3

[a] Data obtained by Yellow Springs Instruments Glucose Analyzer

3 MULTIENZYME ELECTRODES

The enzyme immobilized on nylon fabric approach can be used for multienzyme systems.[4,8-10] Apart from the above example of the co-immobilization of glucose oxidase and peroxidase[8] the approach has been exploited for setting up enzyme electrode systems for sucrose,[4] lactose[9] and starch.[10]

3.1 Enzyme Electrode for Sucrose

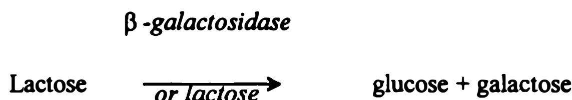
A trienzyme electrode based on a membrane of nylon with immobilized enzymes has been made from a stock of invertase, mutarotase and glucose oxidase in the ratio of 2000:1000:200 enzyme units (where 1 unit = 16.67 nanokatals).[4] Mutarotase is necessary

to ensure α-D-glucose conversion to its β-anomer, such that the glucose oxidase catalysed reaction can function. The system exhibited good linearity (0.001 to 1 mM) towards sucrose, rapid response times (10 to 20 s), robustness (just 6% reduction in signals after >14 h continuous flow of fresh 1 mM sucrose), and good storage stability (38 days, stored in 0.1 M pH 7 phosphate buffer at 4 °C). This electrode, also set up in a modified Stelte cell, was selective to sucrose over various sugars (fructose, arabinose, sorbose, galactose, raffinose, maltose, and lactose), except, of course, for glucose.[4] In any case, glucose in samples may be determined along with sucrose, by setting a glucose oxidase electrode and current monitoring unit upstream of the sucrose/glucose sensitive trienzyme electrode.

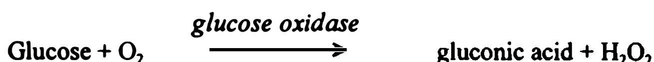
It is only ascorbic acid that interferes from among the organic acids (gluconic, ascorbic, citric, lactic and acetic acids) studied. Again, as mentioned above, the ascorbic acid interference could be overcome by using hexacyanoferrate(II) mediator,[8] such that a lower applied potential (-100 mV rather than +600 mV) can be employed.

3.2 Enzyme Electrode for Lactose

A tetraenzyme membrane electrode, made of galactose oxidase, lactase, mutarotase and glucose oxidase was felt to be appropriate for lactose analysis by the following reactions:



Scheme 1



Scheme 2

Scheme 3

However, the expected two-fold increase in sensitivity from such an electrode, compared to the trienzyme electrode with lactose, mutarotase, glucose oxidase in the ratio of 600:200:200 units where 1 unit = 16.67 nanokatals did not materialise[9]. The trienzyme electrode was superior in terms of response to lactose over the linear range (0.003 to 2 mM). It exhibited rapid response times (15 to 20 s), good temperature stability, a long lifetime (just 5% reduction in signal after 18 h continuous flow of 1 mM lactose), and good storage stability (*ca.* 8 months in 0.1 M, pH 8 phosphate buffer at 4 °C) with intermittent use.[9]

The electrode was selective to lactose over sorbose, fructose, mannose, galactose, maltose, raffinose and sucrose[9]. As for the case of sucrose, discussed above, glucose gave a response and this may be co-determined in samples as described for glucose with sucrose. Likewise, it was only ascorbic acid that interferred from among the organic acids (gluconic, lactic, ascorbic, citric and acetic acids and sodium benzoate) examined.[9]

On the applicability of the trienzyme electrode for lactose, comparable results to those of a soluble enzyme test kit (Boehringer Mannheim spectrophotometric) method were obtained for various processed milks[9] (Table 2).

Prior to the determination of lactose, the samples were pre-treated with Carrez I and Carrez II solutions in order to remove protein. Also, any glucose present in the samples was determined with precalibrated glucose electrode and the glucose found subtracted from the total glucose determined with the trienzyme lactose electrode.[9]

Table 2 *Lactose in processed milks. The enzyme electrode and Boehringer Mannheim test-kit methods were used for analysis*[9]

Food Sample	Lactose Content g per 100 g of sample	
	Enzyme Electrode	Test-kit Method
Fluid skimmed milk (UHT)	5.04	5.31
Pasteurised milk	4.87	4.89
Canned evaporated milk	9.87	10.17
Canned unsweetened condensed milk	8.69	9.13
Dried skimmed milk	27.41	27.64

3.3 Enzyme Electrode for Starch

A trienzyme electrode made from amyloglucosidase, mutarotase, and glucose oxidase in a 2000:100:100 units ratio, where 1 unit = 16.67 nanokatals, is effective for monitoring starch preincubated with α-amylase at room temperature for 1 hour.[10] Preincubation is necessary since α-amylase included in the multienzyme membrane gives electrodes with weak signals.[10]

The system calibrated from 10^{-4} to 0.1%, and was thermally stable over 30 to 70 °C. When pretreated 0.1% starch was pumped over a newly prepared electrode for 60 hours there was no loss of signal, but continuation of the treatment for a further 36 hours led[10] to a fall to 40%. For operation under normal conditions a freshly prepared electrode was checked for its response to 0.1% starch daily for one week and then at 7-day intervals for 4 months.[10] The response was stable for 3 months before denaturation of enzyme occurred, and thence the activity fell to 50% by day 119.

The electrode suffers from interference from glucose and maltose, and the usual interference, noted above, from ascorbic acid; otherwise various sugars (lactose, sucrose, fructose, mannose, raffinose and galactose), organic acids (gluconic, citric, lactic and benzoic acids), amino acids (lysine, arginine, ornithine and glycine) and bovine serum albumin are without effect[10].

Analysis of various starch-containing samples with the electrode gave results[10] comparable to those for the Boehringer Mannheim starch kit test (Table 3).

Table 3 *Analysis of flour samples. The starch enzyme electrode and the Boehringer Mannheim starch kit were used for the analysis. Data in parentheses have been corrected for glucose content.*

Flour sample	Starch Content g per 100 g	
	Starch Enzyme electrode	Boehringer Mannheim kit
Potato	120 (97)	120 (97)
Corn	140 (98)	130 (95)
Wheat	90	88
Rice	80	79
Tapicoca	100	98

4 DESIGN IMPROVEMENTS

The above shows how membranes of immobilized enzymes on nylon fabric, fitted over a platinum electrode included in a three-electrode amperometric cell as sensing unit in a FIA system, gives robust systems for analysing carbohydrates. It endorses other studies on enzyme electrodes, such as a bi-enzyme electrode for starch, obtained by co-immobilizing glucose oxidase with amyloglucosidase on silk, cross linked with glutaraldehyde and bovine serum albumin.[11] In the manner that some systems have been developed into commercial units, it can be reasoned that it is worthwhile for the starch electrode response to glucose and various other polysaccharides (maltose, maltotriose, maltotetrose, maltopentose, and α- and β- cyclodextrin)[10] degradable by amyloglucosidase and α-amylase in the electrode assay system to be adapted to yield microelectrode arrays of enzyme electrodes for the other sample components. However, short of this, the following relates to other pointers towards improved systems.

4.1 Enzyme Immobilization Directly on the Electrode Surface

Enzyme electrode signals are greatly improved by immobilizing the enzyme directly on the electrode surface. Thus, glucose oxidase immobilized onto an anodized platinum wire electrode silanized with 3-aminopropyltri-ethoxysilane, by linking with glutaraldehyde gave a fast responding electrode permitting increased throughput of glucose samples.[7] However, lifetimes were short, as indicated by a lifetime of just 9 hours for continuous exposure to 2.5 and 10 mM glucose.[7]

A more robust system has been obtained by directly immobilizing the glucose oxidase onto platinized platinum wire.[12] This gives greatly enhanced signals, with longer calibration ranges towards glucose (0.005 to 20 mM) compared to other systems where the platinum has been subjected to pretreatments not involving platinization. The lifetime of 15 hours obtained when the electrode was subjected to a continuous flow of 10 mM glucose considerably exceeded the 9 h observed for the anodized platinum enzyme electrode.[12]

4.2 Studies on Spacer Molecules and Coupling Agents for Immobilizing Enzyme

The extensive use of spacer molecules for enzyme immobilization has been reviewed by Hornby and Morris,[13] who reported that without a spacer in the immobilization sequence, the apparent activity of the enzyme was about half that of the enzyme immobilized without spacer. In a study of the role of various amino acids and two aromatic molecules (*p*-phenylenediamine and *m*-phenylenediamine) as spacers, lysine was found to yield the most active enzyme membranes, the full sequence being lysine > arginine > asparagine > *p*-phenylenediamine > ornithine > *m*-phenylenediamine > glutamine > no spacer.[6]

The coupling agents can be significant in enzyme immobilization. Thus, an assessment of *p*-benzoquinone as an alternative to glutaraldehyde for immobilizing glucose oxidase onto nylon fabric has shown that the resulting enzyme electrodes give higher current signals and an increased glucose detection range, 0.001 to 5 mM glucose compared with 0.001 to 2 mM glucose for glutaraldehyde systems.[6] The spacer agent sequence for *p*-benzoquinone coupling agent was the same as that for glutaraldehyde.[6]

4.3 A System for Incorporating an Electron Transfer Agent in Enzyme Electrodes

The use of electron-transfer agents, such as ferrocene and derivatives, has significantly improved the scope and performance of amperometric probes.[14] Studies have been made to improve this system with trapping electron-transfer mediator.[15] Thus, an electrode of carbon powder and mediator bound together with cellulose triacetate, over which was placed a nylon fabric with immobilized glucose oxidase, led to a robust system.[15] The modified electrode itself had a long lifetime of >2 years. Interference by ascorbic acid was minimal when the electrode was used at +160 mV versus a silver/silver chloride reference electrode.[15]

5 CONCLUSION

The chemical immobilization of enzymes on nylon fabric yields stable membranes for assembling into robust enzyme electrodes for monitoring various carbohydrates in foostuffs. Modifications including the immobilization of enzyme(s) directly onto the electrode surface, the use of alternative linking agent(s) and appropriate trapping of electron transfer agent in the electrode matrix can lead to improved systems. Optimization of such systems can be helped by using simplex optimization procedures[15-17] as demonstrated by the ferrocene modified graphite electrode systems.[15,17]

Acknowledgements

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Biosensors and Biosensing for Analysis of Lactate and Malate in Wines with Electrochemical Procedures

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1 SUMMARY

Analysis of lactic acid and malic acid in wines was carried out using biosensor technology and differential pH measurement. Lactic acid was analysed using a hydrogen peroxide based probe assembled employing a polymeric membrane on which the enzyme lactate oxidase had been previously immobilized. This sensor exhibited a high storage and operational stability, and good reproducibility when used in a wine matrix. The sensor was inserted in a flow-through cell and used for continuous lactate measurement. The low detection limit of this probe and the relatively high concentration of lactate in wine samples required a serial dilution in the range 1:100 to 1:200, thus eliminating all potential electrochemical or enzyme interferences present in the sample.

Malic acid was determined using a differential commercially available pH meter and a new enzymic procedure. Experimental parameters such as pH, temperature, co-factor concentration and enzyme immobilization were optimized. Malate was determined in less than 1 minute using a differential pH meter with good reproducibility. Wine samples and wine musts were assayed with the biosensor procedure, using differential pH measurement. The results obtained were compared with those obtained with a spectrophotometric procedure and correlated well.

2 INTRODUCTION

The quality of red and white wines and their organoleptic characteristics are nowadays well defined when considering the effects of malo-lactic fermentation. In fact a careful control of the malo-lactic fermentation appears to be essential in enology since the formation of lactic and malic acids in the wine clearly modifies its chemical composition. If these phenomena occur before the wine is bottled, they can be controlled accordingly.[1]

Electroanalytical techniques, particularly in biosensor and biosensing technology, appear to match the requirements for selective and rapid analysis of lactate and malate in wine.

Analysis of lactate and malate in wine is currently carried out using spectrophotometric procedures.[2,3] Lactate and malate have been determined with enzyme reactors and an oxygen probe.[4] An electrode based on malate dehydrogenase and NADH oxidase has been constructed and used for analysis in wine.[5] These methods are generally fast and accurate, although the procedure is more complicated and expensive.

Our procedure consists of a reagentless determination of lactic acid with a novel procedure based on differential pH measurement for the measurement of malic acid.

These two procedures are suitable for fast and inexpensive analysis of these two metabolites in wine; in real time they also offer the opportunity of having close control of the malo-lactic fermentation in wines.

3 EXPERIMENTAL

3.1 Reagents and Materials

L-lactate oxidase (E.C.1.1.3.2) from 39 units mg^{-1} solid, malic enzyme (L-malate $NADP^+$ oxidoreductase) E.C.1.1.1.40 from chicken liver, suspension in 2.9 mol L^{-1} $(NH_4)_2SO_4$ solution, 30 units mg^{-1}, and $NADP^+$ pre-weighed vials, were from Sigma Chemical Co., St. Lois MO. All other reagents of pure analytical grade were from Sigma and / or Farmitalia C. Erba, Milan, Italy.

Cellulose acetate membrane, with an approximate molecular weight cut-off (MWCO) of 100 was prepared in our laboratory according to a procedure reported in the literature.[6] Dialysis membrane 15,000 (MWCO) was from Spectra/Pore (Spectrum LA CA). Nylon net having 120 threads cm^{-2}, thickness of 100 μm, and 35% free surface, was obtained from A. Bozzone, Appiano Gentile, Italy.

The hydrogen peroxide probe was a platinum electrode 1.6 mm diameter (model M.F.2012) from BAS (Bioanalytical System) West Lafayette, IN, USA. The flow through cell for the hydrogen peroxide probe (a wall-jet cell mod. 656 electrochemical detector) was from Methrom Herisau, Switzerland. The reference electrode was a silver / silver chloride electrode.

The current output was monitored with an ABD electrochemical detector from Universal Sensors Metaire LA, USA and recorded with an AMEL recorder mod. 686. The peristaltic pump was a Minipuls 3 from Gilson Medical Electronic, SA, France. The instrument used for malic acid analysis was a MICRO CL-10 differential pH meter from Eurochem, Ardea, Rome, Italy.

3.2 Procedures

3.2.1 Lactic Acid Analysis Immobilization of lactate oxidase on nylon net has been previously described[7] and is not reported here.

3.2.2 Sensor Assembly The lactate probe was assembled by securing the enzyme membrane together with a dialysis membrane onto the tip of the oxygen probe with an O-ring. The sensor was then a three membrane probe with a cellulose acetate membrane 100 MWCO as the inner protective membrane, the enzyme membrane in the middle and the dialysis as the outer membrane.

3.2.3 Lactate Measurement A buffer solution consisting of a selected phosphate buffer pH 7.0, 0.1 mol L^{-1} was passed through a cell by a peristaltic pump until the current reached an initial steady state. A solution containing the same buffer plus standard lactate concentrations was then passed through whilst the current variation was recorded. Samples of wine were diluted before analysis with the above buffer in the range 1:100 - 1:200. The procedure for both lactate sensors was exactly the same as described above.

3.2.4 Malic Acid Analysis with pH Measurement Analysis of malic acid was carried out using the following procedure: three solutions were prepared, the first was a phosphate buffer 2.5 mmol L^{-1}, pH 7.4, containing 50 mmol L^{-1} KCl and 0.5 g L^{-1} NaN_3; the second was a solution of 0.1 M $NADP^+$ in water; and the third was a 2 g L^{-1} standard solution of malic acid in water. The last two solutions were adjusted to pH 7.0 with NaOH solution to avoid buffer power variations in the working buffer after their addition. The enzyme was prepared by the dilution of 20 IU of malic enzyme (60 µl) with the working buffer to 1ml and located in the appropriate vane of the differential pH meter. This instrument consists of two flow-through glass electrodes connected with a differential potentiometer and a mixing chamber which can be filled and emptied with peristaltic pumps. The introduction of the sample into the mixing chamber is performed manually by a micropipette; all other data acquisition was computer controlled. Measurements were carried out at the end point (the substrate is totally consumed by the enzyme) or at pre-determined time intervals (kinetic measurement by recording a pH variation versus time).

The determination of malic acid was performed as follows: 10 µl of the $NADP^+$ solution and 5 µl of malic acid standard solution or wine sample was injected into the mixing chamber which was previously filled automatically with a known (1 ml) volume of buffer. The solution was mixed, samples of which were then sent to both the pH electrodes. The instrument recorded the intrinsic difference of potential of the two electrodes and zeroed it. In the second step 0.3 U of malic enzyme was added to the remaining solution in the mixing chamber and, after mixing, was sent to one of the two pH electrodes. This electrode recorded a pH variation due to the enzymic reaction which occurred while the other electrode remained at constant pH due to the presence of the blank solution (without the enzyme). A pH variation was then recorded during a fixed time period (50 seconds) with an initial lag phase of 10 seconds.

4 RESULTS AND DISCUSSION

4.1 Lactate Analysis

The enzyme, lactate oxidase catalyses the following reaction:

Scheme 1

According to this reaction, either oxygen consumption or hydrogen peroxide formation can be monitored with selected lactate probes based on oxygen or hydrogen peroxide detection, and current variations can be correlated to the concentration of lactate present in the sample.

Based on results obtained in previous work[8] we selected the hydrogen peroxide electrochemical transducer as the sensor for the analysis of lactate in wine. This electrode was inserted into a flow through cell. All analytical parameters such as pH, temperature, flow rate and response were optimized. Since the immobilized lactate oxidase has its optimum activity in a range of pH 6-8,[6] we selected a phosphate buffer 0.1 mol M^{-1} pH 7.0. The buffering capacity of this buffer concentration was strong enough to keep the enzyme reaction at the optimum pH when wine samples were diluted 1:100 or 1:200 for the analysis.

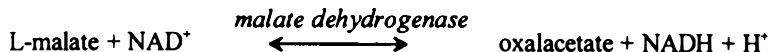
The hydrogen peroxide probe was assembled in a wall jet cell. The working electrode was adapted in this cell by replacing the original carbon electrode provided with the cell. The characteristic of this cell, based on a three electrode configuration and a cell volume of few microlitres, was a fast response time and a fast baseline recovery signal.

We studied the response time and the output current variation at different flow rates by assembling the probe with and without the outer dialysis membrane because it was the first time that a lactate probe based on hydrogen peroxide detection was assembled in a wall-jet cell of this type.

When the probe was used without the outer membrane the current response decreased by increasing the flow rate (Figure 1). When a dialysis membrane was added we observed an almost constant current output at different flow rates (Figure 2). This varying behaviour between two probes was due to the dialysis membrane. In fact, this membrane controls the diffusion of lactate to the enzyme membrane which is not affected by the flow rate variation. The immobilized enzyme acts only on the substrate that diffuses through the dialysis membrane, and the amount of the substrate which diffused is much lower than that which reacts with the enzyme when not protected by the dialysis membrane. This is supported by the fact that the signal obtained for a lactate concentration of 10^{-4} mol L^{-1} was much higher than that obtained with the lactate probe assembled without the dialysis membrane (Figures 1 and 2). A typical calibration plot of lactate determined with a hydrogen peroxide based probe with all parameters optimized is reported in Figure 3.

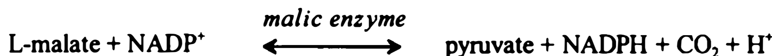
4.2 Analysis of Malic Acid

This compound is usually analysed in wine or wine products using the following reaction:



Scheme 2

This reaction is catalysed by the enzyme malate dehydrogenase (E.C. 1.1.1.37) but the equilibrium favours the side of malate and NAD^+. A trapping reagent for oxalacetate in alkaline medium was used[9] to shift the equilibrium in favour of L-malate oxidation. Another approach was the use of an auxilliary enzyme (GOT) which consumes oxalacetate thereby shifting the equilibrium to the right.[10] We followed a simpler approach which involved the following reaction:



Scheme 3

The equilibrium of this reaction favours the formation of pyruvate at pH 7.4. This reaction produces carbon dioxide and hydrogen ions so a pH variation due to the malic enzyme can be correlated to the malate present in solution. The differential pH meter employed is an advanced multi-parametric automatic analyzer for clinical analysis. The technique has been developed by Luzzana *et al.*[11] and is well documented in the literature.[12,13] The instrument contains two capilliary glass electrodes, a reaction chamber, two groups of peristaltic pumps, a differential amplifier and a microprocessor which controls all the functions of the instrument.

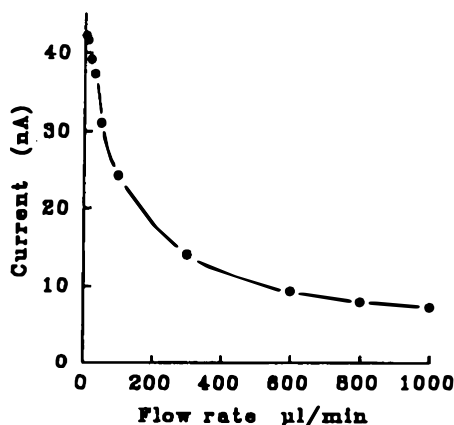


Figure 1 *Lactate probe based on hydrogen peroxide electrode assembled without the dialysis membrane. Response to different flow rates, room temperature, phosphate buffer 0.1 M pH 7.0.*

Since we used a novel procedure, parameters such as the amount of enzyme, $NADP^+$, and working buffer capcity had to be optimized. The buffer capacity we selected (1.2 mM,

corresponding to 2.5 mM of phosphate buffer pH 7.4) was the best compromise to have an appropriate sensitivity using a minimum amount of the enzyme, 0.3 U for each determination. In fact the pH variation is directly correlated to the sensitivity of the measurement; the higher the buffer capacity the higher the amount of the enzyme resulting in the same pH variation. On the other hand, a low buffer capacity could lower the signal-to-noise ratio, so we obtained blank values (measurements without standard or samples) in the range ±0.4 mpH min^{-1}, this was acceptable for our purposes. The effect of $NADP^+$ concentration was investigated and optimized at 0.1 M.

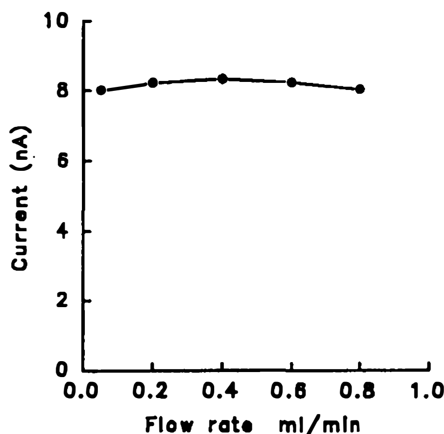


Figure 2 *Lactate probe based on hydrogen peroxide electrode assembled with the outer dialysis membrane. Response to different flow rates, conditions as Figure 1.*

Wine samples were adjusted to neutrality before the analysis to decrease their buffer capacity; samples adjusted in this way did not influence the buffering capacity of the working buffer and results were more reproducible.

Since the instrument operates under computer software control, it can be adapted to any analysis which produces a pH variation. The procedure was therefore adapted for analysis of malate and the instrument automatically measured the malate concentrations.

We found that even better results were obtained using a fixed time measurement. This procedure resulted in more reproducible and much faster signals than those obtained measuring the end point (50 seconds versus 5 minutes). Furthermore, the kinetic measurements are simpler than those performed at the end point. In fact, using the end point measurement the pH variation due to the enzyme itself must be considered and subtracted in an intermediate step of the measurement (a second zeroing stage is necessary

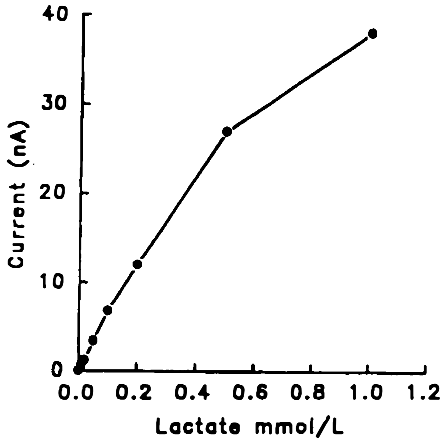


Figure 3 *Calibration curve of lactate using a wall-jet cell assembled with a lactate probe based on hydrogen peroxide electrode. Conditions as Figure 1.*

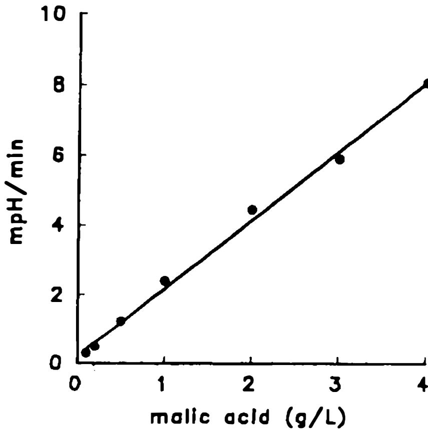


Figure 4 *Calibration curve of malic acid using a differential pH measurement*

as described in the procedure). Using a fixed time procedure with a lag time of 10 seconds the pH variation due to the enzyme is not recorded. The slope (pH/time) measured is independent of the pH variation due to the enzyme the latter being recorded immediately after the enzyme is in contact with the electrode (during the lag time).

Figure 4 shows a calibration curve of malate obtained in the range 0.1-4 g L^{-1} with a linear dynamic range up to 4 g L^{-1} and a detection limit of 0.1 g L^{-1} pH.

4.3 Analysis in Wine

4.3.1 Lactate Acid Analysis. Analysis of 14 wine samples was carried out with the biosensor and spectrophotometric procedures. The results are reported in Table 1, the relative error being in the range 5-10%. Only one sample gave an error of 50% when the two biosensor procedures were compared and an error of 20% when the biosensor procedures were compared with the spectrophotometric method, where the lactate concentration in this sample of wine was the lowest.

Table 1 *Comparative investigation of lactic acid concentrations in wine samples. The results shown are based on a biosensor and a reference spectrophotmetric procedure.*

Sample No	Lactate g L^{-1}		E%
	H_2O_2 probe	Spectrophotometer	B_HS
1	1.22	1.30	6.1
2	1.25	1.18	5.9
3	1.96	1.89	3.7
4	0.18	0.15	20.0
5	0.99	1.01	2.0
6	1.06	1.27	16.5
7	1.86	2.16	13.8
8	0.96	1.08	6.6
9	1.13	1.28	11.7
10	1.42	1.52	6.5
11	1.07	1.18	9.3
12	0.95	0.96	1.0
13	2.37	2.59	8.5
14	1.45	1.62	10.5

Each value is the average of three determinations. B_H = Biosensor based on hydrogen peroxide probe. S = Spectrophotometric procedure.

Table 2 reports the reproducibility of the wine samples as a relative standard deviation of the results for lactate analysis when measured with the two biosensor procedures compared with the spectrophotometric procedure. Results obtained measuring each sample in triplicate show that the biosensor analysis results were more reproducible than those obtained spectrophotometrically.

4.3.2 Malic Acid Analysis. The reproducibility of this method was studied analyzing a malate standard solution and 14 wine samples in triplicate. This results in reproducibilities of 3% for the standard and 5% for wine samples while for the same wine samples the spectrophotometric procedure gave a reproducibility of 8%.

For malic acid analysis recovery studies have been carried out using a pooled sample of red wines in order to minimize any matrix effect which could effect the malate analysis. The results reported in Table 3 showed a recovery in the range 96-106%.

Table 2 *Reproducibility of the hydrogen peroxide probe during the analysis of wine samples. Comparison with the relative standard deviation obtained with the spectrophotmetric procedure.*

Sample No	Relative Standard Deviation (%)	
	H_2O_2 Probe	Spectrophotometer
1	1.6	3.4
2	1.03	4.3
3	0.6	5.4
4	8	6.7
5	0.9	0.6
6	0.7	2.3
7	1.9	4.5
8	0	3.2
9	0	0.4
10	0.7	2.3
11	0	3.2
12	1.4	4.6
13	0	2.7
14	4	9.8

The RSD% was calculated based on triplicate measurements

The recovery of this method has been studied in 4 different musts. Table 4 shows the analysis of malic acid in these musts during the malo-lactic fermentation. The results showed, as expected, a decrease of malic acid concentration over time and when they were compared with results obtained using the spectrophotometric procedure an average error of 8% was obtained. In addition, they showed that lactic and malic acid measurements using electrochemical techniques coupled with enzyme membranes are fast and accurate, and are therefore suitable alternatives to classical methods.

Table 3 *Recovery Studies in a Pool of Red Wines*

Malic Acid Concentration			Recovery
Added g L^{-1}	Expected g L^{-1}	Found g L^{-1}	
-	-	2.4	-
0.5	2.9	3	103.4
1	3.4	3.6	105.8
1.5	3.9	3.9	100
2	4.4	4.3	97.7
2.5	4.9	4.9	100
3	5.4	5.3	98.1
3.5	5.9	5.7	96.6

Samples >4g L^{-1} have been diluted 1:2

Table 4 *Analysis of Malic Acid in Must Samples during Maturation of Grapes. Comparison between spectrophotometric and differential pH measurement procedures.*

No	Sample	Date of Analysis	Malic acid g L^{-1}		E%
			Spectrophotmetric Assay	Electrochemical Assay	
1	Barbera	03/08	18.1	18.8	4
2	Barbera	10/08	10.2	10.2	0
3	Barbera	18/08	5	5.3	6
4	Barbera	24/08	3.8	4.4	16
5	Cabernet	03/08	7.9	8	1
6	Cabernet	10/08	4	3.8	5
7	Cabernet	18/08	2.4	2.6	8
8	Cabernet	24/08	1.8	2	11
9	Riesling	03/08	10.7	10.8	1
10	Riesling	10/08	4.8	5	4
11	Riesling	18/08	2.3	2.6	13
12	Riesling	24/08	1.6	1.9	19
13	Chardonnay	03/08	5.7	6.6	16
14	Chardonnay	10/08	4.9	5.4	10
15	Chardonnay	18/08	2.9	2.9	0
16	Chardonnay	24/08	1.9	2.1	11

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Application of Sensors in the Food Industry: The Role of the TNO Nutrition and Food Research Institute as Systems Designer

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1 INTRODUCTION

The TNO Nutrition and Food Research Institute is involved in sensor research at various levels. Some sensors are newly developed, with or without their corresponding total systems, and existing sensors are being applied to the agro-industrial sector. The work programme involves the development of an integral chain management system for processes and products aided by intelligent sensor systems. To achieve this broad aim, researchers work closely with universities, sensor suppliers, equipment manufacturers and users.

2 SENSOR RESEARCH AND DEVELOPMENT

An example of a newly developed sensor is the 'third-generation glucose sensor,' a biosensor based on enzymes in combination with conductive polymers. This glucose sensor was developed within the framework of C. Koopal's doctoral research (financially supported by the Technology Foundation) in collaboration with Professor R. Nolte, University of Nijmegen.[1] The third-generation glucose sensor is based on the principle of direct electronic interaction between a conductive polymer electrode and a redox enzyme (Figure 1).[2-4] This principle renders the biosensor independent of environmental conditions, such as oxygen tension, which broadens its applicability.[5,6] The third-generation glucose sensor was developed to enable reliable measurements of glucose in complex matrices and processes such as those prevailing in the food and fermentation industries (Figure 2). Furthermore, a feasibility study into on-line measurement of glucose during the fermentation process has been successfully completed.[7] Applications in medicine, aimed at measuring glucose in biological matrices, both *in vitro* and *in vivo*, are being developed. Mass production of third-generation glucose sensors would facilitate integration of this promising biosensor in total systems and its large-scale implementation in the process of glucose measurements. The feasibility of mass production is now being studied.[8] The biosensor principle can also be applied to specific measurements of other chemical and biochemical compounds such as lactic acid. A patent has now been obtained covering the principle of these biosensors.[9]

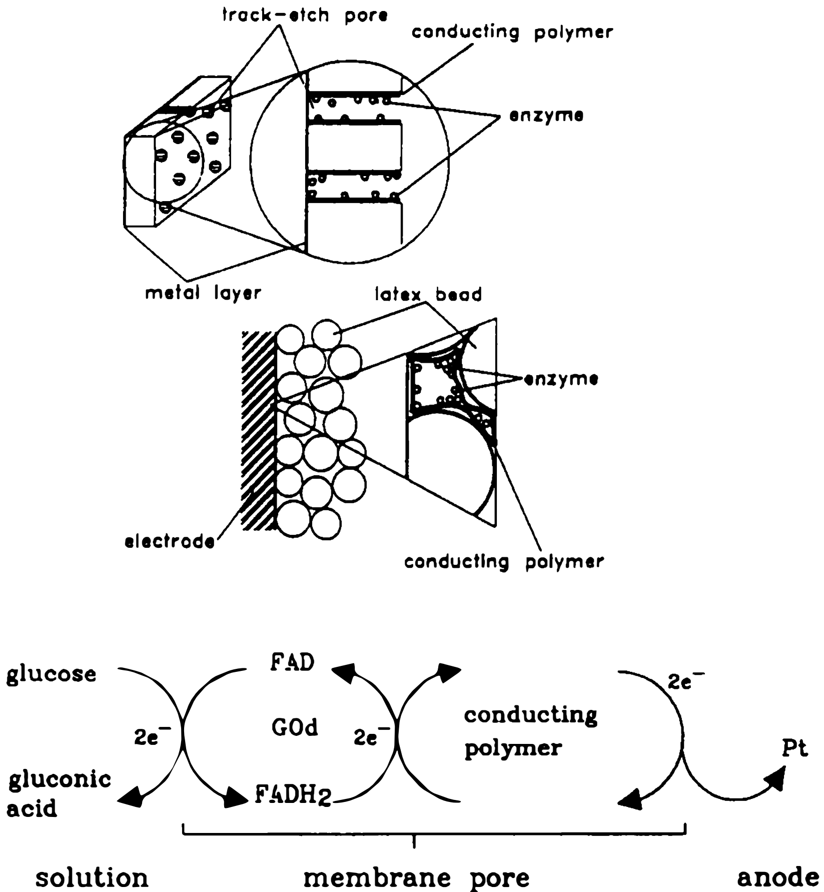

Figure 1 *Schematic drawing of the working principle of the third-generation glucose sensor*

Integration of (bio)sensors in widely applicable measuring systems has the advantage that the development of a sensor can be readily converted to a given need. In many cases flow injection analysis (FIA) provides a solution to the problems which prevent the sensors being directly introduced into existing processes. The FIA system acts as an interface between the complex and highly contaminating process flow and the sensitive chemical sensor. In such a measuring system pre-treatment of samples can take place automatically and without any significant loss of time. Moreover, reagents (such as enzymes or antibodies) can be added to the sample flow, if necessary, and the sensor can subsequently analyse the sample for a given parameter in a measuring time of approximately 1 minute. Such a system can be calibrated automatically on a regular basis with standards and blanks, both being measured at the same time points. Analyses usually carried out in the laboratory can be performed on-line in the production process with a proper combination of FIA and sensor.[10] Some examples of integration of sensors into FIA systems are given below.

2.1 Glucose measurements in bioprocesses

There is a world-wide interest in culturing cells using artificial conditions and the monitoring of glucose concentrations is essential for such processes. C. Koopal is developing another glucose sensor for bioprocesses to satisfy objectives of several projects.

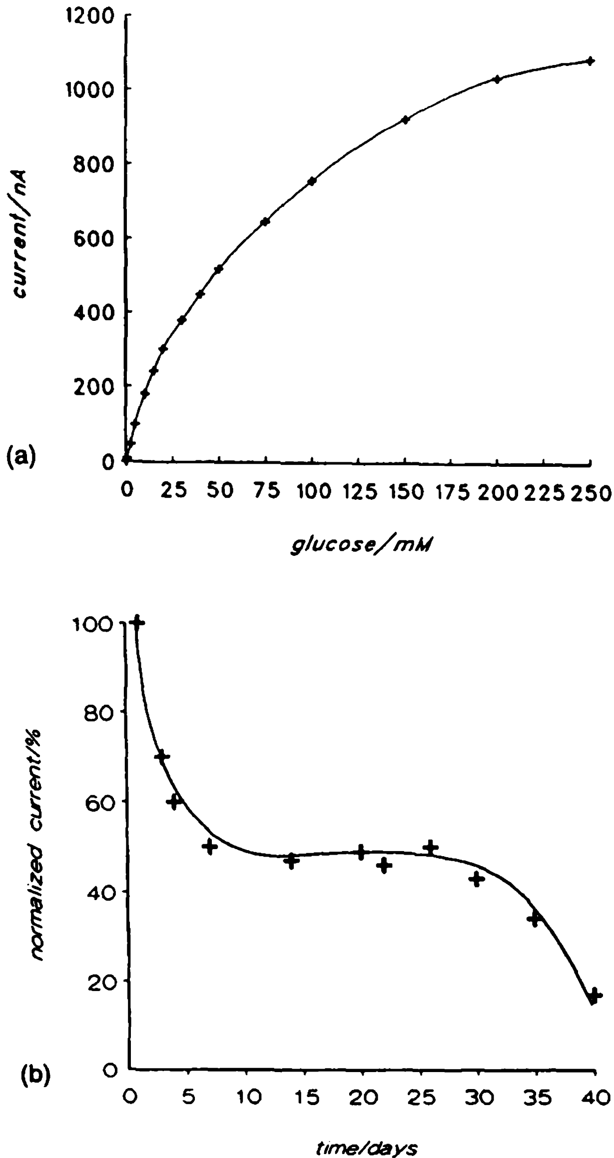

Figure 2 *The flow-injection analysis glucose sensor performance. (a) the dynamic range and (b) the life time under continuous-use conditions (non-optimised sensor)*

This development is based on integration of the third-generation glucose sensor in a FIA system (Figure 2).[8,11] Compatibility with sterilisation procedures, long-term stability, accuracy and the requirement for a high degree of automation are important factors in the use of a glucose measuring system in the complex matrices found in a fermenter. The combination of the biosensor and the FIA system meets these high specifications.

2.2 Immunosensors

In a project co-financed by the European Union, R. Schasfoort is integrating surface plasmon resonance (SPR) immunosensors into an FIA system for the on-line detection of African swine fever (hog cholera - Figure 3).[12] SPR sensors are used for real-time measurements of antibody-antigen interactions and have the advantage of being able to measure selectively, sensitively (low detection threshold level) and rapidly (real-time immunochemical interaction). Figure 4 illustrates the principle and the detection of a hormone. The African swine fever project is a collaboration between European partners; GBF (Brunswick), the Fraunhofer Institute (FRG), IBED and the Gulbenkian Institute (Oeiras, Portugal). Another project with the objective of monitoring on-line surface water for the presence of pesticides in which SPR immunosensors are combined with FIA has been started; this is a joint venture with the universities of Leiden, Lund, Barcelona and Alacala.

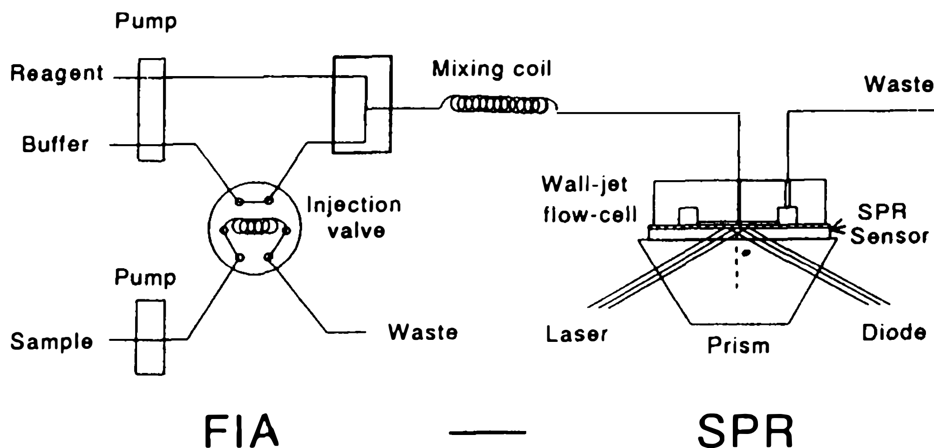

Figure 3 *Schematic drawing of a flow-injection analysis system in combination with an SPR immunosensor*

3 INTEGRATION OF EXISTING SENSORS IN THE FOOD INDUSTRY

Commercially available sensors and sensor systems are usually not directly applicable in the food sector. The complex biological matrices of food products hinder on-line measuring of specific parameters. Research activities have been undertaken aimed at making existing sensors able to make measurments in complex food matrices.

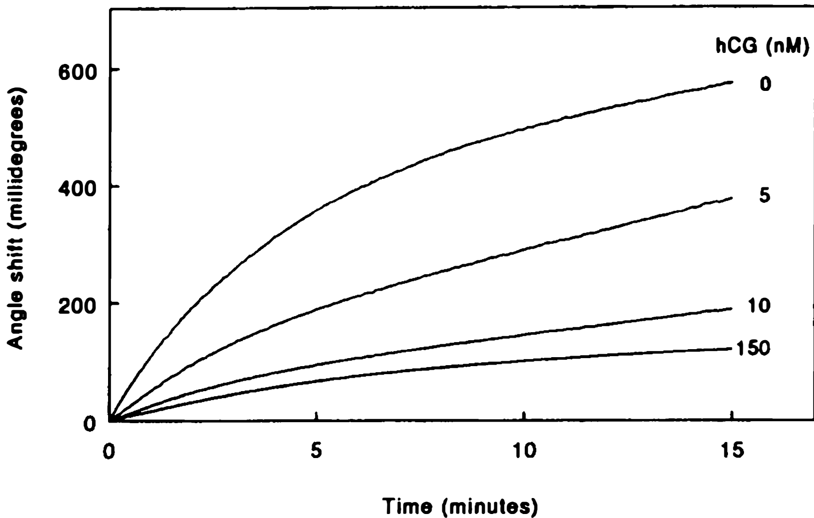

Figure 4 *Determination of the concentration of human chorionic gonadotropic hormone (HCG) by means of an inhibition test with the SPR immunosensor*

There is a tendency in the food industry towards a shift from subjective to objective quality control. A most promising sensor in this area is the 'electronic nose'. Research is being carried out to collect and evaluate all the information and technology required to explore the applicability of commercially available 'electronic noses'. A possible application of the 'electronic nose' to detect 'boar smell' in swine carcasses is now being investigated.

3.1 Stability sensor for meat products

Many factors influence the quality of the final product during the preparation of sausage meat, including gel and fat deposit,[13] the cuttering process (cutting and mixing of meat base materials and additives) is of particular importance in this respect. The process must be stopped at the correct time to avoid quality deviations. The exact moment to stop the process depends on the nature and composition of the raw materials and is the moment when a maximum binding of the sausage batter has been achieved. Until now this right moment has been identified on the basis of experience. However, an objective measurement should be developed to improve quality control. A research project was started to gain insight into the predictive value of the stability of base materials to choose factors to measure in an objective manner during the process. Subsequently, relevant process parameters were modelled in order to define a useful sensor system.[14,15] An in-line sensor was then developed, in collaboration with a sensor manufacturer, consisting of a light reflection meter, a conductivity meter and a thermometer. The sensor system will probably be suitable for determining the right moment for stopping the cuttering process. The result will be an optimal heat stability of sausage batter. The sensor is now being tested in collaboration with some companies for its applicability to various meat products.

3.2 Smoke sensor

R. van Dijk has developed a system for measuring the smoke density reliably under extreme conditions (tar, smoke deposit, high relative humidity and temperature). This measuring system is based on a commercially available optical sensor.[16] The system is now being implemented in collaboration with a German supplier of smoking cabinets (Figure 5)[17] and a European patent for this sensor has been obtained.[18]

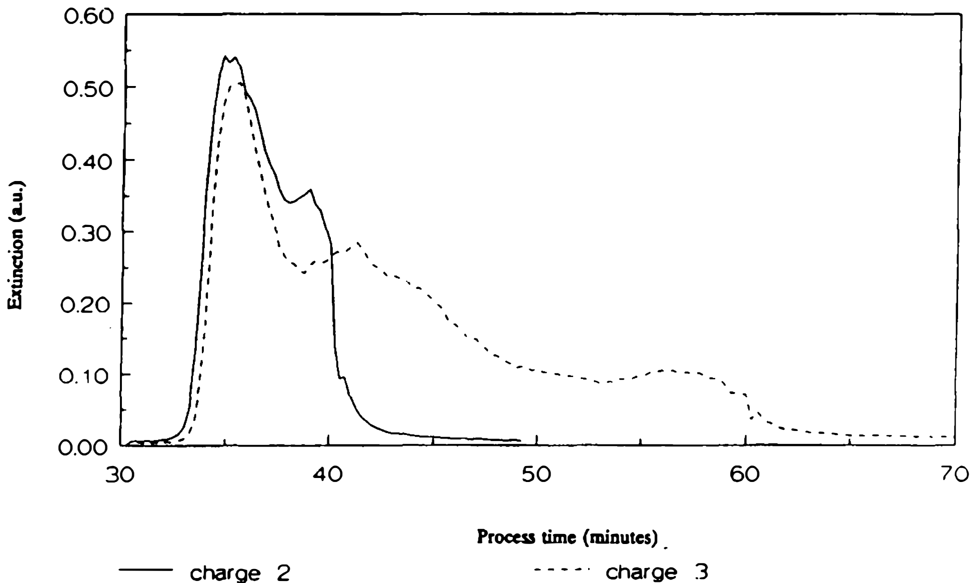

Figure 5 *Output signal of the smoke sensor expressed as the extinction value as a function of process time*

3.3 Ultrasonic sensor

Another example of the application of existing sensors in the food industry is the ultrasonic sensor which is used in many areas including the oil and fat industries.[19] The degree of crystallisation is an important quality criterion for the margarine industry and product quality until now has been usually assessed by operators on a subjective basis. R. Janssens is developing a system for in-line control of the degree of crystallisation (liquid/solid ratio) in fat-containing products such as (low-fat) margarine (Figure 6). The in-line measuring system is based on a commercially available sensor. The use of sensor

data, in combination with other process parameters, in the assessment of products objectively is now being investigated in collaboration with a group of users.

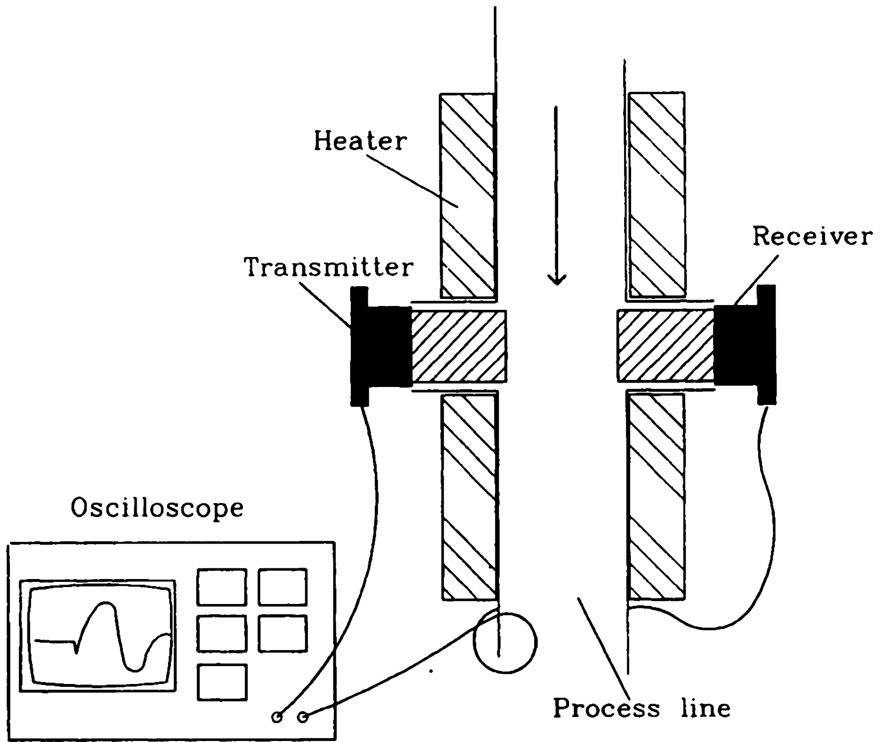

Figure 6 *Schematic drawing of the set-up of an in-line ultrasonic sensor in a process flow*

4 PERSPECTIVES

Intelligent sensor systems will play a major role in the future quality management of food products. In addition to measurements on a process level and quality assurance of food products, integral chain management is in sight. Measuring of relevant parameters in a product or process chain at the right place and at the right time is exactly what intelligent sensor systems are able to offer. Blood is an example of this discussed below. Two other related subjects on quality management, antibiotic measurement and predictive modelling, are also discussed in this final section.

4.1 Blood

Blood is an important source of information, in particular, with respect to an animal's health. The health status of pigs could be established at the breeder's farm or in the

slaughterhouse on the basis of blood analysis for specific biochemical and clinical variables.[20,21] Haematological and clinico-chemical parameters could provide an indication of the animal's health status and hence of stress and meat quality. The erythrocyte sedimentation rate is indicative of the presence of abscesses in the carcass. Enzyme systems provide information on the presence of stress prior to slaughtering, and some proteins are markers of the animal's global health status. The information obtained can be used for decisions for further processing of the meat or for feedback purposes (to adjust breeding and feeding procedures). Research projects in progress are now studying, in collaboration with Utrecht University, the possibilities of monitoring the health status of slaughter animals by analysing blood sampled at random in the slaughter line.

4.2 Antibiotic test

Antibiotics are currently detected according to the EG 4-plates or similar techniques. Results of such agar diffusion tests can be obtained after 13 to 18 hours of incubation. A technique based on ATP-bioluminescence has been developed to provide the results after no more then three hours and the sensitivity of the test is comparable to the new Dutch kidney test, a modified 1-plate agar diffusion test. The ATP/antibiotic test is now being automated in co-operation with some suppliers of ATP measurement equipment. The ATP-antibiotic test makes it possible to screen many samples for the presence of antibiotics in an automated fashion. Antibiotics can be detected in animals for slaughter even before they are transported using this monitoring method.

4.3 Predictive modelling

Shelf-life of meat and meat products is often determined by time-consuming storage quality tests or empirical findings. These methods are rather specific and far from accurate. For several years, therefore, there has been an increasing interest in the development of mathematical models enabling calculation of the growth rate of micro-organisms under different circumstances. Some suitable models are already available, such as the Ratkowski model and the Schoolfield model. When the initial microbial contamination of a product, its storage temperature and other shelf-life determining factors, such as water activity, pH and preservatives content are known, the shelf-life can be predicted accurately by using these models. Furthermore, these models offer the possibility to determine the effect of changes in composition or storage conditions of the product on stability and safety without the need for extensive testing. Also, research related to the shelf-life of new products during development can be simplified considerably. Finally, predictive modelling can contribute to tracing and eliminating weak spots for example in the cooling chain to obtain maximum shelf-life qualities of food products.

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15

A Stop-flow Sequential Injection Analysis Using Immobilized D-Lactate Dehydrogenase for On-line D-Lactic Acid Monitoring during a Fermentation Process

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1 INTRODUCTION

Lactic acid exists in two isometric forms; the L- and D-form. L-lactic acid is produced by several microorganisms and in animal tissues; however, some microorganisms are also capable of producing the D-isomer. There are several reasons for the analysis of lactate including: in dental care,[1] in food science and in bioprocessing *(e.g.* fermentation),[2] and in plastic manufacture.[3] In the food industry, lactic acid is essential for the manufacture of cheese, pickles, yoghurt, fermented meat products and butter milk.[4] Furthermore, D-lactic acid is also an important freshness indicator for vacuum-packed chilled meat.[5] Biotechnological production of lactate covers approximately 50% of the needs today. Fermentation processes are characterized by product inhibition and recovery problems which combine to influence the economics of the process negatively.[4] Several efforts to improve the speed of fermentation have been presented,[6,7] to increase the volumetric productivity and to improve the yields. On-line monitoring of the fermentation process is a step towards a better process monitoring and control and, in a larger perspective, towards a more economic production process.

Methods to determine the D-lactic acid are mainly based on the use of D-specific lactate dehydrogenase (D-LDH) in flow injection analysis (FIA) systems combined with a bioluminescence detector[8] or sequential injection analysis (SIA) system with a spectrophotometer as detector,[9] or by high performance liquid chromatography (HPLC).[10] No method has been presented for the quantitation of D-lactic acid directly without chemical conversion.

A few methods have been published for on-line monitoring of L-lactic acid production during fermentation based on the use of lactate oxidase in a flow injection system.[11,12] For D-lactate to be monitored, lactate oxidase is not a suitable enzyme due to the lack of specificity for the D-isomer. The co-factor dependent D-LDH therefore becomes the choice. When the traditional FIA system was applied with immobilized D-LDH, the costly cofactor, had to be supplied continuously and this high consumption became a main drawback for this application. Recently, a simple system was demonstrated using a SIA system integrated with a reactor with immobilized D-LDH.[9] The consumption of cofactor could be reduced substantially by providing it in a sequentially segemented

mode of operation. The principle of the SIA system was first presented by Ruzicka *et al.*[13] and has subsequently been reviewed.[14] In present work, a similar automatic SIA system was set up to monitor the production of D-lactic acid during batch fermentation of *Lactobacillus delbrueckii* ATCC 9649. Samples were taken from the flux of a membrane filtration unit. This system is to be regarded as a model system for us to test this novel analytical system for on-line bioprocess monitoring.

2 MATERIALS AND METHODS

2.1 Chemicals

D-lactate dehydrogenase (E.C. 1.1.1.28) from *Leuconostoc mesenteroides ssp. cremoris* (specific activity, 350 and 430 U mg^{-1}, two batches of enzyme preparation) was prepared by affinity precipitation with Eudragit-Cibarcon blue complex and DEAE cellulose ion exchange chromatography;[15] L-alanine aminotransferase (Glutamic-pyruvate transaminase, E.C. 2.6.1.2; 76 U mg^{-1} protein) from porcine heart, D-lactic acid, NAD, glutamic acid, and glycylglycine were purchased for Sigma Chemical Co. (St Louis; Mo, USA). The L-lactic acid and UV-enzymic kits for both D- and L-lactate were obtained from Boehringer (Mannheim, FRG). The silica beads were a generous gift from EKA Nobel AB (Surte, Sweden) with an average size of 41 µm, a mean pore diameter of 200 Å and the specific surface area of 300 m^2 g^{-1}, according to the specifications given by supplier. All other chemicals were of analytical grade.

2.2 Immobilization of Enzymes

Silica was modified with *r*-aminopropyltriethoxysilane and later with glutaraldehyde following procedures described by Weetall.[18] D-LDH (550 U) dissolved in 400 µl of 50 mM sodium phosphate buffer pH 7.0 was added to 0.5 g of modified silica. The mixture was left on a blood mixer at room temperature for coupling to take place. After 3 h, 110 U of L-alanine aminotransferase solution was added and coupling continued for another 2 h. Finally, 20 mg of sodium cyanoborohydride was added to reduce the Schiffs bonds formed between aldehyde and enzymes in order to stabilize the coupling. The mixture was left for 1 h at room temperature and then overnight in a refrigerator. The preparation was then washed on a glass filter with 0.1 M phosphate buffer pH 7.0 and then packed in a column (4 mm i.d., 30 mm length) with porous vyon discs at each end. The column was fitted with adaptors for connection to the flow system to be used for analysis. The column, when not used, was stored in 0.1 M phosphate buffer pH 7.0 containing 0.02% sodium azide at 4 °C. The blank reactor was prepared using the same procedure as above but no enzyme was added.

2.3 Microorganism and Growth Conditions

The microorganism used in the study was *Lactobacillus delbrueckii* ATCC 9649. The cells were maintained on MRS agar at 4 °C and subcultured weekly. The medium used for this study had the following composition (g L^{-1}): glucose 20; yeast extract 15 (Difco, Detroit, USA); trypton 1 (Difco, Detroit, USA); KH_2PO_4, 1.0; K_2HPO_4, 1.0;

$CH_3COONa.3H_2O$, 2.0; $C_6H_5Na_3.H_20$, 1.0; trace elements in mg l^{-1}: $MgSO_4.7H_2O$, 100; $MnSO_4.H_2O$, 6.2; $FeSO_4.7H_2O$, 4.0.

The cells were grown in a stainless steel fermenter (Novaferm, Malmö, Sweden) with an initial volume of 3 L. The inoculum used was 200 ml of 15 h culture of *L. delbrueckii*. The cultivation temperature was 37 °C and the pH was maintained at 6.3 by titration with 3 M NaOH. The stirrer speed was kept at 150 rpm.

2.4 Off-line Analysis

The broth sample was taken at regular intervals directly from the fermenter and the sampling vessel (see Figure 2) during fermentation and kept at -20 °C until time for analysis. Cell growth was monitored by measuring absorbance at 620 nm. Glucose was determined by a modification of the dinitrosalicylic method.[16] Cell free supernatants were prepared by centrifugation of samples and then, placed in a 80 °C water bath for 15 min to stop enzymatic reaction before dilution. D- and L-lactic acid was measured by using Boehringer enzymatic kit which measured the amount of NADH generated when incubating the sample with NAD and enzyme.[17]

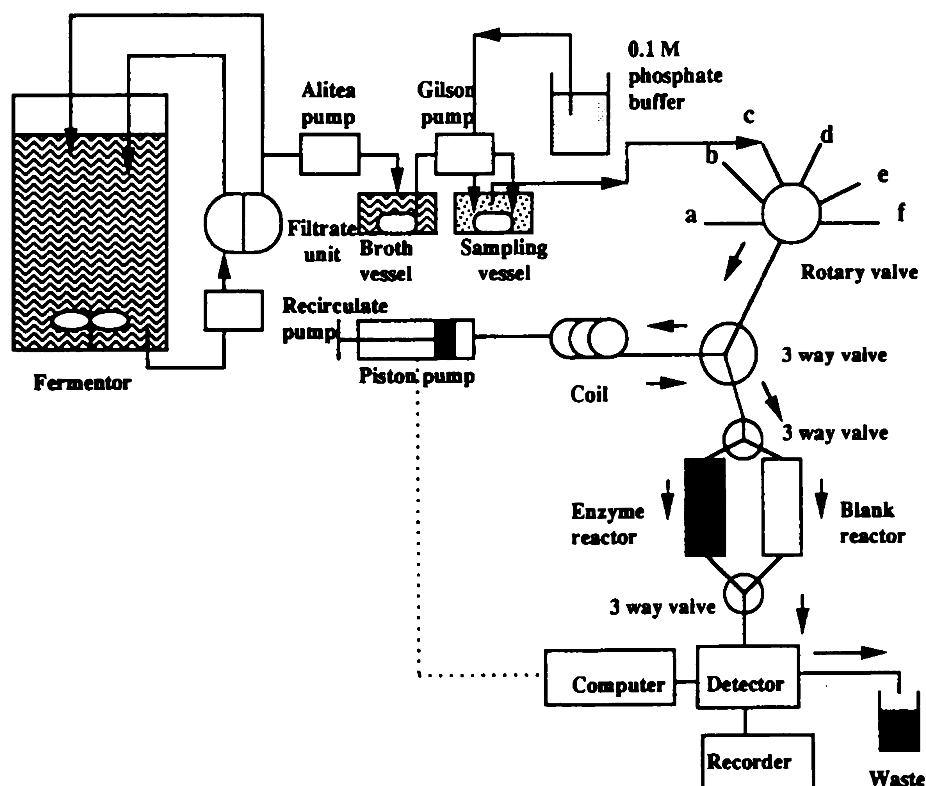

Figure 1 *Schematic representation of the sequential injection system and sampling unit*

2.5 On-line Analysis

The schematic representation of the set up including the SIA system and filtration sampling devices for on-line D-lactate analysis is shown in Figure 1. It included a recirculation pump (Watson-Marlow 520S, Falmouth, UK), a filtration unit with cellulose acetate filter (Sartorius; pore size, 0.2 μm, surface area 63.6 cm^2), a filtrate pump (Alitea C-4, Uttran, Sweden) and a dilution pump (Gilson, Minipuls 2). The broth from the fermenter was continuously circulated (1300 ml min^{-1}) through the filtration unit. The cell free permeate was pumped (69 ml hr^{-1}) from the filtration unit to a small reservoir from which a continuous stream of the broth was pumped to a dilution unit where mixing with an equal volume of 0.1M phosphate buffer pH 7.0 took place. The mixing of broth and buffer was achieved by pumping each solution with the same pump towards each other in identical tubing up to the mixing point. The volume of the reservoir for filtered broth was 0.8 ml. The overflow broth was removed by means of a pump operated at a high flow rate. Homogeneity in the sample was created by stirring on a magnetic stirrer. The diluted sample was analysed using the SIA system. The time delay from fermenter to analysis was around 6 min. The SIA system has been described earlier[9] and a little modification was made. It was constructed by a dual-piston, sinusoidal flow pump (Alitea AB, Uttran, Sweden); a six-port rotary valve (Cheminert 4162510, Valco instruments, Houston, TX, USA); a three-way slider valve with pneumatic actuator (Rheodyne, 5300 and 5301, Cotati, CA, USA); and a spectrophotometer (Shimadzu, UV-120-02, Kyoto, Japan) equipped with

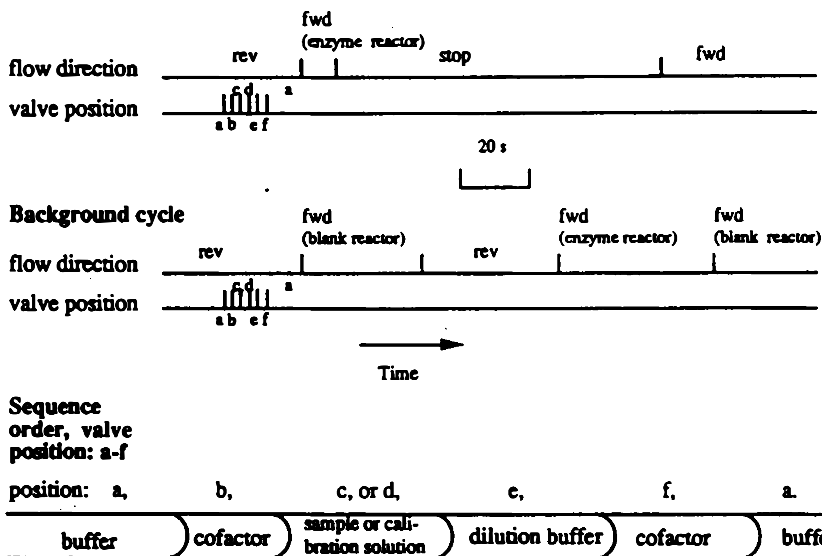

Figure 2 *Time lapses and sequential order of reagents in a measuring cycle*

a 30 μl dead volume and 10 mm light path flow-through cuvette (Hellma, model:178.711, Müllheim/Boden, Germany) for absorbance measurements. NADH was monitored by measuring the absorbance at 340 nm. All the tubings connecting the different units were made of teflon (0.8 mm i.d.). The holding coil, having a volume of 2 ml, served as a buffering volume to prevent the sample and the different reagents used from entering the syringe pump. A blank column without enzyme loading was used to correct for the blank absorbance from the broth.

The on-line analysis is controlled by programming with one blank cycle through the blank column and one calibration cycle per 5 sample cycles. The concentration of calibration solution was 5 mM. The time lapse of the measuring cycle of sample, calibration and the cycle of background in appropriate order of sequence is shown in Figure 2. The total time for one analysis is 182 seconds including 90 seconds incubation time while the flow is stopped when the sample is in the column of immobilized enzyme. The blank absorbance was checked by a blank column with a similar measuring procedure as that for sample analysis (see Figure 2). However, since no significant difference was observed in the reading when varying the length of incubation time during sample analysis in the blank reactor, the incubation was omitted. The two reactors were washed for a short time in order to reduce any accumulation of material causing a background signal and to prevent clogging of the reactors. The measuring cycle was almost the same as previously used but with a modification for calibration solution aspiration and use of a blank column for background correction (for details see reference 9). The principle of the SIA system is to suck the reagents into the system as small liquid segments in a desired order (Figure 2). When all reagents have entered the system, a valve is switched, the flow is reversed and the reagents are forced through the reactor. In this case, the flow was stopped for a certain period in order to increase the conversion, and thus to improve the sensitivity of the analysis. The aspirated volume of wash/carrier buffer, NAD, sample or calibration solution, dilution buffer, NAD, and carrier buffer here are, 1.4, 0.2, 0.1, 0.1, 0.19 and 0.19 ml, respectively. During the whole study, the rate of pump setting was 20 rpm and the arc of cam was driven from 50 to 135 degrees, corresponding to the syringe setting from 2.1 to 4.2 ml with an average flow rate of 3.2 ml min^{-1}. It is to be noted that the pump was stopped whenever the valve was being turned, in order to prevent the pressure from building up which would otherwise affect the reproducibility. Furthermore, the computer control is inevitable, using software to control the pump and valve movements during the measuring and to acquire and process the data from the detector. A micro-switch inside the pump was used to terminate the pumping processes in every analysis cycle (i.e. the starting position of every cycle is the same) to prevent any time errors being accumulated during a long series of measurements.

2.6 Measuring Condition

The optimal condition found for our earlier study was applied here with 0.1 M of glycylglycine buffer pH 10 including 70 mM glutamic acid as carrier buffer, 4 mM NAD at pH 10 as cofactor and 90 second incubation time. NAD solution was prepared fresh for daily use.

3 RESULTS

3.1 Sensor Calibration

A calibration curve for D-lactic acid is shown in Figure 3. A linear relationship between response and concentration was seen in the range from 2 to 25 mM which at higher concentration levelled off. Substrate inhibition may be a reason for this deviation. However, a much better sensitivity and operational range was achieved by operating with a sequential enzyme reaction.

During the on-line measurements, the response of the SIA system was found to decrease slowly. This was interpreted as being mainly due to the inactivation of the enzyme sequence and partly because of the deactivation of the NAD cofactor in alkaline solution. To measure this decrease in response, a standard solution of D-lactate was used to calibrate the sensor throughout the period of fermentation. After an initial period of three assay cycles, we found that the decrease was relatively constant by approximately 1% per hour. In addition, a slight increase of the background response was seen during the on-line monitoring as a result of the changes in the broth during the fermentation process. Both the factors, the decrease in response of standard solution and the increase of background, were taken into account when calculating the D-lactate concentration in the fermentation broth.

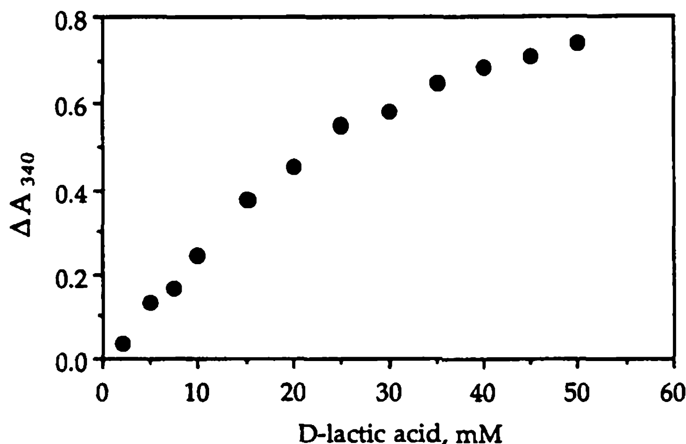

Figure 3 *Calibration curve of the on-line SIA system. Measuring condition: 0.1 M glycylglycine with 0.07 M glutamate pH 10 and 4 mM NAD, 90 sec incubation time*

3.2 Cultivation of *L. delbrueckii*

A complete cultivation cycle of *L. delbrueckii* was followed by off-line analysis. A profile of the glucose consumption, cell growth, and the concentration of the L- and D-form of lactic acid during fermentation is shown in Figure 4. The maximum cell growth was seen after about 14 h. This corresponds to nearly total consumption of glucose and also maximum level, *ca.* 60 mM, of D-lactic acid in the broth. L-lactic acid remained at very

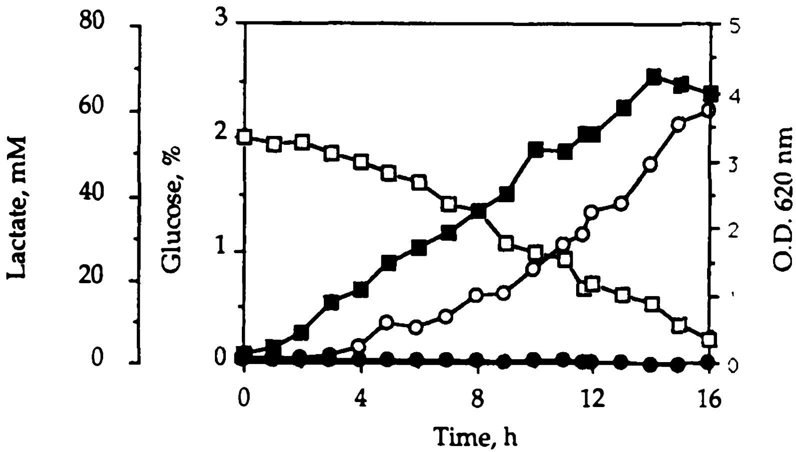

Figure 4 *Profile of the fermentation process. Consumption of glucose (□), cell growth (■), and of the D-lactate (O), L-lactate (●) production measuring off line by Boehringer enzymatic kit during fermentation process*

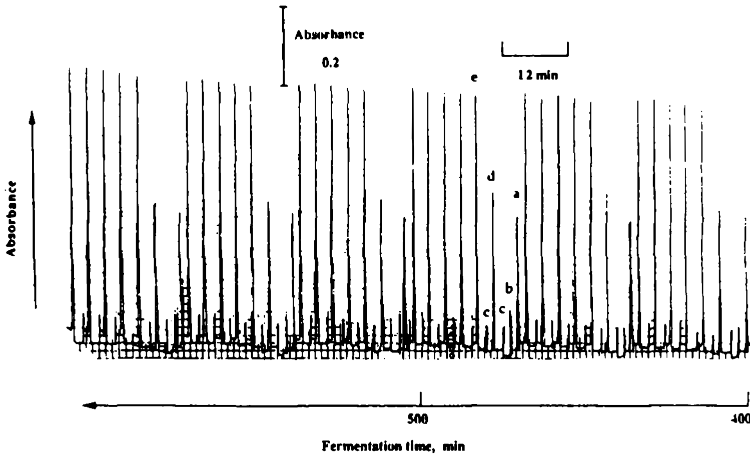

Figure 5 *D-Lactic acid production monitored on-line during the fermentation process. Forty two SIA sequential peaks in chart recorder acquired during the on-line monitoring of D-lactic acid production at 400 to 600 min after innoculation, (a) background peak, (b) washing peak, (c) initial peak, (d) calibration peak, (e) sample peak*

low concentration of about 1 mM in the initial stages and decreased with time during the fermentation. *L. delbrueckii* ATCC 9649 produces mainly the D-form of lactic acid.

3.3 On-line Analysis

Typical SIA peaks registered during a fermentation in the time period of 400 to 600 min after innoculation were recorded as shown in Figure 5. The profile shows a background peak, (a), generated by the background cycle together with two smaller

overlapping ones, (b), which were created by washing the enzyme and blank reactors with buffer in the latter step of the background cycle. Also shown is a calibration peak, (d), followed by a peak caused by improper mixing of adjacent liquid segements, (c). The initial peak was caused by the mixture of leading NAD and the carrier buffer passing through the detector. Five sample peaks, (e), together with initial peak, (c), appeared later after calibration peak, (d). A steady increase of the response signal of sample peak was seen during the fermentation corresponding to the increment of D-lactate in the broth. A slight decrease of the calibration signal can also be seen. The shape of the peaks observed is reproducible and shows that the SIA system works properly.

Table 1 *Comparison of SIA with the other Methods for D-lactate Analysis*

Item	Analysis				Units
	SIA	Boehringer kit	FIA	HPLC	
Sample Handling					
Time for sample preparation	-[a]	*ca.* 20	-[b]	>65[c]	min
Time per sample	*ca.* 3	*ca.* 35	*ca.* 3[d]	>300	min
Consumption of reagents per sample					
Buffer	1.7	1	1.5	-	ml
NAD	*ca.* 0.16	*ca.* 10.6	*ca.* 0.15[d]	0.5	μmol
D-LDH	1.1	109	-[g]	5	u
Note	on-line, automated	batch	with bioluminescence	nmol ml^{-1}	sensitive
Reference	this work	17	8	10	

(a) on-line sampling preparation; (b) no broth sample determined in this method; (c) extraction time for rat liver sample; (d) no mention in the text; 3 min is an estimate from the flow rate and flow system; (e) various kinds of buffer; SIA and Boehringer (0.1 M glycylglycine, FIA (0.5 M pyrophosphate); (f) immobilized enzyme 550 U for 500 cycles; (g) immobilized enzyme.

3.4 Comparison of the Analysis System

A comparison of the on-line SIA method with the off-line Boehringer method, FIA and HPLC methods for D-lactate analysis is shown in Table 1. SIA, as well as FIA, has a much higher sample throughput than batch and HPLC methods. The consumption of reagents, especially enzyme and cofactor, is much less in the on-line SIA method. Certainly, on-line monitoring to acquire quick information about the fermentation is also dramatically different to the batch method. The D-lactate concentrations determined on-line with the SIA system were compared with the results from the off-line samples taken from the dilution vessel and determined by the Boehringer method. A fairly good

agreement was obtained as seen in Figure 6. D-Lactic acid was produced slowly during the first 4 h and then increased steadily until 14 h. The agreement between data from off-line samples taken from the sampling vessel and on-line data is better than the comparison with data from the fermenter. If undiluted broth samples were used in the system, as in the preliminary study, a lower concentration of D-lactate (*ca.* 25-35%) is observed in the on-line system than in the off-line data where the broth concentration is higher than 25 mM (data not shown).

3.5 Specificity of Analysis

The specificity of the SIA system with the D-LDH-ALT reactor is shown in Table 2. The interference of L-lactic acid is less than 1%. The other acids have even less interference except for pyruvic acid. In the situation when D-lactic acid is determined here, due to the rather low pyruvic acid and L-lactic acid concentration in broth sample (0.4 mM and 1 mM, respectively) throughout the fermentation, both of them will not constitute a problem. Thus, the assay has specificity from a practical point of view.

4 CONCLUSION

The present study shows clearly that SIA technology is a promising system to monitor D-lactic acid production on-line during the fermentation process.

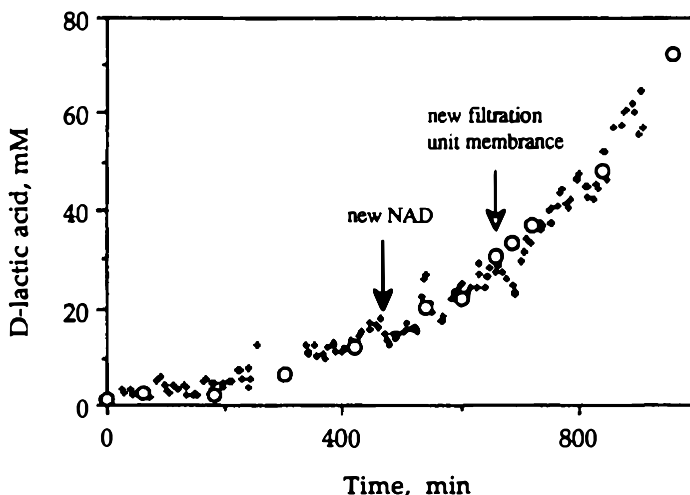

Figure 6 *D-Lactic acid production during fermentation. D-lactic acid content during fermentation as determined by on-line SIA system (+) and by off-line Boehringer enzymatic kit (O)*

Table 2 *Specificity of D-LDH-ALT in SIA method*

Acid	Slope (%) of Calibration graph
D-lactic acid	100
L-lactic acid	0.86
Citric acid	0.59
L-malic acid	0.39
Oxalic acid	0
Pyruvic acid	4.81
Succinic acid	0

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16
The Influence of Halide and Nitrate Ions on Glucose Assay Using a Glucose Electrode

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1 INTRODUCTION

Glucose oxidase (GOD) (E.C. 1.1.3.4) is a widely used enzyme for quantitative determination of glucose. Since 1967, when Updike and Hicks[1] first reported the concept of the glucose electrode utilizing immobilised GOD, a great number of papers on the development and application of enzyme electrodes have been published.

Glucose electrodes consisting of an amperometric oxygen or hydrogen peroxide detecting transducer and membrane with immobilized GOD have been used in batch mode or by incorporation into flow systems.[2-4] These sensors operate linearly below the physiological glucose levels and dilution of blood samples is usually necessary.

One way to overcome this problem is to cover the layer of immobilized GOD with an external polymer membrane to limit diffusion of glucose to the sensor.[6] The disadvantage of using an external diffusion barrier is a prolonged response time of the electrode. Another way is to immobilize GOD, combined with electron transfer mediators, such as polypyrrole[7-8] or ferrocene,[9,10] on a platinum or carbon electrode. These electrodes have linear calibration characteristics up to 30 mM and operate without oxygen interference.

Macholán *et al.*[11] reported that the linear calibration range can be broadened simply in a chemical way by using typical glucose electrodes with borate buffers at alkaline pH. This effect is explained by the rapid complexation of glucose with borate ions.

In our laboratory we have constructed a glucose electrode based on a galvanic silver-zinc oxygen sensor.[12] In this work I report that the linear range of this electrode can be broadened simply by using buffers containing halide or nitrate ions at acidic pH.

2 EXPERIMENTAL

2.1 Materials

Glucose oxidase (*Aspergillus niger* 12 U mg^{-1}) from Merck (Germany), bovine albumin fraction V from Sigma (St. Luis, USA) and glutaraldehyde, 25% aqueous solution,

from Merck (Germany) were used. As a substrate for the enzyme the anhydrous glucose from Polfa (Krakow, Poland) was used. All other reagents were of analytical grade and doubly distilled water was used throughout. At pHs between 3 and 5.5, 0.2 M acetate buffer was used and at higher pH the phosphate buffer of the same concentration was used.

2.2 Apparatus

Oxygen concentration in solution was measured by a galvanic silver-zinc electrode N5972 with oxygen meter N5521 (Mera-Elwro, Wroclaw, Poland) and a linear recorder TZ21S (Laboratorni, Pristrije, Praha, Czechoslovakia). Temperature was maintained at 25 °C with a thermostat U1 (MLW, Medingen, Germany).

2.3 Preparation of Glucose Electrode

The oxygen electrode was prepared as reported earlier.[12] The glucose electrode was prepared by dropping 40 µl of an enzyme solution (mixture of 0.5 ml 8% albumin in phosphate buffer, pH 7; 20 mg glucose oxidase; 40 µl glutaraldehyde solution) onto the electrode. After one hour the electrode was soaked in phosphate buffer (pH 7) overnight before use. The final loading of GOD was 22.6 U cm^{-2}.

2.4 Measurement Procedure

The electrode was placed in 100 ml of 5% Na_2SO_3 solution to set the oxygen meter to zero. The electrode was rinsed and placed in 100 ml of the buffer saturated with air. The meter was set to 100% of saturation after stabilizing the oxygen meter signal. A suitable amount of 1 M glucose solution was added to obtain the desired concentration. The decrease in signal of the oxygen meter was recorded. All measurements were carried out in magnetically stirred solutions. From the recorded results the decrease of oxygen meter signal, I (steady state method), and initial slope, V (dynamic method), were read and expressed in arbitrary units (a.u.). Between measurements the electrode was stored in phosphate buffer, pH 7, at room temperature.

3 RESULTS AND DISCUSSION

In our first paper[12] we reported that the glucose electrode measurements with GOD immobilized in protein gels were largely independent of pH in a broad region from pH 4 to 8 when measured with the steady state method and showed a maximum at pH 5 when measured with dynamic method. This is similar to the maximum measurments with the soluble enzyme.[13,14] Guilbault *et al.*[2] reported that the maximum measurement of the glucose electrode is shifted in more alkaline pH values of 1-1.5 pH units; this shift is attributed to a microenvironmental influence of the polyacrylamide lattice. Another difference between our method and that of Guilbault was that we used buffers without KCl and they used buffers with 0.1 M KCl to maintain conditions similar to those in blood serum. It is well known that Cl^- anions inhibit GOD at acidic pH.[15] This fact led us to investigate, in detail, the influence of Cl^- and other anions on glucose electrode response .

Figure 1 shows the influence of increasing KCl concentration on electrode response in a steady state. It can be seen that addition of KCl strongly diminished the signal at pH values lower than pH 5.5 and heightened the signal at higher values. In the dynamic state the pH optimum is shifted from pH 5, without KCl addition, to pH 6 with 0.1 M KCl and finally to 6.5 in 1 M KCl. A similar effect was observed when KBr and KNO_3 were added. Nitrate anions shift the pH optimum of the dynamic response up to pH 8 in 1 M solution.

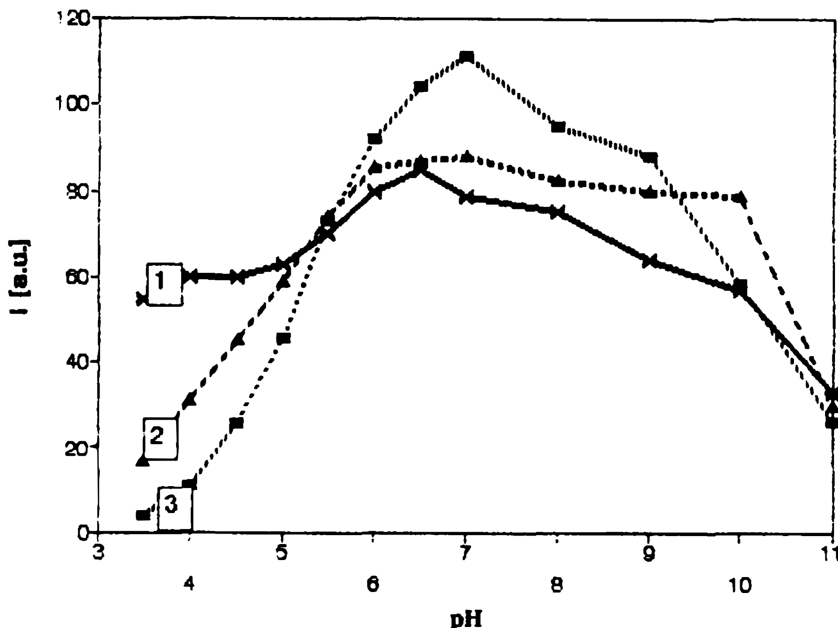

Figure 1 *The pH profile of glucose electrode at 25°C in 0.5 mM glucose. (1) without salt; (2) 0.1M KCl; (3) 1M KCl.*

Figure 2 shows in detail the influence of different anions on the electrode response at pH 4 and 7 in a steady state. In the dynamic state the effect of salt addition is similar but more pronounced. At pH 4 the halide and nitrate anions diminish the electrode response due to inhibition of GOD. The nitrate anions appear to be the strongest inhibitor of GOD. This inhibition is fully reversible. The inhibitory effect of halide anions is attributed to conformational changes affecting the binding of substrate and to changes in flavin reduction.[15] At pH 7 the response of the electrode is increased. The increase in the electrode response at neutral and slightly alkaline pH values can be explained by the response of a galvanic oxygen electrode in an air saturated solution being proportional to the partial pressure of oxygen and independent of the actual concentration of oxygen.[16] The addition of salts lowers the concentration of oxygen in a saturated solution,[17] thus the response of the electode in salt solution is lowered to a greater extent when glucose is added. Figure 3 confirms this explanation showing linear dependence of $\log(I/I_o)$ or $\log(v/v_o)$ on KCl concentration; this is due to the Sechenov relationship.[17] In addition, the fact that the KCl increased the response of the electrode to a greater extent than than KNO_3 and KBr is in good agreement with the salting effect of these salts on the solubility of oxygen.[17]

The inhibition of the GOD by anions is confirmed by the large increase in apparent Michaelis constant K_m values for glucose in solutions saturated with air, when measurements are taken with the soluble enzyme at pH 4 (Table 1). At pH 7, the K_m value

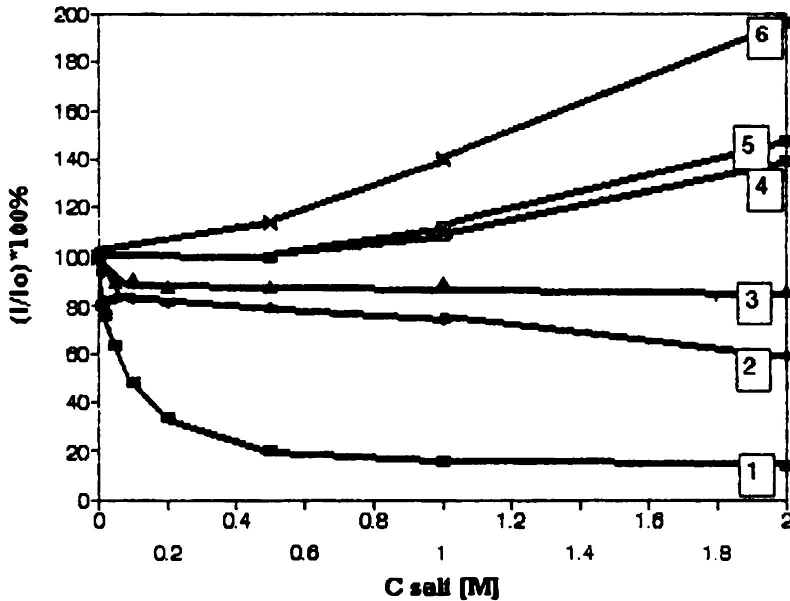

Figure 2 *The influence of salt addition on the steady state response of the glucose electrode at 25 °C in 0.5 mM glucose solution. Value with '0' denote the response in salt free solution; (1) pH 4, KNO_3; (2) pH=4, KBr; (3) pH=4, KCl; (4) pH=7, KBr; (5) pH=7, KNO_3; (6) pH=7, KCl.*

Table 1 *Apparent K_m Values for Glucose Oxidase in Solution Saturated with Air (Oxygen) at 25 °C*

Salt Addition	Km Values (mM)	
	pH 4	pH 7
without salt	20.3	21.4
1M KNO_3	623	25.3
1M KCl	256	11.4
1M KBr	104	11.7

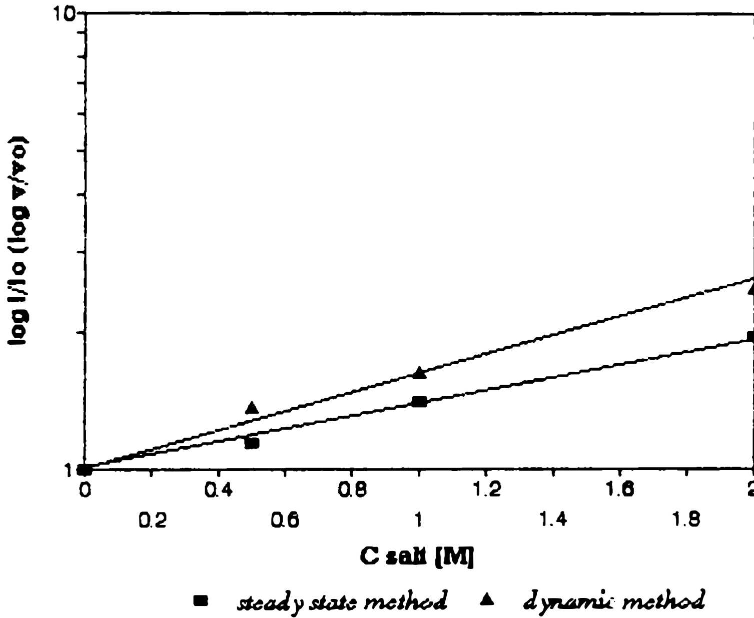

Figure 3 *The Sechenov plots of the glucose electrode response at pH 7 and 25 °C in KCl solution. (1) steady state method; (2) dynamic method.*

Table 2 *Apparent E_a for Glucose Electrode Response Measured for 0.5 mM Glucose Solution*

pH	Salt Addition	E_a (kJ mol^{-1})	
		Steady State Method	**Dynamic Method**
4	without salt	40.3 ± 6.1	63.3 ± 5.3
	1M KCl	57.6 ± 3.5	84.5 ± 5.6
	1M KNO_3	55.1 ± 3.4	84.2 ± 14.8
7	without salt	41.3 ± 1.1	62.3 ± 2.6
	1M KCl	41.1 ± 1.2	61.4 ± 2.0
	1M KNO_3	51.4 ± 1.8	76.9 ± 4.9
9	without salt	40.6 ± 1.5	57.1 ± 2.4
	1M KCl	35.0 ± 3.2	50.0 ± 3.7
	1M KNO_3	39.0 ± 2.4	56.4 ± 4.3

in 1M KNO_3 solution is slightly higher than without the salt addition and for the halide salts it is nearly half that in the absence of the salt.

The electrode response depends on temperature. As both methods of measurement obey the Arrhenius plots, the apparent activation energies, E_a values, could be calculated. The obtained values are summarized in Table 2. It is clear that the apparent E_a values for both methods of measurement are similar at the different pHs. At pH 4, the values of apparent E_a increase when the electrode is working in salt solution. In neutral solution, only the addition of nitrate anions influence E_a. In a slightly alkaline solution, the values obtained for nitrate anions are similar to the salt free medium but lower values are observed in the presence of chloride anions.

The results reported indicate that nitrate anions are very effective inhibitors of GOD. They inhibit this enzyme even at neutral pH value in contrast to halide anions. It can also be confirmed by the results shown in Figure 4 that the response of the electrode with low GOD loading is diminished in the presence of 1 M KNO_3 solution. The response of the electrode is controlled by the enzyme reaction[18] and thus the increase of K_m value could cause this effect. In 1M KCl solution, the responses of electrodes with high and low GOD loading are increased at pH 7 when compared with salt free solution.

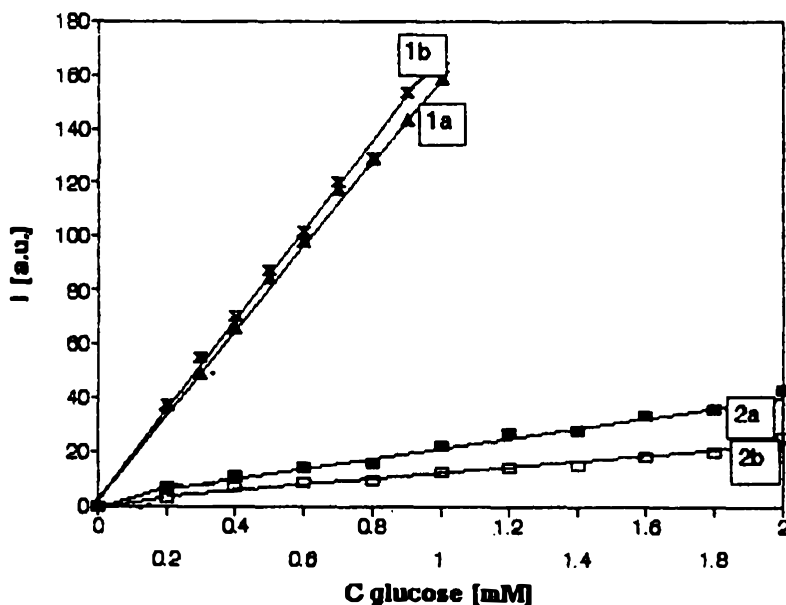

Figure 4 *Calibration curves at pH 7 and 25 °C. (1) GOD loading 22.6 U cm^{-2}; (2) GOD loading 2.26 U cm^{-2}; (a) without salt; (b) 1 M KNO_3.*

The strong inhibitory effect of nitrate anions in acidic solution could be used to broaden the calibration curves of the glucose electrode as shown on Figure 5. Furthermore, the effect of salt addition, especially of KCl at pH 7, could be used for the assay of very

low glucose concentrations (Figure 2) because the concentration range is narrowed by approximately half in 2 M solutions of this salt.

The effect of Cl^- anions both at acidic and neutral pH values must be taken into consideration when glucose is assayed in blood or serum which contains concentrations in the region of 0.1 M of these anions. The use of calibration curves made in solutions without Cl^- could be one of the reasons for measurement error in such assays.

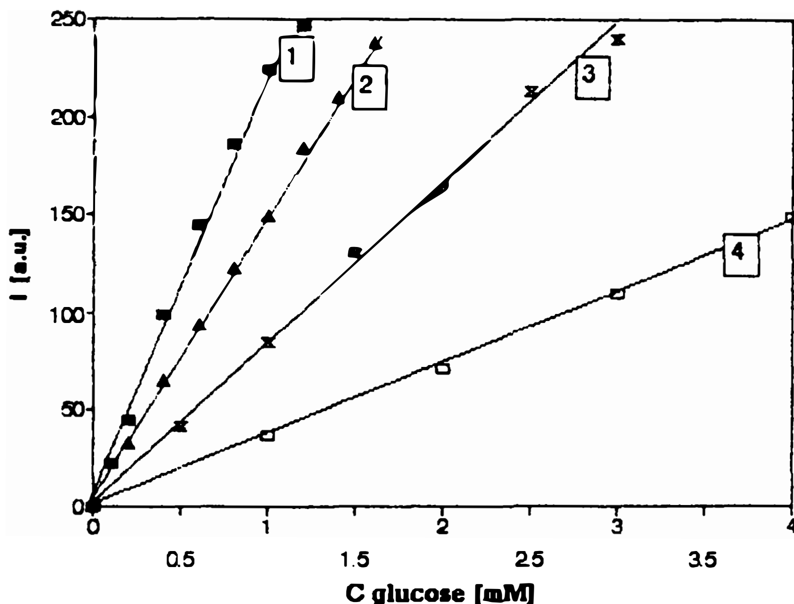

Figure 5 *Calibration curves at pH 4 and 25 °C in KNO_3. (1) without salt; (2) 0.04 M; (3) 0.2 M; (4) 2 M.*

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Applications of Ion Mobility Spectrometry for Food Analysis

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1 SUMMARY

Ion mobility spectrometry (IMS) is a gas phase electrophoretic technique being used to differentiate between fresh and frozen/thawed meats and detect microorganisms in foods.

The selling of frozen food as fresh is a consumer concern; therefore methods to differentiate between fresh and frozen/thawed food are required. It has been found using ion mobility spectrometry that the activity of the enzymes β-glucuronidase and β-galactopyranosidase within the foods increases after freezing. The effects of refrigerated storage (spoilage) and different freezing temperatures were investigated in this study.

In addition, the rapid detection of pathogenic and indicator microorganisms (*i.e.* indicating faecal contamination) in foods are required by the food industry to ensure the safety of the products being produced. Methods using IMS will be described which have the capability to rapidly detect motile aeromonads in foods.

2 INTRODUCTION

2.1 Fresh and Frozen Food

The enzymic differentiation between fresh and frozen/thawed food is based upon the observation that the freezing of food (*e.g.* meat) causes the rupture of membrane bound cell organelles (mitochondria and lysosomes) within the cells of the food. This results in the release of enzymes from within the organelles to the surrounding cell cytoplasm thus giving a higher detected enzyme activity in frozen/thawed meat compared to fresh. It is known that there are two lysosomal enzymes, β-glucuronidase and β-galactopyranosidase,[1] which can be detected by IMS[2] through the liberation of *o*-nitrophenol (ONP) from the substrates ONP-glucuronide and ONP-galactopyranoside, respectively. Experiments were therefore carried out using IMS to establish if there were any differences in the activity of these enzymes in fresh and frozen/thawed chicken samples.

2.2 Bacterial Identification

Ion mobility spectrometry can be used to detect bacteria by following the activity of

different enzymes during metabolism. The activity of β-glucuronidase can be measured by the release of ONP which is detected by the IMS when ONP is linked to β-glucuronic acid. It is known that unlike the majority of microorganisms, *E. coli* possesses β-glucuronidase and can therefore be detected by an ONP-glucuronidase assay. In addition, the time taken to observe the liberation of ONP is also indirectly proportional to the number of *E. coli* in a given food sample.[2] A similar method can be used to enumerate coliforms by substituting ONP-glucuronide with ONP-galactopyranoside.[3] A range of other bacteria can be detected using IMS by monitoring the breakdown of other substrates.[4]

The genus *Aeromonas* comprises two groups; the first being non-motile, unable to grow at 37 °C and is not pathogenic to humans, while the other has the reverse of these characteristics. This second group contains the strains *A. hydrophila, caviae* and *sobria* which have been found in a variety of foods,[5] and have been implicated in gastrointestinal infections.[6,7] There is a further concern as strains in this *hydrophila* group are also able to grow at chill temperatures.[6]

Conventional plate media for isolating and identifying motile aeromonads are numerous, but are both labour-intensive and do not produce a particularly quick result. Suspect colonies would have to be purified before confirmation by a series of biochemical tests for species identification.

A rapid method to recognise the presence of potentially pathogenic aeromonads in foods could utilise the speed and ease of use of ion mobility spectrometry when the samples are tested at 37 °C in an ONP-galactopyranoside-irgasan selective broth. The majority of aeromonads are ONP-galactopyranoside positive and the presence of irgasan (2,4,4'-trichloro -2'-hydroxydiphenylether) restricts the growth of the competing microbial flora. Pseudomonads can withstand irgasan but many strains grow poorly at 37 °C and the majority are ONP-galactopyranoside negative.

Preliminary results presented here show the effectiveness of the IMS system for recognising aeromonads by the *o*-nitrophenyl peak when split from ONP-galactopyranoside. A range of other bacteria show little or no response.

3 MATERIALS AND METHODS

3.1 Ion Mobility Spectrometry

IMS is a rapid and sensitive technique[8] for the detection of a number of volatile organic functional groups (e.g. alcohols, nitrosamines and nitroaromatics). A typical IMS is illustrated schematically in Figure 1.

Spectra were recorded (Figure 2) by coupling the IMS to an IBM PC/AT compatible computer fitted with an advanced analytical signal processing board (ASP) (Graseby, Watford, UK). The areas of the ONP peaks of interest (expressed in units of mV μs) were calculated using an algorithm written in the C language.

An automated sampling system[4] (Figure 3) was used to extract the head space gases above food samples contained within upturned syringes. These gases were then pumped directly into the IMS for analysis. The samples were maintained at 37 °C throughout.

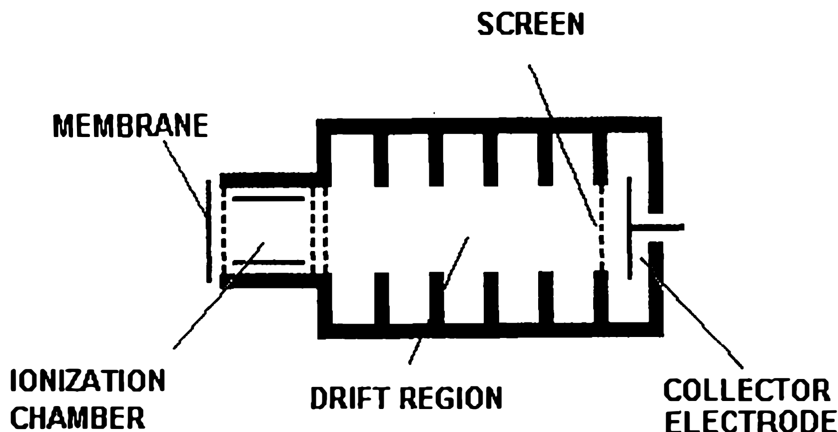

Figure 1 *A typical ion mobility spectrometer. The sample vapour is pumped through the membrane into an ionisation chamber where it is ionised using a nickel 63 radioactive source. These ions are subjected to time of flight measurements in the drift region where the ions migrate under the influence of the applied field to a collector electrode. This migration time is related to the ions mass, size and charge and is also dependent on the temperature and pressure of the drift region.*

3.2 Preparation of Fresh and Frozen/Thawed Food Samples

A 10 g sample of the fresh or frozen thawed chicken was diced into approximately 5 mm squares and then stored in glass beakers at 0 °C. Volumes (15 ml) of 150 mM phosphate buffer (raised to pH 7 by the addition of sodium hydroxide - NaOH) was added to each beaker and the contents stirred for 2 minutes. The food solution was then "whirlymixed" for 30 s and centrifuged firstly at 4000 rpm (1200 g) for 10 minutes. The supernatant was removed and then centrifuged at 16000 rpm (22 000 g) for 10 minutes to remove the lysosomes. Two ml of the subsequent supernatant from each sample was added to the syringes used for automated sampling.

For the glucuronidase and galactosidase assays, ONP-glucuronide and ONP-

galactopyranoside were added to the food samples to give final concentrations of 2 and 1 mg/ml respectively. The IMS automation sampled the head space above the samples every 20 minutes.

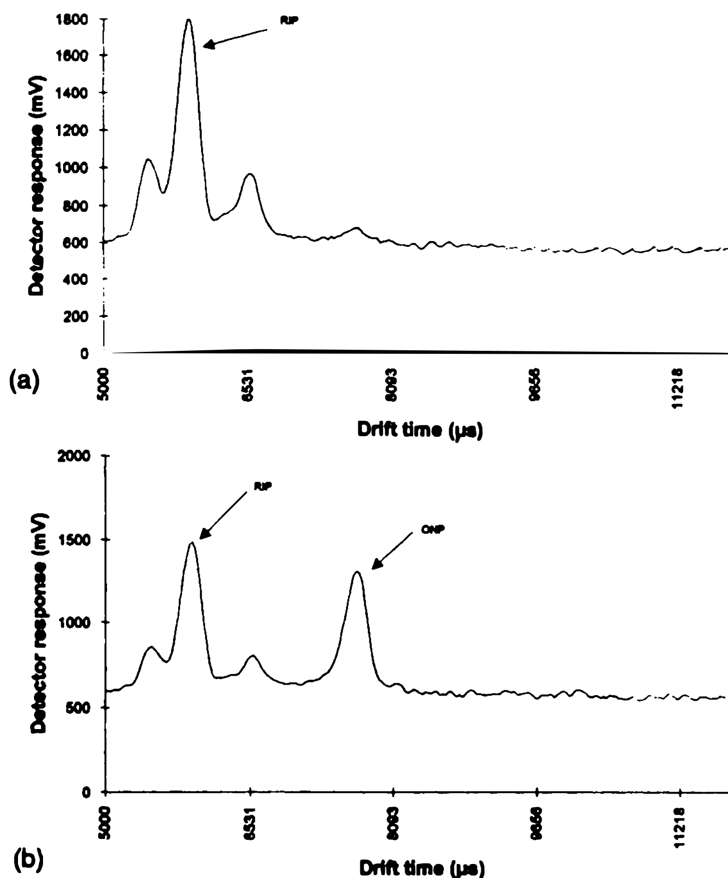

Figure 2 *Ion mobility spectra. (a) clean air - showing the reactant ion peaks (RIP) and (b) clean air with o-nitrophenol (ONP)*

3.3 Fresh and Frozen/Thawed Experiments

Experiments to differentiate between fresh and frozen/thawed chicken breast were carried out. The chicken was split into two, the first half was frozen and stored at -20 °C overnight whilst the other half was stored at 4 °C. The samples were then prepared as above and assayed for β-glucuronidase and β-galactopyranosidase activity on the automated IMS.

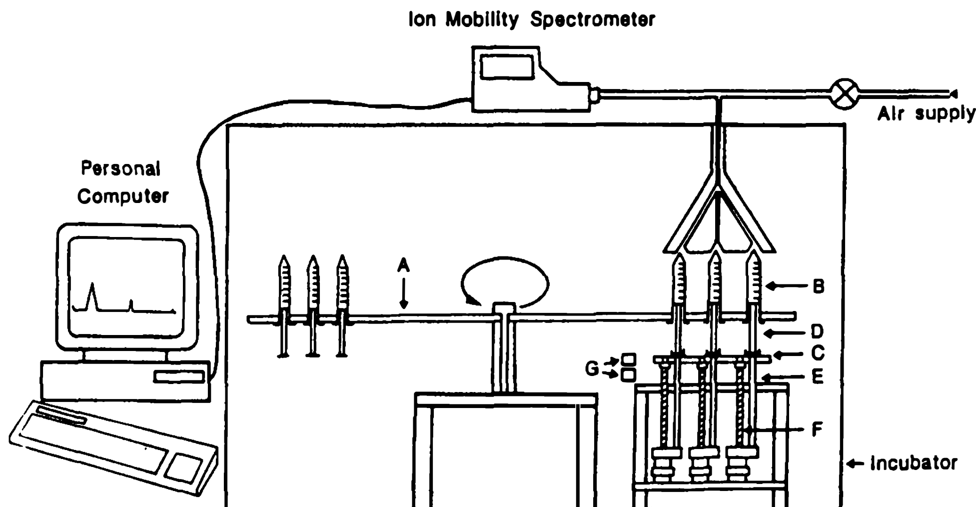

Figure 3 *The automated sampling system of the IMS. (A) the carousel; (B) the disposable plastic syringes; (C) the shoes into which the syringes are located; (D) the syringe plunger; (E) the rod which is attached to the shoes and driven by the lead screw (F); (G) the opto slots which determine the length of the syringe stroke.*

Experiments were also carried out to establish if there were any effects due to different freezing temperatures (-20, -30 and -70 °C). To establish if there were any changes caused by spoilage, samples of chicken were left at 4 °C for up to 8 days before assays were carried out.

3.4 Bacterial Detection Assays

Cultures of various microorganisms (see Table 1) were grown overnight in nutrient broth (Oxoid CM1) at 37 °C. Aliquots (0.01 ml) were inoculated into IMS assay tubes containing 10 ml volumes of ONP-gal-irgasan broth containing: 5 g l^{-1} bacteriological peptone (Oxoid L37); 8.5 g l^{-1} bile salts No. 3 (Oxoid L56); 1 g l^{-1} lactose; 1 g l^{-1} ONP-galactopyranoside (Sigma). This is supplemented with 1 ml volumes of irgasan (0.5% in 0.1 N NaOH) and brilliant green (0.5%). The whole is filter-sterilised (0.45 mm) before storage at 4 °C.

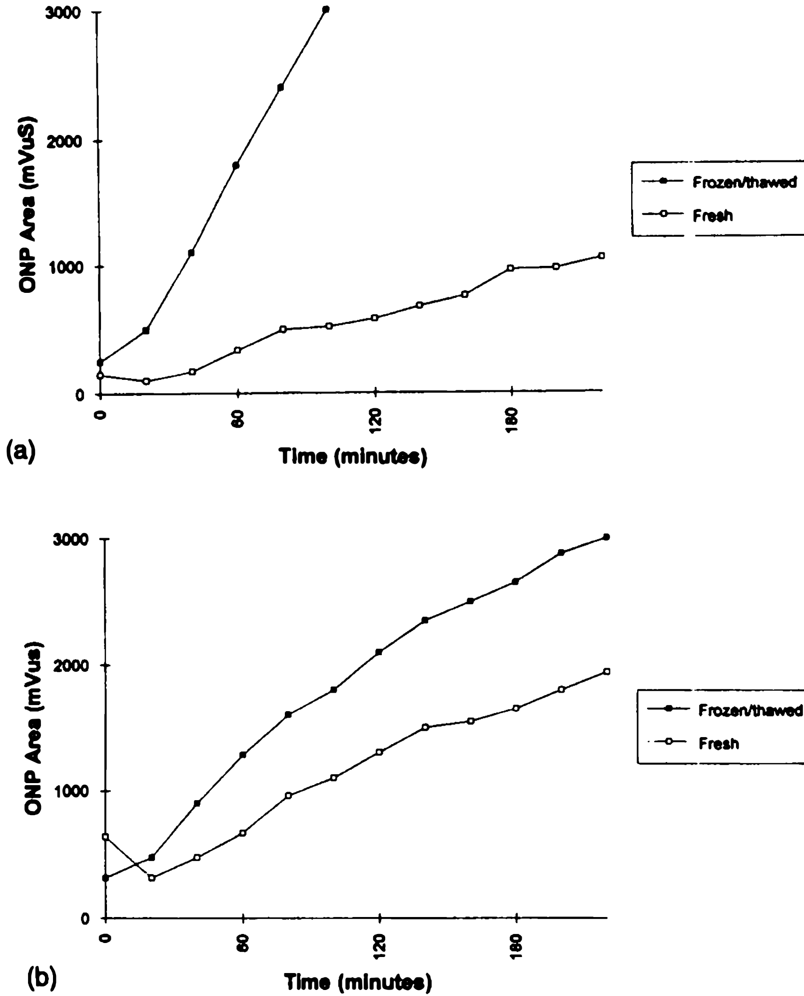

Figure 4 *Production of ONP from fresh and frozen/thawed food samples. (a) β-glucuronidase activity and (b) β-galactopyranosidase activity*

4 RESULTS AND DISCUSSION

4.1 Differentiation between Fresh and Frozen/Thawed Food Samples

Graphs of IMS ONP peak area against time for both fresh and frozen thawed chicken with the substrates ONP-glucuronide and ONP-galactopyranoside are shown in Figure 4. The graphs show that the β-glucuronidase and β-galactosidase activities were significantly

higher for the frozen chicken when compared with the fresh sample. These results agree with the theory that lysosomes are damaged by freezing and thus release enzymes into the surrounding cell cytoplasm.

The results of the experiments to establish whether the spoilage of the chicken had any effect on enzyme activity showed that there did not appear to be any significant differences. It was also found that differences in the temperature of freezing (-20, -30 and -70 °C) had no effect. In all cases the frozen/thawed chicken gave significantly higher β-glucuronidase activity than the fresh samples.

4.2 Bacterial detection

The results of ONP detection by IMS from a range of bacterial cultures are shown in Table 1. This demonstrates that all motile aeromonads tested split the ONP-galacto-pyranoside substrate and were recognised by the IMS technique. There were incidences of false positives from certain *Enterobacteriaceae* but not from pseudomonads. The bacteria giving false positives results were not found to be a problem on conventional Aeromonas agar, and there is some doubt as to the full effectiveness of an old batch of irgasan in current use in this selective broth. A new batch is due to be tested shortly which will hopefully resolve this problem.

Table 1 *Production of ONP by micro-organisms in IMS-Aeromonas selective broth*

Isolate	No of strains	ONP detected
Aeromonas spp	13	13
E.coli	3	1
Citrobacter spp	2	1
Salmonella spp	2	0
Enterobacter spp	2	1
Proteus mirabilis	1	0
Hafnia alvei	1	1
Klebsiella spp	1	1
Yersinia enterocolytica	1	0
Pseudomonas spp	11	0
Listeria spp	4	0
Staphylococcus aureus	3	0
Micrococcus luteus	1	0

4 CONCLUSIONS

IMS is an appropriate technique for determining whether or not chicken has been frozen. The automation of the assay on the IMS allows many samples to be handled simultaneously. However, the preparation of the food samples for the assay is relatively labour intensive and laborious. Other experiments have been carried out which demonstrate that the technique is applicable to pork, cod, lamb and beef.

The method to detect motile aeromonads is showing promise, but further modifications to the medium may be required to eliminate certain false positives. Once this is complete the method will then be used to identify these microorganisms in foods.

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Electronic Noses for Food Control

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1 SUMMARY

An electronic nose is described which consists of a gas sensor array combined with a pattern recognition routine. The sensor array consists of 10 metal-oxide-semiconductor field effect transistor (MOSFET's) catalytically active metal with gates. It also contains four commercially available chemical sensors based on tin dioxide, the so called Taguchi sensors, and a carbon dioxide monitor based on IR absorption. Samples of shrimps, cod roe, ground beef and pork, stored in a refrigerator, were studied. Gas samples were led to the sensor array, and the resulting sensor signals were treated with pattern recognition software based on an artificial neural network. In addition, the classification of various types of cheeses was studied.

2 INTRODUCTION

The application of the electronic nose concept or artificial olfaction has been of great interest in recent years.[1-5] An electronic nose consists of an array of gas sensors with different selectivity patterns, a signal collecting unit and data analysis by pattern recognition software. Several intriguing applications have been developed with practical interest in many important fields such as process and environmental monitoring and medical diagnosis.[3,5] The sample identification is performed through the use of pattern recognition routines operated by artificial neural networks to evaluate data from sensor arrays.

The chemical sensors used in the electronic nose systems which have been developed in our department are mostly based on field effect devices having thin, catalytic metal gates,[6] however, other types of gas sensors such as those based on semiconducting metal oxides are also used.[3] Each application determines the choice of gas sensors, and, if necessary, other types of sensors which are relevant for the application can be added to the array. In this way, we try to make an electronic nose tailor-made for the type of measurements required. Thus, carbon dioxide, oxygen and biosensors, for example for glucose, (in addition to the gas sensors) are also valuable for the investigation of biological material such as food.

Considerable waste occurs during the storage and handling of various types of food.

It often constitutes an ideal growth medium for several groups of bacteria. Assessment of food quality is in the most cases based on various methods of microbial strain identification using a variety of different growth conditions and biochemical tests. These are tedious and cumbersome techniques, requiring skilled staff and are therefore not performed frequently, although in many cases it would be highly beneficial for general manufacturing practice surveillance. Consequently, it appears that there is a large need for a simple, inexpensive and rapid method for measuring the quality of food during storage.

It can be assumed that the composition of volatile compounds evolved from the food reflects the activity and type of microorganisms present, and can be related to the quality of the food. Thus, electronic noses appear to be a useful means of detecting these kinds of changes. Furthermore, they are not in direct contact with the measuring media because they are used for gas phase measurements.

This paper deals with two forms of food quality assessment. The freshness of ground meat, shrimps and cod roe have been monitored during storage in a refrigerator using an electronic nose system; secondly, a similar system has been used to classify various types of cheeses.

3 MATERIALS AND METHODS

3.1 Samples

Shrimps, cod roe, ground pork and beef were purchased fresh from a local dealer; the shrimps being obtained pre-boiled. The ground meat was divided into two sets; each containing ten samples of ground pork and beef, respectively. These samples, containing in average 20 g of ground meat, were then aseptically placed in petri dishes (diameter 56 mm, height 8 mm). In turn each of these were placed in larger petri dishes (diameter 87 mm, height 14 mm) which were partially sealed from the environment using polyethene foil. The large petridishes were placed in a common household refrigerator, the temperature set to 4 °C. A small fan was placed within the refrigerator in order to avoid temperature gradients being set up.

Gas samples to the sensor array were taken from the petri dish via a teflon tubing which penetrated the polyethene foil.

The shrimps were placed in ten 100 ml beakers, each containing three shrimps which had an average weight of 50 g in each beaker. The set of beakers were divided in two groups, each of 5 beakers, one group was immediately placed in the refrigerator, the other group was stored at room temperature for three hours before being placed in the refrigerator. The beakers were sealed with parafilm.

The cod roe was divided up and placed into ten 100 ml beakers which were treated in the same way as the shrimp samples. Each beaker contained in average of 35 g of cod roe.

Gas samples to the sensor array were taken from the beakers via a teflon tubing

which penetrated the parafilm.

Samples of ten different types of cheese were stored at room temperature for 40 hours in 400 ml glass beakers covered with parafilm; each sample weighed 25 g and was cut into four pieces. Each cheese had two identical sample beakers prepared. Gas samples to the sensor array were taken from the beakers via teflon tubing which penetrated the parafilm.

3.2 Sensor Array and Electronic Equipment

The sensor array consisted of ten MOSFETs with thin catalytically active metal gates made from Pt, Ir and Pd, fabricated at the laboratory according to a method described elsewhere.[8] Four Taguchi type sensors, obtained from Figaro Engineering Inc., Japan, were also used in the sensor array. The sensor array is described in more detail in Table 1. The electronic equipment necessary for the measurments by the field effect transistors and by the Taguchi type sensors was constructed at the laboratory. The field effect transistors were placed in a row in a small chamber, volume 1 ml, which was heated by a resistor.

Table 1 *The Composition of the Sensor Array*

Sensor Number	Type of Sensor	Gate Metal Composition	Operating Temperature (°C)
1	FET	Pd, porous	150
2	FET	Pt, porous	160
3	FET	Pd, dense	170
4	FET	Pd, porous	180
5	FET	Ir, porous	190
6	FET	Ir, porous	150
7	FET	Ir:Pt,porous	160
8	FET	Pt:Pd,porous	170
9	FET	Ir, porous	180
10	FET	Pd, porous	190
11	Taguchi	TGS 813	400
12	Taguchi	TGS 800	400
13	Taguchi	TGS 881	400
14	Taguchi	TGS 825	400
15	CO_2		

The Taguchi type sensors were placed in separate cells after the sensor chamber and the carbon dioxide detection was based on infra-red absorption (Rieken Keiki Co., Japan).

Gas samples were pumped from the samples by a membrane pump at a flow rate of

50 ml min^{-1} and injected into a sensor chamber. The injection of gas samples could be performed at the required time intervals by opening a valve operated by a PC. The injection time was 3 minutes for the meat samples, 12 minutes for the cod roe and shrimp samples, and 1 minute for the cheese samples. The PC was also used to collect and manipulate the data from the sensor array.

3.4 Pattern Recognition Routines

The *pattern recognition* routine was based on an artificial neural net (ANN) using the back propogation method as the learning rule. Various network configurations were investigated depending on the application.

The datasets obtained during measurements were usually normalized and divided into a training set, used for learning process of the neural network, and a test set, used for predictions.

Two ANN software packages were used; McBrain from Neurix, U.S.A. and Brainmaker from California Scientific Software, U.S.A.

4 RESULTS AND DISCUSSION

4.1 Ground Meat

Measurements were performed from the second to the fourth day for the ground pork samples and from the second to the eighth day for the ground beef samples.

Typical signal patterns obtained from the various sampes are shown in Figure 1. The changes in the signal patterns with storage time are clearly visible. It is also observed that the signal levels are generally larger for the pork. The last sensor signal in each case gives the carbon dioxide concentration. As expected, carbon dioxide is associated with the growth of microorganisms. It was observed that although the amount of carbon dioxide produced may be a general parameter indicating the growth of microoganisms, it cannot be used alone for estimation of storage time, even if the type of meat (beef or pork) is known. The reason for this is, of course, that different types of microorganisms, and prehistory of the meat, give rise to different rates of carbon dioxide production at a given age of the meat. The total number of patterns obtained from the experiments was 70 (10 times 7). Each dataset, or pattern, consisted of an input set of the 15 sensor signals and an output set of 2 classes, type of meat and number of days storage for each class.

A three layer network was trained to recognize the storage time and type of ground meat. The input layer consisted of 15 units, corresponding to the 15 elements in the sensor array, the hidden layer consisted of 7 units, and the output layer consists of 2 units. The learning (or training) of the net was allowed to cycle over 400 times. The total learning time was around 24 h. Figure 2 shows a summary of the results obtained. A comparison was also made including all sensors except the CO_2 monitor. Thus it was shown that the storage time predictions (especially for beef) were *significantly improved* upon the incorporation of the CO_2 monitor.

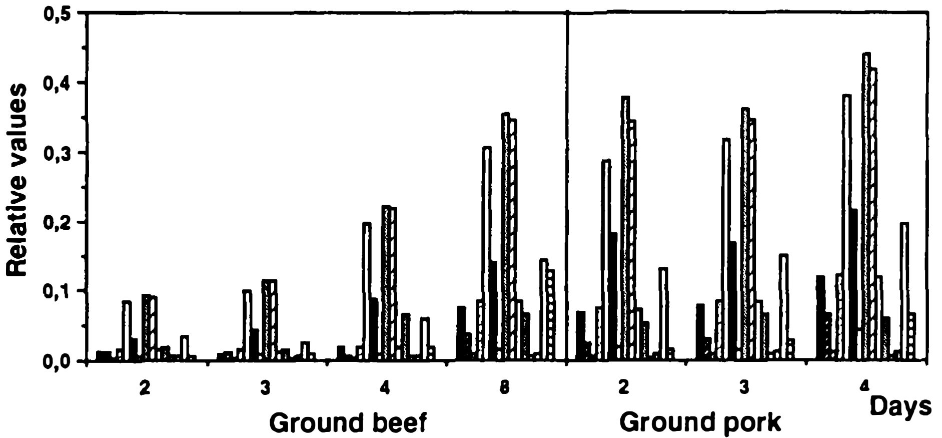


Figure 1 *Signal patterns from various samples of ground meat. Each set of bars on the chart represent the signals from the 15 sensors. From left to right in each set: 10 FETs, 4 Taguchi sensors and 1 CO_2 monitor. A direct comparison between the relative size of the responses can only be made within each class of sensors.*

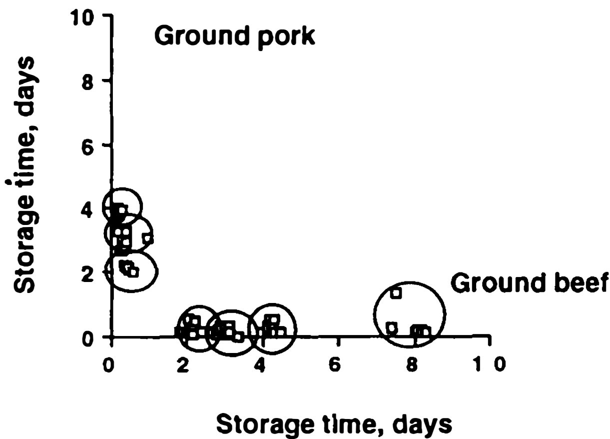
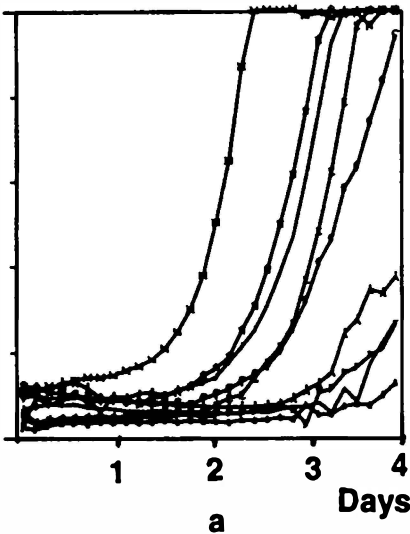


Figure 2 *Summary of results obtained for the classification of ground meat. The drawing illustrates the identification (closeness to the y and x axes, respectively) and the classification according to age of all samples (including both test and calibration samples). Samples belonging to the same group are encircled.*

4.2 Shrimps and Cod Roe

Measurements on samples containing shrimps and cod roe were performed over four days. The samples were divided into two sets; one set being kept in the cold all the time (referred to as 'cold') and the other set being kept at room temperature for three hours before placed in the refrigerator (referred to as 'pre-warmed'). Gas samples from each beaker were taken every 12 minutes, each second hour.

The evolution of carbon dioxide with time is shown in Figure 3a (shrimp samples) and in Figure 3b (cod roe samples). As can be seen, there are large differences in the rate of carbon dioxide production, both within the two groups and between the two groups. The carbon dioxide was markedly lower for the samples of shrimps, which is due to the samples being bought preboiled, which has delayed and reduced the microbial growth. As for the samples with ground meat, the differences of carbon dioxide production within the two groups are due to different types of microorganisms (and prehistory) giving rise to different rates of carbon dioxide production at a given age of the two samples.



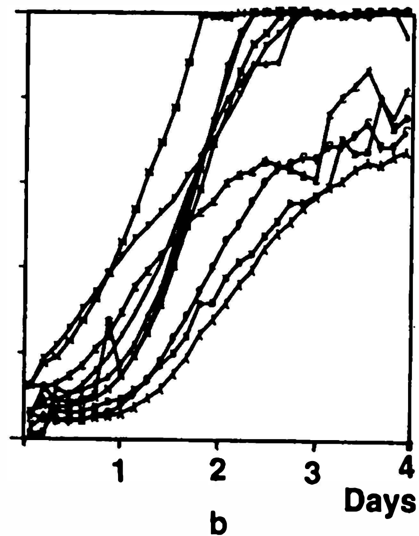


Figure 3 *Production of carbon dioxide by shrimps and cod roe during storage. a) samples of shrimps; b) samples of cod roe. For some samples, the carbon dioxide production has exceeded the measuring range of the detector.*

A three layer network was trained to recognize the storage time. The input layer consisted of 15 units, corresponding to the 15 elements in the sensor array, the hidden layer consisted of 10 units, and the output layer consists of 1 unit, corresponding to the storage time. For the cod roe, the net was allowed to cycle 200 times (making a learning time of 25 minutes) and for the shrimps, the net was allowed to cycle 250 times (making a learning time of 32 minutes). The results for 156 predictions of the storage time for the shrimps are

shown in Figure 4a, and the corresponding results for 150 predictions of the storage time for the cod roe are shown in Figure 4b. It was observed that the predictions for the cod roe are in general better than those for the shrimps. Also, the predictions in both cases appear to improve as storage time increases. This is probably due to more gases evolving as the storage time increases, thus increasing the responses of the sensors and increasing the amount of potentially useful information. The sensor signals originating from cod roe were in general higher than those originating from the shrimp samples, improving the chance of producing accurate predictions for the cod roe samples.

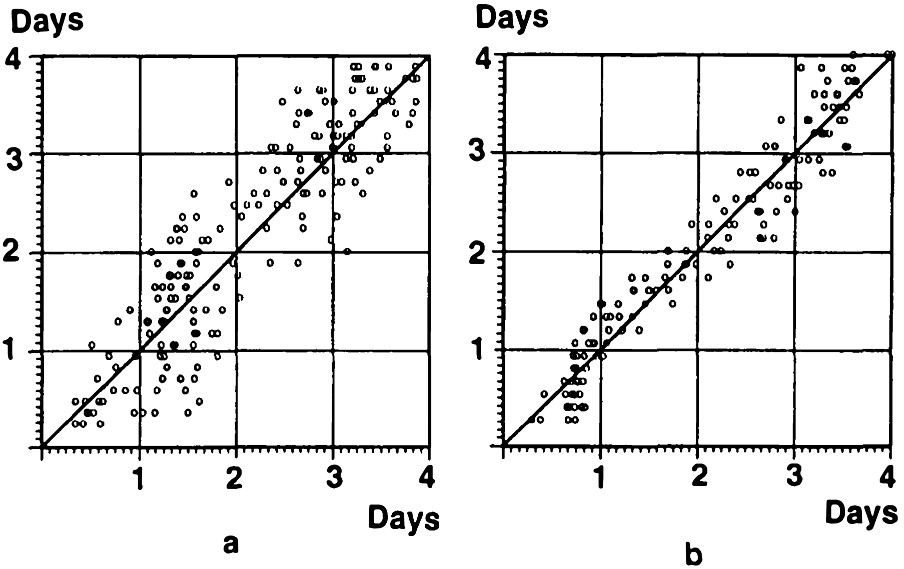


Figure 4 *Shrimps and cod roe : predicted versus actual storage time. Predicted storage time, horizontal axis; actual storage time, vertical axis; (a) shrimps; (b) cod roe.*

A three layer network was also trained to recognize the storage time and the two classes 'cold' and 'prewarm' samples. The configuration of the net was similar to the previous case, but the output consisted of two units, corresponding to the two classes. The net was allowed to cycle 350 times (making a learning time of 40 minutes). It turned out, however, that the predictions of test data were difficult to interpret. However, a general trend was that predictions made with the test data corresponding to short storage times were rather poor, but improved as the storage time increased. Predictions made with test data corresponding to the last day of storage times were, in general, more reasonable.

4.3 Classification of Cheese

The sensor array consisted, in this case, of eight gas sensitive field effect devices and four Figaro type sensors. Samples of ten different kinds of cheese were stored at room temperature for 40 h. After the storage time the headspace gases in each beaker were analyzed by passing the gases over a sensor array for 60 seconds. The response of each of the sensors in the array with clean air was stored and used as a reference for a given

sample. One measurement from each of the beakers was used for the training of neural networks involving all or a selection of the sensors in the array. Table 2 shows the result for a network with twelve input nodes (the sensors), ten hidden nodes and ten output nodes (the different cheeses). It was observed that the predictions were quite good. Apparently, the 'nose' had difficulty in predicting only one of the cheeses. Other networks involving all the sensors gave similar results but the number of correctly predicted cheeses varied. It was found that a smaller network (array) consisting of only six FET devices made a correct prediction of seven out of the ten cheeses and that the four Figaro sensors alone made a correct prediction of three cheeses.

Table 2 *Summary of the Results for the Identification of Cheeses*

Cheese type in test set	**Relative output values from the different output nodes. The sum of the outputs normalized to one for each set.**[1]									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	**0.80**							0.20		
2		0.30				0.02			**0.67**	0.01
3			**1.0**							
4			0.07	**0.72**		0.13		0.05	0.03	
5					**0.94**	0.05				
6				0.12		**0.78**		0.02	0.07	
7							**1.0**			
8			0.01	0.04				**0.94**		
9		0.20				0.09			**0.88**	
10					0.06				0.04	**0.89**

[1] only the most significant numbers rounded off to two decimals are shown.

Cheeses:				
	1	Svarta Sara, Denmark	6	Brie Mareillat, France
	2	Gouda, Germany	7	Brie de Meaux, France
	3	Havarti, Denmark	8	Vachero, France
	4	Gorgonzola, Italy	9	Torparost, Swede
	5	Danablue, Denmark	10	Sheep cheese, Bulgaria

5 CONCLUSIONS

This paper has described how an 'electronic nose' configuration based on a combination of field effect devices with other sensors can be used to classify and control various types of food from its odour. The results show that it can be used not only to determine the storage time of ground meat when kept in a refrigerator, but also its origin (beef or pork). The

storage time for shrimps and cod roe (also kept in a refrigerator) could also be estimated. Furthermore, in some cases it was possible to determine if samples had been warmed to room temperature for a short while (three hours) before being placed in cold storage.

The study also shows that there may be an optimum choice of sensor array composition for a particular application. It is therefore interesting, but not surprising, that the most discriminate sensors were found in three different classes of chemical sensors, namely gas sensitive metal-oxide-semiconductor field effect devices, semiconducting metal oxide devices (Taguchi-sensors) and carbon dioxide monitors. The wide choice of sensors probably optimizes the selectivity patterns necessary for satisfactory analysis of the gases which evolved from the meat. Clear differences in the accuracy of the predictions were found for pork, beef, cod roe and shrimps. We believe this is a consequence of the degree of complication in microbial population of samples, the greater the variety in the population giving a more difficult pattern to analyze.

The 'electronic nose' developed at our laboratory has been used in other situations of practical interest for food application; such as the classification of grain quality, the determination of odours from paper based packing material for food, the classification of various strains of bacteria *etc.* At our laboratory, a small and portable electronic nose, 'a nose to go' has also been developed . This could be used for field studies directly in the factory or in shops.

We have also observed that changes in the sensitivity and selectivity patterns of the sensors in the array do not cause any problems in a large number of the applications tested so far, over a period in the region of 10-30 days during a particular set of experiments, . We believe, however, that one important issue regarding 'electronic noses' is to find an efficient recalibration routine which can be used at the site of installation.

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Biosensors for Food Analysis: Perspectives

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1 INTRODUCTION

The preceding chapters have explained the requirements of food analysts, provided summaries of the various types of biosensors and described both current research and commercially available instruments. In short, it is true to say that the range and availability of instruments commercially does not match the promise which biosensor technology would appear to offer the food industry. This chapter discusses possible reasons for this mis-match and proposes a method of achieving more successful results.

The development of biosensors for the food industry has to be considered in the context of why food analysis is undertaken. The multi-billion dollar global food business has developed as a result of industrialisation; it provides wholesome and safe foods for its customers. It now produces, transports, processes and stores what is often a highly perishable commodity before it is consumed. The nature of the business does not give a high margin return: costs in all parts of the industry are constantly under scrutiny. Food analysis, therefore, is used to ensure the maintenance of the wholesomeness and safety of foods *en route* to the customer and to assist in minimising losses of the perishable materials. It is also a means of ensuring adherence to legislation in the country of sale.

A great number of tests has been in general use for many years or has been developed to ensure compliance with legislation. For new tests to be adopted by the food industry, the industry needs to have a high level of confidence that the novel test will match the results of existing methods or will be accepted by the government agents and the courts of law. This is a tough challenge and for this reason many of the simple routine analyses in the industry are comparatively old because they are tried and tested. Sophisticated tests have generally been developed by government agents or scientific disciplines outside the field of food research such as clinical science and adopted to ensure compliance with legislation. In the current financial climate the food industry normally invests in the development of tests which identify critical safety issues, which confirm compliance with legislative requirements or which will provide individual companies with a competitive advantage. The development of other types of tests are left to researchers, the instrument industry and government agents.

Biosensors fall into the 'other types of tests' category for a number of reasons. Although the end product is simple to use and its potential value is recognised in some

parts of the industry, it is not a simple technology to understand and there is a suspicion that the result may not be reliable. Early experience with the modification of instruments designed for clinical use has not met the expectations of the 'quick fix' raised in the food industry. If the instrument is unreliable the consequence may be the consumption of unsafe food or food which fails to meet legislative requirements. That seed of uncertainty is a major cause of caution. This may also be because the food industry commonly has much experience with less sophisticated technology and does not usually invest in instrumentation development as is common in the field of medicine.

The complex field of biosensor technology is an everyday experience for instrument developers but is a foreign language to those in the food industry. Instruments are developed as a result of a multi-disciplinary collaboration. In the majority of cases this collaboration includes experts in a small selection of the available range of options. The teams seek applications for their complementary set of technologies. The high returns expected in the field of medicine allow a number of such teams of university researchers and instrument manufacturers to approach the same problem with a different set of technologies. The best option or options for the application emerge through trials with proto-type instruments. This approach has served well in meeting the requirements for clinical tests where very large numbers of single analyte tests are commonly carried out on blood or urine. Clinical practitioners are familiar with more sophisticated tests and readily accept the use of this type of technology if the benefits are recognised. Further refinement of sensors is often carried out by the users.

In contrast, the food industry commonly tests for arrays of analytes in a large diversity of materials and sophisticated technology is not widespread. The major difference between clinical tests and food tests is that with the former a large number of the same tests is carried out on the same materials (blood and urine) and with the latter a large number of the same tests is carried out on a wide range of diverse materials (for example vegetables, alcoholic beverages, frozen prepared meals). Once a clinical test is developed for an analyte and is adapted for used with blood and urine it can be made available to all hospitals and general practitioners. Once a food test is developed it needs to be adapted for use with a large range of foodstuffs before it can be made available to diverse users which may include farmers, processors and retailers.

The returns expected from the development of biosensors for the food industry may well be similar to those experienced in the area of medicine but there are many more problems to be solved along the way. In addition, the range of expertise required in solving those problems appears to be greater than that available in the teams currently addressing the problems. It would appear that in the field of food analysis it is far more important to understand the problems to be solved at the beginning, identify likely solutions and select those technologies which might provide those solutions, and then develop the instrument. The current approach appears to be that a team of biosensor developers with a given set of technologies and expertise that tries to meet an apparent need of the food industry. Once a proto-type is developed those with food analysis expertise evaluate the instrument and try to adapt it to the actual needs of the industry. However, in many cases too many design elements have been already decided which limit the options for change. This causes a high

level of frustration in both the instrument developers and the food analysts because neither can satisfy the other's needs at this stage of development.

This chapter will highlight the current status of the development of biosensors for food analysis and identify the issues facing food analysts. Conclusions from this discussion will be drawn with some recommendations for future developments. It is hoped that this will stimulate both food analysts and biosensor researchers to reach a more accommodating understanding and thus lead to new instruments being made available to the industry.

2 BIOSENSOR TECHNOLOGY AND FOOD ANALYSIS

There are four areas of investigation: recognition of the analyte; the transduction of analyte recognition into a signal used to produce a simple, readily readable result; sample diversity; development of instruments for users.

2.1 Recognition of analyte

The recognition reaction between the biological component and the analyte (e.g. protein, sugar) needs to be converted into a continuous signal which is proportional to the concentration of the analyte. This means a specific constituent is identified and quantified; whether a nutrient (e.g. vitamin A) or contaminant such as a single chemical (e.g. a pesticide) or group of chemicals (the aflatoxins) or microbial species (*Listeria monocytogenes*). The two basic types of recognition used in biosensor analysis are catalysis (e.g. enzymes and bacteria) and affinity (e.g. antibodies). Biosensors using enzymes and antibodies have been developed as researcher's instruments and some as commercially available instruments. Enzymes such as ascorbate oxidase, glucose oxidase, lactose oxidase and choline esterase can be used to measure vitamin C, glucose, lactose and pesticides, respectively.

Antibodies can be used to detect food constituents such as vitamins and soya or contaminants such as aflatoxins, pesticides (Chapter 9) and a large range of bacteria and mould as demonstrated by the number of enzyme-linked enzyme immunoassays which are commercially available. Other affinity recognition techniques using DNA and lectins are available but their use not as widely developed.

2.1.1 Enzyme biosensors. There are over 2500 commercially available enzymes which are readily available for use in biosensors. The advantage of enzymes for analyte recognition is their degree of specificity, even to the level of recognising different chiral forms as shown by the differentiation between L- and D-lactate (Chapter 9). Immobilization of enzymes improves both coupling to transducers and their stability during storage and use (Chapters 2, 5, 6 & 12).

An additional benefit of catalytic analysis is the inherent amplification of enzyme turnover. Enzymes bind to a 'substrate' molecule and transform it into a 'product' molecule which leaves the binding site. The enzyme is then free to bind another substrate molecule

and the cycle continues until an equilibrium is reached between the concentration of substrate and product molecules. This biological form of amplification reduces the need for electronic amplification after transduction because a large enough signal is generated to be converted into the final result. When analysis is complete the enzyme does not need regenerating before the next analysis. It may be necessary to expose the enzyme to a 'cleaning solution' (a solution with no analyte present) to return the instrument response to a stable 'zero' base line.

2.1.2 Affinity sensors. As with enzymes, a large number of antibodies is available and their specificity is similar to that of enzymes. Compared to the research on enzyme biosensors there is less breadth and depth of understanding for the construction of affinity biosensors using antibodies and much less for those using DNA and lectins. The majority of experience in developing antibody based biosensors is founded on improving the analytical convenience of immunoassays. The affinity biosensor has focused on quantifying analytes such as pesticides (Chapter 9), African swine fever (Chapter 14) and microbial contamination using both antibodies and DNA (e.g. at Cranfield University, not cited). Detection of low concentrations has been demonstrated with atrazine, a pesticide, which can be measured at parts per billion (ppb) levels (Chapter 9). This is sufficient for most trace analyses of chemicals, toxins and bacteria. However, typical pesticide, toxin and microbial contamination detection there is a requirement for initial screening tests to detect the presence of a range of analytes rather than tests which measure individual analytes.

Monoclonal antibodies (a mass of identical antibodies reacting to a single analyte in the same way) are often preferred by analysts because they have well defined affinity characteristics and are highly specific but it is more labour intensive to obtain them (Chapter 4). Polyclonal antibodies (a collection of different antibodies all reacting to the same analyte in different ways) have less well defined affinity characteristics, but often have a broader range of specificity and production is less labour intensive. The principal difference is that affinity rate constants for monoclonal antibodies are simpler to interpret than those for polyclonal antibodies. The dilemma facing the affinity biosensor developer is whether to use monoclonal antibodies which are analytically preferable but costly and labour intensive to produce or to use polyclonal antibodies which are analytically less preferable but easier to produce at a lower cost. In comparison to commercially available enzymes for catalytic biosensors, it is more difficult to obtain the antibodies for the affinity biosensor. One of the contributory factors for the lack of research data being available for affinity biosensors is that there is a less detailed understanding of antibody binding mechanisms when compared to the knowledge of enzyme reaction mechanisms.

Unlike enzyme reactions affinity binding requires a means of amplifying the binding reaction and regeneration of the affinity molecule. An antibody binds to one molecule and detection relies on the presence of sufficient bound analyte to produce a large enough signal to be converted into a readable result. Amplification of the signal is more important because the reaction is not continuous and a variety of strategies, over and above that of electronic amplification, have been developed to achieve this. One method is to detect the affinity reaction using fluorescent or bioluminescent chemicals which produce large optical signals per unit mass. Alternatively, coupling the affinity reaction to an enzyme enables production of a continuous signal in the presence of a substrate. Once the analysis is

complete then the next analysis requires regeneration of the affinity molecule or the availability of a fresh affinity molecule. The detector surface can have the analyte stripped away so the antibody is regenerated for the next analysis ensuring that it retains its ability to bind to the analyte. Another approach is to immobilise a sufficient excess of affinity molecules for a number of analyses to be performed using the same surface without the need for regeneration to take place.

The range of antibodies available (for bacteria such as Salmonella, toxins such as aflatoxin, meat species, soya, gluten, pesticides, etc.) has been mainly used to develop immunoassays which have the characteristics of wet chemical analyses with the concentration measured by colorimetry. The development of the Pharmacia BIAcore and similar biosensors for clinical analysis provides the basis for food analysis techniques in this format.

Expertise in developing affinity biosensors is readily available but this technique is more difficult than enzyme sensors due to the need for amplification of the recognition reaction and for regeneration of the recognition molecule.

2.2 Transducers

Many suitable transducers enable the translation of the biological recognition reaction into a measurable signal, consequently the form of translation can be matched to the analytical requirements and to the convenience of the application (Chapter 2). Amperometry is popular because it is simple. The hardware is very easy to mass produce making it widely available for instrumentation, especially as chemical mediators between enzymes and electrodes have been widely established. However, the search for the most efficient electron transfer mediator is still a matter of research (Chapter 2). An advantage of amperometry is its low sensitivity to variable levels of oxygen in the sample; this can be important with a number of enzymes. Coulometry requires no calibration: the total charge is independent of reaction kinetics and is suitable for capillary fill devices which provide a small volume for a measurement to be taken in a reasonable time. Potentiometry in general is costly and gives poor performance. Ion selective electrodes, based on potentiometry, have in the past proved unsuitable for food analysis because of the presence of interfering constituents but there is now a broader range of electrodes which can offer a high degree of specificity. The field of optics has advanced rapidly and offers a variety of options which can be independent of electrical interference. Developments in optical gratings to measure refractive index has increased the range of possibilities and the development of silicon-based optical chips should lower the cost of this type of component. Piezoelectric mass measurement is simple but a more recent development. Thermometric transduction is also a new development; however, it is important to take sufficient precautions to prevent thermal interference.

2.2.1 Enzyme biosensors. The most commonly used transduction mechanism for enzyme biosensors is the measurement of oxygen or hydrogen peroxide which is commonly consumed or produced during the enzyme reaction (Chapter 3). The enzyme is immobilised on a membrane (Chapter 3), sandwiched between two membranes (Chapter 8) or attached directly to the electrode surface (Chapter 12) using techniques which allow the

reaction to take place. The gas is depleted or generated in an area around the enzyme and detected by the electrode, normally located very close to the enzyme. When oxygen is measured it is important that variations in oxygen concentration, due to diffusion or to consumption by irrelevant reactions in the sample mixture, are controlled and prevented from interfering with the biosensor measurement. The control of interfering chemicals such as protein, ascorbate (Chapter 12), halide and nitrite ions (Chapter 16) is also important. The commercially available Yellow Springs Instrument (YSI) biosensors are based on the measurement of peroxide. There is a large body of experience in using this form of transduction for producing commercially based instruments. These instruments are designed around flow injection analysis and although compact and easily transportable they are not pocket sized instruments.

Research data concerning amperometry transduction are also readily available, often based on chemical mediators (e.g. ferrocene derivatives) which couple the enzyme to the electrode (Chapter 2). This is the basis of the pocket sized Medisense glucose sensor which is now widely available to diabetics (Chapter 2). There is, therefore, expertise in the development of research instruments and their transformation into miniaturised sensors which are commercially available. However, there are large differences in the problems which occur when analysing food and when analysing blood from different patients; e.g. sugars in soft drinks and cake compared to blood sugar measurement. Despite this, the transition from medical to food analysis biosensors can be made. The principle of the Medisense glucose sensor transduction mechanism has been used to develop the prototype knife sensor for bacterial load estimation on the surface of meat and the sterilisable glucose biosensor for monitoring glucose in fermenters (Chapter 2).

2.2.2 Affinity biosensors. Chapter 4 describes a commercially available device using optical transduction based on surface plasmon resonance. The affinity molecule, in this case an antibody, is immobilised on a surface and will 'capture' analyte present in the sample. There is a significant body of research knowledge at Pharmacia concerning the development of this type of affinity device and making the technology commercially available. Currently, only clinical diagnostic applications for blood samples have been developed. Other common transducers for affinity systems are based on electrochemical, calorimetric and acoustic signal generation. The TNO Nutrition and Food Research Institute (Holland) has also used the optically based surface plasmon resonance transduction system in a flow injection analysis format for detecting African swine fever and pesticides (Chapter 14). Piezoelectric crystal transduction is another means of detecting affinity reactions at very low concentrations of analyte, for example atrazine (Chapter 9).

2.3 Sample diversity

The majority of biosensors developed for food analysis are water based systems. However, Cranfield University (Chapter 2) and others have developed a biosensor format using an enzyme recognition system which works in organic solvents to measure phenol vapours, sulphur dioxide and methane. In principle, therefore, analytes soluble in both water and fatty substances can be measured with biosensors.

The commercially available biosensors from Yellow Springs Instruments, all water based analysis systems, have been shown to be useful with a wide range of foods (Chapter 8). This includes raw materials such as cereals, vegetables, and aspartame; a wide range of finished products such as: ice-cream, peanut butter, luncheon meats, cheese, whey, beers and wines, infant formulae, food sauces, salad dressings, seasonings, and soups; as well as effluent. The research papers have also demonstrated their use with a further range of foods in addition to those mentioned: raw materials such as potato, cereals, milk (from skimmed to condensed), raw meat, baked goods, honey, jam, rapeseed, syrups, molasses, flour, malt drinks, wine (Chapters 2, 3, 9, 10 and 12-15). They can also be used in and monitoring processes such as fermentation (Chapter 2), ensuring optimum sausage formulation during manufacture (Chapter 14) and environmental samples such as river water (Chapter 9). This evidence illustrates the promise that biosensors have for food analysis.

Yellow Springs Instruments require only simple sample preparation and have a facility for automation of sample handling (Chapter 8). This moves analysis away from the time consuming and labour intensive nature of traditional techniques. The new membranes developed for YSI instruments will reduce the number of inaccurate results due to the many interfering constituents present in food (Chapter 8).

2.4 Development

Development of biosensors for food analysis has a number of attractions because extreme miniaturisation is unnecessary, unlike the requirements for clinical applications. Destructive testing is tolerable and continuous measurement is unnecessary. Accuracy and precision is a variable requirement (± 10% now is often preferable to 0.1% tomorrow) and there is consumer and regulatory demand for testing. This provides a solid basis on which existing technology already available in the field of medicine can be further developed. However, there are difficulties to be overcome. There are a large range of food analytes. There is a common requirement for sensors which detect more than one analyte. In addition, there is a perception that novel instruments for food analysis must be low cost because of the current availability of industrial food engineers and technologists with vast experience makes process monitoring largely unnecessary (Chapter 2). This may change in future as the number of skilled operators is likely to diminish.

The initial problem for commercially available instruments was to develop a biological recognition element which had a reasonable period of use. The two approaches adopted were: (i) making the cost of the biological element (e.g. an enzyme membrane) low enough to make it disposable; (ii) to develop immobilisation techniques which increase the biological element lifetime in use to be long enough for the cost to be acceptable. Both approaches have been taken by YSI. The latter approach has been the subject of research at the University of Leeds and the achievements have been adopted by YSI to improve enzyme based biosensors (Chapters 5 and 6). The use of artificially constructed enzymes, 'synzymes' is an additional solution to the problem of enzyme instability; for example the lipophilic amide of trifluoracetylaniline can mimic the activity of alcohol dehydrogenase (Chapter 2). Research papers record a range of immobilisation techniques which have been developed, although they are not always understood, and

experience has taught researchers which techniques are appropriate for different applications (Chapter 3).

Affinity biosensors have additional problems, the main one being the regeneration of the affinity reaction. A fixed number of affinity molecules are used for biological recognition so repeat assays require the analyte to be removed from the binding site whilst maintaining the binding activity. Pharmacia has developed a regeneration protocol in the BIAcore system which enables antibody binding to be regenerated after the removal of analyte. In addition, there is a sufficient excess of immobilised antibodies to compensate for small losses of antibody during regeneration so the analytical response remains largely unaffected. The need for regeneration can be eliminated by immobilising a sufficient number of affinity molecules to the detector surface so a large number of analyses can occur before they are all used up.

The conditions for optimum performance of the biological recognition element require a large number of interacting factors to be studied simultaneously. The effectiveness of the transducer coupling and the signal generation also require optimisation. Statisticians have identified a means of investigating a number of factors simultaneously with a reduced number of experiments. These techniques are based on methods such as those developed by Tagushi, sometimes referred to as a 'design of experiments' technique. Such methods are not widely utilised by the majority of researchers so the computer programme to aid researchers in the design of their experiments to optimise biosensor performance is a significant advance (Chapter 7). This should certainly shorten the development time of an effective biosensor and improve its performance.

To date, biosensor development for food analysis has been a piecemeal approach as a result of the food industry being interested in 'modifying' existing instruments designed for clinical analysis. There have been some partial successes leading to the availability of instruments from Yellow Springs Instruments and companies in Germany, with reports in food technology journals of commercially available instruments in Japan. Biosensor researchers and food industry users have both been disappointed with research results because achievements have not met the initial expectations identified by both parties. This has reduced the interest in funding research; it is not surprising, therefore, that the number of biosensors currently available commercially is low.

2.5 Acceptance, availability and use

Examples of different achievable biosensor formats have been demonstrated for food analysis which include the commercially available flow injection analysis approach (Yellow Springs Instruments and others) and the knife prototype sensor for microbial loading on meat and the steam sterilisable fermentation biosensor (Cranfield University) (Chapter 2). The antibody affinity biosensor from Pharmacia, developed for clinical analysis, could well be suitable for food analysis. These technologies are available; therefore the major question is: why has the use of these biosensors and prototype instruments not been more widely adopted? Is it because the analyses provided are not the most important? If this is the case then what are the priorities in food analysis which

should provide the thrust for developing instruments for which there is widespread demand?

Commercially available biosensors developed for food analysis are primarily those based on the Yellow Springs Instrument system. These include analysis for glucose and sucrose in cereals, baked goods, vegetables, peanut butter and ice cream; aspartame; lactate in cooked food and luncheon meats; alcohol in beers and wines; monosodium glutamate in foods; choline in infant formulae.

There are more developments in research laboratory instruments; a large number of applications concern sugar analysis and detection of bacteria but have also included a broad range of other analytes of interest in food analysis. These include bacterial contamination in milk (L- and D- lactate) and organophosphate and carbamate pesticides (total anticholinesterase activity) (Chapter 9); sugars in cocoa; glutamate in seasonings; isocitrate, acetic acid and gluconolactone in fermentation, fish freshness (putrescine, spermidine and cadaverine), essential fatty acids in oils; and lactose and butyric acid in milk; glucosinolates in rapeseed (Chapter 10). Instruments to measure sugars are commonly developed because enzymes for sugar substrates are readily available and are often more stable than other enzymes. The presence of constituents of food which interfere with the analysis thus giving false results is a common problem. False results with sugar analysis are often as a result of interference by ascorbic acid, protein and other sugars. This interference can be overcome with simple pre-treatments to improve the accuracy of results in foods such as ice-cream, Horlicks, flour, syrup, and skimmed, evaporated and condensed milk (Chapter 12). Biosensors have also been developed for lactate and malate in wine (Chapter 13), fermentation monitoring of glucose (Chapter 14) and lactate (Chapter 15) and for detecting the difference between fresh and frozen meat (Chapter 17). Combinations of enzymes can also be used to measure aspartame in the YSI system (Chapter 8) and in other sensors to measure starch in potato and corn, wheat and rice (Chapter 12). There is therefore wide experience in research groups around the world which have in depth knowledge of constructing sensors together with an understanding of the causes of poor performance; e.g. the effect of halide and nitrate anions on glucose analysis (Chapter 16).

Commercial aspects of biosensors include size, robustness, ease of use and costs. The Yellow Springs Instrument format is not a pocket device like a Sony Walkman radio cassette but it is as portable as a small microwave oven. It is robust enough to be sited close to the factory line but like computers it needs to be housed in a process control room to maintain sufficient cleanliness, and in an environment of controlled heat and humidity for efficient continuous operation. Analysis is rapid enough to produce results in minutes but is not instantaneous and a modest amount of sample preparation is required to bring the analyte into solution. Appropriate dilution of this sample can overcome the potential interferences of food components. Sample handling can be automated to increase the ease with which a large number of results can be obtained during daily use. It requires a basic skill level to operate and the enzyme membrane is simple to replace. For most food industry and government requirements this is a major advance over sending samples to a laboratory and getting results hours or days later, and it reduces the costs significantly. An example given in Chapter 8 is a choline test which traditionally takes 5 days at a cost of $85 per test, which can be reduced to 5 minutes at a cost of approximately $2. Similar

sensors have been developed in Germany (Sensomat B10 for alcohol; Microzym-L for lactate); the USA (Orion system for sugars, lactate and alcohol); and Japan (Glu-11 for glucose) (see Chapter 2). The lifetime of the active detection element in these systems varies but is becoming reasonably acceptable; changing biologically active elements once a month is tolerable as long as the disposable element is reasonably inexpensive. Other prototype commercial biosensors include the knife system for estimating microbial load on the surface of meat and the steam sterilisable glucose sensor which can be used for long periods (Chapter 2). In addition, the sensitivity of the enzyme sensing element in a fermenter can be protected when the sterilising regime is operated by retracting the probe from a microbially safe sheath.

3 CRITICAL ASPECTS OF FOOD ANALYSIS

The concerns of the food analyst are: food safety, cost effective manufacturing and compliance with legislation. These concerns involve similar analyses in a large number of different food matrices, often screening for analytes followed by confirmatory tests as well as confirming the presence of 'live' and problematic microbial species as opposed to those which are 'dead' or have no health or safety concern. The requirements for screening for analytes and the particular issues relating to microbial species are of particular concern to the food industry.

It is a common requirement to screen foods for a class of contaminants which comprise a wide variety of chemicals. For example, the primary question concerning pesticide analysis is: are there any pesticides present? Traditional analysis involves two screening runs using gas chromatography, one with a polar column and the other with a non-polar column. Once a pesticide is detected the presence of the suspect analyte(s) is confirmed using gas chromatography-mass spectrometry. Although this requires extensive sample preparation and sophisticated instrumental analysis it provides the answer to the question. To be of use the biosensor needs to screen the sample for the same range of analytes, this probably requires a battery of enzymes or antibodies to detect organochlorines, organophosphates and the carbamates. This is a complex task for a biosensor; in fact the enzymes or antibodies require a broad specificity which will bind to family groups of pesticides rather than specific chemicals. There are enzymes like acetylcholine esterase which is inhibited by organophosphates and carbamates (Chapter 9). In the case of antibodies, both monoclonal antibodies and polyclonal antibodies may be used.

For microbial species, it is only necessary to detect viable bacteria or fungi which can grow under the right conditions. These include: a minimum water activity, at the right temperature, and the absence of inhibitors such as acidity, preservatives or antibiotics. In addition, it is important to quantify the number of viable spores (dormitory bacteria) and viable bacteria, whilst ignoring those fragments of microbial species such as cell walls and DNA (the non-viable breakdown products of viable organisms) which still retain characteristics that can be recognised by affinity probes. Affinity biosensors based on antibody or DNA recognition can react with the fragments of disrupted microbial organisms. These fragments will not multiply to increase the number of organisms and will

not cause deterioration or safety problems with food. Biosensors which detect the organism may be unable to detect vegetative spores, which can also multiply under appropriate conditions to cause deterioration and safety problems. Spore surfaces are a different construction to the normal microbial organism and form a protective layer to cope with adverse conditions. Microbial species, including vegetative spores, may occur in small concentrations in the food, and under normal circumstances do not cause problems, but when storage conditions are abused the organism can rapidly multiply within a day or a few days to cause serious health problems. It is important to prevent the occurrence of false positive results and also to detect small concentrations within a large bulk material: in food this can be tonnes of material. The current solution to these issues is to take a large sample of food, homogenise it, filter the whole sample through an affinity column or membrane to concentrate the small number of microbial organisms and then put the filtrate on a growing medium to see if the bacteria grow and then identify which bacteria are present. With *Salmonella* this takes approximately 5 days. As shown previously with *Listeria* (Chapter 1), it is also important to identify *Listeria monocytogenese* as opposed to the other less harmful 6 family members which could be present.

These issues are best considered at the outset of designing a biosensor for food analysis and not once the major elements of the design have already been decided upon.

4 KEY ISSUES

It is clear from this symposium that technology and experience for biosensor development is readily available in the research community and the instrument industry. The key issues fall into three categories: instrument development, sample diversity and commercially important analyses.

4.1 Instrument Development

There are six stages of sensor development which need to be recognised by the parties involved:

a) identify the target analyte and target foods;
b) identify the critical user issues for the target analyte and target foods;
c) develop the analyte recognition and transduction system;
d) overcome the problems with the target food matrices;
e) address the critical user issues in format design;
f) refine the commercial aspects of the instrument.

Identifying the target analyte and target foods and identifying the critical user issues for the target analyte and target foods are very important for a successful outcome (stages a and b). If the focus of the system is not identified at the start a successful application is very unlikely. A review of the issues facing the measurement of a target analyte in the target foods will identify the critical requirements for the analysis. These critical requirements will have an effect on the choice of biological recognition system (type, specificity and target range); the coupling system to the transducer; the type and form of

transducer and the overall design of the instrument. It would appear that a large number of criticisms of current biosensor systems is based on biosensors being modified to fit the analysis rather than integral design elements addressing the critical issues of the analysis. It may mean that food analysts need to revise their requirements to meet the actual needs rather than basing their needs on current assumptions. In addition, it may mean that biosensor technologists should change their approach to overcome the issues rather than to modify their current systems to carry out yet another analysis.

Developing the analyte recognition and transduction system and overcoming the problems with the target food matrices (stages c and d) require the application of the extensive experience of biosensor technologists in selecting the appropriate elements of the system, optimising the performance of the individual elements, constructing the basic design and addressing the food matrix effects which would cause false positive and false negative results.

Addressing the critical user issues in format design (stage e) is as important as identifying the critical user issues for the target analyte and target foods (stage b). Stage e will follow up stage b and identify the most important requirements for validation and general adoption of the test by the user. This will involve critical re-evaluation of the design to ensure it satisfies the user requirements. Refining the commercial aspects of the instrument utilises the expertise of instrumentation companies in design, electronics and microprocessing technologies.

A large number of biosensor developments begin after scant attention is paid to stage b. This means that two issues have not been addressed. The first is that biosensors are built with convenience for the designer as the prime concern and not convenience for the user. In other words, the compromises made in design relate to biosensor operation and not with the primary purpose in mind, that of food analysis. Secondly, the pragmatism of food analysis over the years is not taken into account. The implications of the first issue are self evident. The second is not so clear to those outside the food industry. There are many aspects to this; however, two examples will suffice.

Precise analysis is time consuming, requires significant expertise and is expensive. Shorter, more convenient, lower cost analyses which have been developed over the years are often less precise but the results, although compromised in analytical terms, have been related to manufacturing practice. Within the industry these results are relied upon for commercial judgements; examples include: wet chemical analyses for reducing sugars and oven techniques for measuring water content, which is used as an approximation for water activity. Methods developed to replace these existing analytical methods and which provide accurate results cannot easily be related to years of commercial experience with the existing methods and their approximations. Therefore convenience of analysis and increased precision are at odds with each other. Continuing with the inconvenience of current methods is therefore more acceptable in commercial practice than adopting new methods which are an unknown quantity.

Secondly, there are many analytical requirements for screening tests with precise results. The accurate quantification of a single pesticide in any food is largely irrelevant.

Legislation lists a large number of maximum residue limits (MRL's) which have to be met. It is necessary to be sure that all the levels are met; anything less than an accurate screening test for pesticides is regarded as useless to anyone but the farmer, who sprays the crops. However, as he prepares the spraying solution, his needs are limited to those of verifying the solution concentration and of verifying the dosage on the crop. It is important that there is confidence in the results of the analysis because if the result is wrong there could be legal consequences. For similar reasons there is a requirement for screening tests to detect microbial organisms but in this case the consequences of getting this wrong can be fatal.

In the majority of cases, therefore, biosensor developer needs to pay greater attention to the issues which concern the user. It is the user who has the experience necessary to address these issues and not the biosensor developer. The user does not have the resources to make the experience available to the instrument manufacturer because analytical development is not the food industry's business. Moreover, the industry has experience of instruments being developed for food analysis which have not met expectations and therefore is highly sceptical about new instruments. This is the crux of the problem which needs to be resolved.

4.2 Analysis

The harvest and manufacture of food products require estimations of analyte concentrations in large quantities of material or product. Estimates are required for concentrations of analyte in tonnes of material and the biosensor works best when it is presented with milli- to micro-grammes of sample to analyse. In addition, the same analysis needs to be carried out on a variety of foodstuffs. A solution to these problems could be a two-stage process for the biosensor analysis.

The first stage is a sample handling system that can reduce kilogrammes of material to a homogeneous solution / colloidal suspension at the right dilution for a sample of milli / microlitres to be taken for analysis. In the case of microbial analyses, this may have an additional filtering / affinity stage to ensure that any organisms present in the large sample volume are isolated. The second stage is the biosensor analysis of a standard sample prepared by the sample handling system. This type of biosensor format can be optimised to handle samples from a wide variety of food sources. The sample handling system would provide two major benefits: samples can be pre-treated to improve the uniformity of the constituents in solution prior to analysis; secondly, the food industry and government agencies would have greater confidence in results from the samples because they are representative of the bulk material.

4.3 Commercially Important Analyses

Biosensor research scientists and instrument companies are eager to understand the priorities of the food industry for food analysis. This priority list is thought to relate directly to the commercial value of the instruments developed to make those analyses. Table 1 is a provisional short list which may provide some of the analyses of importance for food. Suitable candidates for biosensor analysis of general appeal include: the pathogens, pesticide screening, limited general microbial screening such as Total Viable

Count, yeasts and moulds, sugars and acidity. It is crucial that the issues and concerns of the food industry are clearly established and addressed in the selection of analysis strategies and that the instruments are designed with the user in mind. It may be very important to use the two-stage analysis process described in the previous section. Both of these just take a few minutes, thus providing a rapid analysis. Such a two-stage analysis system, once established as reliable, could then be used to develop other analyses of interest to more specific sectors of the food industry. These include analyses for: alcohol, starch, aspartame, flavours and preservatives such as sulphite, nitrite and benzoate (Table 1).

Table 1 *Commercially Important Analyses*

Analyses of General Interest to the Food Industry	
Pathogens :	particularly *Samonella, Listeria, Escherichia coli* 0157, *Clostridium botulinum*
Microbial screening tests	particularly Total Viable Count, yeasts, moulds, faecal coliforms, Bacillus cereus, Staphylococcus aureus
Pesticides	Total screening test
Moisture	Total water and water activity
Sugars	Individual sugars and reducing sugars
Acidity	
Nutritional values	
Proximate analysis	analyses such as protein, minerals, carbohydrate, fibre
Analyses of Specific Interest to Sectors of the Food Industry	
Alcohol in beverages	Needs to be precise because taxation is levied
Fat content	In dairy foods
Starch and fibre	Cereals
Aspartame & sugars	Soft drinks
Preservatives	Soft drinks, chilled foods
Flavours	Specific analyses for specific companies

5 CONCLUSIONS

The evidence provided by the contributors of this symposium confirms the promise for biosensors in food analysis. There are commercially available biosensors but the current appeal is limited and they have not been widely adopted by the industry. The major reasons for the poor progress in development is the lack of understanding found in the food

industry concerning biosensor technology and the lack of understanding in the biosensor community concerning the requirements and conditions of food analysis. In general biosensor formats are being devised for other purposes such as clinical analyses and then being modified for food analysis. This has limitations because the problems encountered in clinical or environmental analyses and those encountered in food analyses are very different. It would appear that a clearly focused approach needs to be adopted for developing biosensors for food analysis.

To be most effective, development of biosensors for food analysis requires a multi-disciplinary approach (Chapter 2). No one institution or instrument company has all the high quality expertise required. This includes: food knowledge across the disciplines of food science, technology and macro- and micro-analysis for all aspects of cultivation, processing and retail; biochemistry; electrochemistry; optics; electronic and general engineering; design and fabrication; micro-processing. This appears to be a daunting task. However, the way forward out of this apparent impasse would be for food analysts and biosensor developers to collaborate and adopt the following strategy:

1) select a short list of opportunities (as in Table 1);
2) identify the critical user requirements for these opportunities;
3) evaluate the technologies available and identify those which would appear to be suitable for the shortlisted opportunities;
4) identify the potential problems that are likely to be encountered in the analyses;
5) design analysis strategies and compatible biosensor formats which would overcome these problems;
6) develop laboratory models for the elements of the designs to prove the principles identified in (5);
7) design an instrument putting together the elements which proved successful in (6);
8) provide the proto-types developed in (7) to food industry analysts to evaluate the performance.

An integral part of the initial strategy for successful biosensors for food analysis would appear to be the adoption of a two-stage process; sample preparation, then analysis. Sample preparation would include homogenisation, bringing the analyte into solution or colloidal suspension and appropriate dilution; attention may need to be focused on resolving the particular issues related to microbial detection in foods. The second stage would be the biosensor analysis of the solution after sample preparation. There is no reason why both stages should not take less than five minutes and be coupled together if speed is an important requirement.

In summary, therefore, biosensors have a future in food analysis so long as the food analysts and biosensor researchers can develop a common understanding, communicate freely, set the priorities and work together. The question is, will this happen?

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