

Technological Trends and Needs in Food Diagnostics

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Foreword

The study, Technological Trends and Needs in Food Diagnostics, was jointly initiated by Tekes, the National Technology Agency of Finland, and Partners for Life, an EU-funded project for activating SMEs in the food, agro and biotechnology fields to participate in EU research programmes. Recent hazards in the food sector have raised a question of improving food safety. Safer and healthier food is also one of the priority areas of the coming 6th Framework Programme. The objective of this study was to find the newest technology available, to provide a market outlook as well as to chart the business opportunities in the field.

As the writers state in this Technology Review, an increased number of tests does not alone improve food safety, but the problem is more holistic by nature. However, new, improved and faster methods are needed as part of the total food safety system and management. As stated in the review, a large number of new methods can be applied to the food industry as well. How good a business opportunity this is, is somewhat controversial. The food industry, contrary to the pharmaceutical field, is more price-sensitive and may not be as attractive for the diagnostic industry as the traditional diagnostics market is. On the other hand, there may be new business opportunities particularly in the service sector. The new technology may also offer further applications for process control, which will lower the barrier to acquire it.

In addition to the Technology Review, the report offers an account of validation procedures and organisations both in Europe and in the United States.

We hope that readers will find this review useful. We would be very delighted, if this report could promote food safety and create and encourage new business in the area. We would like to thank the writers, Malin Brännback and Gabriela von Blankenfeld-Enkvist, for their excellent and thorough work, as well as all those who have in one way or another contributed to the completion of this report.

August, 2002

Tekes

Acknowledgements

The report at hand is a continuation of a study that was started in October 2001 and was concluded with a background report on food safety in January 2002¹.

We would like to thank all the people who generously shared their time and expert knowledge with us (see Appendix 1). We hope that the report will help to set a frame for further discussions and form a basis for a constructive dialog in the field.

We would also like to thank the following persons at the National Technology Agency of Finland (Tekes), Raimo Pakkanen, Liisa Rosi, Auli Pere, and Pirkko Suhonen. We greatly acknowledge the contributions of Markku Järvenpää (Ministry of Agriculture) and Sabine Herlitschka (BIT, Austria) and the entire group of Partners for Life who acted as efficient catalysts in initiating this study.

Finally, we would like to extend our warmest gratitude to research assistant Johanna Ylitalo whose assistance has been of help during the entire process of completing this report.

¹ Wiklund P., Orava M., Brännback M., and de Heer A. J., (2002): The Present State of the Field of Food Diagnostics. Turku School of Economics and Business Administration/Innomarket (<http://www.tukkk.fi/markkinointi/innomarket/Reports/9752.pdf>)

Executive summary

Food diagnostics as a business area is relatively new and can still be characterized as an emerging one, partly because the industry and the market is not well defined. At this stage it is not clear what specific kinds of business models will prevail as profitable and viable, because it is dependent on whether we are concerned with food analysis, food diagnostics, microbiological testing, or microbiological food testing. The business of food analysis is expected to grow through the increasing demand for food safety, however, it is unclear whether this will lead to actual market growth of food diagnostics. In Europe, the public interest, which appears to be primarily of political character has been driven by a need to re-establish consumer confidence into the public food safety systems.

It is often expected that more testing, especially for food pathogens, will result in an increased safety of the product. However, the concept of food testing is limited in guaranteeing food safety and therefore the HACCP concept has been applied to guarantee process safety rather than product safety. There is a clear corporate and public communication challenge, which needs to be addressed. The question of whether a change of method will lead to a significant improvement in food safety or will mainly have impact on costs has to be further analyzed.

The field of rapid methods is growing fast with numerous analytical options being available for different testing needs. These new rapid methods have the potential to offer considerable advantages concerning sensitivity, speed and potential for automation, allowing increased sample throughput for analytical laboratories, and ease of use.

Many commercial systems that allow fully automated testing, mostly based on ELISA immunoassays are already on the market. The use of new recombinant antibodies and molecular imprinting techniques will improve the sensitivity and versatility of the technique. The development of homogenous assays and automation has improved the usability of the Polymerase Chain Reaction for routine applications in food laboratories. New assay formats are developed that can be further exploited for food diagnostic applications. They often allow simultaneous detection of several analytes.

The major benefit of rapid methods, such as PCR, might be the increased capacity for sample throughput and automation. Consequently, the technique might be more suitable for large analytical laboratories. Immunomagnetic separa-

tion techniques will be further developed to enhance the speed and sensitivity of different detection methods. The automation of this method can be expected in the near future.

The combination of homogenous assays with multiplexing capacity and potential for automation will lead to further decrease in the time to result, increased sensitivity, improved sample throughput and user friendliness.

There is a clear need for better methods that allow real time monitoring of the success of cleaning procedures. The development of real time rapid methods for hygiene monitoring will have an impact on problems that are associated with biofilm formation. This might lead to a reduction of product contamination through biofilms and offer benefits in terms of improved food safety. Methods based on ATP bioluminescence, Adenylate kinase but also biosensor applications might be further developed for this area.

Viable cell counts, as coliform counts, faecal coliform counts, yeast and mould counts, will remain an important tool for the assessment of safety and quality of food products, and real time methods to assess these parameters should be developed. Alternative methods should be cheap, robust and provide analysis results in real time. Microscopic methods based on DEFT, flow cytometry, and biosensors could be developed for this application.

The most promising breakthroughs are to be expected in the area of sensor technology, that will allow the creation of on-line or on-site, sensitive, low-cost devices for routine-use. Biosensors have a high potential for automation and allow the construction of simple and portable equipment for fast analysis. These properties will open up many new applications within quality and process control, control of the fermentation process, and quality and safety control of raw materials. These new applications should be further explored and technologically evaluated.

The current testing practice is hampered by the fact that it is slow, with the result of the microbiological testing normally being available after several days. This is too slow to allow an effective use of the testing results, and valuable time is lost before corrective actions can be established. This can lead to large costs for spoiled raw materials and products, product recalls, and might even damage the reputation of the company. Increasing the speed of the testing method will improve that situation.

Fast microbiological testing methods are needed to give timely information on critical microbial parameters for process control and to be integrated for verification of HACCP systems for food safety. A real improvement of the current methods is taking place through the development of the first real one-shift methods where results can be obtained in less than 8 hours.

The market for food diagnostics is small in absolute terms, but also in comparison to the global market for food analysis. There are approximately 50 established companies, with no apparent market dominator. The companies are either specialized in food diagnostics, sometimes with environmental or veterinary applications, or human in vitro diagnostic companies that use their technologies for applications within food diagnostics.

The European market for food analysis has a value of €490 million with rapid tests worth €86 million. The market share of rapid methods compared to traditional methods is still low. In Europe, it is about 15% for pathogen testing, 17% for spoilage organisms, 40% for chemical contaminants, and 10% for process chemicals. As shown a number of rapid methods are being developed but the acceptance and penetration of these methods is still slow. As a result, the incentives to adapt new technologies to the special requirements of the food industry are low. The future development of the field thus critically depends on a better market acceptance for new methods.

A lack of a validation procedure was commonly considered a major obstacle for the establishment of rapid methods by our interviewees in the food industry. A validation procedure offers the advantage of that the diagnostic company can prove the performance of their new method in comparison to well established methods that are known by the user. This is very valuable information to the user for the decision process of which method to use for each particular purpose. Conversely, a lack of validation data when introducing a new method necessitates that more tests have to

be performed by the food laboratory, requiring time and systematic work effort. It is obvious that this adds to the costs of establishing a new technique in a food laboratory, and moreover, not all laboratories will have the means to perform the necessary studies at all.

The validation process also offers important information to official food control agencies, and allows a degree of control over the quality of the method being offered on the market. The performance results from the validation process can be used by regulatory agencies to accept a new technique as official testing methods. Today, conventional methods have to be used to confirm positive test results obtained with rapid methods. Clearly this does not increase the incentive to adopt new technologies.

With the existence of numerous validation bodies, and in the absence of a mutual recognition, most diagnostic companies that strive for validation of a method will have to deal with several separate regulatory bodies. Moreover, it will decrease confidence of the end-user in the whole validation process, if no common acceptance standards can be created. A validation process with mutual recognition between different validation bodies resulting in global acceptance would be a major step towards the opening of a common market for rapid methods within food diagnostics.

More private service providers are needed in Finland and some degree of consolidation of municipal laboratories as well. Furthermore, encouragement of small firms to establish themselves into the service sector should be promoted. A technological evaluation of the commercial potential of biosensors will also be needed since it has a market potential for different markets. The focus should necessarily not only be on food pathogens, from a market point of view. Other applications should be considered. Finally, there is a need for a strategic decision on whether pathogen testing should be re-integrated into in-house food laboratories or if it should be encouraged that this sector should be outsourced and developed as a private service sector.

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1 Introduction

The aim of the study was to investigate technological trends and needs in Food Diagnostics and to understand the factors that shape and influence the development of this field. Food Diagnostics is an emerging field which applies “modern” methods developed mostly in the field of human in vitro diagnostics to food. It is concerned with the detection of bacteria, viruses, parasites, chemicals, biotoxins, heavy metals and prions in all steps of the food chain from raw materials to end products.

The first part of the report introduces the concept of food safety management tools to provide the frame to understand the role that Food Diagnostics can and should play in the broader concept of Food Safety. We will also discuss the statistical limitations of microbiological testing. The second part of the report will give a concise overview of the methods that are commonly used today and of promising methods under development, their potential field of application, and their current limitations. We will discuss general methodological requirements, and give an introduction into biofilms in the food industry and the problems associated with them. We will mainly focus on methods used for the detection and identification of food pathogens and spoilage organisms. The third part of the report will present a market analysis and discuss the factors that influence the future of the food diagnostic field, with emphasis on method validation. GMO markets are influenced by different factors and will thus be discussed separately. The picture presented in the study was obtained through discussions with key persons from the food industry, research institutes, food laboratories and governmental agencies and by background research using secondary sources².

1.1 Food safety

According to the Codex Alimentarius Commission on food hygiene, food safety is *the assurance that food will not cause harm to the consumer when it is prepared and/or*

*eaten according to its intended use*³. This definition is, however, hampered by the caveat that an absolute level of food safety cannot be obtained. It has therefore been recognized that instead, an acceptable level of risk has to be defined.

Throughout the world, food production, preparation and distribution have become increasingly complex, and raw materials are often sourced globally. Changes in food processing techniques, food distribution and the emergence of new food pathogens have changed the epidemiology of food-borne diseases. Food-borne microorganisms are continuously changing due to their inherent ability to evolve and their amazing capacity to adopt to different forms of stress. New primary production technologies and food manufacturing practices are introduced all the time, food consumption patterns and the demographic structure of many countries continue to change. The implementation of Food Safety should be seen as an ongoing process, which is influenced by environmental, socio-economical, political and cultural factors. Food safety issues need to be managed on a continuous basis, from a regional, national, European and global point of view.

New, flexible tools are required for evaluating and managing new food safety challenges. It is beyond the scope of the report to discuss all the aspects that might be relevant to food safety. We would like to refer to the comprehensive report recently published by the Institute of Food Technologists (IFT) (2002)⁴.

The report “*European Policy on Food Safety*”⁵ (2000) provides a relevant discussion on concerns regarding food safety quality standards and public health policy making and an analysis on the Commissions proposal on the “*White paper on Food Safety*”.

In the following, we will focus on food safety management tools, as they directly relate to the issue of food microbiological testing.

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- 2 We would like to particularly refer to the following sources used for background information: Forsythe S.J. The Microbiology of Safe Food (2000) Blackwell Science Ltd.; Encyclopedia of Food Microbiology (1999) Ed. Robinson, R.K., Batt C.A., Patel P., Academic Press; and the web pages of the Institute of Food Technologists (IFT) (<http://www.ift.org/publications/>)
 - 3 Codex Alimentarius Commission (1969): Recommended International Code of Practice General Principles of Food Hygiene. CAC/RCP 1-1969, Rev. 3 (1997)
 - 4 Institute of Food Technologists (IFT)(2002): Emerging Microbiological Food Safety issues, Implications for control in the 21th century. (<http://www.ift.org/govtrelations/microfs/>)
 - 5 Trichopoulou A., Millstone E., Lang T., Eames M., Barling D., Naska A., and Van Zwanenberg P., European Parliament, Directorate General for Research, Directorate A (2000): European Policy on Food Safety”. (http://www.europarl.eu.int/stoa/publi/pdf/99-indu-envi-03_en.pdf)

1.2 Food safety management tools

End-product testing and microbiological criteria were traditionally used to evaluate the safety of a food product and to set criteria for deciding about rejection/acceptance of a food batch. This approach, however, has some limitations, especially for testing of pathogens that occur in low numbers and are non-randomly distributed.

To guarantee the safety of foodstuffs producers have therefore shifted their focus towards the use of food safety management tools, most important HACCP (Hazard Analysis Critical Control Point), and the consequent application of hygienic measures, based on Good Manufacturing/Hygienic practice (GMP/GHP). Food safety management tools use input of scientific information to identify critical contamination points in the food chain and the production process, and design measures to control them. However, the lack of reliable data is often limiting the usefulness of this approach and therefore data collection is one of the priorities for future food safety strategies. In the absence of relevant data, other strategies might still have to be used to control food hazards. The integration of different food safety tools is required for the production of safe foods from “field to fork”.

As shown in Figure 1 quality assurance and safety tools can be combined in an approach that integrates quality control and quality assurance with a commitment to food safety.

1.2.1 Good agricultural practice

It is not possible to produce crops that will be completely free of harmful microorganisms due to the many possible routes for contamination, but risks can be minimized through certain practices. Important preventive strategies include the management and control of manure and irrigation water.

1.2.2 Good manufacturing practice and good hygienic practice

Good Manufacturing Practice (GMP) and Good Hygiene Practice (GHP) describe the basic measures that have to be applied during production, processing, handling and distribution, storage, sale, preparation and use. They form the basis for other approaches, most importantly HACCP.

Requirements for GMP/GHP have been developed by the Codex Alimentarius Commission on Food Hygiene and the food industry, and cover the following general requirements⁶:

- the hygienic design and construction of food manufacturing premises
- the hygienic design, construction and use of proper machinery
- cleaning and disinfection procedures
- the microbial quality of raw foods
- the hygienic operation of each process step
- the hygiene of personnel and their training.

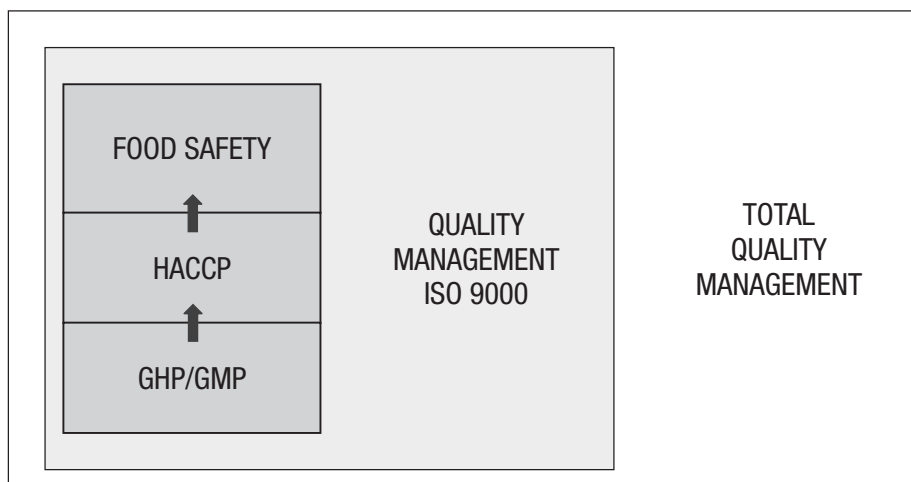


Figure 1. Food safety management tools.

⁶ Codex Alimentarius Commission on Food Hygiene (1969) General Principles of Food Hygiene Vol. 1A CAC/RCP 1, Amendment 1999

1.2.3 Hazard analysis critical control point

Traditionally, the microbiological safety of food was controlled through testing of the end-product for pathogens or their toxins. This approach, however, has limitations to guarantee that safe foods are produced, as discussed in detail below. The adoption of Hazard Analysis Critical Control Point (HACCP), based on GMP and GHP as the main approach to ensure adequate food safety has been accepted world wide. HACCP is a systematic, scientific approach to identify specific hazards and establish appropriate control systems. The focus is on the prevention of problems. The HACCP system does therefore not rely on end-product testing for pathogens. Instead, analyses are performed at points along the production chain, particularly to verify that Critical Control Point (CCP) are under control. Random sampling and analysis might be useful in the HACCP verification step. Fig. 2 shows the seven HACCP principles⁷.

Hazard: a hazard is defined as a physical, chemical or biological agent in a food, or condition that might cause an adverse health effect.

Critical Control Point (CCP): a step at which control can be applied and that is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level.

Critical limit: a maximum and/or minimum value to which a biological chemical or physical parameter must be controlled at a CCP.

HACCP principles can also be applied to other parts of the food chain, under the condition that a critical control point can be identified. The introduction of the HACCP system allows regulatory agencies to shift their control efforts towards inspection of the HACCP system. The HACCP system can be combined with food safety objectives to develop quantitative process performance criteria.

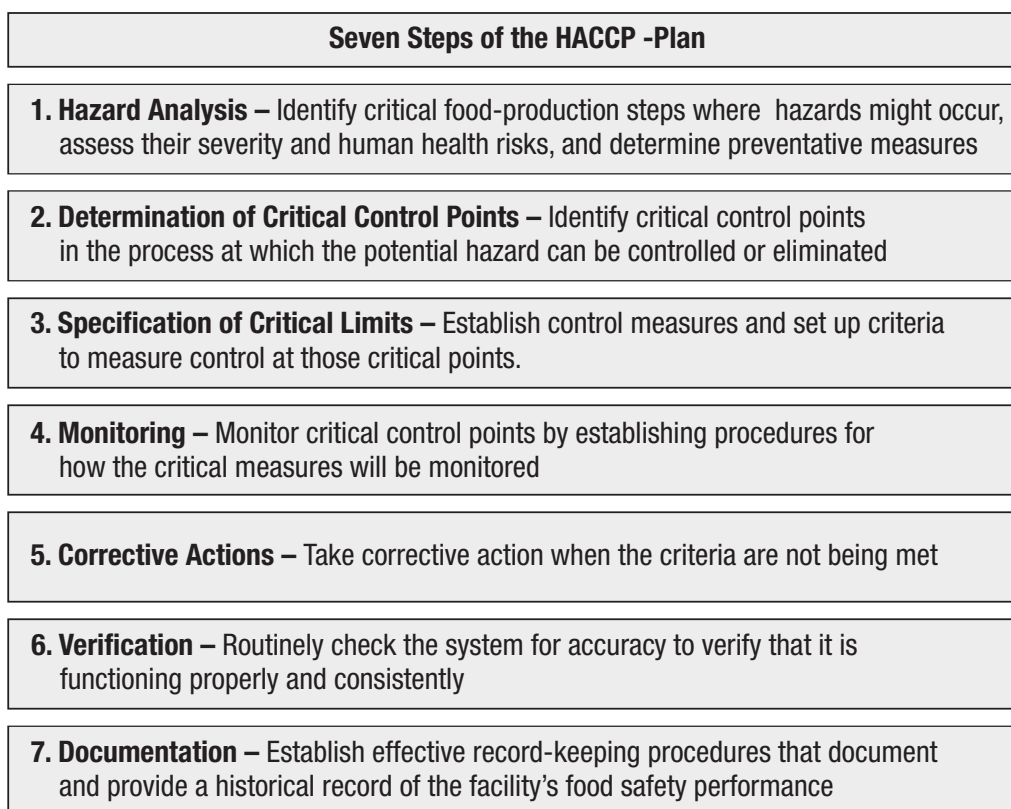


Figure 2. The hazard analysis critical control point system.

7 Codex Alimentarius Commission (1997): Hazard Analysis Critical Control Point (HACCP) System and Guidelines for its Applications. Annex to CAC/RCP 1-1969, Rev. 3 1997

1.2.4 Quality management systems

The aim of quality assurance is to ensure that a product continuously conforms to standard. The ISO 9000 series of Standards contains 5 different standards for quality management and quality assurance that can be used for total quality management and HACCP. They represent a generic management system which means that the same standard can be applied to any organization irrespective of its products and sector of activity.

The three core standards are: the ISO 9000 Quality management system – Fundamentals and vocabulary; the ISO 9001 Quality management system – Requirements; and the ISO 9004 Quality management system – Guidelines for performance improvement. The ISO 9000 were published in 1987 and completely renewed in 2000. In the future, ISO 9001:2000 will be the only standard through which an organization can be certified.

1.2.5 Total quality management

Total Quality Management (TQM) is a structured system for satisfying internal and external customers and suppliers by integrating the business environment and continuous improvement. It aims at long-term success through customer satisfaction.

1.2.6 Risk analysis

Risk analysis (RA) is a management tool for governmental bodies to define an appropriate level of protection and establish guidelines to ensure the supply of safe food. *Risk* in this context is defined as a *health effect caused by a hazard in a food and the likelihood of its occurrence*. Risk Analysis is useful to decide which hazards should be prevented, eliminated or reduced to acceptable levels.

Risk analysis can provide the input to develop food safety policies that are based on scientific data to define *acceptable levels of risks*. Food Safety Objectives can translate public health goals into quantitative targets to define appropriate levels of control. They are also important to evaluate the equivalence of different processes in respect to their safety performance.

RA consists of three elements:

1. *Risk assessment* is the use of scientific data to identify, characterize and measure hazards, assess exposure and characterize the risks involved with a food. In food safety regulations and policy development, risk assessment should consider the likely impact of a particular food safety problem and protective measures, and the urgency and controversy surrounding an issue. The final risk estimate should always contain information about assumptions made, and the degree of the variability and uncertainty in all steps of the risk assessment procedure. The Codex Alimentarius Commission has developed guidelines for risk assessment⁸.
2. *Risk management* is defined within the Codex as the process of weighing policy alternatives in the light of the results of risk assessment and selecting and implementing appropriate control options. The outcome of the risk management process is the development of standards, guidelines and other recommendations for food safety. If necessary, controls and regulatory measures are adopted.
3. *Risk communication* is defined as an interactive process of exchange of information and opinion on risk among scientists, policy makers, and the public during the risk assessment and management process.

1.2.7 Food safety objectives

A Food Safety Objective (FSO) is a statement of a maximum frequency and/or concentration of a microbiological hazard in a food at the time of consumption that provides an appropriate level of consumer protection^{9,10}. For example, the health goal of reducing the incidence of a food borne illness by 50% from 10% to 5% per year could be translated into an FSO of 100cfu/g (colony forming units/mass) as maximum bacterial number at the time of food consumption. By projecting the possible growth of bacteria between production and consumption, a processing safety objective at the time of the production can be established. This can be used to define performance criteria for the production process, which would be the level of reduction that the process has to obtain to meet the processing safety objective. Now the process criteria or product criteria that will be needed to achieve this performance can be defined. The process criteria might be time and temperature of a processing step, a product criterion might be the pH of a

8 Codex Alimentarius Commission (1999): Principle and Guidelines for the Conduct of Microbiological Risk Assessment. CAC/GL30

9 International Commission on Microbiological Specifications for Foods (ICMSF)(1997): Establishment of Microbiological Safety Criteria for Foods in International Trade. World Health Stat.Quart. 50: 119-123

10 International Commission on Microbiological Specifications for Foods (ICMSF)(2002): Microbiological Testing in Food Safety Management. Kluwer Academic, N.Y.

product. The critical limits of the process will be monitored using HACCP. The transition from microbiological criteria to Food Safety Objectives will also increase the choice for different methods of analysis.

1.2.8 Microbiological criteria

Microbiological criteria have historically been used to accept/reject food lots with unknown history at the port of entry and are subject to the same limitations as end-product testing.

Microbiological criteria usually define^{8,11}

- the microorganism of concern and/or the toxins and the reason for concern related to these
- the microbiological limits for the foodstuff at a specified point in the food chain
- a sampling plan and a decision criteria to accept/reject, usually in the number of samples that have to conform to the limits
- the analytical methods used for detection and/or quantification.

In 1985, the Food and Nutrition Board Council (FNB/NCR) addressed the subject of microbiological criteria and found that such criteria were of limited use¹². This is particularly true when safety assurance is the goal and therefore HACCP should be applied wherever possible. However, microbiological criteria are often demanded by governmental agencies, or between companies in a supply chain.

1.3 Limitations of microbiological food testing

Pathogens of concern often have a low incidence rate in a food-stuff or raw materials and are frequently not equally distributed. This results in statistical limitations for the usefulness of food testing to confirm or reject the presence of a pathogen. Not all food from a lot can be tested for the presence or absence of a certain pathogen. Instead, samples have to be taken, that should be representative for the whole lot. So called “lot-acceptance sampling plans” are often used to establish criteria for acceptance or rejection of a food lot and whether a product meets a certain set of specifications. The number of samples and the % occurrence of the contaminant (% defective lots) determines the probability that a defective lot is accepted (Table 1). For example, if 30 samples are collected from a lot with a defect rate of 1%, the probability for accepting is as high as 74%. The higher the prevalence of the contamination, the higher the probability to detect a defective sample.

This is of practical relevance for many food pathogens. E.coliO157:H7 in ground beef, for example, has a prevalence rate of less than 1%. Also the prevalence rate for listeria monocytogenes in ready-to-eat foods was reported to be between 1.08% to 4.91%¹³.

Non-random distribution of a pathogen adds to the problems of detecting a contamination when occurring at low rates. The effectiveness of testing is further influenced by sample preparation and the sensitivity and reliability of the analytical method.

Table 1. The probability of accepting a defective product using a 2-class sampling plan (differentiates between accept/reject and is often used for pathogens with zero-tolerance for occurrence) in relation to the number of samples taken³.

Composition of lot	Number of Samples						
	10	20	30	50	100	200	300
Percent defective							
1	0.90	0.82	0.74	0.61	0.37	0.13	0.05
5	0.60	0.36	0.21	0.08	0.01		
10	0.35	0.12	0.04	0.01			

11 Codex Alimentarius Commission (1997): Principles for the Establishment and Application of Microbiological Criteria for Foods, General Requirements (Food Hygiene). Suppl. to Vol.1B, CAC/GL 21
 12 Committee on Food Protection, Food and Nutrition Board Council, National Research Council (FNB/NCR) (1985): An Evaluation of the Role of Microbiological Criteria for Foods and Food Ingredients. National Academy Press, Washington, D.C.
 13 Food and Drug Administration/Center for Food Safety and Applied Nutrition U.S. Dept. of Agriculture/Food Safety and Inspection Service, and Centers for Disease Control and Prevention FDA/CFSAN, USDA/CDC (2001) <http://www.FoodSafety.gov/~dms/lmrisk.html>

The predictive value of a test is influenced by the prevalence of the defect, and additionally by the sensitivity and specificity of the test³.

Sensitivity is the probability of a sample testing positive if a contamination is truly present.

Specificity is the probability of a test being negative if a contamination is truly absent.

With a test sensitivity and specificity of 95%, a pathogen with a high prevalence of 60% (e.g. campylobacter in poultry), the test will be positive in 96.6% of all cases where the sample is truly contaminated. This means that testing results will correspond well to the true level of contamination.

With a test sensitivity and specificity of 95%, and a defect rate of 1 % (e.g. E.coliO157:H7 in ground beef) the sample will be truly contaminated in only 16.1% of all positive test results.

The high rate of false-positive and false-negative results limit the value of testing in food with low defective rates. In this case, it can not be concluded that food that has been sampled for a certain pathogen and has tested negative is actually safe for consumption. This will also create an unnecessary high expenditure for testing with inadequate benefits in terms of increased food safety.

As the defect rate of the product becomes low, it is more efficient to implement control systems that have been validated for the pathogens of concern. These limitations should also be taken into account when methods for the detection of pathogens with low occurrence are developed. Food safety under these circumstances will be more efficiently accomplished using other tools than e.g. online testing.

2 Methods

Safety and quality of a product are best controlled through effective management of those processing steps where hazards may arise. Microbiological contaminants in food are still the most important problems in terms of economical costs and epidemiological impact. We will therefore focus on methods used for microbiological testing.

Establishing critical limits within the processing steps and monitoring of the relevant parameters allows for control of the process. It is not possible to use microbiological parameters as critical control points, since the current methods are too slow to give the fast feedback that is required within a HACCP system. Even testing of raw materials and end-product testing is hampered by the fact that the methods are too slow. Therefore, much effort is put into developing methods which will allow the assessment of the microbiological quality and safety of foods in shorter time.

Food is a difficult test matrix since it is extremely varied in its chemical composition, contains many different ingredients, and often contains an intrinsic microbial flora with varying amounts of shelf-limiting bacteria and even pathogens. To account for the differences, the Association of Official Analytical Chemists (AOAC) recommends as many as 14 different food groups to be included in the validation procedure for Salmonella tests.

Furthermore, food is processed with varying technologies and stored under different conditions. During processing bacteria might be damaged sublethally with subsequent problems of detection. As a result, different enrichment protocols might be necessary.

Microbiological methods are divided into *traditional* or *conventional* methods and *rapid* methods. Conventional methods are still considered the *gold standard* and are often required by national and international regulatory agencies as official control methods.

The term *Rapid Methods* is used for different types of tests including miniaturized biochemical kits, antibody-based tests and nucleic acid hybridization-based tests. They can be manual, semi-automated or fully automated.

Ideally, the rapid methods should enable a quick estimation of the microbial parameters of the food to allow for immediate corrective actions during the manufacturing process. However, the majority of rapid methods do not presently meet this demand. They do offer advantages in analysis

time but also give the possibility to eliminate labour intensive steps and give the potential for automation. Due to the speed-up of the sampling process, sample treatment, detection/enumeration procedure, the output of the laboratory can be improved. However, a real shortening in the time-to-result can only be obtained if the lengthy incubation procedures can be replaced by faster techniques or even omitted.

In this chapter we will give a short overview of the methods that are commonly used today and of promising methods currently in development, their potential field of application, and their current limitations. We will discuss general methodological requirements, introduce the concept of hygiene control or environmental testing and give an introduction into biofilms in the food industry and the problems associated with them. We will also discuss the limits of microbiological testing. Usually, quality control of foods is obtained through viable cell counts, which represent the most commonly performed operation in food testing. Therefore alternative methods for viable cell counts are described in more detail. All the methods described for pathogen testing can also be used for detection of spoilage organisms but will be mostly discussed in respect to the more demanding pathogen testing. The demands on the method and their precise adaptation might vary greatly depending on the specific application.

2.1 Traditional methods

Traditional or conventional methods for isolation and identification of microorganisms involve the homogenization of the food, its inoculation into selective media and incubation for a predetermined period. Samples can be solid, liquid, air or surface samples. The sampling methods depend on the material, the surface structure, and the expected contamination level. Food samples are currently almost exclusively taken manually by destructive techniques such as excision or scraping.

Diluting or enrichment steps might be necessary depending on the level of the microorganism. To substitute for the enrichment step techniques such as filtration, centrifugation, or magnetic separation might be employed. PCR can also be used for target amplification.

After enrichment the broth is streaked on to solid media to yield isolated colonies that can be identified on the basis of

their biochemical and serological characteristics. Identification relies on cultural enrichment to increase the number of the target organism and to allow for resuscitation of injured cells. Followed by selective plating, these methods provide discrimination of the target organism from the background microflora but are non-enumerative. Enumeration methods are based on the ability of a bacterial cell to multiply in a nutrient-rich medium. Sometimes selective agents are added to favour the growth of a specific group of organisms, but most of these methods are quite unspecific.

There have been a number of developments to speed up or automate the procedure, including the addition of colorimetric/fluorimetric substrates, the development of rehydratable nutrients eliminating the requirement to pour nutrient plates, and the development of biochemical identification kits.

2.2 Rapid methods

Recently, a comprehensive review on “Rapid methods and Automation in Microbiology” has been written by Fung (2002)¹⁴. An introduction to rapid methods can also be found in the Bacteriological Analytical Manual Online (BAM)¹⁵.

Rapid methods can be used for isolation, early detection, characterization and enumeration of microorganisms and their metabolites. According to Fung¹³ the development of methods can be described as follows:

- 1965–1975: the age of miniaturization and diagnostic kits development
- 1975–1985: the age of immunological kit development
- 1985–1995: the age of genetic probes, molecular testing systems, and polymerase chain reaction (PCR) applications
- 1995–: the age of biosensor, computer chip and microarray systems.

Most rapid methods try to replace the selective and differential plating step of conventional methods with more rapid techniques. Pathogen testing requires very sensitive methods because for many pathogens in food there is a zero tolerance, namely that less than 1 cfu (colony forming unit) is allowed in two parallel 25g food samples. To obtain such sensitivity, all current methods need at least one enrichment step, usually incubation for a minimum of 6 up to 48 hours.

A new method must have high sensitivity, high specificity, high precision (repeatability), be rapid, robust and cheap. There is currently no method that will fulfil all requirements. Antibody-based methods as represented by the ELISA assay and DNA based methods as the polymerase chain reaction are the most widely used technologies in food diagnostics today. Also Immunomagnetic separation (IMS) is exploited in a number of commercially available kits and will become an even more important technology in the future. We will therefore give a more thorough description of the present techniques including the currently available commercial assays that represent the most advanced developments in their field. A number of interesting novel methods are currently being developed and will be introduced in more detail in section 2.3.

While some microarray based systems are commercially available, most of the biosensors and microarray based applications are still on the level of prototypes. For other promising methods with future potential, but with less momentum for commercial development specifically in food diagnostics, as flow cytometry, bacteriophage technology or adenylate kinase, only a basic description of the method is given together with brief background information on the current stage of its development. Since it is not possible to explore all methods, assay formats and commercial kits in this rapidly growing area in the context of this report, we have chosen to highlight promising technologies instead of giving a complete picture of the field. A list of Rapid Test kits, including information on the recognition status is available from the AOAC¹⁶ pages. The level of information given might vary considerably between different methods described, reflecting our opinion of their relevance for current or future applications in food diagnostics.

2.2.1 Advances in viable cell count methods

One of the most common tests is the *viable cell count*, the enumeration of viable microorganisms. It gives information on food quality, food spoilage and food safety. Even if they cannot be considered to be a “rapid method”, they will be covered due to their importance for routine food testing. Originally, these methods were introduced to perform total viable counts, coliform counts, faecal coliform counts, yeast and mould counts, with the subsequent introduction of differential counts and pathogen counts. Although standard plate counts are easy to perform and cheap, they are time-consuming in terms of operation and data collection.

14 Fung D.Y.C., Comprehensive Reviews in Food Science and Food Safety (2002): Rapid methods and Automation in Microbiology. (<http://www.ift.org/publications/crfsfs/crfsfs-20001206.pdf>)

15 Bacteriological Analytical Manual Online (BAM): www.cfsan.fda.gov/ (CFSAN)

16 AOAC www.aoac.org/testkits/microbiologykits.htm

Table 2. Alternative methods for viable cell counts.

Product	Company	Principle
Autoplater	Spiral Biotech, Bethesda, Md. USA	Spreads a liquid sample on a surface of a Petri dish with a concentration gradient. Count manual or electronic. Completely automatic system.
Whitley automatic Spiralplater	Microbiology International, Rockville, Md. USA	See above
Petrifilm system	3M Co., St. Paul, Minn. USA	The liquid sample is directly applied on a film with embedded rehydratable nutrients, incubated for 24-48 hours and counted using a Most Probable Number (MPN) conversion table.
IsoGrid system	QA Laboratories, San Diego Calif. USA	The probe is filtered through a square filter with hydrophobic grids and incubated to allow colony growth and counted.
SimPlate system	Biocontrol, Bellevue, Wash. USA	The liquid sample is added and mixed with nutrients and indicators and evenly distributed in a microplate. During incubation fluorescent enzyme substrate is hydrolyzed by the bacteria. The number of fluorescent wells is counted and analyzed using a MPN conversion table.
Redigel system	3M Co., St. Paul, Minn. USA	The probe is added to a ready-to-use tube containing a liquid pectin gel with nutrients. Pouring into a calcium-coated Petri dish leads to hardening of the complex resembling normal agar, that can be incubated and counted.

Traditional total viable counts take three days to complete and utilize high amounts of reagents and consumables. For that reason, there is a considerable interest in the development of alternative methods for viable cell counts. Some of the alternative methods developed are described in Table 2. More methods based on growth and metabolic activity are available, like optical methods using specific chemical and physical changes connected with microbial growth that results in a colour change of the medium, turbidimetry, thermal methods and electrical methods based on impedance or conductance changes, that are not further described here.

2.2.2 Microscopic methods for bacterial counts

A number of rapid methods using microscopic detection have been developed to employ bacterial counts that often can be performed in less than 1 hour. It is possible to investigate the total level of viable microorganism, specific bacterial groups or specific types of microorganism. These methods have already been utilized for counting bacteria in milk, milk products, water, beverages, raw meat, fish, poultry and food contact surfaces.

The Direct Epifluorescent Filter method (DEFT) is based on membrane filtration and detection either by fluorescent staining with acridine orange, which stains all cells, or by BAClight, which differentiates between viable and dead cells. The sensitivity of the method is 10^4 cfu/ml sample. The cells are then counted by eye or in semi-automated imaging systems. A similar method test can be completed within 20 minutes is currently implemented in Irish Food factories¹⁷. Specific sample preparation methods have to be developed for each type of food. By using pathogen specific antibodies, specific microorganisms can be detected and the sensitivity of the method is about 10^3 cfu/ml sample.

The Chemunex Scan RDI system (Monmouth Junction, N.J. USA) uses filtration and subsequent staining with a fluorescent dye, and analysis in a scanning chamber.

The Microstar System (Millipore, Bedford MA, USA) uses a technology that traps bacteria in the matrix of the filter, with subsequent growth of colonies. By this method, only viable cells are counted. The filter is sprayed with a permeabilizing reagent to release ATP, which can subsequently be detected using a bioluminescent agent and analyzed using a CCD camera system.

17 Duffy G., Kilbride B., Fitzmaurice J., Sheridan J. J., The National Food Centre Teagasc, Dublin (Ireland) (2001): Routine Diagnostic Tests for Food-borne Pathogens, (<http://www.teagasc.ie/research/reports/foodprocessing/4681/eopr-4681.pdf>)

2.2.3 Miniaturization and diagnostic kits, biochemical identification techniques

The identification of microorganisms with conventional methods using different biochemical test is labour intensive, with high usage of consumables and reagents. Miniaturized systems in the form of diagnostic kits have been developed that are based on the use of dehydrated growth substrates, or on ready-to use media. These techniques are usually termed modern biochemical identification techniques, are widely used and have a high importance for daily laboratory practice.

Pure cultures are grown in a variety of liquid or solid media and detected by a colour change, gas formation or enzyme-induced changes of the colour of the substrate. The results can be compared to a diagnostic chart, or analyzed with a database.

A number of diagnostic kits are on the market for the identification of pathogens, spoilage organisms, starter cultures, etc.¹⁸. The systems offer visual analysis of the results or databases might be used to interpret and analyze the results in semi-automated and automated systems. In a comparison with conventional methods the use of kits was considered accurate, efficient, time-, space-, labour-saving and cheaper than conventional methods. They have a widespread use and conventional methods are often mainly relevant for reference testing.

2.2.4 Antibody-based methods

Immunochemical analysis is a well-established technique with application to many different fields. Antibody-based methods have proven to be simple, rapid and sensitive for detecting and quantifying different types of food contaminants.

Antibodies can be used to detect pathogens and spoilage organisms such as viruses, bacteria and moulds, but also low molecular weight food contaminants, such as mycotoxins, pesticides and veterinary drugs. However, the development has been constrained by the availability of high performance antibodies against the selected target analyte. The specificity of an immunoassay is largely determined by the intrinsic specificity of the antibody used for detection. There are three different types of antibodies available for assay development: polyclonal antibodies, monoclonal antibodies and recombinant antibodies. Recombinant antibodies are a very powerful new approach which offers many

advantages. Also the emergence of molecular imprinting techniques opens possibilities for new applications.

Recombinant antibodies and molecular imprinting

Recent advances in gene technology have also greatly facilitated the genetic manipulation, production, identification and conjugation of recombinant antibody fragments. The antigen binding domains of monoclonal antibodies can be rescued from the hybridoma cell lines and produced in heterologous systems for "Hybridoma Immortalization". Genetic fusion and recombinant expression allows the development of completely new heterologous fusion proteins for research, diagnosis and therapy.

Molecular imprinting is the process of template-induced formation of specific recognition sites (binding or catalytic) in a material where the template directs the positioning and orientation of the material's structural components by a self-assembling mechanism.

The material itself could be oligomeric (the typical example is DNA replication process), polymeric (organic MIPs and inorganic imprinted silica gels) or 2-dimensional surface assemble (grafted monolayers).

The selection of the appropriate antibodies allows the construction of tests with broad respectively narrow selectivity. Usually, an enrichment step is necessary. Tests can be single-use format like dip-sticks or done in miniplates, microplates or special formats. Only a few basic forms of antibody assay formats exist, but many different modifications are on the market. Both homogeneous and heterogeneous assays are widely used, but homogeneous assays are considered superior in their ease-of-use.

The different assay format include:

Latex-agglutination test: antibody-coated coloured latex-particles or colloidal gold particles are used for quick serological identification or typing of pure culture isolates of bacteria. With presence of the antigen, agglutination that can be visually inspected occurs. Reverse latex agglutination tests for soluble antigens and is often used for toxins.

Immunodiffusion test format: an enrichment sample is placed in a gel-matrix with the antibody. If antigen is present, a visible line of precipitation is formed

ELISA (enzyme-linked immunoabsorbent assay) (Fig. 3). The ELISA test exists in different formats, but usually consists of a sandwich procedure. The antibody is bound to a

18 Systems available on the market include: API systems (bioMérieux, Marcy-'Etoile, France), Enterotube Minitek (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), Crystal ID System (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), MicroID (Organon Teknika, Durham, NC, USA), RapID Systems (Remel, Lenexa, KS, USA), Biolog (Biolog, Hayward, Ca, USA) and Vitek (bioMérieux, Marcy-'Etoile, France).

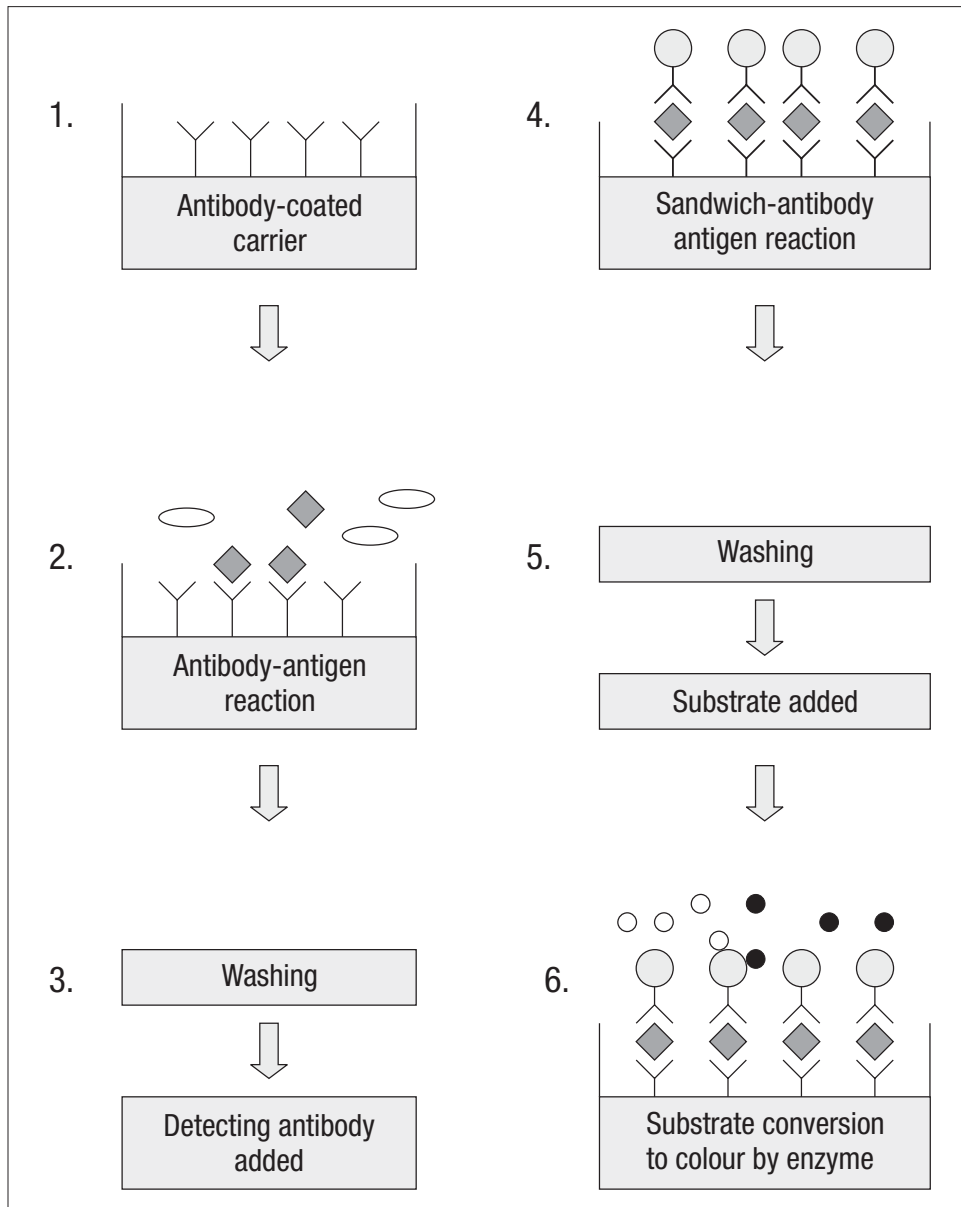


Figure 3. The test well is coated with the primary antibody against the target-analyte (1). The sample is added and upon presence, the analyte binds to the antigen and forms an antibody-antigen complex. Only the specific analyte is bound (2). The test well is washed several times to remove unbound antibody (3). The secondary antibody (detecting antibody) is added that recognizes the antigen-antibody complex. It is coupled to an enzyme for the detection procedure (4). Unbound antibody is washed away (5). The substrate is added. The Enzyme coupled to the secondary antibody converts the substrate into a coloured end product that can be visually inspected (6)

Table 3. Automated ELISA systems.

System	Company	Description
VIDAS	bioMérieux, Marcy-'Etoile, France	Uses a sensitive fluorochrome for detection and is called ELFA. More than 13.000 units in use worldwide. Available for Listeria, Listeria monocytogenes, Salmonella, E. coli O157, staphylococcal enterotoxin and Campylobacter.
Assurance EIA	BioControl, Bellevue, Wash. USA	Adapted to automation in high-volume testing. Available for Listeria, Salmonella, E. coli O157, Campylobacter.
Transia Elisamatic II	Diffchamb, Västra Frölunda, Sweden	High-throughput, fully-automated
Detex	Molecular Circuitry Inc. King of Prussia, PA, USA	Electrochemical immunoassay biosensor, performs 27 tests per run. Analyzes different pathogens during one test run and can test samples simultaneously for two different pathogens.

solid matrix, usually a 96 well plate, but also dipsticks, paddles, pipette tips or other solid matrixes have been used. The sample is added, and upon presence of the antigen binding occurs that can be monitored using a secondary antibody coupled to an enzyme. Upon adding of substrate, a colour reaction occurs. The principle of an ELISA is shown in Fig. 3.

The detection limit of an ELISA is between 10^4 – 10^6 cfu/ml, an overnight incubation to detect pathogens with low occurrence is therefore necessary. Many companies market systems that are based on ELISA assay formats. Recently, a number of fully automated systems have been introduced. Table 3 shows some important systems.

Immunochematography

This is also a test format using a “sandwich” procedure but the secondary antibody is coupled to latex beads or colloidal gold. The enrichment sample is transported through a series of chambers, and no washing is necessary. This is the technology used for home pregnancy tests. “Lateral flow technology” is the newest development based on this principle. The test system usually consists of 3 reaction units (regions). The first well contains the antibody attached to colour particles. The liquid sample is added and upon formation of the antibody-antigen complex, the complex will migrate by capillary action to the second region where it is captured by a second antibody and a visible line will form. Excess antibody will migrate to the third chamber where it is captured with a third antibody and forms a control band to show that the assay has worked. The test is completed within 10 minutes, but requires overnight incubation of the sample.

The Reveal system (Neogen, Lansing, Mich. USA) and the VIP system (BioControl, Bellevue, Wash. USA) are on the market for the detection of E.coli O157, Salmonella and Listeria. Eichrom Technologies (Darmstadt, Germany) markets the Eclipse system for the detection of E.coli O157.

Some recently developed immunoassays make use of the growth of the pathogens in the actual assay. An example is the Unique Salmonella detection system developed by Tecra (Roseville, Australia). An overnight incubation is also necessary for this method. The sample is added to the first tube and a dipstick coated with Salmonella antibodies is added for 20 minutes to capture Salmonella and transferred into a second tube where it remains for incubation to allow further growth of the captured bacteria. Newly produced bacteria will also be captured by the antibodies. The dipstick is then placed in 3 more tubes for reaction with the secondary antibody, washing and the colour reaction. The system can be used in laboratories with small volume throughput.

2.2.5 Immunomagnetic separation

Immunomagnetic separation (IMS) techniques can be used to replace or supplement and speed up the enrichment step that is usually necessary before the detection of pathogens. IMS differs from the assay format described above, as it is not a detection method in itself. It can be combined with different end-detection methods. A number of kits are exploiting this technique with examples given below.

For IMS, paramagnetic beads coated with various antibodies are used to concentrate target bacteria selectively. Two companies currently produce antibody-coated magnetic particles for the use in food diagnostics: Dynal Biotech (Oslo, Norway) and Vicam (Watertown, MA, USA).

Dynal beads are uniform, super paramagnetic polystyrene microspheres. The hydrophobic surface allows the absorption or coupling of different molecules. Paramagnetic and superparamagnetic beads only have magnetic properties when exposed to a magnetic field and can therefore be easily resuspended without aggregation in the absence of a magnetic field.

For the separation procedure the sample is mixed with the antibody-coated paramagnetic particles, and after incubation, the bead-bacteria complex is extracted through application of a magnetic field with a magnetic device. The complex is washed once or twice. Bacteria remain viable after IMS and do not need to be detached from the beads prior to cultivation. The procedure is not really quantitative due to differential recovery rates of the bacteria-bead complexes and the possibility of several bacteria attaching to one bead. Recovery rates range typically between 25 to 50%. An advantage is the reduction of the background microbial flora that shortens the time required for selection of suspect colonies for confirmation.

Under optimized conditions pre-enrichment times as low as 4 to 6 hours allow the detection of 1–2 bacterial cells/g of the original food sample.

There are a number of commercial available test kits that combine IMS with various detection methods.

The EHEC-Tek system (Organon Teknika Corporation, Durham, North Carolina, USA) uses IMS to capture E.coli O157:H7 prior to the ELISA detection procedure, which leads to enhanced assay sensitivity.

The Origen assay system (IGEN Inc. Gaithersburg Maryland USA) is based on immunomagnetic electrochemiluminescence.

For the PATHIGEN test available for Salmonella, Listeria, Campylobacter and E.coli O157, the technique is used in a sandwich immunoassay format. A pathogen specific antibody is immobilized on the magnetic particle. The second antibody is labelled with a light-emitting compound, the ORI-TAG label. With the pathogen present, both antibodies bind to it and form a complex which is then transported to a flow cell. A magnet captures the beads on the surface of an electrode in a flow cell. The label (Ru(bpy)₃²⁺) and a coreactant (TPA) are oxidized at the surface of the electrode. The chemical reactions produce an excited label that emits light which is measured by a photodetector. The amount of the pathogen correlates to the intensity of the emitted light and determines the concentration of the pathogen present in the sample.

The measurement takes less than 1 minute. A pre-enrichment of about 6 h is still necessary. This technique is thus a real one-shift method with test results available in less than 8 hours. The sensitivity of the system is about 1-2 cfu/g of the initial food sample.

IMS used in combination with PCR has several advantages. Often, PCR assays which are directly applied to food have a significantly decreased sensitivity which is based on the unspecific inhibition of the DNA polymerase by different food components. Through removal of these inhibiting food components from the sample, PCR ready DNA is eas-

ier obtained and the sensitivity of the PCR assay can be improved. Moreover, PCR assays use very small sample sizes of 1 to 20 µl. IMS allows the concentration of the sample by a factor 5 to 10 and thereby increases the sensitivity of the assay.

A technique called magnetic capture-hybridization PCR (MCH-PCR) involves lysing the bacteria to release DNA and hybridization with pathogen-specific gene sequences using biotin labelled DNA probes. Following capture of the hybrids by streptavidin coated magnetic particles, the bound DNA is used for PCR amplification.

A disadvantage of the IMS based techniques is the need to optimize for each type of sample since the performance of the method is determined by a number of factors e.g. the concentration of coated antibody per mg particle, the number of particles per test, the incubation conditions, type of sample and washing regimes, and the antibody-specificity. Under optimum condition, a recovery rate of 90% has been reached.

IMS has proven to be a useful tool to enhance the speed and sensitivity of different detection methods. All of the steps have currently to be performed by hand, but an automation of the method can be expected in the next future.

2.2.6 Nucleic acid-based assays

Nucleic acid-based assays can be either performed directly, or after amplification of the target sequence. Polynucleotides with a sequence complementary to the single-stranded DNA or RNA are used as probes. DNA sequences that are homologous to all members of the family of interest, or unique sequences that are particular to a species can be chosen for the construction of the probe. Probes that target different genera, species or different strains can thereby be constructed. Genes associated with virulence are often used for detection of pathogens. The ribosomal RNA represents an attractive target for probe design since it is present in multiple copies in most organisms, and databases with sequences for several thousand microorganisms are available. Ribosomal RNA contains stretches of conserved sequences interspersed with variable sequence regions, providing the scope to design single-stranded DNA probes for broad-range or specific target detection.

The binding of the probe to the complementary cellular DNA/RNA is called hybridization. In order to detect hybridization, the probe needs to be labelled. Among others, Biotin, Digoxigenin and different fluorochromes have been used for labelling.

Since there are many different assay formats available which are produced by many different manufacturers, only some will be introduced below.

For detection of food pathogens, the GENE-TRAK system (Hopkinton, MA, USA) uses direct hybridization in a sandwich hybridization format. The capture probe directed to specific rRNA sequences is linked to a solid support and is designed to hybridize with the target sequence. A second probe is used that hybridizes to a sequence adjacent to that site. This probe is labelled with fluorescein on both ends. The hybridization product is detected with secondary antibodies labelled with horseradish peroxidase that can be visualized after a chemical color reaction. A number of other manufacturers produce similar tests.

Amplification-based methods

Molecular assays have become faster, less expensive and through the development of homogenous assays easier to use, making them more attractive for routine use in food laboratories. There are a number of methods available that al-

low the amplification of the target sequence or the detection signal, including branched DNA technology (bDNA), Nucleic Acid Sequence-based Amplification (NASBA), Q β Replicase, the Ligase Chain Reaction (LCR), Transcription-mediated amplification, strand displacement amplification (SDA) and rolling circle amplification (RCA). In a recent Tekes technology review¹⁹ a short introduction into methodology and an overview over the diagnostic companies developing these methods can be found. While the PCR method has been most widely accepted, other assay formats that have yet not reached practical applications in food diagnostics, have been developed. For a recent review see also²⁰

One promising method is the *Rolling Circle Amplification* (RCA) method that uses both signal and target amplification. The technique allows direct amplification on a solid phase, and is therefore suitable for the combined use with microarrays.

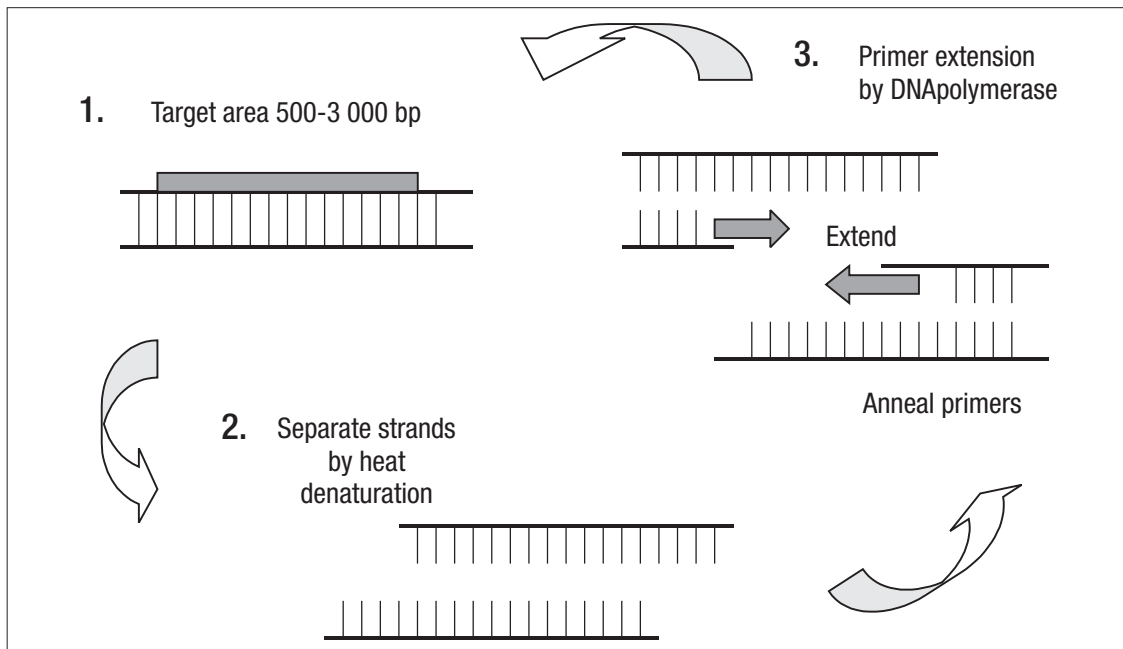


Figure 4. The Polymerase Chain Reaction.

The target area on the DNA comprises a stretch of 500 to 3000 base pairs (1) The double-stranded DNA-molecule of a target pathogen is denaturated to form single strands (2). Two oligonucleotide primers anneal to the complementary sequences on opposite strands of the DNA. The temperature is lowered to allow hybridization. The primers are extended by enzymatic polymerization with the thermostable Taq DNA polymerase using nucleotides present in the solution to form a sequence complementary to the target sequence (3). The three steps form a thermal cycle that is repeated between 20 to 40 times. Each cycle will double the number of copies produced resulting in a million fold amplification of the target sequence within one hour. The identity of the amplification product has to be confirmed by a detection method.

19 Siitari, H, National Technology Agency (Tekes) (2002): Nucleic acid diagnostic market: Unmet Needs and Product Potentia., Technology Review (125/2002)

20 Schweitzer, B. and Kingsmore, S. (2001) Combining nucleic acid amplification and detection. Current opinion in Biotechnology 12: 21-27

The *Nucleic Acid Sequence-Based Amplification* (NASBA) amplifies RNA and DNA targets as single-stranded anti-sense RNA. Reverse Transcriptase, Rnase H and T7 polymerase and two primers are needed.

PCR is now an accepted method to detect pathogens by amplification of the target DNA and detecting the target PCR products (Fig. 4).

Detection of the amplification product has previously been done by gel electrophoresis, which is not a very convenient technique for routine use in a food laboratory. Recent advances have led to the development of homogenous assays for real-time detection which allow greater sensitivity and easy detection of PCR products that make PCR more suitable for routine-use in food laboratories. A major disadvantage of the PCR technology is that it will detect both viable and non-viable bacteria. The decreased sensitivity of the PCR reaction directly applied to a food sample is well known and can also lead to false-negative results. The inclusion of a short pre-enrichment step solves these issues, but also makes the analysis much slower. Several methods of sample preparation have been proposed to overcome this disadvantage including IMS, filtration, centrifugation, the use of organic solvents and detergents and sample dilution.

The use of intact RNA as target has been proposed to discriminate between viable and dead cells. The preparation and isolation becomes more difficult as RNA is much less stable. However, new specific isolation procedures for m-RNA are currently being developed.

The BAX-system (Qualicon, Inc., Wilmington, DW., USA.) was the first commercial PCR system for the detection of food-pathogens. The FSIS (Food Safety and Inspection Service) USA has recently adopted this system for screening of ready-to-eat food, meat poultry and egg products for *Listeria monocytogenes*.

The BAX-system works directly from an overnight enrichment of the target organisms without a separate DNA isolation step. All PCR reagents required for the test are incorporated into a single tablet. The principle of the TDF-PCR detection is based on an intercalating dye, SYBR green I, that binds to the double-stranded PCR product. The assay is an *in situ* process, simultaneously amplifying the target DNA and directly detecting the increasing fluorescence signal during the annealing/elongation phase of the PCR reaction. One additional thermal cycle is run consisting of a denaturation step and a product annealing step. Fluorescence is monitored directly in the PCR reaction tubes during this final cycle. The rate of increase in fluorescence over time during the product annealing step yields a characteristic pattern for positive samples that can be differentiated from negative samples.

The TaqMan system (Applied Biosystems, Foster City, CA., USA) also allows data evaluation directly in the reaction vessel, and through online detection of the amplification reaction a quantification of the amount of target sample present in the DNA probe. The probe is an oligonucleotide with both a reporter fluorescence dye and a quencher dye attached. When both dyes are attached to the probe, reporter dye emission is quenched. During each extension cycle, the Taq polymerase cleaves the reporter dye from the probe, which subsequently emits fluorescence. Online following of the production of the amplification product allows quantification of the amount of DNA in the original sample.

The Molecular Beacon Technology (Stratagene, La Jolla, CA., USA) uses a hairpin-shaped hybridization probe. The probe contains a reporter fluorescence dye and a quencher dye attached to respective ends of the probe sequence. In the absence of the target PCR products the beacon remains in a hairpin shape and the fluorescence is quenched. During the generation of target PCR products, the beacons will attach to the PCR products and cause the hairpin molecule to unfold, and fluorescence will be emitted, allowing real-time detection of target PCR products. In contrast to the TaqMan system, the amplification product is measured directly. Using several different fluorochromes, several PCR products can be followed in the same reaction tube.

2.3 Future technologies

2.3.1 Sensor technology

The most promising breakthroughs of the development of on-line or on-site, sensitive, low-cost, rapid methods for routine-use are expected to be made in the area of sensor technology. Many prototypes for food diagnostic application in the food and drink industry are currently being developed. They have high potential for automation and allow the construction of simple and portable equipment for fast analysis. These properties will open up many applications within quality and process control, control of fermentation processes, quality and safety control of raw materials, and for HACCP monitoring.

Sensor Technology covers a wide area of diverse techniques, including opto-chemical sensors and biosensors. Biosensors are a subgroup of chemical sensors where the analytical devices are composed of a biological recognition element such as enzymes, antibodies, receptors, proteins, oligonucleotides, or even a whole cell coupled to a chemical or physical transducer. A transducer measures the changes that occur when the sensor couples to its analyte. The sensitivity of the system is determined by the type of transducers employed. Biosensors can be used for the detection of very different analytes such as pathogens, pesticides and toxins. Biosensors can be grouped according to their

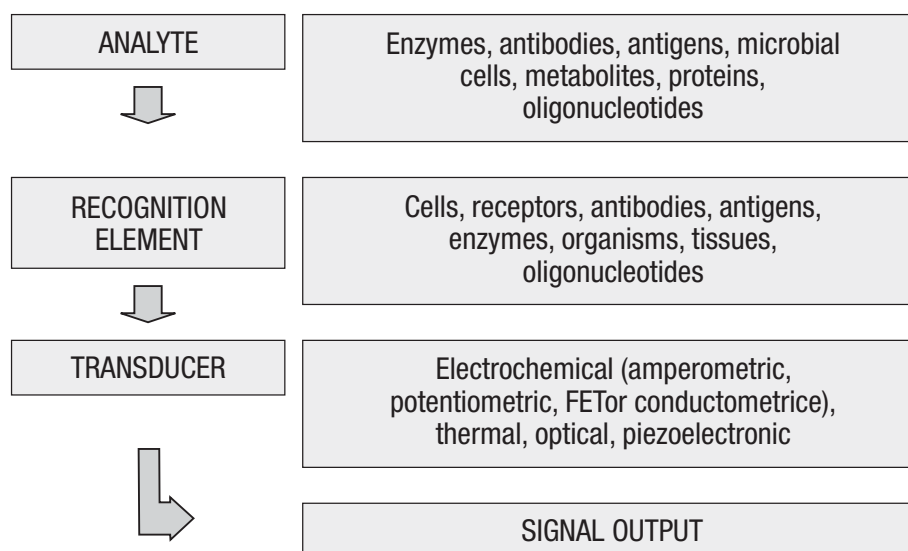


Figure 5. Show the principle elements of a biosensor. Binding of an analyte to a recognition element leads to a signal change at the transducer. Numerous different components or technologies can be used at the various stages of detection.

biological recognition element into immunosensors using antibodies and hybridsensors using DNA or RNA probes. Microarrays are constructed of a high number of parallel hybrid receptors and can therefore be considered biosensors. For the construction of low density microarrays suitable for the use in food diagnostics, electrochemical transducers have proven to be a cheap and efficient choice. Electrochemical transducers include potentiometric, amperometric, piezoelectric, capacitive and conductive instrumentation.

Optical immunosensors are most popular for bioanalysis and optical transducers comprise the largest group of transducers used for biosensors today. Optical transducers include fibre optic transducers and those without fibre optics as spectrophotometers, fluorimeters and luminometers. Due to the diverse nature of the technologies used in the field, we will focus on a description of microarrays and optical sensors and include some highlighting examples of commercial systems and prototypes available for applications within food diagnostics. For recent reviews covering research activities in this rapidly growing field see ^{21,22}.

However, only a limited amount of methods are combined and currently exploited for their use in food diagnostics. As recognition elements, bioaffinity based receptors that use the selective interaction between ligand and receptor, antibody or nucleic acid are most widely used. As transducers, electrochemical and optical systems have gained practical importance.

2.3.2 Microarrays

Microarrays often are also referred to as biochip, DNA chip, DNA microarray or gene array, and allow to conduct many analyses in parallel. The size and complexity of such arrays varies considerably. High-density microarrays (up to 100.000) are mostly used for research, as gene expression profiling and microbial genotyping, and drug development purposes. The use of microarrays as research tool was described in a recent review²³. Medium (some hundreds to thousands of different analytes) and low-density microarrays (some 10 to hundred different analytes) are more suitable for mass production and can therefore be applied for diagnostic purposes due to their considerably lower cost.

The immobilized molecule is termed the *probe*, while the solution-phase molecule is termed the *target*. Probes can consist of cDNA, oligonucleotides, proteins and are immobilized in a regular arrangement on the surface of a solid substrate. As solid substrates, a. a. glass, nitrocellulose, nylon, polypropylene, and silicon have been used. Probes can be deposited on the surface of the substrate by deposition of presynthesized probes using specially developed printing systems (microspotting) or by *in situ* synthesis at individual locations on the array.

To do an analysis the unknown sample is incubated with the microarray, allowing the hybridization of complemen-

- 21 Mello L. D. and Kubota L. T. (2002) Review of the use of biosensors as analytical tools in the food and drink industry, Food Chemistry, in print,
- 22 Lippa, P. B, Sokoll, L.J. and Chan D.W. (2001) Immunosensors-principles and applications to clinical chemistry. Clinica Chimica Acta 314, 1-26.
- 23 Lucchini S., Thompson A., and Hinton J.C.D. (2001), Microbiology, 147: 1403-1414.

tary sequences. For high density microarrays fluorescent probes are mostly used for the detection of the hybridization reaction. For medium and low density microarrays, electrochemical detection offers a cheap and sensitive detection system.

An alternative assay format are the so called *virtual arrays*. Beads are used for the attachment of the probes. Since the beads have no fixed location, the identity of the probe attached to the bead has to be encoded differently. Luminex Corporation (Austin, TX, USA) uses polystyrene as the matrix material, with 100 spectrally distinguishable beads analogous to a 100 spot microarray.

GeneScan Europe (Freiburg, Germany) offers a microarray for the detection of gene manipulated organism. The first version of the GMO Chip contains a total of 14 tests and analyses. The GMO Chip Kit can recognize DNA from respectively: plants and virus species, commonly used construction elements in the field of genetic engineering, or specific genetically-engineered modifications (for identifying approved and non-approved GMOs). The NUTRI-Chip (Scil Diagnostics GmbH Martinsried, Germany) offers the simultaneous qualitative identification of *Listeria monocytogenes*, *Salmonella* spp. and *Campylobacter jejuni/coli*. For further reviews and links see ^{24,25,26}

A prototype based on a low-density microarray with electrochemical detection is currently being developed for the assessment and quality control of yeast based bioprocesses (Scanbec Oy, Oulu, Finland). The device consists of a low-density microarray allowing parallel detection of 16 probes. The microarray can be used for the detection of spoilage organism in the brewery process. The sensitivity of the method is 106-107 DNA copies. An additional application is possible through the investigation of the gene expression in yeast with microarrays. By investigating characteristic changes in the expression of certain yeast genes, critical stages in the fermentation process can be identified and used for better control of the fermentation process. Also the quality of the yeast for the brewery process can be analyzed with this method.

2.3.3 Optical biosensors

Optical biosensors have been developed for rapid detection of contaminants in foods, including pathogens, and several have evolved into commercial prototype systems. For a recent review see ²⁷. Optical transducers have gained from

advances in optical fibre and laser technology. They are based on methods such as bio- or chemiluminescence, reflectance, scattering, and refractive index, and UV-vis absorption. The basic principle utilizes electronic or optical transduction technology to monitor a parameter of the reaction between a biomolecule and an analyte into a quantifiable electrical or optical signal. This signal can be related to concentration. In Fibre-optic biosensors a fibre-optic probe serves as the transducer. The analyte in the food interacts with the bioactive molecule, usually an antibody. Antibodies can be immobilized directly on the fibre, either on the blunt end or along the sides of a fibre tip. The binding of antibody and analyte is detected as a change in an optical signal measured through the fibre-optic assembly. The light from a laser travels to the fibre tip and penetrates into the area outside the tip. A fluorescently labelled complex binds to the antibodies on the tip. The fluorescent signal then radiates in all directions, and some of it travels back up the fibre tip to the detector. Non-selective adsorptions and interferences due to background fluorescence have been the major drawbacks for the development of this sensor type.

So far, only a few commercial systems are available on the market. Additionally, some optical biosensor prototypes have been developed.

The first commercial applications have been obtained with the surface plasmon resonance (SPR) sensor. Biacore International AB, (Uppsala, Sweden) technology offers this assay for determination of water-soluble vitamins and for veterinary drug residues, antibiotics and growth promoters.

The SPR technology monitors biomolecular interactions in real time. The detection principle relies on surface plasmon resonance, an electron charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under specific conditions. In Biacore systems, SPR is used to monitor molecular interactions occurring on a biospecific surface; the changes in molecular weight as molecules bind. An increase in molecular weight on the surface coating of the sensor chip causes a corresponding increase in refractive index which alters the angle of incidence required to create the SPR phenomenon (the SPR angle), which is detected as a response signal. The system is based on a microfluidic device in which the analyte flows past the detector surface. Detection of molecules in solution can be made either by direct binding to the biosensor coating molecules, or by competition binding with soluble capture molecules added together with the sample.

24 A list on suppliers for prefabricated microarrays is available from: http://www.epa.gov/nheerl/epamac/links_prefabricated_arrays.htm.

25 The <http://www.GMOchips.org> page for the EU- commission research project "New technologies in food sciences facing the multiplicity of new releases GMO" with introduction into several projects, including the development of low-density biochips, links to related products and publications.

26 In www.biotech-europe.de/rubric/products3/P02-01-A4.pdf an overview over commercial pre-made DNA arrays is available.

27 Rand, G.A., Ye, Y., Brown, C.W. and Letcher S.V. (2002) Optical Biosensors for Food Pathogen Detection. Food Technology Vol 56: 32-39.

Future applications might include protein quality and the detection of allergens genetically modified (GM) proteins, BSE prions, pathogens and biocide residues

Another SPR-based biosensor system has been developed by Texas Instruments (Dallas, TX., USA). and packaged into handheld sensors for small molecular analytes that permit on-line and real time analysis.

The Georgia Tech Research Institute (Atlanta, GA, USA) has introduced a system based on an integrated optic interferometer. The antibodies are coupled to urease and ammonia is produced upon binding of the antigen in the presence of urea. The ammonia gas induces measurable changes of the optical properties of the sensor using a laser detection system. This optical biosensor has been tested for the detection of 12 different bacterial species and has a sensitivity of 500 cfu/ml sample.

A sensor using liposomes has been developed at the Cornell University (Ithaca, NY, USA). Upon binding of the fixed antibodies, the liposomes rupture, thereby releasing dye or other markers.

The Raptor is a portable, automated fibre-optic biosensor that is commercially available from Research International (Woodinville, WA, USA). Four different antibodies are immobilized on separate fibre probes. Binding to the appropriate probe generates a fluorescent signal within a few minutes. The assays are fully automated for simple operation and data analysis. It has been tested for monitoring water quality and testing for both toxins and bacteria

The Fibre Optic and Biosensor Research Group at the University of Rhode Island (Kingston, RI, USA) developed several sensors that use vibrating quartz crystals or fibre optic probes. Newer versions use antibody-coated magnetic beads that are magnetically focused in front of an optical fibre. Pierson Scientific (Andover, MA, USA) recently converted the membrane biosensor laboratory system into a portable prototype device.

2.3.4 Flow cytometry

Flow cytometry has mostly been used as a research tool for the study of microorganisms. However, the development of cheaper instrumentation should make it more useful for routine applications in the future. Flow cytometric studies

of microorganisms are based on their staining with a fluorochrome. Stained cells in suspension are injected into a fast-moving carrier fluid. The cells arrive in a single file in the measuring area where a light source is focussed on the fluid stream. The individual cells pass the light beam very rapidly. The stained cells will be excited and emit light at a specific wavelength, which will be measured by a detector. It is possible to analyse the cells using staining with different specific fluorochromes or to monitor their light scattering properties. A particle will deflect or absorb light in a characteristic way depending on its size and structure and the angle of the light. Multiparameter analysis with up to eight different parameters is possible with certain instruments.

Flow cytometers can also be equipped with cell sorting devices allowing to sort out cells with a certain profile. By this method, *Staphylococcus aureus* could be separated from *E. coli* to a purity of 95%. The technique is called FACS (Fluorescence activated cell sorting). When staining cells for analysis, it is possible to use nucleic acid stains, protein stains, antibody and dyes for membrane potential measurement and enzymatic activity. The technique can provide rapid information on live and dead cells and information on their physiology and enables both qualitative and quantitative analysis of microbial cells in liquids. Due to the complexity and inherent cost of the equipment, the practical use of the method is still limited to research. However, with the appearance of cheaper systems on the market, it is considered to be a promising technology for the future²⁸.

2.3.5 Bacteriophage-based techniques

Bacteriophages are viruses that selectively infect a certain range of bacterial hosts. This property which allows discrimination between different bacterial strains has been exploited in "phage typing" of bacterial samples. Recently, a new method for the detection of food-borne pathogens has been developed where the bacteriophages are engineered to carry the *luxAB* gene, expressing the enzyme luciferase. The infected cell will produce light that can be measured with a photodetector. Viability is essential for the light production, so that the assay can discriminate between viable and non-viable cells. The assay is more rapid due to the faster replication cycles of the virus compared to bacteria, generating a 20 to 100 fold increase of the virus particle within 30 minutes. At present, there are no commercial kits available using this technology.

28 Commercial flow cytometers are a.o. offered from Becton&Dickinson (Cockeysville, MD, USA), Bio-Rad Laboratories (Hercules, CA, USA), Chemunex (Paris, France), Coulter (Miami, Fla., USA), Ortho Diagnostics Inc. (Raritan, NJ, USA) Partec GMBH (Münster, Germany), and A/S Foss Electric (Slangerupgade, Denmark).

2.4 Hygiene and environmental testing

2.4.1 Biofilms in the food industry

The hygiene of all surfaces, machinery, pipes and instruments have an impact on the quality and the safety of the food produced.

The microflora of the processing plant is composed of microorganisms from the air, water, raw materials, dust, dirt, and people. Food can be contaminated during processing due to cross contamination via contaminated raw materials, personnel, aerosols, malfunctioning or improperly cleaned equipment, and other major contamination sources in the plant. Hygienic design and proper cleaning have therefore a direct effect on the quality of the food produced. Work to provide design standards is being performed by the European Hygienic Equipment Design Group (EHEDG), the 3-A Committee, International NSF (formerly National Sanitation Foundation), the European Committee for Standardization (CEN), Safety in Biotechnology Standards, and the International Standards Organization (ISO). The Codex Alimentarius Committee on Food Hygiene has produced several hygiene standards⁵.

Hygiene testing or environmental monitoring involves testing for possible contamination of working surfaces to monitor the success of the cleaning procedures. One big problem in the food industry is the formation of biofilms on a number of different surfaces. Biofilms can build up on any surface in the food industry. Amongst the most contaminated surfaces are floors, drains, and some conveyor belts. Less contaminated surfaces are walls, ceilings, and the equipment. For a surface to be colonized by bacteria, conditioning is needed. This occurs when a wet surface forms and soluble molecules concentrate on the surface. In the food industry spontaneous conditioning occurs through adhesion of food molecules and/or cleaning agents and disinfectants.

Biofilms develop when microbes attach to these conditioned surfaces and start to secrete a protective extracellular matrix containing polysaccharides and glycoproteins. The adhesion process seems to trigger a number of characteristic genotypic and phenotypic changes, so that bacteria in a biofilm differ significantly from bacterial cells in solution. These changes might also underlie their high resistance to all antimicrobial agents. In addition, biofilms will occur under unstable environmental conditions exposed to different types of stress as fluctuating access to nutrients, chemical shock and desiccation. The coprotection towards other forms of stress that often is acquired in

the process might additionally protect from those chemicals used in hygiene operation. Several commercial alkaline cleaning products were shown to detach 10-90% of a biofilm grown under laboratory conditions but did not produce a significant decrease in the number of adhering cells in a naturally grown biofilm.

Removal of biofilms through cleaning is very difficult, but cleaning will effectively reduce fresh contaminations from pathogenic microorganisms through external sources and prevent establishment of those microorganism in biofilms.

The hygienic design of the plant and equipment is hence the basic tool to prevent the establishment of biofilms. Dryness of surfaces and air help to efficiently inhibit the establishment of biofilms.

Biofilm pieces that occasionally detach might be sources of contamination for a food product with pathogens. This contamination will be difficult to detect due to the sporadic and random occurrence of the process. Fast regrowth of the bacteria in the biofilm due to only partial removal after the cleaning cycle leads furthermore to a decreasing quality of the food product. For that reason, fast and robust methods are needed to monitor cleaning efficiency and to establish efficient sanitation operation procedures. As for microbial methods within HACCP system, those methods need to be fast, and should ideally provide results in real time.

2.4.2 Rapid methods in hygiene monitoring

Hygiene testing is currently based on conventional cultivation after sampling of the surfaces using contact plates or swabbing. Comparison between microscopic evaluation of biofilms and culturing shows an order of magnitude difference in the total number of the organisms. Due to the strong adherence of the biofilms it is necessary to use substances that induce detachment. SprayCult from Orion Diagnostica (Espoo, Finland) is a product that is used for the detachment of biofilms.

ATP bioluminescence

The method is based on the firefly luciferase reaction which emits light in the presence of ATP. This phenomena is called bioluminescence.

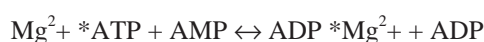
ATP occurs in all living cells and is therefore useful to indicate the presence of microbial organisms and food residues. The presence of ATP on a surface can therefore be used to indirectly assess the cleanliness of a surface. There are several ATP monitoring systems on the market. They

involve swabbing of the tested surface using a surfactant and measuring the presence of ATP after addition of Luciferin and Luciferase in a luminometer. The reaction is very fast and provides readings within 2 minutes. The major limitation of the method is the relatively low sensitivity. According to theoretical limits, a reliable ATP measurement should be obtained with at least 1000 bacterial or 10 yeast cells in the sample. A further limitation is the swabbing procedure which is used for sampling, and which might only remove 1 to 10% of the contaminants from a surface. To address that problem the BioProbe system (Pall Corporation - Life Sciences Ann Arbor, MI, USA) consist of a luminometer connected to a suction cup which allows measuring directly at the surface. Currently there are quite many companies offering ATP instruments²⁹.

Adenylate kinase

The limited sensitivity of the ATP bioluminescence method might be overcome in the near future by an assay developed by DERA Porton Down (Salisbury Wiltshire, UK) that is using adenylate kinase rather than ATP as a cell marker.

Adenylate kinase is a constitutively expressed enzyme that catalyses the reaction



This reaction serves to rephosphorylate the AMP produced in many reactions. ADP can be further phosphorylated to ATP. However, by adding excess ADP, the reaction can be driven in the other direction to produce ATP, that can be measured using the luciferin/luciferase reaction. The reaction creates about 40 times more ATP for the bioluminescence reaction than would be possible for the ATP naturally present on its own. The Adenylate kinase (AK) assay offers therefore a 10 to 100 times higher sensitivity than the ATP bioluminescence assay. At its present state of development at DERA Porton Down the AK assay has been shown to have a detection limit of 10 E.coli cells within 5 minutes.

AK technology is simple to use and does not require the development of new detection principles or instrument systems. Since the AK content of bacterial cells is much more constant than their ATP content the new method yields results that are directly comparable between different species.

The AK assay can be made both highly specific and ultra-sensitive when used in combination with specific binding techniques such as solid phase capture by antibodies. Kits are to be expected in the near future.

2.4.3 Riboprinting and pulse-field gel electrophoresis

Riboprinting and Pulse-field gel electrophoresis are used for the genetical characterization of microbial cells. These methods are able to produce characteristic DNA fingerprinting patterns that can be used for surveillance programmes for food borne diseases. By comparing patterns collected in central databases can be connected to sources of contaminations can be traced even for geographically separated incidents. With the same technique it is also possible to trace sources of contamination in a food production plant. A fully-automated Ribo Printer Microbial Characterization system is available that can identify organism to genus, species and subspecies level automatically (DuPont Qualicon, Wilmington, DE, USA). The use of these methods is currently limited to research laboratories. Many contract research organizations (CROs) offer the application of these methods for detection of contamination sources as a service to the food industry.

2.5 Applications and limitations of rapid methods

The field of rapid methods is growing fast with numerous analytical options being available for different testing situations. These new rapid methods have the potential to offer considerable advantages regarding their sensitivity, rapidity and potential for automation, allowing increased sample through-put for analytical laboratories, and ease-of-use. Most of the current rapid methods detect single target, but more and more methods with multiflexing properties are developed. Rapid methods are well suited for screening purposes for large numbers of food samples. Since most methods lack the required sensitivity, culture enrichment is still necessary before analysis. Benefits of enrichment include dilution of inhibitors, recovery of sub-lethally damaged cells and differentiation between viable and non-viable cells.

29 e.g. Lumac (Landgraaf, the Netherlands), BioTrace (Plainsboro, N.J. USA) Lightning (BioControl, Bellvue, Wash. USA), Hy-Lite (EM Science, Darmstadt, Germany), Charm 400 (Charm Sciences, Malden, Mass., USA), Celsius system SURE (Cambridge, UK), and Zylux (Maryville, TN, USA)

3 Validation

“Standardization is the unique, defined solution of a repetitively occurring task in the light of the scientific, technological and economical possibilities available at any given time”

– Otto Kinzle, co-founder of the German Institute for standardization

The aim of a *validation* procedure is to ensure that a new method performs according to specified requirements. It always contains an evaluation of the specificity, sensitivity, precision and accuracy and kit performance data of the new method. The validation process is usually coordinated by an independent validation body, which also has to develop the rules for this process. The actual validation is performed by different laboratories, in the form of peer-reviewed and collaborative laboratory studies.

The validation of a rapid method has to demonstrate that it produces results that are comparable to the results obtained by a reference method³⁰. There are different validation and standardization bodies as ISO (International Organisation for Standardization, Geneva Switzerland), CEN (European Committee for Standardization, Brussels, Belgium), AOAC (Association of Official Analytical Chemists, Gaithersburg, MD, USA), NMKL (Nordic Committee on Food Analysis, Oslo Norway), and AFNOR (Association française de normalisation, Saint-Denis La Plaine Cedex, France).

AOAC is the validation body that is responsible for the validation of rapid methods in the U.S. NordVal and MicroVal are the nordic and European bodies for the validation of rapid methods for microbiological analysis.

A validation procedure offers the advantage of that the diagnostic company can prove the performance of their new method in comparison to well established methods that are known by the user. This is very valuable information to the user for the decision process of which method to use for his particular purpose. Conversely, a lack of validation data when introducing a new method necessitates that more tests have to be performed by the food laboratory, requiring time and systematic work effort. It is obvious that this adds

to the costs of establishing a new technique in a food laboratory, and moreover, not all laboratories will have the means to perform the necessary studies at all.

A good validation procedure implies that a method has acquired a certain degree of standardization before it is implemented into practical use in food laboratories. It is not an easy task to maintain the reliability of a standard while following the ever-increasing pace of technological and scientific development. This is clearly an inherent problem to this area. For official control there is a need to maintain and rely on certain established standards to be able to guarantee the quality of the control, but this will invariably hamper technological progress and innovation. On the other hand, technology developers need to create markets for their new products quickly to be able to create incentives for further developments.

The validation process also offers important information to official food control agencies, and allows a degree of control over the quality of the method being offered on the market. The performance results from the validation process can be used by regulatory agencies to accept a new technique as an official testing method.

This will have a positive influence on the penetration of rapid methods, since they currently cannot be used as official control methods. Clearly this does not increase the incentive to adopt new technologies.

With the existence of numerous different validation bodies, and in the absence of a mutual recognition, most diagnostic companies that strive for validation of a method will have to deal with several separate regulatory agencies. In most cases no new laboratory studies will be required, and the various agencies might just end up cross validating each other. This will not lead to any added value for the method in question, but will increase costs and prolong timelines. Moreover, it will decrease confidence of the end-user in the whole validation process, if no common acceptance standards can be created. A validation process with mutual recognition between different validation bodies resulting in global acceptance would be a major step towards the opening of a common market for food diagnostics.

30 A reference method is a method that is internationally recognized and accepted ISO, CEN, AOAC and NMKL methods, and certain national standards.

In practice, the major problem with validation procedures is that they require a very significant effort from the laboratories involved, measured both in time and absolute workload. Currently, the AOAC approves 12 to 18 official methods annually. This indicates that the capacity for regulatory acceptance procedures of validation is limited and can easily become a bottleneck. New tools are needed to guide the selection and evaluation of new rapid methods, and to ease exchange of experience with various new test methods.

3.1 MicroVal

MicroVal is a European certification organization for the validation and approval of alternative methods for microbiological analysis of food and beverages. The MicroVal procedure aims also at the recognition of validated rapid methods as official control methods. The lack of a common validation procedure was perceived a major obstacle for the establishment of rapid methods by both the diagnostic companies and the food industry. An Eureka project was started to develop a certification scheme describing the methodology and the organization to be used³¹.

The interest from both diagnostic companies and manufacturers for a European validation for rapid methods is also reflected in the composition of the general committee (MGC):

- **Manufacturers:** Organon Teknika (UK), Laboratories 3M Santé (France), R. Biopharm (Germany), Diffchamb (Sweden)
- **Users:** Nestle (Switzerland), Unilever (the Netherlands), UNIR (France)
- **MicroVal third parties:** AFNOR (France), DIN Certco (Germany), Campden & Chlorleywood Food res. Ass. (UK).

MicroVal has recently started its activities in form of a pilot project. The target is to conclude a validation procedure within one year. Discussion are ongoing between AOAC and MicroVal for a reciprocity agreement.

3.2 NordVal

NordVal is the Nordic committee for the validation of alternative microbiological methods, including analyses of food, water, feed, animal faeces and food environmental samples. They have so far evaluated 8 different rapid test kits. The validation procedure is aimed at the acceptance as official control method, but is not implicitly built into the validation process³². A secretariat will be established at the Institute of Food Safety and Toxicology within the Danish Veterinary and Food Administration.

3.3 AOAC International

The AOAC International³³ (Association of Official Analytical Chemists, Gaithersburg, MD, USA) is an independent, non-profit organization that focuses on the coordination of the development and validation of analytical methods in the area of analytical chemistry and microbiology. The AOAC official methods have been incorporated into the FDA methods. Accepted methods are published in the AOAC periodicals *Journal of AOAC* and *Inside Laboratory Management* and *The Referee*.

Currently, the AOAC approves 12 to 18 official methods annually. Compared to the amount of methods and test kits developed it is becoming increasingly obvious that this is not enough to keep pace with the fast development of rapid methods. To fill that gap, a new project was introduced in June 2002 called "e-CAM" (electronic-Compilation of Analytical Methods). "e-CAM" consists of a collection of information about methods in a database available on-line. The idea is to collect all the available information on a method in one database to ease the selection of the best suitable methods for a user. It also aims to promote exchange of experience with certain methods and make that information more readily available. Different validation status are considered such as regulatory methods, harmonized collaboratively (fully) validated methods, multiple-laboratory validated methods, single laboratory validated methods, and research non-validated methods with some information on their quality.

31 The validation protocol was accepted by CEN/TC 275: Food analysis-horizontal method and further developed into a European standard: prEN/ISO 16140. Through the CEN/ISO "Vienna agreement" this standard will also be adopted as ISO-standard.

32 For more information, see, <http://www.nmkl.org/NordVal/NordVal.htm>

33 <http://www.aoac.org/>

4 The market for food diagnostics

4.1 The global market for food diagnostics

The market size³⁴ for food diagnostics can be assessed through different estimates about the number and types of microbiological tests, but no direct statistical data are available. As to be expected, the estimates differ depending on how the market was segmented, and some of the data are not completely congruent.

We will use the following definitions, which might differ from those used elsewhere:

Food Analysis: all food testing, including both traditional and rapid methods. The market can be segmented into, spoilage, pathogens, chemical contaminants, and process chemicals (chemical analysis).

Food Diagnostics: food testing with rapid methods, same market segmentation as above.

Microbiological Testing: all testing for microorganisms (bacteria, viruses, moulds, yeasts) in food and beverages, pharmaceuticals, water, and environmental testing.

Food Microbiological Testing: all testing in food and beverages for microorganisms.

Additionally, what is considered a rapid method is not clearly defined. Nevertheless, trends for the global market size, for different regions, test methods and applications can be obtained.

The market data show that traditional methods still clearly dominate the market of routine test for quality control, with different forms of total plate counts as the most performed test. Rapid methods are still a comparably small market. The market penetration of rapid tests is greater in the U.S. than in Europe.

The world market for food analysis totals €1.1 billion. This includes testing for pathogens, spoilage organisms, chemical contaminants and process chemical tests (mostly different chemical analyses of food compounds). The world market for all microbiological tests is around €1.1 billion, with 755 million microbiological tests performed in 1998. 56% of those tests were performed on food, 10% on beverages, 30% on pharmaceuticals, and 4% for environmental testing. Food microbiological tests created global sales of around €565 million, and an estimated 440 million food tests were performed in 1999. In the US sales for food microbiological testing were about €209 million compared to €170 million in Europe in 1999. North America and Western Europe together make up about 2/3 of the world market.

Table 4 shows the global sales of traditional food microbiology tests versus rapid methods by region. With an annual growth of 9% the world market for food microbiology is expected to reach €864 million in 2005. Whereas the market share for rapid methods is 31 % (compared to total sales) in the US, in Europe only 17 % of the sales come from rapid methods. This shows the smaller market penetration of rapid methods in Europe compared to the US.

Media and reagents still make up the largest share with €350 million or 62% of the market. In comparison, rapid methods generate global sales of €115 million or a 20% total market share. ATP methods for hygiene monitoring have a market share of 9% and generate €50 million of sales (Table 5).

Within rapid methods, immunoassays still stand for 60 % of all the tests sold and generate sales for €70 million, followed by DNA methods (PCR) methods with €25 million and a 22% market share (Table 6).

34 The following sources were used: a Theta study: Food Safety testing: Opportunities for Microbiological & Genetic in vitro diagnostics, Report No. 1031 April 2000, a market analysis by the food diagnostic company Diffchamb (Diffchamb AB Västra Frölunda, Sweden) with information on the European market (www.diffchamb.com), the review "Rapid methods and Automation in Microbiology" (2002) by D.Y.C. Fung with estimates by the author and information on two studies by Strategic Consulting Inc. and "Industrial Microbiology Market Review" (1998) "Pathogen Testing in the U.S. Food Industry, 2000. They recently published an update and extension to the former report "Food diagnostics" (2002).

Table 4. Worldwide sales of traditional food microbiology tests versus rapid methods by region.

1999	Traditional Methods			Rapid Methods		
Region	Sales (€ millions)	Market Share (%)	Growth per year (%)	Sales (€ millions)	Market share (%)	Growth per year (%)
North America	140	35	5	63	55	16
Western Europe	115	29	4	23	20	27
Latin America	61	15	8	15	13	17
Japan	56	14	7	12	10	20
Rest of the world	28	7	4	2	2	12
Total	400	100		115	100	

Table 5. Worldwide food microbiology sales by technology.

Year	1999			2005		
Technology	Sales (€ millions)	Market Share (%)	Growth per year (%)	Sales (€ millions)	Market Share (%)	
Media/ID reagents	350	62	5	460	53	
ID systems	50	9	5	65	8	
Rapid Tests	115	20	19	244	28	
ATP Hygiene	50	9	15	95	11	
Total	565	100	9	864	100	

Globally, 370 million enumeration tests were performed which correspond to 84% of all food microbiological tests and a market value between €185 and 280 million.

Forty four million tests for pathogens were performed worldwide in 1999. Most of the pathogen tests (35 million) were done using traditional methods, while only 9 million tests were performed using automated ID methods or rapid methods. 90 % of all pathogen tests are made for 5 food borne pathogens: Salmonella, Listeria, E.coli, Campylobacter and Staphylococcus. The world market for these tests accounts for €220 million, with rapid tests generating a sales volume of €45 million.

Table 6. Worldwide food microbiology sales by rapid methods.

Technology	Sales (€ millions)	Market Share (%)
ELISA/EIA	70	61
DNA	25	22
Impedance	10	9
Flow Cytometry	5	4
ATP	5	4
Total	115	100

4.2 The European market for food diagnostics

The European market for food diagnostics totals 490 million € with rapid methods amounting to €86 million (Table 7). In Europe around 240 million tests worth €220 million for spoilage microorganisms (e.g. coliform bacteria, yeast, mild) and 28 million pathogen tests to a value of €98 million test are performed annually.

Rapid tests for pathogen detection generate sales volumes of around €13 million, corresponding to 13% of the market. For detection of spoilage microorganism rapid tests generate sales of around €38 million, which corresponds to 17% of the market. The market size for chemical contaminants as mycotoxin, food allergens, hormones, antibiotics is €53 million in Europe, with rapid tests representing 40% of the market or €20 million.

Table 7. European food analysis/diagnostics sales.

	(€ million)	Rapid methods (€ million)	% Rapid methods
Food pathogens	98	13	15 %
Spoilage micro-organisms	220	38	17 %
Chemical contaminants	53	20	40 %
Process chemicals	120	16	10 %
Total	491	87	

5 GMO

The world market for GMO (Genetically Modified Organisms) is treated separately from the food microbiology market. For background information on GMO safety see ³⁵. Even if similar methods are used and developed for GMO testing, such as PCR, ELISA and DNA microarrays, the testing market is influenced and shaped by different factors.

All our interviewees expressed uncertainty about the future of GMO testing. In Finland to date no products are on the market labelled “containing GMO” or “GMO free”, respectively.

The world market for GMO testing is still relatively small; the global market was €21 million in 1999, with sales worth only €3 million in Europe, corresponding to a 14% market share. 62% of the market for GMO tests consist of immunoassays, mostly rapid dipstick tests for qualitative yes/no results at grain elevators as grain is prepared for shipment in the U.S. The costs for sampling are in the range of €2–10 for rapid tests based on immunoassays and between €100–300 for highly sensitive PCR tests per sample.

Growth rates in the range of 100% were predicted for DNA based tests and the market is estimated to grow to €70–100 million in 2005.

GMO testing is supposed to guarantee that the raw material and the end-products are free of gene-modified protein or DNA. The current requirement through “The Novel Food regulation” in the European Union requires labelling of food products containing more than 1% products of gene-modified origin.

With low defect rates to be detected through sampling programmes, testing for GMO is subject to the same limitations as discussed in 1.3. Also here, it is more efficient to guarantee the process for separation of non-biotech foods than to guarantee the product.

The market is currently still sorting out developing with regard to methods of verification and price premiums for non-biotech foods. This has led to the import of certified GMO-free food to Europe. Both domestic and international marketing functions in the U.S. have begun to differentiate markets for biotech and non-biotech products and

this has created a new business opportunity. It is estimated that 24% of the grain elevators will separate biotech corn and 20 % will segregate biotech soybeans. The European market is willing to pay a price premium for non-biotech food. In a survey, 53% of the European consumers stated that they would be willing to pay more for non-GMO foods. However, experts question the ability of handling and distribution systems in the U.S. to maintain a separation that could achieve a threshold level of 0.1% or even under 1%.

In July 2001, the EU commission adopted two proposals; new regulations on GMO traceability and the labelling of GMOs and products produced from GMOs, and a proposal for a regulation in food and feed.

The new labelling system will additionally require labelling of:

1. All foods produced from GMO irrespective of whether there is DNA or protein of GM origin in the final product.
2. All genetically modified feed.

Currently no labelling requirements are in place for feed or for highly refined soy or corn oil that are free of traces of protein or DNA.

If these proposals are implemented, problems will arise with labelling required for foods which contain neither DNA nor gene-modified protein and therefore cannot be tested for it at all to control compliance to regulations. The whole testing concept might be put into question if this caveat is not adequately addressed.

Also with feed products some problems are to be expected, since separation cannot be maintained without significantly higher costs. Is the market willing to pay a higher premium for non-biotech feeds as it is in the case of non-biotech foodstuffs?

In reality, food containing GMO, even if only in trace amounts, has long been present on our tables. The estimate is that 70% of all processed foods contain ingredients derived from corn or soy and often they serve as basis for secondary ingredients that are not listed on the label. The future of labelling requirements and the market for GMO-testing are therefore highly uncertain.

6 Conclusions

6.1 Business outlook of food diagnostics

Food diagnostics as a business area is relatively new and can still be characterized as an emerging one, partly because the industry and the market is not well defined. The business of food analysis is expected to grow through the increasing demand for food safety, however, it is unclear whether this will lead to actual market growth of food diagnostics. Especially in Europe the growing political interest into food safety issues has been driven by a need to re-establish consumer confidence into the public food safety system, which was badly disrupted as a consequence of recent food contamination incidents, e.g. Bovine Spongiform Encephalopathy (BSE), enterohemorrhagic *Escherichia coli*, multi-drug resistant strains, Dioxin, and GMOs.

At this stage it is not clear what kinds of business models will prevail as profitable and viable, because it is dependent on whether we are concerned with food analysis, food diagnostics, microbiological testing, or microbiological food testing.

In terms of trends and needs it is, however, clear that food safety has become a public issue. This need is most visible in the growing *public* concern, i.e. consumers and political actors. This is not to say that the corporate community is unresponsive to this need, but there is an evident conflict between what can be done with respect to what is economically viable and what is publicly desirable. It is expected by the public that more testing, especially for food pathogens, will result in an increase of food safety of the product. However, the concept of food testing is limited in guaranteeing food safety and therefore the HACCP concept has been applied to guarantee *process safety* rather than *product safety*. There is a clear corporate and public communication challenge, that needs to be addressed. The question of whether implementation of rapid methods will lead to a sufficient degree of improvement in food safety or will have more impact on cost savings has to be further analyzed.

It is not clear whether the consumers in general are willing to pay a premium price for food safety tests, on something

which they already assume to be safe. Therefore, despite the growing public concerns over food safety, the economic incentives to develop new rapid methods are not exactly compelling. However, it is obvious that food safety has a very high impact on the image and branding of food processing companies and thus has a clear long-term corporate profit impact. In light of that it might be better to consider the issue of food safety and food diagnostics as an element of corporate communications.

The business area of food diagnostics is currently a derivative of the much larger medical diagnostic industry and other diagnostic areas (veterinary, environmental, agricultural), because new technologies are developed there, which has implications for both technology and market management in terms of economies of scale and scope. There has been an increased and renewed interest in the US after September 11, 2001 to develop new methods for pathogen testing resulting in an increase of R&D funding in that area, see for example, our description of several biosensors developed into prototypes mainly carried out in universities or spin-off companies. Diagnostic technologies can be applied in a variety of different areas either directly or after modification thus seemingly widening the commercial potential. Although there are apparent synergies between the different diagnostic sectors at the R&D level, the overall benefits might be meager as the markets are dramatically different, requiring for example modified product development processes, an entirely different sales force, technology transfer, and are subject to different regulatory requirements.

The food industry itself is not expected to increase their R&D expenditures, which in most cases is currently keeping pace with inflation. R&D activities listed by importance are new product development, product safety research, clinical trial or support, quality control, quality assurance, ingredient technology, process development, market research and nutritional research analysis. Areas of food science in order of importance are healthy food, functional food, food safety from a process perspective, development of natural food, food safety from an ingredient point of view, organic food, reduced fat food, and methods development for quality control.

Hence, with respect to business outlooks for the field of food diagnostics we have to address these issues on several levels. First, there is the industry level which defines the market and its constituencies, which sets the corporate framework – the strategy – for business activities in general. Second, there is the technological level or product level, which sets the framework for what is currently available, what is being developed, and what future development trends can be seen. Finally, there is the societal level consisting of governmental agencies, consumers, and other stakeholders who directly or indirectly are affected by actions taken and try to affect actions taken on the prior two levels. In this report we have so far focused on the second level and in this section we will look at key issues rising from the first level. The final level is outside the scope of this report and although it is extremely interesting and important it requires an entirely separate study.

From a purely competitive perspective this means that the area of food diagnostics is already a highly competitive area, especially as many diagnostic companies are already well established in traditional diagnostic areas. On the other hand food diagnostics in terms of market attractiveness is competing with other potentially more attractive markets such as pharmaceutical technologies and services measured in terms of market potential. Food diagnostics is a fairly small market in absolute terms, but also in comparison to the whole market for food analysis. There are approximately 50 established companies with some market leaders but no clear market dominator. The small market size has implications for market attractiveness, which may not provide incentives enough to develop new technologies or to adopt new technologies developed in, e.g. *in vitro* diagnostics to the special requirements for the food industry. The future development of the field is therefore critically dependent on the diffusion and acceptance of new technologies. Here, for example, the creation of a common validation procedure and acceptance for official testing purposes is paramount.

In the field of food diagnostics a number of different businesses exist. There are the already mentioned technology developers, which focus primarily on human *in vitro* diagnostics. Additionally, there are service providers, which are private, public or university-based research institutions. In Finland, municipal public laboratories are currently serving this sector. The proportion privately owned and public service providers vary between countries and where the tests are performed is also dependent on the type of test. In Finland, pathogen testing is rarely performed on-site as this necessitates holding control cultures of pathogens and having specialized facilities available for

handling infectious bacteria. Therefore, for example, 81% of *Listeria* and *Bacillus* pathogen testing of the Finnish food industry is performed in municipal laboratories, whereas 14% are carried out in-house and less than 5% are performed by private laboratories³⁶. Other tests as, for example chemical testing, are clearly less dependent on close proximity to service providers. In contrast to the situation in Finland, New Zealand has a considerable number of university-based service providers and a strong tendency to establish commercial spin-offs. Moreover, the service providers have focused their service offerings through product packaging of services, which makes it easier to commercialize the services. This seems to be a viable business concept. Product packaging of services is also common praxis among established CROs. Finnish municipal laboratories are so called general purpose laboratories, which is not economically sound in the long run.

If we look at rapid methods for pathogen testing, which has been one primary focus of this report, we find that market acceptance has been slow with ELISA based methods currently dominating the market. The major benefit of rapid methods, such as PCR, might be the increased capacity for sample throughput and automation, and might ultimately be cost saving. They are considered to be more demanding than conventional methods and require higher technical expertise for implementation and might therefore be more suited for use in larger analytical laboratories than for routine use in the food industry. It is important to realize that market acceptance is likely to be much higher when the methods implementation is a part of the development process. Again, this has market opportunity consequences for private laboratories and contract research organizations, which perform a wide range of analyses and also have the capacity to carry out work-related research and investigations, with modern technology, high expertise, and high volume throughput. Additional advantages are lower costs for implementation with higher expertise, expertise can be shared, and lower costs with higher sample throughput. Disadvantages are the need for close proximity between service provider and user.

The pace of biotechnological scientific and technological advances is constantly growing and has made the industry extremely knowledge intensive. This fact has strategic business consequences as it requires firms, whether technology developers or service providers or food processing companies performing tests in-house, to ensure constant knowledge development in order to stay competitive. Therefore, increasing consolidation activities as well as collaborations is expected in the future.

36 Tolvanen R. Elintarvike- ja ympäristöhygienian laitos, Helsingin Yliopisto (2002): *Listeria*- ja *Bacillus*-Näytteiden Tutkimusvalmiudet Elintarviketeollisuudessa.

6.2 Future outlook on methods

Viable cell counts

Viable cell counts, as coliform counts, faecal coliform counts, yeast and mould counts, will remain important for the assessment of safety and the quality of food products in the food industry. Alternative methods should be cheap, robust and provide analysis results in real time. Microscopic methods based on DEFT, flow cytometry, and biosensors could be developed for this application.

Hygiene monitoring

The development of real time methods for monitoring of the cleaning process in the food industry will have an impact on problems that are associated with biofilm formation and might therefore directly improve food safety in the food industry. Methods based on ATP bioluminescence, Adenylate kinase but also biosensor applications might be further developed for this area. Again methods should be kept in-house and developed to integrated real-time methods.

Immunoassays

Many commercial systems that allow fully automated testing, mostly based on ELISA are already on the market. The use of new recombinant antibodies and molecular imprinting techniques will improve the sensitivity and versatility of the technique.

Nucleic acid based assay

The development of homogenous assays and automation has improved the performance of the PCR methods for routine use in larger food laboratories. They often allow detection of several analytes (multiplexing). New assay formats are developed that can be further exploited for food diagnostic applications.

Biosensors and microarrays

The most promising breakthroughs are to be expected in the area of sensor technology, that will allow the creation of on-line or on-site, sensitive, low-cost devices for routine-use. Biosensors have a high potential for automation and allow the construction of simple and portable equipment for fast analysis. These properties will open up many new applications within quality and process control, control of the fermentation processes, and quality and safety control of raw materials. The new application possibilities offered should be further explored and technologically evaluated.

Immunomagnetic separation

IMS will be further developed to enhance the speed and sensitivity of different detection methods. The automation of the method can be expected in the next future. The combination with homogenous assays with multiplexing capacity and potential for automation will lead to further decrease in the time to result, increased sensitivity, improved sample throughput and user friendliness.

6.3 Recommendations

As becomes evident it is too nascent to recommend with certainty any specific business model, which will be economically viable. However, it is clear that more private service providers are needed in Finland and some degree of consolidation of municipal laboratories as well. Furthermore, small firms should be encouraged to establish themselves into the service sector.

A technological evaluation of the commercial potential of biosensors will also be needed since it has a market potential for different markets.

The focus should necessarily not be only on food pathogens, from a market point of view. Other applications should be considered.

Finally, there is a need for a strategic decision on whether pathogen testing should be re-integrated into in-house food laboratories or should it be encouraged that this sector be outsourced and developed as a private service sector.

Appendix 1

The interviewees:

Aaltonen, Tuula, Director, EELA, Milk and Hygiene Unit
Ahokas Tuula, Research Director, Ingman
Breitenstein Antje, Researcher, University of Oulu
Eerola Riikka, Microbiologist, Ruoka-Saariainen
Haikara Auli, Group Manager, VTT Biotechnology
Hatakka Maija, Food Agency, Senior Officer, Food Control
Headman Klaus, Headman Oy, Managing Director
Heiskanen Seppo, Manager, Finnish & Food and Drink Industries Federation
Hielm Sebastian, Assistant Professor, University of Helsinki
Honkanen-Buzalski Tuula, General Director, EELA
Järnström, Stefan, BioNobile, President, Managing Director
Johansson Tuula, Researcher, EELA
Julin Marja, Glomega, CEO, Managing Director
Kalkman-Spruyt, Pauline, MicroVal secretariat
Kallio Jarmo, Aboatech, Managing Director
Korkeala Hannu, Professor, University of Helsinki
Laaksonen Teppo, Labmaster, Managing Director
Lövgren, Timo, Professor, University of Turku
Majjala Riitta, Director Risk Assessment group, EELA
Mannonen Leena, Food Agency, Senior Scientific Officer
Mäntynen Vesa, Satalab Oy, Head of Laboratory
Matti-Sandholm Tiina, Research Professor, VTT Biotechnology
Neubauer Peter, Professor, University of Oulu
Niskanen Aimo, Anibiotech, Managing Director
Pensala Olli, Customs Laboratory, Head of Division/Microbiological Quality
Raaska, Laura, Group Manager, VTT Biotechnology
Siemens, Volker, Research Scientist, VTT Chemical Technology
Söderlund, Hans, Research Professor, VTT Biotechnology
Tuomola Mika, Researcher, University of Turku
Uosukainen Johanna, Quality Manager, Nestle
Vanhatalo Riitta-Leena, Director of the Food Laboratory, HK-Ruokatalo
Wirtanen Gun, Group Manager, VTT Biotechnology

The interviewees for the first part of the study:

Armfelt Riitta, Helsinki University
Iitiä, Antti Innotrack Oy
Jalkanen Laura, Food Safety Centre
Kaitaranta Jukka, Raisio Group
Kallio, Heikki Turku University
Karppinen Matti, Hes-Pro Finland Oy
Kilpi Petteri, Hes-Pro Finland Oy
Lammimäki Jorma, AnalyCen Laboratoriot Oy
Lavi Jukka, Raisio Group
Reini Raine, AnalyCen Laboratoriot Oy
Salmela Heikki, Hes-Pro Finland Oy
Sivelä Seppo, Valio Oy,
Taimisto, Anna-Maija Valio Oy
Tiistola, Esa Hes-Pro Finland Oy

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