Session 8
The Future

Chairman and
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THE IMPACT OF MOLECULAR BIOLOGY ON IMMUNODIAGNOSTICS

R A BADLEY
Unilever Research, Colworth Laboratory, Sharnbrook, Bedford, MK44 1LQ, UK

ABSTRACT

To date the impact of modern molecular biology on immunodiagnostics, including both nucleic acid and protein facets, has been minimal. The historically observed lag between scientific developments in this area and significant, usually commercial, exploitation suggests that within five years the fruits of antibody engineering, antigen understanding and combinatorial libraries for both antibodies and antigens should begin to be seen.

INTRODUCTION

Immunodiagnostics exploits the natural interaction of antibodies and antigens - the two halves of the diagnostics equation. Commercial exploitation requires the successful addition of detection of binding and readout, often referred to as delivery or format.This paper focusses on the principal elements, antibodies and antigens, but the importance of the delivery must be kept constantly in mind during any such discussion. Choice and behaviour of these components in assays are both very frequently determined in crucial ways by the other elements of the assay "system". For example, the adsorption of antibodies to plastic surfaces can result in loss or retention of binding activity depending on both choice of antibody and plastic. Indeed, and this may be relevant to an embryonic plant immunodiagnostics arena, there have been too many ventures in the much more developed medical area, especially springing from academic enterprises, which have produced interesting antibodies and which have failed through a lack of appreciation of the importance of "delivery".

In looking at likely developments for antibodies and antigens I will draw on the full range of applications so far. There does not seem to be any intrinsic reason why lessons from medical diagnostics cannot be applied to plants. There will be no attempt to be comprehensive, rather, areas of high enterprise and potential will be highlighted and implications drawn where possible. Some key references, usually pertinent reviews, will be given.

What is "molecular biology" in relation to antibodies and antigens? I will take it to mean the consideration of these entities in molecular terms and their manipulation in ways understood in such terms also. This, of course, includes nucleic acid and genetic based information, but not exclusively. As we shall see, protein and peptide structures are becoming increasingly important also.

An historical perspective.

Immunodiagnostics can be said to have emerged as a new analytical discipline in the 1970's. It resulted from developments in antibody production and in inventive formats. The latter often involved labelling either antigen or antibody with some form of detectable tag.

The polyclonal antibodies initially available, were followed, but not entirely replaced by, monoclonal antibodies. We are now considering the likely impact of new antibody based binding molecules arising from molecular biology approaches.

In parallel, new assay principles were being elaborated, such as competitive and immunometric formats. Based on such considerations Freytag was moved to write in 1991 that his "first prediction about the future of immunodiagnostics.....if the fundamental discovery has not taken place today, then we shall not see widespread utilization of the technology before the year 2000". This prediction was based on the historical record of the product development cycle in this area. Some of the evidence is presented in an updated and expanded version in Table 1.

Table 1. Some key events in the evolution of immunodiagnostics and the time delay from discovery to widespread use.

Technology	Earliest paper	commercially available and successful	Ref.
Polyclonal antibodies	pre-1950	pre-1950	-
RadioImmunoAssay (RIA)	1959	early 1970's	1
Enzyme Labelled Immuno-Sorbent Assay (ELISA)	1971	early 1980's	2,3
Enzyme Modulated Imunoassay Technology (EMIT)	1971	early 1980's	4
Fluorescence Polarization ImmunoAssay (FPIA)	1961	early-mid 1980's	5
2-site immunometric assay	1970	mid 1980's	6
Monoclonal antibodies	1975	mid 1980's	7
2-site simultaneous immunometric assay	1979	mid 1980's	8
Cloning and expression of antibody genes	1984	when?	9-11
Antibody fragments expressed in *E. coli*	1988	when?	12

References. 1 (Yalow and Berson, 1959), 2 (Engvall and Perlmann, 1971), 3 (van Weeman and Schuurs, 1971), 4 (Rubenstein and Ullman, 1971),5 (Dandliker, 1961), 6 (Habermann (1970), 7 (Kohler and Milstein, 1975), 8 (Davis and Porter, 1980), 9 (Morrison *et al.*, 1984), 10 (Boulianne *et al.*, 1984), 11 (Neuberger *et al.*, 1984), 12 (Skerra and Plückthun, 1988)

Thus we can have some ideas about the newly available components arising from the ability to clone antibody genes. First attempts used available monoclonal antibody producing cells as the source - one clone giving one set of genes for a single specificity. These have been turned into a variety of new molecules, some small e.g. binding domains and others larger e.g. combined with other molecules such as enzymes. More recently, the genetic material from whole collections of cells, such as spleen or blood lymphocytes, has been turned into libraries of potential antibodies.

The impact of new molecules, so far.

Despite much activity over the last five years or so, Table 1 suggests we should not expect significant exploitation of new antibody molecules just yet. This is indeed the situation. There are no really significant commercial uses of genetically engineered antibodies for immunoassays. As for antigens, the basic scientific discoveries have mostly been too recent to allow for significant exploitation so far.

ANTIBODIES AND THEIR DERIVATIVES

The genetic engineering of antibodies is based on the complex manner by which the final antibody protein structure is pieced together from the genetic building blocks. In particular the binding site is made up from two chains, V_H and V_L, derived from the heavy and light chains of the whole antibody. Within each of these there are three hypervariable regions and interposing less variable sequences. The overall structure of the antibody, the way it has been traditionally broken up by proteolytic enzymes and the genetically engineered fragments that can be made are shown in Verhoeyen and Windust (1995), a recent review of advances in antibody engineering. Of particular importance here are the structures based on the variable fragment (Fv). This is the smallest unit containing all the binding site structures of the antibody although there have been some rare examples of smaller units, especially V_H showing significant binding activity themselves. The V_H and V_L chains can be unlinked or linked. If the linker is engineered as a short peptide chain, such units are referred to as single chain (sc) Fv's. The antibody engineer now has the capability to take all these various pieces and put them back together again, more or less in any way desired.

Fv antibody fragments (Verhoeyen and Windust, 1995)

By far the most work has been done on Fv's and their derivatives, compared to other possible combinations of pieces of the antibody structure. Whilst Fv antibody fragments can be grown in mammalian cells, the preferred route has been in *Escherichia coli*. There has been much hard work done to try and control and increase the levels of Fv expression. It is probably the case now that, because diagnostic manufacture requires relatively small amounts of Fv, sufficient can be made even for a substantial commercial assay application using current technology. However expression is becoming possible in a wider variety of organisms. Thus, *Bacillus subtilis*, *Staphylococcus carosus*, yeasts and fungi have all been used successfully. Even higher plants are proving to be of interest and may end up a route of choice when scale is an issue.

Issues for diagnostics

There are a number of features of Fv's that are likely to prove beneficial or disadvantageous in immunoassays, which should be borne in mind when we consider their possible use in the future.

Stability An obvious consequence of the Fv construction is that the Fc region of the whole IgG antibody is lost. Although difficult to prove, the Fc has been felt to contribute significantly to various forms of non-specific binding in immunoassays which can lead to false assay results. Time will tell if this is generally true. Such binding usually sets the sensitivity limit for a two-site assay - "signal-to-noise". In contrast, the Fc probably plays a significant role in the adsorption of IgG's to plastic surfaces, one of the commonest ways commercial manufacturers "stick" antibodies in to their kits. There is evidence that some Fv's may be less stable to the denaturing adsorption process and thus less suitable for this rather convenient manufacturing process (Badley, 1994). Again, time will tell.

Size Since Fv's are smaller than whole IgG's it should be possible to pack more binding sites into a given area or volume. Since binding site density is one of the factors controlling the capture of antigen by antibody there may be a benefit from using Fv's; provided their activity can be retained after capture.

Tails (Ford *et al.*, 1991) A major benefit of Fv's is the control available over a number of functions, by engineering appropriate tails to the terminii of V_H or V_L. This is done by adding appropriate bases to the genes to code for the desired peptides. Tails can provide a number

of functions. Purification by affinity chromatography using antibodies against the tail peptide has ben achieved for several different tails. Similarly, poly-histidine tails allow metal chelate affinity chromatography (Hochuli *et al.*, 1988). Both these interactions could conceivably be used within immunoassays. Tails have been designed to modulate non-covalent binding by adding hydrophobic or hydrophilic residues (Davis *et al.*, 1989; Berry *et al.*, 1989). They act as useful tags in assays for measuring the amount of Fv. Potential covalent coupling sites, e.g. cysteine or lysine, can be incorporated to try and direct coupling. Again, the jury is out on the general usefulness of all these procedures.

Stability enhancement (Glockshuber *et al.*, 1990; Reiter *et al.*, 1994) It has already been suggested that some Fv's can be less stable than their IgG counterparts. A number of strategies has been used to enhance stability. Thus, use of scFv's can confer significant stability against heat or pH extremes, for example, compared to Fv's. Alternatively, the V_H and V_L can be held together more firmly by engineering in a disulphide bridge between them. This has met with success but how general it will be remains to be seen.

It is becoming clear that despite many claims of high expression levels, there remains a problem that significant work is often needed to obtain useful quantities of Fv's e.g. for an immunoassay development programme. For diagnostics, it can be expected to be possible, if the development work is put in.

Bivalent antibody fragments (Verhoeyen and Windust, 1995)

Since most antibodies used in immunoassays are IgG's they have two binding sites per molecule. Despite the fact that we often represent our immunoassay reactions as single site (one binding site plus one epitope), in practice, some assays will only work usefully if both binding sites are involved together - the so called avidity effect. A typical example of this would be where an antibody conjugate against a bacterial surface antigen was being used to detect the bacterium. For an antigen repeated across the surface, at distances compatible with the spacing of the two IgG binding sites, a large increase in apparent affinity can often be observed due to simultaneous binding of both IgG sites to two epitopes fixed to the same surface (that of the bacterium). Intuitively it is clear that the dissociation rate of the antibody must be slower if two links have to be broken rather than one and hence the increase in affinity. Thus, avidity with the desired general properties of Fc removal, scale, cost and control, can be a motivation for making bivalent antibody fragments.

There have been demonstrations of the avidity effect with antibody fragments. The general need to have both sites able to bind to the same surface dictates that they can point in approximately the same direction. The successful methods have employed structured tails which can make strong non-covalent interactions, creating a flexible link and allowing the required geometry, unlike some of the two binding site structures described in the next section. The principal results have been from Andreas Plückthun's group.

leucine zipper (Pack *et al.*,1992)	bivalent
4-helix bundle (Pack *et al.*,1993)	bivalent
tetrazipper(Pack *et al.*,1995)	tetravalent

Bispecific antibody fragments

There have been quite a number of demonstrations over the years of antibodies containing two different specificities (reviewed in Verhoeyen and Windust, 1995). Whilst the principle was demonstrated and many imaginative uses were suggested and some even patented, none of the production routes yielded material of sufficient purity or quantity. However genetic engineering technology has made the development of a new generation of bispecific

antibodies possible (Holliger *et al.*, 1993; Whitlow *et al.*, 1994).

In principle, any of the methods used for making bivalent antibody fragments could be used to make hetero-dimers (bispecific) but the problems of purity still remain. It has been found better to engineer in the bispecificity from the start, leading to a new class of antibody based molecule, termed "diabodies" or multivalent Fv's. These molecules are made from two chains each containing mixed scFv's *i.e.* V_H and V_L for the two different specificities: $V_H(A)$-$V_L(B)$ + $V_H(B)$-$V_L(A)$, preferentially coexpressed in a single cell. The mixed Fv's spontaneously dimerise to form functional, bispecific molecules. Generally speaking, such structures will have their binding sites pointing more away from each other than in the same direction and hence their utility is likely to be different from the molecules described above. One obvious use is to form a cross linker, say between a target antigen and an enzyme - a self assembling detector conjugate. Although such molecules have not been used in commercial assays, one possible route is suggested by our own work on the use of antibody fragments for controlled delivery of therapeutic agents (Verhoeyen *et al.*, 1995). In this case a bispecific antibody fragment (anti-*Streptococcus* + anti-glucose oxidase) was used to initiate the spontaneous self assembly of glucose oxidase- *Streptococcal* surface antigen complexes in the presence of glucose, iodide and peroxidase in solution.This arrangement killed the bacteria very rapidly and specifically (an 8 log kill in 2 minutes) by means of the local high concentration of hypohalide produced. This was much faster than that obtained even with chemically coupled whole antibodies and enzymes, demonstrating the benefits of proximity (closer to bacterial surface) and self assembly. It would be surprising if analytical techniques using similar principles do not appear before too long.

Fusion proteins - bifunctional antibodies (Verhoeyen and Windust, 1995)

Genetically engineered fusion proteins - two genetically joined functional protein domains from different sources - have been known for many years. Immunoglobulin domains have been fused with a variety of other proteins such as enzymes, metal chelating proteins and toxins. It is perhaps surprising how successful such constructs have been. It is also surprising that there are no significant immunoassays using them. An obvious use would be to replace enzyme-antibody conjugates in assays e.g. ELISA's. It may be that conventional chemical conjugation works well enough in most cases. There may also be issues of avidity and storage stability. For the future, more subtle molecules are beginning to appear where variable parts of the Fv have been replaced. Again, here, there must be implications for new assay concepts.

Libraries for new antibodies (winter *et al.*, 1994)

Of all the recent developments this is perhaps the one which will have the most impact in the shorter term (2-5 years). Here I do not want to deal with the technical issues of how the results are achieved, these are succinctly reviewed in Verhoeyen and Windust (1995), but more to offer some pointers on the implications.

Two parallel developments are being combined in such a way that it is likely the way antibodies are generated in the future will be revolutionised. On the one hand it is increasingly possible to clone the antibody repertoire by the polymerase chain reaction (PCR). On the other, a powerful new screening methodology is evolving to allow the interrogation of the libraries produced by cloning. Some of the reasons why this is important are:

larger numbers of candidate antibodies (initially as fragments) will be available.
combinations of genes not normally present in the body can be provided
specificities normally unavailable e.g. self antigens can be searched for
animals can be avoided, apart from initial library construction
chosen clones can be further manipulated for function.

The screening concept that has evolved is that the library of antibody fragments is expressed on the surface of bacteriophage particles (a virus living on *E. coli* bacteria). The key feature is that the protein binding molecule carries with it the genetic information for making more of itself but of course this also means the protein sequence can be deduced as well. Thus if the phage particle, with the desired antibody fragment on its surface, can be selected by its binding capability then, in principle, it can be separated out from all the non-binders and grown up to provide the specific genes needed. They can be put into whatever organism is desired for further growth or engineering.

The screening is normally done by "biopanning" *i.e.* allowing binders to bind to antigen in a coated vessel and washing away the non-binders. Repeated cycles of this procedure can result in enormous enrichments of specific binding phage. A whole variety of screening criteria can be introduced at this stage to ensure desired characteristics of the final antibody fragment are built in. Obvious ones are affinity, temperature stability and specificity.

There are, perhaps not surprisingly, some complicating issues.

The body's immune system has a built in mechanism for generating high affinity antibodies derived from those originally selected in the early immune response. This can be a key issue for many immunoassays. So far, the libraries produced do not contain all of that information but some contain part, especially if immunised animals are used as the source of cells.

The size of the library (number of copies of each specificity) can be an issue. In practical terms it depends on how many phage can be contained in the panning vessel *i.e.* on phage titre and volume.

The diversity of the library (number of different specificities) is also important. The different cells used to make libraries will inherently have different numbers of specificities available. This diversity can be reduced when the phage-bacterium system does not reproduce all the possible members initially present.

There is an enormous effort proceeding on all three of these topics and it can be expected that improvements will be made in short times (1-2 years). However, at present, the largest library used contained about $6x10^{10}$ different specificities (Griffiths *et al.*, 1994) and the best affinities obtained were around 4nM. Current results support the notion that there is a direct correlation between library size and affinity strength. Whilst a 4nM affinity may be suitable for many immunoassay applications, commercial experience suggests there are many others where it needs to be ten-fold higher. Some success has been obtained in improving affinities by site directed mutagenesis (altering specific amino acid residues in fragments by genetic engineering means). So far this has been rather limited. It is probably better to get the right affinity first *i.e.* put the effort into the library rather than into the mutagenesis. An indicator of progress is that creation and searching of libraries is now a commercial enterprise (e.g. Cambridge Antibody Technology).

It might also be expected that specificities of antibodies could be altered by engineering changes in their binding sites either at random or by design. Whilst this will almost certainly

happen it is probably several years away. However with the current rapid pace of change in this whole area, it is perhaps dangerous even to predict that it will take that long.

We should not forget that the very areas of difficulty highlighted above are just those where the normal immune response can provide solutions, provided the analyte of interest is one where a response can be obtained. Furthermore, there are two decades of experience backing up the monoclonal antibody route. We should not expect to see the new techniques completely replacing the old. Until, or even if, the issues listed above are dealt with, the monoclonal route will continue to be used, especially for commercial assays, where a robust, practical, working antibody is a prerequisite for success. The new technologies will probably emerge in those areas where monoclonals have not succeeded e.g. difficult specificities.

ANTIGENS (Clackson and Wells, 1994; Cortese *et al.*, 1995)

Although there is a massive amount of new and very creative work going on utilising molecular biological approaches to understanding, creating and manipulating epitopes and antigens, much of it is directed towards pharmaceutical or vaccine applications. As such it is not of direct relevance to this discussion but the very pressure it is creating will have an impact on immunoassay and indeed the first signs are already visible (for example van Amerongen *et al.* 1994).

The key concept underlying much of this work is one held in common with the antibody libraries discussed above - the combinatorial approach allied with efficient screening and selection. From the immunoassay point of view it has now become possible to find peptide sequences which can bind to most antibodies with lower or higher affinity. If the sequence is part of the natural antigen then it is referred to as an epitope. It does not preclude other interactions between the antigen and antibody but represents the minimum preserving the specificity of the interaction. If the sequence is not present in the antigen or the antigen is a non-peptide structure, it is called a mimotope. There are a few well documented and surprising mimotope sequences not just for antibodies but, for example, for the biotin binding site of streptavidin (Devlin *et al.*, 1990). There are a whole variety of techniques that are being used to discover peptide binding sequences. They break down to a combination of chemically or biologically synthesised libraries which can cover random sequence combinations for a given peptide length or contain overlapping sequences stepping along a known sequence. The chemical synthesis of the latter on plastic pins is known as pepscan (Meloen *et al.*, 1994). More recently, these ideas have been extended to libraries made up from other polymerisable building blocks e.g. sugars, bases and even non-biological molecules. Any of these may have diagnostic implications in the future.

Experience shows that only a proportion of antibodies have a naturally linear epitope sequence in the native antigen - perhaps a quarter of mouse monoclonal antibodies. The easiest way to find these is by the so-called pepscan method, provided the antigen sequence is known. It may even discover some mimotopes. If the antigen sequence is not known then a random library method is more likely to succeed. This could be by looking for lead tripeptide sequences showing just a little binding using pepscan technology and gradually building up in length during several rounds of optimisation. It could also be by creating large libraries of peptides of uniform length, either chemically or biologically, and searching for the rare binding events. Success has been achieved by both chemical routes e.g. bead libraries (Lam *et al.*, 1991) and phage display libraries (Cortese *et al.*, 1995). Most of the comments applied to phage display for antibody fragments apply here also. There is a physical limitation to the diversity of such a library imposed by the container and panning conditions. In practice libraries greater than 10^8 members become difficult to handle. To put this into context, a

library containing all possible octomers of all twenty amino acids would contain 2.6×10^{10} members. Typically epitopes contain from 3 to 10 amino acids.

A powerful additional technique, once having established a "lead" sequence is to use the pepscan to perform an amino acid replacement net *i.e.* replace each, or selected, residues one at a time by others. Binding affinity and specificity can often be optimised by such means.

What are the consequences of all this for immunoassays? There are a number of practical ones. There are antigens used in assays which can be difficult to deal with. They may be non-identified as molecules, difficult to obtain, unstable or dangerous. The availability of a substitute can be an aid in assay development and a benefit when used as a kit standard in an assay.

They can, at least in theory, provide some new assay components, perhaps leading to new principles or formats. So far, very little has appeared academically or commercially. A feature we might expect to see exploited has its parallel in the antibody fragment area *i.e.* control. By manipulating the molecular biology, here the specific sequence, it should be possible to control both affinity and specificity for assay reagents.

The power of the techniques is well illustrated by our discovery of the linear epitopes, as seen by a rabbit, on the surface of the milk protein beta-lactoglobulin. These are sequences which in pepscan bind antibodies present in immunised animals. It is interesting to note that all the sequences discovered so far reside at the surface of the native molecule, perhaps as might have been anticipated for an immune response generated by injection of an antigen.

SUMMARY AND CONCLUSIONS

I have described a series of new molecules arising as a result of molecular biological approaches to antibodies and antigens. Very little of this new knowledge is being exploited yet, especially for commercial assays.

For new antibody based molecules there are some key issues to resolve *i.e.* how to achieve the required:

- affinity
- stability
- expression levels.

In addition, the molecules will need to bring a new dimension to the current practice or there will be no commercial or academic incentive to employ them. It is not yet clear where this will be but some contenders have been suggested.

For new antigens, issues are

- affinity (again)
- non-linear epitopes
- new principles/formats to exploit.

Taking up Freytag's 1991 hypothesis, discussed at the beginning, we should expect that within five years the fruits of antibody engineering, antigen understanding and combinatorial libraries will be seen in commercial immunoassay systems. There is no reason to assume other than that plant related immunoassays will be included in that transformation.

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BIOSENSORS

C R LOWE

Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT

ABSTRACT

This review describes the potential applications of biosensors in the agricultural, horticultural, environmental and food industries. The principles, current status and likely future impact of biosensors are discussed together with their advantages and limitations.

INTRODUCTION

Dramatic developments have occurred in chemical sensor technology over the last decade such that now devices offer the prospect for substantial improvements in the spatial and time resolved estimation of specific chemical species (Lowe *et al.*, 1990, 1992). The principal impetus for the development of chemical sensors has undoubtedly arisen from the rapid advances in health care biotechnology. For example, it is now accepted that measurement of blood gases, ions, metabolites, proteins and hormones allows some appreciation of regional metabolism and thus underlies progress in diagnostic and prognostic medicine. Reliable and inexpensive sensors could prove invaluable in monitoring key indicators in the central laboratory, surgery, outpatients and home, on samples derived from biological fluids. Similarly, continuous real-time monitoring of patients could reveal rapidly changing biochemical events which could be missed by conventional discrete *in vitro* sampling. Such continuous *in vivo* sensors could not only provide prolific real-time data on regional or local biochemical changes but also furnish sufficient data to direct drug release via a closed-loop feedback system.

Two distinct modi-operandi of sensors can thus be envisaged (Lowe, 1989): Discrete off-line devices coupled to analytical instrumentation to assay discontinuous samples, and, in-line devices which follow continuous changes in the concentrations of target species in real time. Continuous measurements are particularly appropriate in the fermentation and biotechnology industries where feedback control is a desirable option. Chemical sensors may also be expected to impact agricultural, horticultural and food research and, subsequently, the industries which these sectors serve. This report reviews the likely analytes, the sensor requirements and the novel technologies likely to satisfy these needs and speculates on the future uses of these devices in the agri-food industries.

THE NEED FOR SENSORS IN THE AGRI-FOOD INDUSTRIES

The measurement of environmental variables such as temperature, light intensity and humidity, either proximal to a single plant or a whole crop, are of current concern to the agricultural and horticultural industries. More recently, however, there is increasing interest in more localised measurements of the chemical potential of water, minerals, nutrients and gases in the air or soil, close to root, shoot or leaf surfaces and within plant tissue. The glasshouse industry requires these variables to be measured both in the aerial environment and, especially in hydroponics, in the nutrient film solution. In

arable farming, such target analytes could be measured in distributed optical or electrochemical sensors which may be interrogated individually in order to give meaningful population or environmental averages. In livestock farming, many of the requirements will parallel those in human health care and thus would be expected to benefit from the rapid advances occurring in the biomedical arena. It is anticipated that eventually, selective sensors will find their way into farm management, where the routine monitoring and control of the environment, feed and body conditions of the animals could provide valuable data for livestock management.

The principal requirement for sensors in the food processing and beverage industries which add value to agricultural products is in process control. A variety of analytes including moisture, fats, protein, carbohydrates, pH, chemical species and whole microorganisms can be monitored in-line or on-line and provide real time data for feed-back or feed-forward devices. Improvements in process control could beneficially impact product quality, yield, tolerance to variations in raw materials, plant performance, energy efficiency and plant flexibility and thereby reduce operating costs. Table 1 summarises typical target analytes of potential interest to the agri-food industries.

Table 1 Analytes of interest to the agri-food industries.

Humidity, temperature, water content
pH, ionic strength
Gases (O_2, CO_2, NH_3, ethylene)
Ions (NO_3^-, Po_4^{3-}, Na^+, K^+, Ca^{2+})
Minerals (Cu, Zn, Mg, Ca)
Heavy metals (Hg, Cd, Pb)
Raw materials (fats, carbohydrates, proteins)
Metabolites (glucose)
Pesticides / herbicides
Hormones (growth hormone, progesterone)
Microbial contaminants (plant/animal viruses, salmonella)

BIOSENSOR ARCHITECTURES

All biosensors exploit a close liaison between a selective biorecognition system and a transducer which translates the biorecognition process into a usable signal (Lowe, 1984, 1985). The biocomponent is typically an enzyme, sequence of enzymes, lectin, antibody, receptor or binding protein, organelle, bacterial, plant or animal cell or whole tissue slice. This component is responsible for both the selectivity and sensitivity of the final device; a discrimination ratio of $\geq 10^7$ - 10^8 may be required to recognise the target analyte in the presence of a complex mixture of other, potentially interfering, substances.

Biorecognition systems can be categorised into three principal types: biocatalytic systems such as enzymes, organelles, whole cells, tissue slices or other 'active' organs, where the selective binding sites 'turn over'. Such systems are appropriate for monitoring analytes in the mM-μM concentration range such as ions and metabolites where continuous sensing in real time is a possibility. 'Irreversible' or 'affinity' sensors exploit antibodies, binding protein or receptor systems where high affinity interaction sites can become saturated and the devices are more appropriate to 'one-shot' or 'single use' disposable devices. Analytes such as hormones, drugs, toxins and whole cells with concentrations in the μM-pM range are commonly monitored with these devices. Finally, 'amplified' systems represent a hybrid configuration which exploit an antibody or other appropriate high affinity system as the initial selective binding event followed by a biocatalytic amplification step based on enzymic catalysis, substrate or coenzyme cycling, cascade or bioluminescent systems linked to a transducer. Such systems are capable of monitoring analytes in the pM-aM concentration range, although response times can be long.

BIOCATALYTIC SENSORS

Enzymes are particularly appropriate for inclusion in biosensors since they are known to create physico-chemical changes as part of their catalytic modification of the target analyte. One of the most promising approaches exploits electron exchange between enzymatically-generated electroactive species and an electrode surface. Early amperometric devices monitored oxygen consumption or hydrogen peroxide production accompanying the oxidation of target analytes by their respective oxidases. In the best known example, the hydrogen peroxide formed by the oxidation of β-D-glucose by glucose oxidase is monitored by oxidates at a platinum or graphite electrode. Problems are associated with a dependence on ambient oxygen concentrations and interference from electroactive interferants found in the sample and oxidised at the high potentials required for electron exchange. Substitution of an artificial electron acceptor designed to shuttle electrons between the enzyme and electrode for the natural electron acceptor, oxygen, circumvents most of these problems and has formed the basis of a commercial instrument for glucose measurement in whole blood. However, despite these innovations which have reached commercial fruition, amperometric biosensor technology is developing apace. It is now possible to create devices capable of promoting direct electron exchange between redox enzymes and electrodes, for example, by incorporating enzymes into conducting organic salts, glasses or polymers. Thus, entrapment of enzymes into the electronically conducting polymer, polypyrrole represents a means of immobilising biologically active molecules on defined electrodes of any size or geometry, with potentially good electronic communication and to facilitate the fabrication of reagentless amperometric sensors (Foulds & Lowe, 1986; Yon Hin & Lowe, 1992; Wolowacz *et al.*, 1992; Yon Hin *et al.*, 1993; Yon Hin & Lowe, 1994). This approach has also been shown to be suitable for the co-entrapment of enzymes with mediators, substrates and coenzymes and for the fabrication of multi-analyte microelectronic biosensors (Foulds & Lowe, 1988). Alternatively, chemical derivatisation of enzymes with redox centres has allowed enzymes to be 'hard-wired' into electrodes and thereby achieve very efficient electronic communication.

There is an increasing demand for disposable devices and some amperometric sensors have been fabricated using planar thick-film technology. Sensors based on thick-film

electrodes can be made small and inexpensive and display excellent reproducibility and long term stability. Thin film silicon microfabrication presents an alternative approach which has been successfully exploited to construct enzyme-sensitised field effect transistors, conductimetric devices and amperometric systems (Cullen *et al.*, 1990; Yon Hin *et al.*, 1990). Indeed, the design, fabrication and operation of fully integrated amperometric devices offers exciting prospects for both *in vitro* and *in vivo* sensors for some key analytes. A fully integrated amperometric glucose sensor comprising electrodes, digital potentiostats and some signal conditioning has now been fabricated and assessed.

AFFINITY SENSORS

A significant proportion of analytes of interest to the agri-food industries are present at concentrations <μM and thus require highly selective and sensitive devices based on immunological recognition systems. The construction of 'one-shot' direct immunosensors which require only the addition of the complementary antigen in the sample to elicit a response represents a major challenge. Early attempts at fabricating an immunologically-sensitised field effect transistor (Immuno-FET) have not matched expectations of its performance, despite the introduction of a number of elegant device materials and configurations. Indeed, it is now widely recognised that immunologically-sensitised potentiometric devices are never likely to be constructed in view of the technical difficulties in creating an ideal polarised interface at which such measurements could be made.

Immunological reactions can be monitored directly by exploiting optical measurements, particularly evanescent field detection in an optical waveguide. When light irradiates an interface between two optically transparent media of different refractive indices, impinging from the medium of higher refractive index at an angle greater than the critical angle, total internal reflection occurs within the optically denser medium. An electromagnetic waveform is generated in the optically less dense medium close to the interface and penetrates a fraction of a wavelength into the less dense medium. This evanescent wave may be exploited to 'sense' immunological interactions taking place at, or close to, the interface and with minimum interference from substances present in the bulk medium. Evanescent field detection forms the basis of a number of novel immunoassay formats based on surface plasmon resonance (SPR), the resonant mirror, planar waveguides, surface relief gratings, fibre optics, fluorescence capillary fill devices and tapered monomode fibres (Carlyon *et al.*, 1992; Davies *et al.*, 1994; Tubb *et al.*, 1995). The possibility of miniaturising conventional optical techniques using inexpensive laser diodes, integrated optical waveguides or various fibre optic formats offers exciting prospects for the future.

An alternative to monitoring optical changes associated with immune complex formation exploits sensitive mass-to-frequency transducers based on piezoelectric materials. Quartz crystal microbalances coated with thin films of selective adsorbents have found substantial applications in gas and volatile monitoring. However, damping in fluid media has proven a difficult obstacle to introducing this approach into a biosensor format. For example, difficulties were encountered with damping, non-specific adsorption and sensitivity when human IgG was monitored in aqueous samples with a goat (anti-human) IgG-coated surface acoustic wave (SAW) device. Acoustic plate mode (APM) devices comprising interdigitated transducers deposited on the lower face of a lithium niobate ($LiNbO_3$) crystal and a biological layer on the upper face have proven more successful. However, whilst these systems prove more adaptable in

aqueous media, the sensitivity of the device is limited by the fact that the majority of the acoustic energy lies within the bulk of the crystal. A preferred approach exploits a Y-cut quartz surface skimming bulk wave (SSBW) device coated with a thin (0.1 - 2.5μm) polymer overlayer to generate a Love plate acoustic sensor. This novel approach represents the acoustic equivalent of the planar optical waveguide since the majority of the acoustic energy is contained within the overlayer and therefore sensitive to mass changes at its surface. The Love plate device has been shown to be suitable for monitoring the adsorption of proteins, whole cells and low molecular weight analytes (Gizeli *et al.*, 1992; Gizeli & Lowe, 1995).

AMPLIFIED SYSTEMS

Direct immunosensors are conceptually simple but suffer several major disadvantages: they are susceptible to non-specific adsorption, are limited in sensitivity by the affinity of the antibody, are applicable mainly to higher molecular weight analytes and are severely restricted by the kinetics of diffusion at low analyte concentrations. Some of these deficiencies can be circumvented by coupling labelled immunoassays to appropriate physico-chemical transducers; amplified systems thus represent a hybrid configuration of biocatalytic and affinity sensors and can achieve sensitivities down to aM. Traditional biological amplification systems, including substrate and coenzyme cycles, cascades and ion flux triggers, have been exploited to enhance the sensitivity even further. For example, in a classic example, the enzyme label, alkaline phosphatase, of an immunoassay, hydrolyses these 2'-phosphate from $NADP^+$, whence the product, NAD^+, enters a coenzymatic cycle driven by two counter-acting oxido-reductases, alcohol dehydrogenase and diaphorase, whence the electron flow from the NAD^+/NADH cycle is coupled to a metal electrode via a ferricyanide-mediated exchange. This system is potentially able to detect one hundredth of an attomole (10^{-20} mole) of the analyte. These systems are more appropriate in applications where sensitivity, rather than time or cost, is the principal consideration.

PERSPECTIVES IN BIOSENSOR RESEARCH

Over the last decade or so, biosensor research has concentrated on coupling well established biorecognition systems to innovative physical transduction techniques. More recently, there has been a trend to examine the recognition component in more detail in order to develop systems with more sensitivity, selectivity or stability. Thus, natural sources of novel biological activities of microbial, plant or animal origin have been screened in order to identify new catalytic or binding systems. Similarly, the techniques of protein engineering could be used to generate novel recognition molecules with improved specificity, affinity, reversibility, kinetics or with anchoring groups to facilitate immobilisation or orientation on the sensor surface. However, 'biomimetic' techniques involving imprinting polymers, artificial enzyme, catalytic antibodies, ribozymes or chemical combinatorial techniques are likely to find increasing application, providing of course, that the enhanced durability of such systems is not offset with a diminution in selectivity and/or sensitivity.

Interest is also likely to be maintained in the further refinement of established transducer technologies. In particular, modern integrated optics, monolithic silicon and micromachined systems, when coupled to digital signal processing circuitry could dramatically improve device versatility, reliability and sensitivity. Indeed, multifunctional chips incorporating separations, sensors and signal processing techniques on a single device are likely to become the norm within 10 years or so and find major applications in the agri-food diagnostics markets.

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TOWARDS "ON-LINE" PCR

D I GRAY AND N J C STRACHAN
Central Science Laboratory, Food Science Laboratory, Torry, PO Box 31, 135 Abbey Road, Aberdeen, Scotland, AB9 8DG, UK

ABSTRACT

Traditional methods for the recording and verification of PCR amplified products are at present time consuming processes which are unsuitable for most applications outside of the laboratory environment. Combining the amplification and verification processes would significantly reduce the time and effort required to perform an analysis. New developments in biosensors and thermal cyclers will allow more rapid, portable and user-friendly devices to be manufactured.

INTRODUCTION

The polymerase chain reaction (PCR) for the amplification of DNA sequences was first introduced in 1985 by Saiki *et al.* and without doubt has been one of the most revolutionising discoveries to be made in molecular biology. This technique is enormously versatile and coupled to its other qualities of speed, simplicity, sensitivity as well as its low cost, has increased the efficiency of research in the molecular sciences. The PCR has now become one of the most widely used routine methods to be developed, finding applications within many diverse disciplines. In microbiology the PCR has allowed the rapid identification and typing of low numbers of micro-organisms from environmental samples. This has benefited research in microbial ecology and systematics, as many micro-organisms are nutritionally fastidious and are unable to be grown by conventional culturing techniques. In medicine it has been used to screen for genetic disorders, HLA typing, presence of HIV, as well as the diagnosis of diseases such as cancer. It has even found uses in archaeology, pathology and forensic science to amplify DNA from archival specimens and biological materials found at crime scenes. Basic molecular research now depends upon the PCR for the production of sequencing templates and gene probes, the cloning of genes, screening clones as well as their mapping and sub-cloning.

Until recently the PCR process was regarded as a two stage process, the amplification reaction followed by a detection step. However, automated closed and flow-through systems have been developed to perform the amplification and detection steps within a single machine (Findlay *et al.*, 1993). This has reduced the requirement for the handling of PCR products in an open laboratory environment and the associated risks of product carryover. The next step in the automation of this process would be to perform a sensitive, and specific, detection and vertification procedure actually during the amplification phase of an assay. This would result in a considerable time saving compared to the same analysis performed post-PCR.

THE MECHANISM OF THE PCR

The PCR is a rapid technique for the in vitro enzymic amplification of a specific target sequence from within a complex of nucleic acids. This amplified sequence is usually less than 2 kilobases in length. The specificity of the PCR is governed by the choice of the oligonucleotide primers which anneal to separate DNA strands and bound the target sequence which is to be amplified.

The starting DNA is denatured by heating to 94°C in the presence of excess deoxynucleotide triphosphates (dNTPs), 2 oligonucleotide primers, a heat stable DNA polymerase enzyme and appropriate buffer and salts. The primers are present in great excess when compared to the sequence to be amplified and are annealed to opposite strands at homologous sites at a temperature which determines the stringency of the hybridisation. The primers are then elongated by the DNA polymerase in a 5'→3' direction, at a temperature of 72°C. This DNA synthesis occurs and extends across the segment between the two oligonucleotide primers. During the first few cycles of denaturation; annealing and extension the PCR consists of products produced by primer extension. These are intermediate in length, with single stranded DNA being synthesised from each site where a primer is able to bind in the original starting DNA and these products accumulate arithmetically. The exponential amplification phase of the PCR will then produce two single stranded products of equal size which together form the final PCR product. Each strand of this product acts as a template during further rounds of amplification and this product accumulates exponentially according to the formula $N = N_0 I^x$ where x is the number of cycles, N_0 is the starting number of copies and I is the efficiency of the reaction expressed as the number of completed target molecules produced per cycle from each template molecule. The theoretical maximum for I is 2 but experimental evidence has shown it to be less than 1.7 (Li *et al.*, 1988).

When attempting to use the PCR process to detect potential plant or animal pathogens it is possible to obtain target DNA from dead or lysed cells, rather than viable cells. This contamination of amplification reactions will lead to false positive results. More complex amplification strategies such as the nucleic acid sequence-based amplification (NASBA) process (Compton, 1991) have been developed to deal with the problem of cell viability. NASBA technology is able to directly amplify RNA targets even in the presence of native genomic DNA.

Another problem area which is being addressed in numerous laboratories is the separation, concentration and purification of target organisms (or DNA) from different matrices. Because the PCR process is performed in reaction volumes of 100 µl or less, obtaining sufficient quantities of pure enough target material can be difficult. Chemical contaminants which are introduced into the amplification reaction together with the target can poison the amplification reaction. Purification of the target is frequently necessary for environmental, food or plant samples and can represent a significant cost in both time and resources in any PCR-based analysis (Simon *et al.*, 1996).

PCR THERMAL CYCLERS

Commercial thermal cycling machines, of varying levels of sophistication, are available in a variety of different reaction formats. These instruments are usually controlled using an on-board microprocessor with associated key-pad and electronic display. Internal instrumentation accurately monitors the temperature at each stage of the heat cycle. Cooling methods vary between different instruments with internal refrigeration, Peltier, forced air or water being the most common methods. Some instruments have interchangeable or add on reaction formats, allowing for 0.5 ml/0.2 ml microtubes, 96-hole microwell plates or glass slides (for *in situ* PCR) to be controlled from a common unit.

A major disadvantage of thermal cyclers which incorporate metal heat blocks is that as the thermal mass of the block is reduced, to facilitate more rapid transitions between different temperatures during cycling, the temperature uniformity is compromised and increased well-to-well variations occur. By performing the PCR within a heated chamber and containing the reaction components within thin-walled glass or plastic capillaries 10 µl amplification reactions

can be performed within 15-30 min, as compared to 90-120 min on a standard metal heat block instrument. Air is ideally suited as a heat transfer medium because of its low density and by heating and cooling samples using high velocity air, temperature transitions of up to 5-10°C sec^{-1} are easily achieved (Black *et al.*, 1995, Douglas *et al.*, 1995).

RECORDING OF REACTION PRODUCTS

Standard Methods

The most common method of detecting and analysing the products (amplicons) of a PCR is by gel electrophoresis. A small quantity of the post-PCR product is mixed with a loading buffer containing a tracking dye and loaded in to an agarose or polyacrylamide gel, together with an appropriate DNA standard. Electrophoresis is then performed until the products are suitably separated. The DNA bands of the agarose gel are made visible by ethidium bromide staining and observed under UV light conditions. A permanent record, in the form of a photographic or digital image, is then usually made of the gel, from which the sizes of the amplicons and their quantities can be deduced. Digital imaging of gels is increasing in popularity as the stored images are more easily manipulated and with appropriate software, analysis of the gel can be performed automatically prior to a final hard copy being produced (Douglas *et al.*, 1995).

Many other techniques exist to detect products produced during the PCR. The incorporation of fluorescent dyes (eg fluorescein, rhodamine), low energy radioactive isotopes and non-radioactive labels (eg enzymes: alkaline phosphatase, horseradish peroxidase; haptens: biotin, digoxigenin; fluorescent agents: luminol, acridinium esters) into the products during the PCR have all been used in various end-point detection systems (MacCallum, 1995). However, the determination of the presence, or size, of a PCR product is no guarantee that the correct product has been obtained. Non-specific amplification of DNA can occur with the PCR process particularly where the process parameters or primers have not been fully optimised. Membrane based detecting methods (Southern, dot and slot blotting) can be used when identification of a particular PCR product is required (Sambrook *et al.*, 1989). One or more probes (which can be generated and labelled using the PCR process) are hybridised to membrane immobilised PCR products and then made visible using a detection system. The main disadvantage with blotting and probe-based methods is the time and extra resources required to perform such assays, which can commonly take 1-2 days to complete.

Rapid biosensor methods

Recently, sensors which utilise the evanescent wave principle (Graham*et al.*, 1992; Strachan and Gray, 1995) surface plasmon resonance (SPR) (Nilsson *et al.*, 1995) or resonant mirror devices (Watts *et al.*, 1995) have been developed to detect DNA hybridisations.

Evanescent wave fibre-optic sensors work on the principle that when light is propagated down a fibre-optic by internal reflection an electromagnetic wave is also generated close to the fibre but within the medium that surrounds it. This is the evanescent wave and because it penetrates beyond the optical interface it can be used to stimulate fluorescent labels which can be either attached to or in very close proximity to the surface of the glass fibre. As the fluorescence is propagated back up the fibre it is detected by a solid state sensor. Strachan and Gray (1995) utilised this type of device (Fig.1) to demonstrate its general applicability to specifically and rapidly identify PCR products by their hybridisations with internally located complementary sequences which had previously been bound to the glass fibre.

Surface plasmon resonance (SPR) devices detect changes in refractive index over time at a sensor surface. Such changes are proportional to the mass of molecules attached at the surface. A resonant mirror device is also an evanescent wave sensor which by measuring changes in the resonant angle can be used to monitor binding and dissociation of molecules at its surface. Both of these devices have been used to study DNA-DNA hybridisations in real-time and in addition SPR sensors have also been used to monitor solid-phase gene assembly; performance of cleavage endonucleases; DNA synthesis, using T7 DNA polymerase or the Klenow fragment and DNA minisequencing (Nilsson *et al.*, 1995).

In experiments performed using an evanescent wave fibre-optic sensor (Fig.1) amino-terminated oligonucleotides (M2980 and M2979, Table 1) were covalently bonded onto the fibre as described by Graham *et al.* (1992). A 200 bp PCR product derived from the *fla*A gene of *Listeria monocytogenes* NCTC 11994 (Gray and Kroll., 1995) using FAM- (fluoresceine) and biotin-labelled primers. The fluoresceine-labelled DNA strand was isolated and mixed with a pre-hybridisation buffer and passed into the flow cell. A steady increase was observed in fluorescence (Fig.3 a & b) when hybridisation occurred with M2980, where the 20-distal bases of this oligonucleotide were complementary to an internal sequence of the single stranded PCR product. When the non-complementary 20 bp oligonucleotide M2979 was used no significant increase in fluorescence was observed (Fig. 3c).

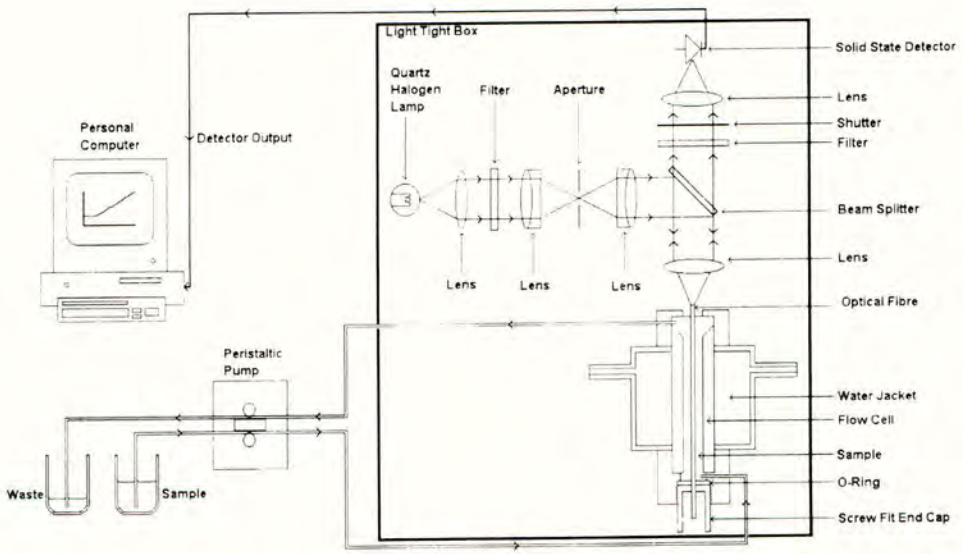

Fig.1 Fibre-optic fluorimetric sensor.

To perform hybridisation assays an instrument was constructed using a fluorimeter sourced from Sapidyne Inc. (Idaho, US) and configured as in Glass *et al.* (1987). The output voltage from the sensor was digitised and stored on a personal computer. The glass fibre-optic element was encompassed by the sample cell which consisted of a specially designed and manufactured combined, glass jacket and stainless steel flow cell with screw-on end caps for interfacing. The temperature was monitored by means of a thermocouple controlled by pumping pre-heated water through the glass jacket from reservoirs. Hybridisations were performed at 60°C and reversed at ≥80°C.

Table 1. Amino-labelled olyonucleotides which were immobilised onto glass and used in hybridisation experiments.

Sequence Number	Sequence	Comment
M2980	5'-AMINO-[GTTCTCTTGATGA-CGCTGCT]*-CAATCTTGCAACGT-ATGCGT-3'	40-mer with 20 distal bases complementary to the central portion of the single stranded PCR product.
M2979	5'-AMINO-[GTTCTCTTGATGA-CGCTGCT]*-3'	Negative control

*Spacer sequence consisting of non-complementary sequence to the *fla*A gene region under study.

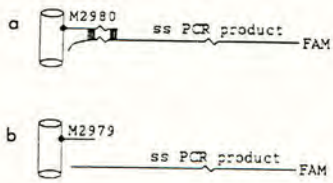

Fig 2 Experiments Performed using a fibre-optic sensor.
(a) Hybridisation of an internal 20 bp region
(b) Negative control.

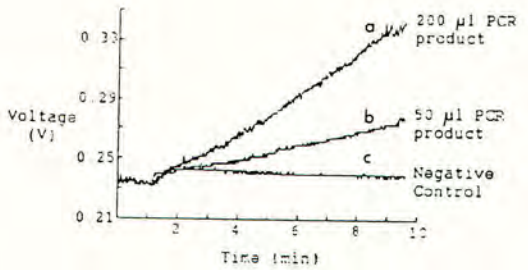

Fig. 3 Biosensor output.

(a & b) Voltage from sensor increases as hybridisation occurs.
(c) No significant increase in voltage sensor as specific hybridisation is not possible.

Monitoring of the PCR process in real-time

On-line, or near on-line, detection of the PCR process using very sensitive detection systems would be advantageous as the presence of the appropriate PCR product would be detected after fewer cycles of amplification. The requirement to remove the amplified end products and apply a separate detection system would also be eliminated. The overall analysis time would be greatly reduced and there would be no requirement to handle amplified products thus minimising the possibility of contaminating the laboratory. By combining the amplification and detection process into a single machine a more portable approach to the PCR process could be developed. The requirement for the preparation of the reaction components without the introduction of contamination could be eliminated by their lyophilization into pre-sealed reaction tubes. These and other modifications will lead to a more user-friendly process which could be applied outside of a molecular biology laboratory. Agriculturists could use such equipment to detect plant

pathogens on crops prior to their spraying or harvesting and environmental health officers could analyse suspect samples directly without having to involve the services of a specialist laboratory.

An indirect method to monitor the PCR process in real-time using an evanescent wave biosensor has been evaluated by Gray and Strachan (unpublished results). The availability of a fluorescently labelled primer in a PCR mix was monitored by observing the fluorescence from an amplification reaction. Over time a decrease was observed as less fluorescently-labelled primer was available to anneal with its complementary sequence which had been previously covalently linked to the glass fibre-optic element.

Preliminary experiments involving a more direct approach are also being attempted. Two standard PCR primers, one carrying a fluorescent label, are used to amplify a specific target region. A third sequence is immobilised onto a glass fibre element held within the PCR mix. This oligonucleotide is complementary to a short internal sequence on the single stranded DNA product which carries the incorporated fluorescent label. During the cycling process a proportion of this single stranded DNA will anneal to its complementary sequence and be detected due to the evanescent wave principle.

This approach does not prevent the detection of false positives, but it uses labelled primers which are relatively cheap and easily obtained. It also offers the possibility to interface with the currently available generation of very fast thermal cyclers, based upon high velocity air for heating and cooling. These machines are already able to complete a 30 cycle amplification reaction in less than 30 minutes, and if coupled to an *in situ* fibre-optic evanescent wave device would give real-time output of the PCR process. Newer designs of such cyclers will also allow different formats, such as microtiter plates, to be rapidly cycled. However, the interfacing of large arrays of fibre-optics from numerous samples and then multiplexing them to a detector represents a major engineering challenge.

The PCR process could also be monitored in real-time using fluorogenic probes consisting of both a reporter dye (fluorescein) and a quencher dye (rhodamine) attached. Holland *et al.* (1991) have described an assay whereby the 5'→3' exonuclease activity of *Taq* DNA polymerase is used to detect specific products of a PCR. The DNA polymerase is able to cleave a probe which has previously hybridised to the template during the PCR. In the intact probe the proximity of the reporter dye to the quencher dye causes a suppression of the reporter dye fluorescence. Cleavage can only occur if the intact probe has annealed to target DNA and results in an increase in the reporter fluorescence signal which can be detected using fluorimetry. This system has several advantages in that there is no requirement for any post-PCR hybridisation to labelled probes. As the fluorescence signal increases in direct proportion to the initial copy number it is also quantitative. No fluorescence signal is possible, due to the non-specific amplification of DNA, so no false positives are possible. Finally, the use of multiple probes within a single PCR is possible because different fluorescent dyes can be used for each reporter probe. A significant disadvantage of this system is that at the present time the cost of synthesising each doubly labelled probe is very high.

CONCLUSION

If sensitive and specific detection and identification processes can be successfully coupled to the PCR to give on-line information considerable benefits will result. New developments in biosensors, thermal cycler design and reagent presentation will allow this amplification procedure

to become more user-friendly and portable. This will result in new users outside of the laboratory environment being able to apply this technology.

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APPROACHES TO A SENSOR SYSTEM FOR THE EARLY DETECTION OF SOFT ROT IN STORED POTATO TUBERS.

B P J de LACY COSTELLO, R J EWEN and N M RATCLIFFE.

Faculty of Applied Sciences, University of the West of England, Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY.

ABSTRACT

The focus of this paper is the detection of key volatiles which are released by the action of the bacterium *Erwinia carotovora* on potato tubers. To this end, a wide range of vapour sensors were tested in the headspace above potato tubers inoculated with the bacterium *Erwinia carotovora.* The responses, when compared to those obtained upon exposure to fresh potatoes and water vapour, were significantly different. The most promising sensors from these preliminary tests have been combined to form a prototype multi-sensor array type device. It is considered that an array of this type, when coupled with pattern recognition software, would have applications in the early detection of soft rot in stored potato tubers.

INTRODUCTION

The action of bacteria on a variety of foodstuffs releases volatile compounds which constitute the characteristic malodours associated with the rotting process. The pattern of vapours is often representative of both the type and severity of any infection on a given substrate. There are, however, many common vapours released by the action of fungi and bacteria which could be present in the environment, or in lower levels in fresh produce. The analysis of a specific pattern of vapours is usually necessary to distinguish infection from other factors, especially where no one vapour is indicative of the rotting process.

Bacterial soft rot, caused mainly by the bacterium *Erwinia carotovora,* is a major problem in the bulk storage of many vegetable crops, especially potato tubers. Reported work (Varns & Glynn, 1979) on the analysis of *Erwinia carotovora* infections of Russet Burbank cultivar and Kennebec cultivar potato tubers by GC-MS, cites an increase in the concentration of organic vapours above the infected tubers. The volatiles emitted in the highest concentrations were ethanol, acetone and 2-butanone. Additional volatiles detected in the headspace of the diseased tubers included acetaldehyde, methyl acetate, ethyl acetate, propanethiol, hydrogen sulfide, dimethyl disulfide, n-propanol and isobutanol. Detection of these volatiles would permit the early identification of any infection allowing remedial action to be taken.

The ideal detection system would be sensitive to low levels of vapour (typically 1-10ppm), portable (to permit multi-location testing) and inexpensive. Common laboratory methods for the detection of vapours, such as GC-MS, although accurate and sensitive, are expensive and require auxiliary equipment for operation which limits portability. Currently soft rot detection in storage facilities is undertaken using thermographic monitoring techniques to identify the increased heat associated with the infection. This method is largely ineffective as any infection has spread to a substantial part of a store before detection is possible. The emphasis must be on the early detection of an infection to reduce financial losses.

Vapour sensors constructed from ceramics such as tin dioxide have been utilised for the detection of ethanol (Sberveglieri, 1992), ethyl acetate (Persaud & Dodd, 1982), acetone (Nenov & Yordanov, 1992), acetaldehyde (Sberveglieri *et al.*, 1993), butan-1-ol, propanol (Gardner *et al.*, 1992) and H_2S (Schiebaum *et al.*, 1991). Work has been reported on the successful detection of vapours released due to the action of *Penicillium* infections on citrus fruit, using sensors of this type (Cowell *et al.*, 1994). Vapours such as ethanol, ethyl acetate, and acetaldehyde previously studied in this project are also found in the headspace above diseased potato tubers.

Conducting polymer based sensors have also been utilised for the detection of organic compounds (Miasik *et al.*, 1986). They are used in arrays incorporating pattern recognition software for monitoring the quality of foods such as fish (Hatfield *et al.*, 1993) and beverages, such as beer (Pearce *et al.*, 1993).

This work aims to combine both ceramic sensors and polymer sensors in an array type device. The recent advances in sensor technology should help provide a solution to the problem of bacterial soft rot in potato storage sites.

MATERIALS AND METHODS

Method used for the inoculation of potatoes with *Erwinia carotovora.*

The potato tubers were thoroughly cleaned and surface sterilised with alcohol. A sterile cork borer was used to remove part of the flesh. Into the resulting cavity 0.5 ml of an *Erwinia carotovora* suspension was added (bacteria stored at -70°C). The removed tissue was replaced and sealed with vaseline/paraffin wax (50/50 m/m). The inoculated tubers were stored in polythene bags in a moist environment at 20°C for 14 days. The potato tubers were then removed and cut in half to reveal the soft rot and used in sensing trials. When the tubers were not being used they were stored in a refrigerator at 4°C.

Analysis of potato tubers using Gas Chromatography - Mass spectrometry.

Potato tubers infected with *Erwinia Carotovora* were analysed via two separate analytical techniques, solvent extraction and solid sample injection at 60°C. The fresh potato samples were also analysed in the same way.

The method used for solvent extraction was as follows: 10 g of potato tissue was extracted using 2 ml of dichloromethane/methanol 1:1. The crude extract was filtered through a glass pipette which had been lined with glass wool. The filtered extract was then evaporated to a volume of 0.1 ml using an inert gas flow. The resulting extract was analysed by GC-MS using a Hewlett Packard Mass Selective Detector (HPMSD) which was fitted with an HP-Innowax column (0.25 mm internal diameter, 25 m in length) where the stationary phase was a cross bonded polyglycol phase. The column temperature was 200°C.

The solid sample injection technique was far simpler, and possessed the advantages of a lower operating temperature of 60°C and a lower chance of identifying compounds extracted from the tissue of the tubers.

Sensors used for gas sensing experiments.

The ceramic sensors used were commercially available (Envin Scientific Ltd., UK.) TGS800 sensors (sensor type 1) which were used as supplied. These sensors required heating to a temperature of 350°C

prior to use. Sensors used requiring no external heating fell into three categories: (1). sensors based on mixtures of tin dioxide and polypyrrole (sensor type 5) (de Lacy Costello *et al.*, In Press a). (2). Sensors based on colloidal polypyrrole films fabricated on PMMA (perspex) substrates (sensor type 4) (Ratcliffe, 1990). (3). Sensors based on composites of polypyrrole with thermoplastic binders such as PVC (sensor type 3) and polystyrene (sensor type 2) (de Lacy Costello *et al.*, In Press b).

Testing of sensors to model compounds.

Each sensor type was tested in the headspace of pure vapours which had been identified in the headspace above rotting potato tubers. The vapours tested included ethanol, acetone, 2-butanone, ethyl acetate and ammonia (0.02 M solution). However, the high concentrations of vapour in the headspace are not representative of those encountered in the headspace of actual systems. For this reason it was decided to disperse the organic liquids of interest in an inert non-volatile liquid matrix dimethyldigol prior to sensor testing (a 5% solution of each was used). The sensors were placed 4 cm above the liquid and the change in current measured over a two minute period using in-house designed testing equipment (de lacy Costello *et al.*, In Press a). Promising sensors from this screening were then tested in the headspace vapour of the infected tubers, fresh tubers and water vapour.

Testing of sensors to potato tubers.

25 g of fresh potato tuber was cut and placed into a two litre polythene sample bag. The sensor was then introduced and the change in current monitored for two minutes. This was repeated for all sensor types. After an equilibration time of ten minutes the sensors were tested to *Erwinia* infected tubers and subsequently to a saturated headspace of water vapour. This was repeated three times to test the reproducibility. The results from preliminary testing were analysed using principal components analysis (PCA). The best five sensors from preliminary testing were combined to form an array type device.

Determination of the presence of volatile amines.

Our analysis using GC-MS failed to identify amines in the headspace above rotting potato tubers, and no references to the presence of such vapours could be found in the literature. However, it is likely that the micro-organisms would decarboxylate amino acids to give amines. The majority of these amines would remain within the tuber as protonated salts due to the relatively low pH. To test this theory, 25 g of fresh and diseased potato tuber were taken and treated with 5 ml of 2M NaOH. The treated potato tubers were then each placed in a 2 litre bag, and the sensors exposed to each headspace. The results obtained were compared with those obtained previously from rotting and fresh tuber samples. It was expected that the sensors based on polypyrrole would be most likely to respond, since polypyrroles are highly sensitive to amines, particularly ammonia (Gustafsson *et al.*, 1989), whilst giving much lower responses to organic vapours (Hatfield *et al.*, 1994).

As a further test of whether amines were being released, a series of pH sensitive immobilised dyes were utilised. The dyes used were bromophenol blue (BPB), bromocresol green (BCG) and bromocresol purple (BCP) which are known to change colour from yellow to blue or green at a range of pH values. The pKa values of the dyes are 5, 6 and 10 respectively, thus BCP was expected to give the best indication of whether alkaline vapours were emitted by the potato tubers. These dyes are used extensively for detecting changes in pH (Kostov, 1992) and have been used previously for ammonia detection (Sadaoka, 1993). For the experiment 2 g of both fresh and diseased tuber were placed in Petri dishes containing the immobilised dyes. The colour change and time taken to change in each case was recorded.

RESULTS AND DISCUSSION

The GC-MS results showed the vapour profiles of *Erwinia* infected potato tubers to be significantly different to those of fresh potatoes. The key volatiles detected above the *Erwinia* infected tubers were acetaldehyde, ethanol, 1-butanol, acetone, and 1-propanol, whilst there were only small amounts of ethanol and acetaldehyde detected above the fresh tubers. These results correlate well with the GC-MS data of Varns & Glynn (1979) performed on *Erwinia carotovora* infected tubers.

The results from the test vapours (Table 1) show that the five sensor types identified gave differing response profiles. Sensor 1, based on a heated ceramic system, was most sensitive for organic vapours whilst giving a relatively low response to ammonia vapour. The polypyrrole-based sensors type 2-4 gave larger responses to the ammonia vapour than to organic vapours. The interactions of ammonia with polypyrrole-based systems and the resulting high sensitivity has been reported extensively in the literature (Gustaffson *et al.*, 1989). Even sensors 2 and 3, which varied only in the polymer used to bind the polypyrrole, exhibited subtle variations in response patterns. Although the vapour concentrations used were considerably higher than those encountered above infected tubers (typically in the range 10-50ppm total organics above tubers), the results are useful for assessing the response pattern of a range of sensors to the volatiles above infected potato tubers.

All sensor results are displayed in terms of percentage change in current calculated using the following relationship: $100(I_g-I_o)/I_o$, where I_g was the current two minutes after the exposure to the headspace of the vapour, and I_o was the stable baseline current prior to the exposure.

Table 1. Results of initial screening experiments of sensors to key vapours (dissolved in dimethyldigol) known to be found in the headspace above infected tubers.

	Percentage change in current (- indicates a decrease in current)				
Vapour	Sensor 1	Sensor 2	Sensor 3	Sensor 4	Sensor 5
Ammonia 0.02M*	617	-639	-861	-760	2900
Ethyl acetate (5%)	4000	-217	-226	-16	600
Acetone (5%)	18000	-54	-94	-20	400
Ethanol (5%)	10000	-94	-46	-21	200
Butan-2-one (5%)	14000	-181	-264	-12	300

* dissolved in water.

The next stage was to expose the sensors to the headspace above infected and fresh tubers. The sensors were tested for their response to water vapour as this increases substantially above diseased tubers. This was to ensure that response profiles obtained were not just due to increased humidity but were actually as a result of vapours released during the *Erwinia carotovora* infection. The results displayed in Table 2 show the percentage change in current to each system. This raw data was analysed using principal components analysis (PCA). PCA is a data reduction technique which can be used (Slater *et al.*, 1993) to group similar data sets when no obvious correlation is apparent in the raw data. The results displayed in Figure 1 were calculated using the covariance matrix. The first three principal components for the three sets of combined sensor outputs have been plotted and can be seen to separate the data into three distinct groups.

Table 2. Percentage change in current when exposed to infected and fresh potato tubers.

	Soft rotted potato	Fresh potato	Water vapour
Sensor 1	1000	74	100
	850	82	120
	1190	79	122
Sensor 2	8.2	1.0	4.2
	9.7	4.0	3.6
	15.0	3.7	3.1
Sensor 3	12.8	0.9	2.8
	13.2	3.2	3.5
	15.1	3.5	3.5
Sensor 4	-13.6	-0.7	-3.8
	-17.9	-0.9	-2.7
	-30.3	-0.7	-2.9
Sensor 5	29.9	1.1	82
	32.1	2.3	75
	27.2	1.6	73

Future work will involve the incorporation of these sensors into a neural network device. A neural classifier allows discrimination between complex odours to be performed via computer processing of the signal patterns arising from the sensing elements. The sensors are interfaced to the software via an analogue to digital converter. Devices of this type have been utilised for complex odour analysis in a number of applications. The sensors we have identified will be interfaced to existing software written in-house. The array may also incorporate other sensor types still under development.

As mentioned above, no amines were detected via GC-MS. However, the experiments using the pH sensitive dyes suggested strongly that alkaline vapours were emitted from the diseased tubers. All three dyes changed colour when exposed to diseased tubers but not when exposed to fresh tubers. BPB changed from yellow to an intense blue in about thirty seconds. BCP changed from yellow to blue-green in five minutes, and BCP changed from yellow to dark green and subsequently purple in twenty-five minutes. These results are indicative of ammonia vapour or other basic vapours. It is likely that *Erwinia* infections would give rise to amines as there is sufficient nitrogenous material available. *Erwinia carotovora* does not require a nitrogen source for growth and is capable of fermenting many carbohydrates that exist in vegetables. Although surface sterilisation of the potatoes was carried out prior to inoculation, the possibility remains that the malodour detected may have been partly due to the action of invading micro-organisms which entered after the destruction of the outer plant barrier by the pectinase-producing *Erwinia carotovora*. This process would mirror very closely that occurring in a potato tuber storage facility. The sensing system should still give adequate warning of infections before widespread damage occurs.

Further evidence of amine production was obtained by sensory analysis of potatoes treated with sodium hydroxide. The treated infected tubers when tested against the five sensor types gave responses typically 3 times greater than the untreated equivalents. In the case of sensor type 4, which is extremely sensitive to amines, the difference was 10 times greater. These results appear to confirm the speculation that the majority of amines produced remained within the tuber as protonated salts due to the relatively low pH; basifying with sodium hydroxide then catalysed their release. Thus, despite the GC-MS data, from these investigations it would appear likely that ammonia and other amines are released during the

rotting process. A number of the sensors utilised display high sensitivity to amines and can detect extremely low levels below the threshold for the human nose.

CONCLUSIONS

A study of the interactions of vapours from *Erwinia carotovora* infected tubers with immobilised dye sensors has shown that alkali vapours are released during the infection process. Other organic vapours, such as ethanol, butan-1-ol, acetaldehyde and acetone, were detected in the headspace above infected tubers using GC-MS, in agreement with those reported in the literature. An initial screening of sensors to model vapours resulted in the identification of five sensor types giving high sensitivity to vapours emitted from *E. carotovora* infected potato tubers. These sensors were then screened against infected potatoes under laboratory conditions and were found to give distinct patterns of response when compared directly to the patterns obtained for water vapour or fresh potato tubers. It is therefore envisaged that the combination of these sensors with existing neural network software will produce a device capable of early detection of soft rot in stored potato tubers. Further work is required to produce a sensor system capable of the detection of soft rot in potato storage sites.

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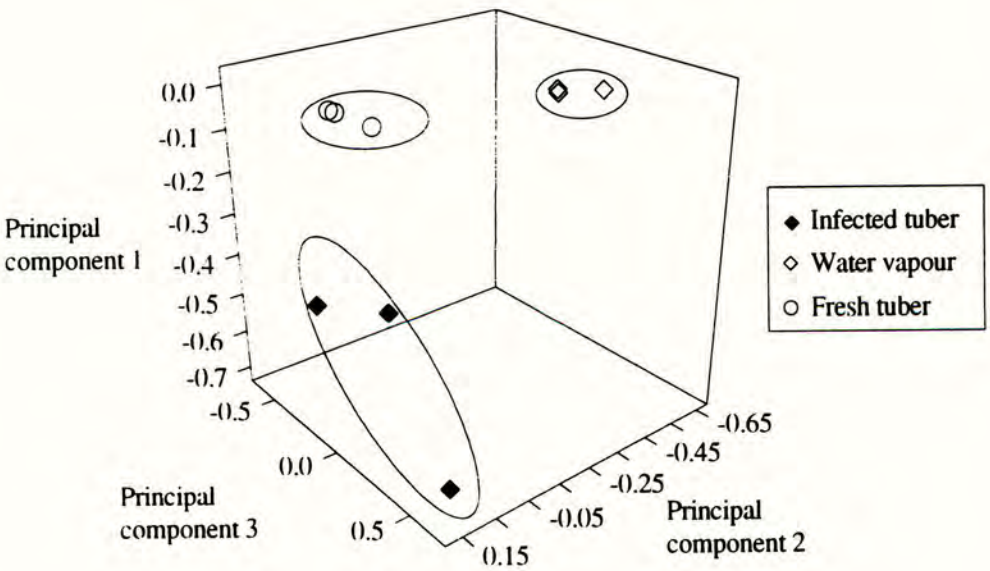

Figure 1. Plot of the first three principal components illustrating the grouping of sensor outputs into three distinct sets (data from Table 2).

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MEETING MARKET NEEDS: DETECTION, PREDICTION AND CONTROL OF PRODUCT QUALITY

R C RIGHELATO
Ashbourne Biosciences, Innovation Centre, University of Reading, Reading RG6 6BX

ABSTRACT

Measurements of "fitness for purpose" are increasingly being built into pricing and buying decisions made by food manufacturers and retailers. The ability to do this is determined by (i) understanding the parameters of raw materials that determine quality for the customer, and (ii) the available detection and measuring technology, which must fit modern packing storing and processing operations and cope with the sampling problems presented by the dispersed, heterogeneous nature of most agricultural produce. A wide range of technologies are being explored, which could increasingly provide information on produce quality in real time, some of which may be developed for use in the field, in storage and in packing houses.

INTRODUCTION

Agriculture is changing from being essentially production driven to an industry driven by the needs of customers. As support systems are dismantled and the food manufacturers and retailers exert their buying power to specify the raw materials best suited to their processes and their consumers, markets often only exist if strict quality criteria are met.

Quality, however, in its many manifestations, is often difficult to monitor and to control and there is a growing need for measurement technology that is commensurate with the scales and speeds of the operations of harvesting, storage, processing distribution and retailing.

This paper assesses the developing needs for quality measurement in the field and factory, and discusses some of the technologies that may be brought to bear.

CONSUMER DRIVERS

Consumers have a hierarchy of requirements of their food: eating quality, nutrition and safety are supplemented by other, sometimes idiosyncratic, factors such as national or regional origin, methods of production, composition and contamination.
Eating quality or expectation that a consumer has of a food is complex, a mixture of visual, olfactory and tactile stimuli when buying, as well as the organoleptic properties

discovered on eating. Retailers and processors increasingly seek to apply objective, physical methods to define key quality parameters in the produce they purchase, and, technology permitting, seek to buy produce screened to fit the requirements (Table 1). Whilst instrumental techniques are available for many of the parameters of interest, most are still laboratory based and many do not have the speed required for on line or at line use.

Table 1 Consumer driven quality parameters

Consumer requirement	Parameter	Possible rapid measurement methods
Eating Quality	shape, size	mechanical grading image analysis
	colour	light spectroscopy
	texture	acoustics, light scattering, rheology, fracture mechanics, magnetic resonance imaging (MRI)
	sweetness	polarimetry, enzyme biosensor
	acidity	pH
	flavour	"electronic nose", GC
Nutrition	composition	FTIR, MRI
Safety	mycotoxins microbial pathogens	ELISA, phage tests, gene probes
Contamination	chemical residues, microorganisms	ELISA, bioluminescence, conductance, volatiles (eg IMS, electronic nose) etc
Authenticity		
composition	species, GMO	ELISA, gene probe
production methods	animal life history organic products	various detection methods + smart cards chemical residues ELISA

MANUFACTURER-DRIVEN REQUIREMENTS

In addition to meeting their customer needs, manufacturers may have requirements of their raw materials to fit storage needs, processing operations and the distribution chain (Table 2). Breadmaking quality, for example, assessed by simple rheological measurements of the flour, is long-established as a criterion for purchase of wheat. Other tests are used for recoverable sucrose from beet, malting quality of barley, freezing quality of peas *etc*. Tests are often based on mimics of part of the manufacturing process, almost all are off-line and often require hours or days to complete. Thus they cannot readily be integrated into modern manufacturing operations, whose competitiveness relies on fast throughputs and low inventories.

Current research on the molecular and genetic bases of the properies of food materials is beginning to provide alternative analytes, such as specific proteins or DNA sequences, which may be monitored more rapidly off line, and in some cases compositional criteria which can be monitored spectroscopically, on-line.

Hygienic quality of raw materials is important in much food processing: specifications usually cover total microbiological load and specific pathogens. With increasing numbers of foods sold as fresh or minimally processed products, often eaten without further cooking, the need for real time monitoring and control of microbiological status of raw materials is growing. The widespread introduction of ATP bioluminescence techniques has provided a rapid way of assessing gross contamination but it is difficult to use to detect pathogens which may be a small part of the population or to use on live plant or animal tissue.

TECHNOLOGY NEEDS AND DEVELOPMENTS

Clearly there are needs for new detection and measurement methods which can be applied to a range of quality criteria in the food chain. Typically measurement is an area of technology whose development is largely science-driven, that is there are many potential applications awaiting solutions. However, there are several problems:

- finding appropriate analytes which are responsible for, or are indicators of, the quality of concern;
- developing methods capable of response times that fit current processing technology (often tens to thousands of items per second through a production line);
- detection of dispersed, discrete targets: *eg* a single rotten fruit in a silo or on a process line, or very low numbers of a specific microorganism in the presence of many more non-target organisms.

There is a wide range of technologies being explored which may provide solutions to these problems. The biodiagnostic techniques (*eg* gene probes, ELISAs) are suitable for many off-line uses, and current research may provide the sensitivity increases needed for real time pathogen detection by, for example, combining immunorecognition with massive signal amplification triggered by the metabolism of viable cells (Aojula, Zamani and Clarke, 1993); this may soon lead to detection of single viable bacteria in the short timescales needed for positive acceptance or release of products. Biorecognition using antibodies is increasingly used for identifying quality-related proteins (table 2) and may be extended to flavour compounds and other non-protein quality indicators.

Imaging technology with the power to inspect the internal structure of food items is based on acoustics and nuclear magnetic resonance (Duce and Hall, 1995). The decreasing cost of computing power, and magnets for MRI, is making these methods more accessible but they suffer from intrinsic limitations of resolution of small objects and time for data accumulation.

Table 2 Manufactuer driven quality parameters

Example Quality requirements	Possible parameters	Possible rapid measurement methods
Processing		
grain quality for milling, malting *etc*	specific grain proteins	ELISA
fruit concentrates	sugars content wall composition	biosensors, polarimetry FTIR, ELISA
Storage		
various fruit potatoes/onions	mould volatiles volatile indicators of sprouting *etc*	ion mobility spectrometry electronic "nose", GC *etc*
Hygiene		
total microbial contamination	on-line: volatile metabolites	IMS, electronic "nose", GC *etc*
pathogens	antigens/DNA	novel, high amplification ELISAs, gene probes

There are many light-based techniques which are generally very rapid; thery are used for size, shape and colour measurement and sorting, and can sometimes be applied to detection of subsurface features. For example, infra-red reflectance, which is used to observe disease defects in potatoes may also detect sub surface bruising; chlorophyll fluoresence (Greaves and Wilson, 1987), which indicates the health of photosystem II, gives a measure of oxyradical induced stress which may be related to post-harvest qualities of leaf vegetables; time-resolved light scattering can give information on sub-surface texture. Because of the speed of most light-based measurements, these techniques hold the greatest promise for field and factory monitoring and robotic operations.

The problems of detection or monitoring in stores or loads of discrete items may be addressed by detection of volatile indicators of the condition of concern. "Sniffing" technologies (Gardner and Bartlett, 1994) have been developed based on gas chromatography, mass spectroscopy, semi-conducting polymer arrays, *etc*, and are beginning to be applied to microbiological detection problems (Ogden and Strachan, 1993).

Biosensor or ELISA arrays, using antibodies or cloned receptor proteins, for recognition of individual flavour volatiles and flavour profiles, which may offer greater specificity and sensitivity than current sniffing technology, may also be worthy of exploration.

It is anticipated that some of these detection and monitoring technologies, developed often in the defence sector and for clinical medicine, will provide the solutions to many of the problems of detection and quality measurement in the food chain, and allow on-line or at-line use in field robotics, packing and factory operations.

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