

Rapid Methods

FOR FOOD AND FEED

QUALITY DETERMINATION

edited by:

A. van Amerongen
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quality determination

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*Wageningen Academic
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Foreword

There is an ever-increasing need for rapid methods and instrumentation in the field of food and feed quality. Key issues dealt with in the food and feed industry include: monitoring of processes at all stages; showing due diligence in the control of food and nutritional quality; achieving rapid results for detecting (micro)biological, chemical and physical deterioration of food and feed; detecting rapidly and reliably food authenticity and/or adulteration; and finally, implementing quality tracking & tracing.

The term 'rapid method' is used variously in discussions of rapid methodology and instrumentation. It should be noted that 'rapid' is not a goal in itself. In addition to increased speed, rapid methods must also take account of other criteria such as sample preparation and pre-treatment, accuracy and sensitivity, data analysis, total costs proportionate to the benefits, etc., eventually leading to methods best suited for use.

The chapters in this book summarise some important developments in the shift from slower, traditional measurements to more rapid methods for food and feed quality determination, including testing of food and nutritional quality, quality deterioration and spoilage, authenticity and adulteration, and quality tracking & tracing and rapid testing.

The methods and techniques presented here, in their varying degree of complexity, will be a valuable resource for researchers and professionals from the food and feed industry as well as from the scientific community. This book is an ideal supplement to 'Rapid Methods for biological and chemical contaminants in food and feed' as published in 2005.

The editors gratefully acknowledge the contributions by all authors, who generously gave their time and expertise to this book.

The editors

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Electronic sensing: food and feed applications

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Abstract

There is a requirement for rapid early detection of mould activity in food throughout the food chain as part of a quality assurance programme and to enable critical control points to be effectively monitored. The rapid development of electronic nose technology has resulted in examination of the potential of using this qualitative approach to enable decisions to be made about the status of grain and in bakery products. Since moulds produce characteristic odours when growing on different substrates the opportunity exists to use these volatile production patterns to improve decision support systems for making decisions about food quality. We have examined the use of electronic nose systems for the discrimination between different spoilage fungi *in vitro* and *in situ* in grain and bread. A real-time monitoring system was developed which enabled a sample to be evaluated in < 10 minutes to provide diagnostic information on whether it was 'good', 'bad' or 'intermediate'. Subsequent studies in bakery products have shown that it is possible to detect and differentiate spoilage mould growth on bread within 24-36 hours of inoculation, prior to visible growth. It was also possible to discriminate between non-microbial tainting, e.g., lipoxygenase, from microbial spoilage by filamentous fungi, yeasts and bacteria. Other traditional methods such as enzymes and colony forming units could only detect changes much later. Potential exists for using electronic nose systems in quality assurance and for monitoring critical control points as part of a hazard analysis critical control points scheme. The recent development of electronic tongue technology with applications for liquid foods and slurries will also be summarised.

Keywords: early detection, volatile fingerprints, micro-organisms, sensor arrays, electronic sensing, food quality

1. Introduction

Quality assurance (QA) systems within a hazard analysis critical control points (HACCP) framework are becoming the norm in developing effective prevention strategies in food production processes. This requires effective identification of

the critical control points (CCPs) and effective monitoring of these points in the food chain. Thus reliable and accurate methods are required for enabling management of the CCPs to be implemented (Tothill and Magan, 2003). The diagnostic tool required depends very much on the type and amount of information required. For example, is qualitative 'yes' or 'no' information required? Is a quantitative measurement needed? What is the sensitivity required? For example, does the monitoring require detection of 10^2 or 10^4 cells in a food raw material? How often does a measurement need to be made and does it need to be on line or at line? The economics of implementation in the food industry are determined by all these factors.

Generally, two factors determine the use of the technique: the time required to complete a specific test and the time needed for the result. For example, traditional serial dilution or washing techniques, which depend on agar media and an incubation period, require 1.5-2 hours of labour and perhaps 48-72 hours for an answer. Enzyme-linked immunosorbent assays (ELISA) techniques may require less time and give an answer much more rapidly (< 30 minutes). Lateral flow devices require simple sample preparation and give an answer in a few minutes (Danks *et al.*, 2003). Today, the pressure is for relatively cheap diagnostic tools, which can be used routinely giving results within a few minutes at most.

There are a number of indicators of microbial spoilage in food matrices. These include analyses of microbial populations, changes in enzyme activity, respiratory losses, changes in chemical components such as chitin, ergosterol or ATP, immunofluorescence, immunosensors and DNA probes, lateral flow devices and microbial volatiles. This chapter is predominantly concerned with the potential of using volatile fingerprints, so called electronic nose (e.nose) systems, produced by microbial contaminants as a tool for determining microbial quality of food and feed. Gardener and Bartlett (1999) defined this as 'an electronic nose is an instrument which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern recognition system, capable of recognising simple or complex odours'. Table 1 summarises the development of such sensor arrays from a historical perspective. This chapter will consider (1) measurement and data analyses, (2) type of sensor formats used, (3) food and feed applications and sensitivity of detection, and (4) electronic tongue systems.

Table 1. A brief historical perspective on development of sensor array systems for odour detection and development of e.nose type approaches.

1962:	Stereochemical theories of olfaction
1978:	Structure-activity relationships in human chemoreception
1982:	First development of a model nose using three sensors with broad sensitivity (Persaud and Dodd, 1982)
1988:	Term 'electronic nose' used for 1 st time
1990s:	First commercial devices developed using conducting polymer sensor arrays
1990s:	Applications in food, environment, and medicine using generic research-based devices
>2000 onwards:	More targeted approaches of e.nose systems for specific use in food, environment, and medical applications

2. Measurement and data analyses

The system consists of three basic building blocks: the vapour phase flows over a sensor array, the interaction with the sensor surfaces occurs, and this response is analysed using software for an output and interpretation. There are a number of different types of sensor arrays, which are used. Sensor technology has developed rapidly over the past decade and this has resulted in a range of different sensor formats and the development of complex microarray sensor devices. In the specific area of e.nose systems, several different physicochemical techniques have been used to produce sensor arrays for odour characterisation. There is a large body of literature on all these sensor technologies (Turner, 1999), but the key to the devices under consideration here is the fact that total specificity is not required. Using multifactorial approaches, it is simply enough for the elements of the array to react differently to various analytes, enabling discrimination to be made between samples. Each of the different sensor formats is described briefly below.

2.1. Conducting polymer sensors

Conducting polymer sensor arrays consist of unique polymers with different reversible physicochemical properties and sensitivity to groups of volatile compounds. These compounds interact with and attach to the polymer surface,

changing the resistance under ambient temperature conditions. This in turn changes the signal, which is monitored for each sensor type, enabling an array to be constructed that has overlapping detection ranges for different groups of volatile compounds. Sample presentation is crucial for this type of sensor to avoid humidity and drift problems.

2.2. Metal oxide sensors

The oxide materials in these sensors contain chemically adsorbed oxygen species, which can interact with the volatile molecules, thus altering the conductivity of the oxide. The selectivity of these sensors can be changed by using different amounts of noble metals or by changing the operating temperature. They are very sensitive, robust and resistant to humidity and ageing effects, although they can suffer from drift over time.

2.3. Metal oxide silicon field effect sensors

These sensors are related to metal oxide sensors but the output signal is derived from a change in potential when the volatile molecules react at a catalytic surface. The operating temperature for these sensors is in the range of 100-200 °C. They are sensitive to many organic compounds.

2.4. Piezoelectric crystals

Sensors containing piezoelectric crystals use the radio frequency resonance of quartz materials coated with acetyl cellulose or lecithin membranes. The adsorption of volatile molecules onto the membrane produces a change in the magnitude of the resonance frequency that is related to the mass of the volatile analyte. The selectivity of these sensors is dictated by the thickness of the coatings.

2.5. Surface acoustic wave devices

These devices are an alternative to the above sensors that are based on waves emitted along the surface of a crystal by the electric field of surface-deposited aluminium electrodes.

2.6. Optical sensors

These sensors are based on a light source that excites the volatile analyte, and the signal can be measured in the resulting absorbance, reflectance, fluorescence or chemiluminescence.

2.7. Electrochemical sensors

These sensors contain electrodes and an electrolyte. The responses generated are dependent on the electrochemical characteristics of the volatile molecules that are oxidised or reduced at the working electrode, with the opposite occurring at the counter electrode. The voltage generated by the reactions between the electrodes is measured, and has been used to measure CO, SO₂ and H₂S.

3. New sensor technologies

New approaches are being tested to provide better sensor shelf-life, greater sensitivity and more consistency under variable conditions of temperature and humidity for commercial applications. Surface plasmon resonance can be used, in which changes in the optical properties of the polymer materials are used in sensors or the resonance of associated cantilevers which can be monitored based on changes in the mass of the volatile compound being analysed. Discotic liquid crystals, which consist of an aromatic core surrounded by hydrocarbon side chains, are very sensitive to the presence of volatile molecules and insensitive to humidity, and could provide advances. Materials based on metalloporphyrins, which can detect a broader range of fingerprints because of the diversity of metal ions and substituted porphyrins available. Recently, an expanded colourimetric sensor array system based on metallated tetraphenylporphyrins and chemoresponsive dyes has been developed which has good sensitivity, with thresholds of detection for amines, carboxylic acids and thiols that are better than the human nose. Preliminary data with bacteria using this technique have been promising (Suslick *et al.*, 2002).

The usefulness of e.nose type devices depends on two crucial components. First, the sample must be presented in the correct format to optimise the interaction of volatiles in the headspace with the sensor array. Sample analysis should be consistent and the sample should be presented in the same way. Thus, humidity, temperature and sample size must be standardised to ensure that data sets can be compared and analysed with confidence. This also minimises drift problems over

time, although these can be overcome by using a set of appropriate standards for calibration. Second, pattern recognition must enable large data sets to be analysed rapidly to obtain appropriate and useful results (see Pearce *et al.*, 2003). Normally, volatile odour pattern data are received in the form of normalised data sets based on the divergence, area, adsorption or desorption components of the individual sensor responses. This generates a significant amount of data and requires effective data management. The techniques used to analyse such data sets have included simple supervised techniques such as discriminant function analysis (DFA), which can parametrically classify an 'unknown' or 'random' sample from a population or group. DFA has been successfully used to detect bacteria, yeasts and some filamentous fungi (Needham *et al.*, 2005). A simple unsupervised multivariate method such as cluster analysis has also been used to identify a volatile odour class without prior information on the nature of the volatile fingerprint. Principal components analysis (PCA) is a popular non-parametric technique that makes no assumptions about the data, and which groups related data in three dimensional space of a multivariate system where the parameters are partially correlated and the results are displayed in a two dimensional pattern recognition map.

Where real-time data analyses is required neural networks (NNs) which consist of a series of algorithms that are more appropriate for non-linear sensor systems are used. This enables a specific system to be developed for a specific target at the required level of sensitivity. By having enough background (control) data and by using so-called backpropagation approaches, sensor drift and non-linear data sets can be taken into account and effectively used for effective prediction of the group into which a real sample falls (Magan *et al.*, 2001). Of course, a large number of training sets are often required to develop appropriate NN systems. Where there is still an overlap between groups, the potential exists for the use of 'fuzzy logic' NNs. These are more flexible, and can be trained rapidly with large amounts of sensor array data from samples to provide a foundation of healthy background volatile fingerprints. This subsequently makes differentiation of contaminated samples easier and more rapid, often in a matter of minutes. However, it is clear that for different microbial contaminations specific NN analysis systems might need to be developed. Some could be qualitative only and useful for screening, whereas others could be semi-quantitative and give more information for treatment of the disease (Turner and Magan, 2004).

4. Food and feed applications

This approach has received much attention because it requires no sample preparation and is thus non-invasive, can use a larger representative sample, and the sample presentation can be standardised easily. The disadvantage is that it is qualitative and at most semi-quantitative, and sensor systems can be prone to drift. However, new generation sensors may significantly reduce these problems. Table 2 shows the range of food products for which this approach has been examined, either for research or in practical applications. This demonstrates the broad range of food products, which have been examined.

5. Raw materials contamination

It is well known that microbial contaminants produce a range of volatiles. These volatile fingerprints vary with individual micro-organisms. For example, using gas chromatography-mass spectrometry (GC-MS) it has been shown that the volatile profiles produced by *Pseudomonas fragi*, *Saccharomyces cerevisiae* and *Penicillium verrucosum* are very different (Needham *et al.*, 2005). It has been shown that raw materials such as grain which is poorly stored has off-odours

Table 2. Summary of the range of food matrices and types of spoilage which have been examined using e.nose systems.

Food product	Types of spoilage
Grain/flour	moulds/insects/toxin
Bread	bacteria/yeasts/moulds
Milk	bacteria/yeasts
Cheese	moulds
Coffee	bacteria/yeasts/moulds
Fish	microbial taints/freshness
Tea	cultivars/speciality coffees
Beers	tainting
Wines	tainting/yeasts
Nuts	quality/toxins
Fruit	quality/disease
Meat	microbial taints/freshness

due to alcohols, esters, ketones, mono- and sesquiterpenes, and aldehydes. The dominant volatiles in grain were found to be 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone (Magan and Evans, 2000). Studies with grain having different levels of moulding and natural samples showed that by using a standardised grain amount, a conducting polymer array of sensors and a radial based-neural network it was possible to carry out real-time analyses of grain samples in 10 minutes (Evans *et al.*, 2000). This enabled a decision to be made on whether grain was acceptable or not. Interestingly, results were very comparable with those produced by an odour panel classification. This approach was based on acceptable or unacceptable and showed a small group of samples in a so-called zone of uncertainty, which suggested that they were in the process of going mouldy and thus needed further investigation. This study also investigated the potential for differentiation between mould contamination and mite contamination by using both a conducting polymer sensor array combined with a metal oxide array for the pest infestation. This suggests that the opportunity exists to combine such systems for real-time analyses of different types of contaminants from a single sampling.

6. Sensitivity for microbial detection in food and feed

Recent studies have attempted to differentiate between non-microbial and microbial spoilage. Needham *et al.* (2005) used a bacterium, yeast and filamentous fungus to inoculate bread for examination after different time periods (24, 48, 72 hours) at 25 °C. The results were compared with uninoculated bread and lipoxygenase containing bread to simulate non-microbial spoilage. In this study a conducting polymer sensor array was used. Figure 1 presents the cluster analysis dendrogram. It appeared to be possible to discriminate between non-microbial volatiles and those produced by a *Penicillium* species, but not between the yeast and bacterial contamination after 48 hours prior to any visible growth being present.

Studies with milk-based media have been carried out to try to differentiate between different bacterial and yeast contaminations. *Bacillus cereus*, *Kluyveromyces lactis*, *Staphylococcus aureus* and *Candida pseudotropicalis* at initial levels of 10⁴ CFU/ml were analysed after 5 hours of incubation using a conducting polymer sensor array. The data were subject to cross-validation using individual samples of each treatment as unknowns. The results are presented in Figure 2, which shows it could be successfully done accounting for 83% of the data (Magan *et al.*, 2001).

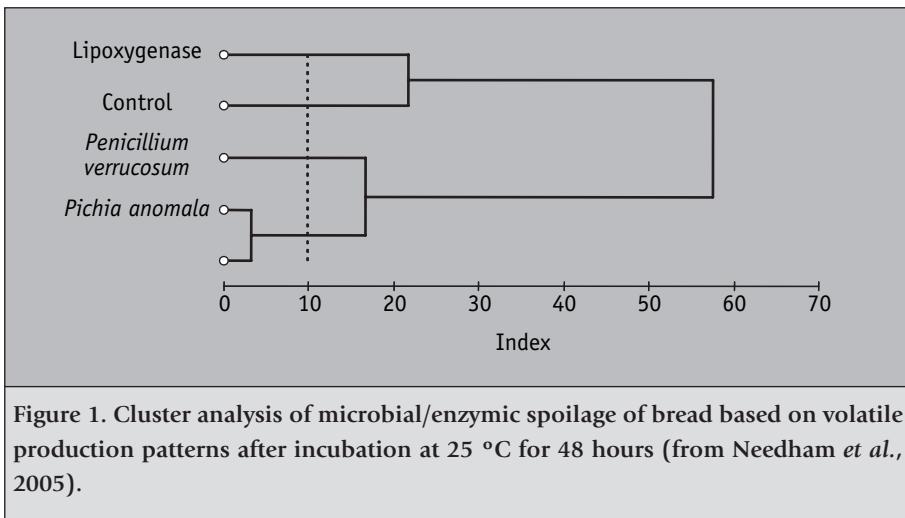


Figure 1. Cluster analysis of microbial/enzymic spoilage of bread based on volatile production patterns after incubation at 25 °C for 48 hours (from Needham *et al.*, 2005).

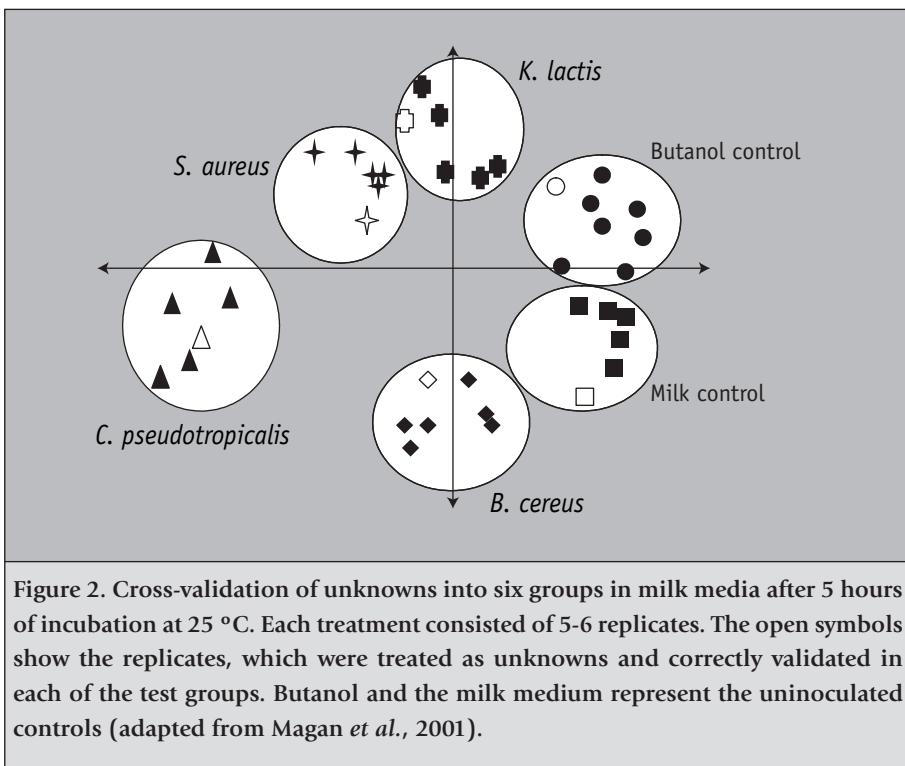


Figure 2. Cross-validation of unknowns into six groups in milk media after 5 hours of incubation at 25 °C. Each treatment consisted of 5-6 replicates. The open symbols show the replicates, which were treated as unknowns and correctly validated in each of the test groups. Butanol and the milk medium represent the uninoculated controls (adapted from Magan *et al.*, 2001).

Studies with different micro-organisms and food products suggest that the minimum number of cells which can be detected in food matrices within 24-48 hours of incubation is $10^3\text{--}10^4$ for grain and milk, and 10^3 for bread and cheese. Studies by Keshri *et al.* (2002) showed that it was possible to detect moulds in bread matrices within 48 hours, much earlier than that using enzymatic assays or traditional serial dilution methods. Where detection of 10-100 CFUs is needed it may be possible to achieve this by the addition of specific enzymes to enhance volatile headspace generation. This was successfully achieved in medical applications for detection of *Mycobacterium tuberculosis* (Pavlou *et al.*, 2004).

Recent studies have used a MS-based e.nose system to examine the mould contamination of Spanish bakery products. PCA, DFA and Fuzzy ARTMAP approaches and solid phase micro-extraction to concentrate the volatiles produced by the moulds were used (Vinaixa *et al.*, 2004). These authors successfully (98%) discriminated mould growth from control in 48 hours, again prior to any visible growth. After 96 hours 88% discrimination between a range of spoilage species was possible. Potential thus exists for applications in bakery production plants to monitor batches. Each measurement using this type of system would require 20-25 minutes. This could quite easily be included in a QA system provided the initial investment gives a relatively rapid return.

7. Electronic tongue systems

There has been recent interest in using arrays of electrodes with different selectivity and sensitivity by deposition of different electroactive surfaces. Usually electrochemical detection by comparison with reference electrodes or cyclic or square wave voltammetry is used (Figure 3). This approach has been used in relation to monitoring liquids and slurries and thus may have both food and feed applications. Recently, it has been examined for analysis of port wine age, discrimination of orange juices, Spanish red wine, mineral water, mould discrimination in media, food matrices and olive oil. This approach has potential for examining adulteration of high value food products rapidly.

Recent studies of port wine ages from 10 to 40 years old showed that the ages were successfully discriminated using an electronic tongue approach (Runitskaya *et al.*, 2005). Studies of mould growth in liquid media showed that measurements with an electronic tongue system could be used to discriminate between 1, 3, 7 and 10 days of growth (Soderstrom *et al.*, 2005). Varying the medium content with different nutrients also influenced the results. These studies show that some

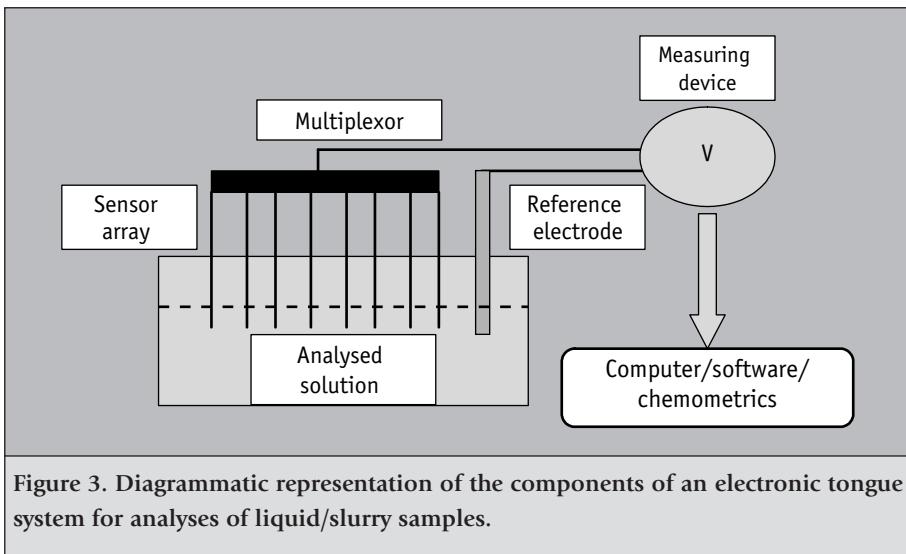


Figure 3. Diagrammatic representation of the components of an electronic tongue system for analyses of liquid/slurry samples.

potential exists for effective utilisation of these types of electronic systems for food and feed applications.

8. Conclusions

Overall, e.nose systems and the development of electronic tongue systems have been demonstrated to be very useful for use in monitoring and QA, especially where a yes/no answer is required rapidly. For applications in the food industry where cost/benefit considerations are important, the economic cost of introduction of such systems may be critical. However, sensor technologies are evolving very rapidly and we can expect better sensitivity, stability and shelf-life for food/feed applications. Generic systems have usually been employed for a range of applications. In the future, development of specific systems for each application coupled with appropriate NNs and associated software will be required to take full advantage of the technology.

The opportunity now also exists to take data gathered remotely at different sites and use advanced information-technology approaches, satellite communication and web-based knowledge systems to analyse information rapidly giving results from a central point within minutes. This would open up the potential for using these systems throughout a food chain, perhaps also to monitor more effectively,

foodborne pathogens. However, from my experience with the bakery and dairy industry where non-invasive and shelf-life testing is of particular interest the following factors need to be considered: flexibility, in an at-line environment, ease of operation and maintenance, appropriate software, and time and labour savings. From an economic point of view the price should be in the region of 10 to 15K euros, because margins are very small in these industries. The technology does have potential for effective management systems where rapid diagnostics techniques are advantageous for conserving and/or improving QA.

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Proteome and metabolome analyses for monitoring food quality and authenticity

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Abstract

Proteomics and metabolomics are progressively being recognised as key tools for characterising biological systems since they represent the downstream consequence of gene expression changes either from biotic or abiotic stress. Several strategies have been developed for profiling proteomes and metabolomes, and these profiles can be used for monitoring food and environmental safety, food authentication and food processing. A brief overview of current strategies in proteome and metabolome analyses is presented and its potential in food authentication is discussed.

Keywords: proteins, metabolites, post-genomic, adulterants, food quality, authenticity

1. Introduction

Post-genomic technologies are typified by simultaneous high-throughput measurement of several analytes at the level of gene products, i.e. transcripts (transcriptomics), proteins (proteomics) and metabolites (metabolomics). The underlying theme in these analyses is the large scale parallel detection of several of these analytes (transcripts, proteins, metabolites) simultaneously or within an analysis, so that a 'holistic' picture of the biological system under study emerges, more than would be afforded when analysing for specific components individually.

Reliable and rapid authentication of species of origin (especially with meat and meat products), authentication of geographical origin of plant products, monitoring adulteration/contamination, and authentication of organic produce and genetically modified products are some of the issues concerning food authentication (Lees, 2003). A common objective in all these is the identification of suitable markers or group(s) of markers to characterise the authenticity of

foods or their potential adulterants/contaminants and to use these to resolve authenticity issues.

Food, processed or packaged, is generally of plant or animal origin. Proteins and metabolites form a substantial fraction of organic matter, usually with significant functional import in most foods. Several macro- and micronutrients, anti-nutrients, plant toxins, secondary metabolites and allergens are proteins or metabolites. They are thus suitable candidates as (bio)markers and have the potential to be ideal candidates as indicators of the functional status of food and for characterising it. The issue of genetically modified crops has been a subject of intense debate ever since field trials were introduced in the 1980s, and is a major issue in food traceability. Here again, analysis at the level of proteomes and metabolomes will be useful for monitoring and characterisation purposes.

The following paper presents the rationale for analysing at the level of proteomes and metabolomes and gives an overview of strategies currently being pursued in different areas of application. A brief discussion on the potential application of some of these techniques and strategies for monitoring food authenticity is also presented.

2. Analyses beyond the genomes - proteomes and metabolomes

The usual route to biological characterisations and one that is traditionally thought to have a stable basis is the analysis of the genetic make-up of biological systems. However, genomic information alone does not completely characterise biological systems and is certainly not sufficient for understanding biological processes; moreover for some foodstuffs (e.g. oil) DNA has been removed by the processing of the raw material into the product. With the sequencing of the human genome, it is now known that humans have only three times as many genes as a worm, share 50% similarity to the genome of a fruitfly, 85% to that of our canine friends and 99% to that of chimpanzees. These suggest that more than the genetic make-up, the contextual combination of gene products confers complexity and diversity to the functional genome. Consequently, there is greater emphasis on technologies that would elucidate the functional aspects of genes and gene products, in the post-genome era. Proteins and metabolites are at the 'business end' and carry out the activities of the cell at the biochemical level (Figure 1). Whilst the genome sequence can give information on which proteins the cell has the potential to make, and the transcript profiles can indicate which

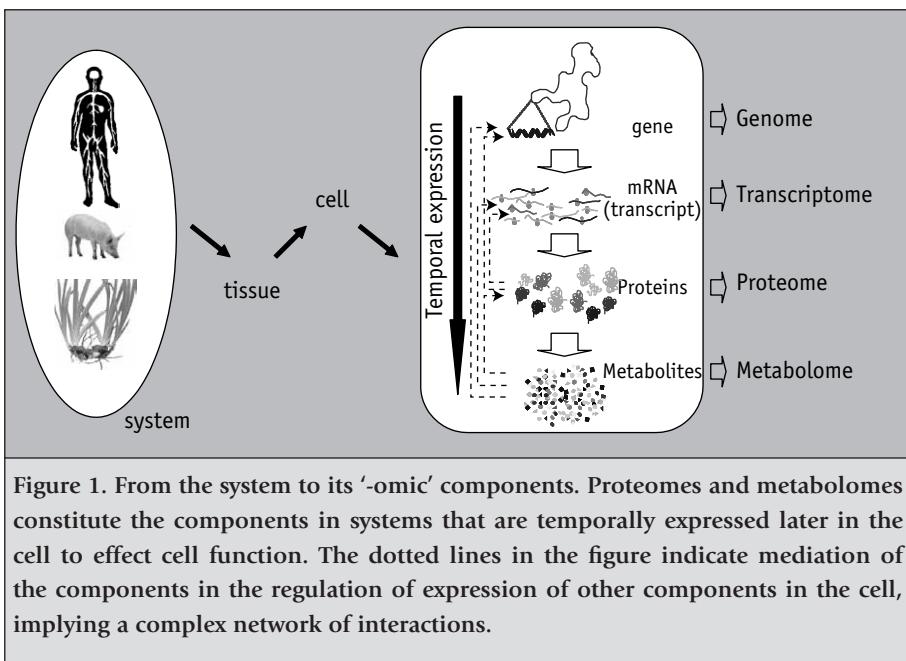


Figure 1. From the system to its '-omic' components. Proteomes and metabolomes constitute the components in systems that are temporally expressed later in the cell to effect cell function. The dotted lines in the figure indicate mediation of the components in the regulation of expression of other components in the cell, implying a complex network of interactions.

proteins are being produced, analyses beyond the genomic and transcriptomic levels are required to gain information on what the expressed proteins do to carry out cellular function at the molecular level.

The argument for monitoring proteomes and metabolomes is further substantiated by the following observations: (1) the existence of an open reading frame does not necessarily imply the existence of a functional gene; (2) the relationship between genes and gene products is not necessarily linear, with more proteins being expressed by a given gene; (3) mRNA levels do not always correlate with protein levels; (4) biochemical events that occur post-transcriptionally and/or post-translationally, such as alternate splicing, regulation of enzyme activities, distribution of metabolites between cellular compartments are not accounted for while monitoring at the genomic or transcriptomic level alone; (5) complex network interactions at the level of proteomes and metabolomes mediate several cellular activities in response to physiological signals that will be hard to account for at the level of genomes and transcriptomes, as genomes and transcriptomes are fairly conserved between cell states and types, within a system; (6) proteins and metabolites are more closely associated with cellular function as they are

instrumental in translating the genetic information to phenotypic response, and are consequently ideally suited to provide information on cellular activities and the observed phenotypes.

3. Proteome analyses

Proteome analysis as originally conceived (Kahn, 1995; Wasinger *et al.*, 1995; Wilkins *et al.*, 1996) involved the detection and quantitative identification of expressed proteins (expression proteomics). In recent years, it has been extended to include characterisation of the structural, functional and contextual aspects, such as post-translational modifications, protein-protein interactions and subcellular localisations. Three major streams thus emerge (Figure 2A): (1) expression proteomics, (2) functional proteomics, and (3) structural proteomics. Although not mutually exclusive and consisting of overlapping areas, these three streams aim to address different aspects of the proteome. In a general sense, proteome analysis translates to mapping the cellular proteins in a spatial and temporal manner.

Expression proteomics involves the detection of proteins in a complex mixture of analytes in the cellular milieu, identification of the detected proteins, quantifications in maximum numbers with high efficiency, and assessment of differential expression in a qualitative, semi-quantitative or fully quantitative manner between different cells in a given state or between different states of a given cell. Three major analytical biochemical strategies have evolved in expression proteomics (Figure 2B): (1) two-dimensional sodium dodecyl polyacrylamide gel electrophoresis (2D-SDS PAGE) based separation followed by mass spectrometric (MS) identification of separated proteins, (2) (multidimensional) liquid chromatography (LC) or capillary electrophoresis (CE) based separation of proteins/digested peptides, followed by MS based identification, and (3) protein analytical microarray technology. In addition, there is a fourth strategy that involves genetic engineering based approaches.

2D-SDS-PAGE is the most widely employed tool in expression proteome analyses. Here, proteins in cell extracts are separated based on charge (*pI*) in the first dimension using isoelectric focusing and by size (*Mr*) in the second dimension using SDS-PAGE (Figure 2B). The separations are usually carried out in slab gels. The gels can be used at an analytical scale to look for prominent signals, or at a micro-preparative scale for specific identifications and further characterisation of the separated proteins. In a general 2D-SDS-PAGE analysis, the full complement

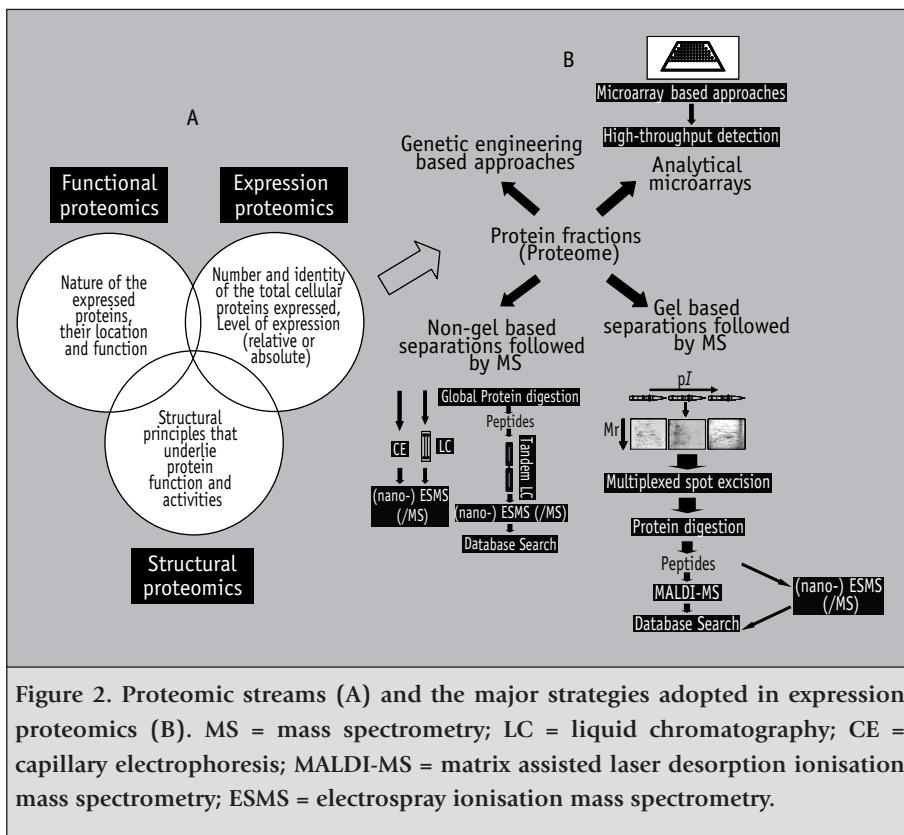


Figure 2. Proteomic streams (A) and the major strategies adopted in expression proteomics (B). MS = mass spectrometry; LC = liquid chromatography; CE = capillary electrophoresis; MALDI-MS = matrix assisted laser desorption ionisation mass spectrometry; ESMS = electrospray ionisation mass spectrometry.

of the proteome is seldom seen due to the limited dynamic range of analysis, poor detection of proteins of low abundance and membrane-bound proteins, multiple spots for a protein as a result of post-translational modifications, and the limits in resolution resulting in the presence of multiple proteins per spot despite two dimensions of separation. Specific strategies are available to counteract each of these limitations and can be adopted (e.g. Rabilloud, 1999; Bae *et al.*, 2003; Tastet *et al.*, 2003). The employment of multiple gels that address specific limitations or that cover wider pI and Mr characteristics can be helpful in comprehensive analyses, but this necessitates a more labour intensive activity.

Another approach to proteomic analysis is the application of high efficiency capillary separation techniques such as capillary liquid chromatography (CapLC) or capillary electrophoresis (CE) based approaches (Romijn *et al.*, 2003; Shen

and Smith, 2002). CapLC separation efficiencies have dramatically increased over recent years and efficiencies greater than 10^5 plates/column are now achievable with capillary columns packed with $1.5\text{ }\mu\text{m}$ particles. Improvements in column manufacture and operating pressure have also contributed to the developments. CE includes both dynamic-state electrophoresis, such as capillary zone electrophoresis (CZE), and static-state electrophoresis, such as capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CITP), the former two preferred for analytical separations and the latter usually for preconcentrations.

Proteome analyses by LC or CE based separations can be effected by employing either the 'top-down' (Reid and McLuckey, 2002) or the 'bottom-up' strategy (Bogdanov and Smith, 2005). The top-down approach involves the separation of intact proteins from complex mixtures, followed by subsequent analysis of separated proteins. The bottom-up strategy involves global digestion of proteins using specific proteases, separation of peptides and subsequent analysis of the separated peptides to infer proteomic information. LC separations can also be applied prior to 2D-GE as a clean-up or pre-fractionation operation (e.g. Champion *et al.*, 2003), or for further separation of co-migrating proteins from 2D-GE resolved protein spots. LC separations can also be effected by employing multiple configurations in tandem, using different principles of separation, such as polarity, ion-exchange, size-exclusion, affinity, etc. Increased peak capacities (number of individual components resolved) and load capacities (amount of material that can be run maintaining good chromatographic resolution) can be realised in such hyphenated approaches. For example, the combination of strong cation exchange (SCX) with RP-HPLC has been demonstrated to be useful in separating over 3,000 peptides from human hemofiltrates (Raïda *et al.*, 1999). The same combination in a single biphasic column (Link *et al.*, 1999) results in a more efficient separation developed as multi-dimensional protein identification technology (MudPIT), and has been demonstrated in the analysis of the yeast proteome (Washburn *et al.*, 2001).

Developments in mass spectrometry with respect to the discovery of 'soft' ionisation methods (in particular, matrix-assisted laser desorption ionisation (MALDI) (Hillenkamp and Karas, 1990) and electrospray ionisation (ESI) (Fenn *et al.*, 1989)), and improvements in mass resolution, sensitivity and accuracy, have significantly enabled identification and quantification of proteomic expression. Consequently, a majority of the strategies and techniques in expression proteomics are dependent on mass spectrometry. Peptide mass fingerprinting

(Henzel *et al.*, 1993) (which involves the digestion of separated protein with a protease, usually trypsin, followed by analysis of the eluted peptides by MALDI-MS and database match of the peptide patterns) and tandem mass spectrometry (usually employing nano-ESI and involving fragmentation of a selected peptide in a collision cell followed by the analysis of the fragment ions in a second analyser) are some MS strategies employed in identifying proteins.

Microarray-based approaches involve miniaturisation of standard assay procedures in multiple arrays to allow simultaneous analysis of multiple determinants/analytes. Large-scale assessment of protein profiles can be carried out by the use of immunoassays on microarrays. Antibodies immobilised in an array format onto specifically treated surfaces act as 'baits' to probe the sample of interest to detect proteins that bind to the relevant antibodies, using for example fluorescence detection.

Although high-throughput techniques for large-scale absolute quantification of the expressed proteins are yet to appear, several approaches for relative quantification have emerged (Righetti *et al.*, 2004; Sechi, 2004). Relative quantifications with 2D-PAGE can be effected by difference gel electrophoresis (DIGE), which involves a two-colour fluorescent labelling system (with the spectrally distinct fluorescent dyes, Cyanine-2 (Cy2), Cyanine-3 (Cy3) or Cyanine-5 (Cy5)) that allows two proteomes (say controlled and perturbed) to be differentially labelled and analysed in the same gel. Alternatively, methods based on stable isotope labelling open the possibility of quantification using MS, in a more reproducible and accurate manner. The methods rely on the principle that stable isotope incorporation shifts the mass of the peptides by a predictable amount. The ratio of the analyte between the isotope incorporated and the non-incorporated state can then be determined accurately by the measured peak ratio between the underivatised and the derivatised sample. Isotope labelling can be introduced pre-experiment, at the growth phase (metabolic or *in vivo* labelling), or post-experiment (chemical labelling) *in vitro* at the pre- or post-proteolytic digestion stage. More recently, the development of a quantification method that involves the design, expression and use of artificial proteins derived from concatamers (genetic sequences coding for the peptides linked end-to-end) of standard Q peptides, generated by gene design *de novo*, in an approach termed QCAT (Beynon *et al.*, 2005) shows promise for absolute proteomic quantifications.

Functional proteomics involves mapping the cellular proteins with respect to their interactions in the cell in effecting cellular activities. It is concerned with the detection and identification of the nature of proteins in the cellular context leading to the definition of its role (function) in the cell that in turn helps in understanding cellular activities. It provides information regarding such aspects as protein-protein interactions, protein localisations, and post-translational modifications.

It is now known that many cellular activities are carried out by multi-protein complexes, and that protein-protein interactions mediate many aspects of cell behaviour. Protein-protein interaction networks can be used to predict protein function (Vazquez *et al.*, 2003). Currently, three major strategies can be identified for analysing protein-protein interactions: (1) the yeast two-hybrid system, (2) functional protein microarrays, and (3) affinity capturing methods coupled to MS-based protein identification techniques. Chromatographic separation, density gradient centrifugation, epitope-tagging and immuno-localisation (Kumar *et al.*, 2002) are some of the techniques used in subcellular proteomics, in addition to those employed for general protein profiling and protein complex identifications. For the assessment of protein-biochemical activities, functional microarrays can be constructed by arraying purified proteomes/sub-proteomes and screened for biochemical activities, as has been demonstrated by Snyder and colleagues (Zhu *et al.*, 2000).

Post-translational modifications (PTMs) are protein processing events in which the nascent (translated) protein is modified covalently in order to confer or abstract functionality, allowing for diversity in the regulation of protein function. These include phosphorylation, glycosylation, acetylation, methylation, ubiquitination, etc. Four basic strategies that can be used to varying degrees of success have been identified (Mann and Jensen, 2003) as being currently available for monitoring PTMs. These are (1) 2D-GE based separations followed by MS identification, (2) affinity-based enrichment of modified proteins followed by MS of protein mixtures, (3) (LC)LC-MS/MS of enzymatically digested proteins (peptide mixtures), and (4) selective derivatisation of peptides followed by affinity purification and MS. Sequence-based MS forms a vital part in current techniques for identifying PTMs.

Structural proteomics involves the prediction of biochemical function of uncharacterised proteins based solely on structural homology to another protein of known function, in a genome- or proteome-wide scale (Sali *et al.*, 2003).

Current experimental methods of characterisation in structural proteomics include X-ray crystallography, NMR spectroscopy, electron microscopy, electron tomography, and fluorescence resonance energy transfer (FRET).

4. Metabolome analyses

The metabolome can be defined as the metabolite complement (the low molecular weight components and intermediates involved in metabolism) of a cell, tissue or organ in a particular physiological state (Oliver *et al.*, 1998; Tweeddale *et al.*, 1998; Vaidyanathan *et al.*, 2005). Metabolites can be endogenous (arising from within the cell), exogenous (arising from outside the cell), primary (central to the metabolic needs of the cell), or secondary (arguably of less importance to the cellular machinery).

Individual metabolites can be analysed using the traditional methods of chemistry and biochemistry. Usually this is accomplished by isolating the metabolite of interest from the sample milieu and analysing it. However, when the objective is to capture changes in the overall metabolite composition of the cell, the exercise of analysing several metabolites in parallel in a sample milieu that is chemically heterogeneous becomes challenging. In addition, rapid quenching of cellular activities is essential if quantitative levels of metabolites are to be captured in the analysis. In general, current biochemical strategies for metabolome analyses are an extension of those used to analyse specific metabolites and involve a combination of analyte separation techniques, such as gas or liquid chromatography or capillary electrophoresis, followed by detection using techniques such as NMR or MS (Figure 3).

The coupling of gas-chromatography (GC) with mass spectrometry has been the preferred route to analyse plant metabolomes (Hall *et al.*, 2002). Rapid sample throughput is achievable with the use of TOF-MS coupled to GC separations and databases of up to 500 metabolites have been generated (Wagner *et al.*, 2003). It has been shown that the number of peaks detected can be increased by appropriate optimisation of the instrumental conditions. The application of 2D-GC or GCxGC ((Dalluge *et al.*, 2003; Phillips and Beens, 1999) coupled with TOF-MS, should increase analyte resolution and enable high-throughput quantitative determinations (Van Mispelaar *et al.*, 2003). Liquid chromatography (LC) coupled to electrospray ionisation mass spectrometry is another approach in metabolite profiling. Reverse-phase and hydrophilic interaction chromatography have been investigated. It is possible to generate over 2000 mass signals from

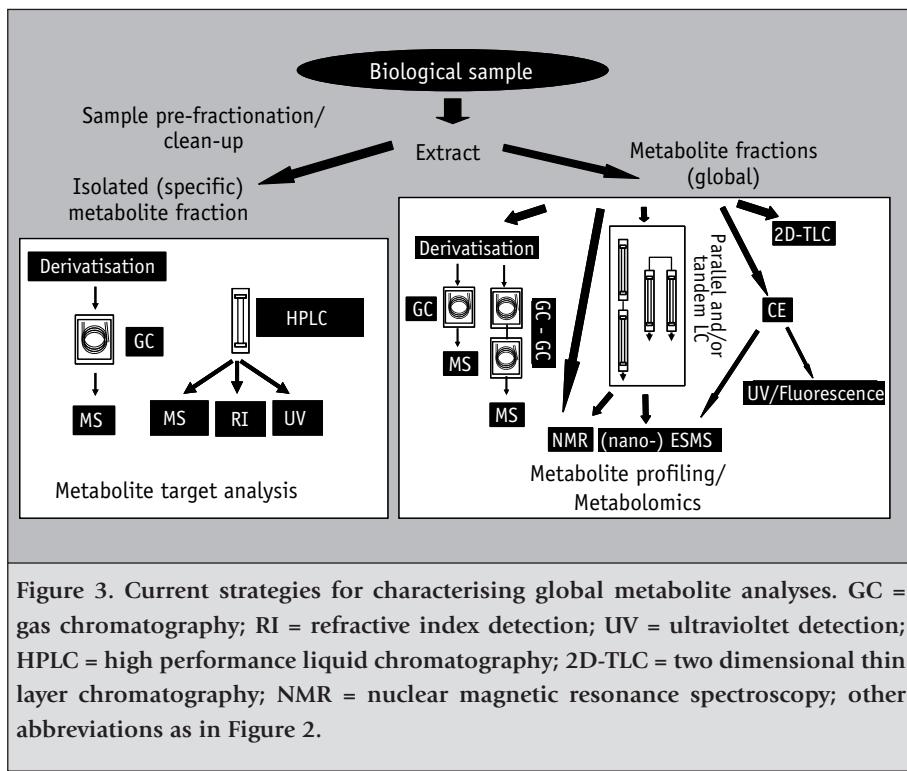


Figure 3. Current strategies for characterising global metabolite analyses. GC = gas chromatography; RI = refractive index detection; UV = ultraviolet detection; HPLC = high performance liquid chromatography; 2D-TLC = two dimensional thin layer chromatography; NMR = nuclear magnetic resonance spectroscopy; other abbreviations as in Figure 2.

LC-MS analysis of metabolic extracts as has been demonstrated in the analysis of plant extracts with capLC techniques (Von Roepenack-Lahaye *et al.*, 2004) and human metabolome (Zhou *et al.*, 2005). NMR can also be coupled to LC separations to derive comprehensive metabolomic information. The combination of LCs in tandem or in parallel offers scope for increase in analyte throughput, as is miniaturisation. In capillary electrophoresis (CE), metabolites can be separated based on size and charge, by applying voltage across buffer filled capillaries. Detection is usually by UV/Vis or fluorescence techniques. A more productive approach is the coupling of mass spectrometry to CE. The concurrent use of three CE-MS techniques, each for analysing cationic metabolites, anionic metabolites and nucleotides has been shown to be useful in detecting over 1500 metabolites in microbial cell extracts (Soga *et al.*, 2003).

Two-dimensional thin layer chromatography (2D-TLC) has also been shown to be useful in characterising metabolomes (Ferenci and Maharjan, 2005).

Compared with the other techniques discussed above, 2D-TLC is a fairly simple analytical technique affordable by most laboratories. It can be useful in monitoring prominent metabolomic changes, although it is limited in resolution for comprehensive high-throughput determinations on metabolomic scales; and since densitometry is used, it is generally considered semi-quantitative. Other techniques include the application of electrochemical detection in combination with LC separations and MS (Meyer *et al.*, 2005; Rozen *et al.*, 2005).

In addition to the quantitative levels of metabolites, the metabolic reaction rates or metabolic flux through a pathway also need to be considered for gaining insight into metabolic networks, for a more holistic perspective on metabolism. Isotope tracer techniques have been in use for such analyses (Sauer *et al.*, 1997).

5. Rapid methods - fingerprinting (and footprinting) approaches

Approaches that adopt short and simple protocols, which can still provide the necessary information, are highly desirable in instances where a rapid turnover of information needs to be monitored. Such analyses are useful for large-scale screening of samples for more elaborate investigations to follow, and the identification/monitoring of biomarkers representative of specific physiological or disease states, including food authentication. The rapid characterisation of prominent changes in a reproducible manner would be useful in such instances. Techniques capable of handling a large number of samples (high throughput) with minimal sample preparation would be ideal. Identification/quantification may not be required, but a consistent pattern needs to be generated. Such approaches will be useful in establishing deviations outside statistically relevant confidence thresholds that will be indicative of safety concerns or loss of authenticity.

Fingerprinting involves capturing changes in intracellular components and footprinting (Kell *et al.*, 2005) involves the analysis of changes in extracellular components (secreted into the immediate environment of organisms, cells or tissues, in response to physiological alterations in the system). These analyses ideally do not involve elaborate separation or sample clean up operations. Instead the system is directly observed using detection techniques that may be non-invasive (e.g. infrared spectroscopy, NMR, Raman spectroscopy) or invasive (e.g. MS). Since analytical resolution will be poor compared to more comprehensive methods of analysis, chemometric techniques (Lavine and Workman, 2002) and

machine learning approaches (Mitchell, 1997) are used to extract or deconvolve the relevant information from the spectra. There are currently three major 'holistic' techniques that do not involve analyte separations and that provide the kind of information and type of measurement associated with fingerprinting and footprinting. These are MS, NMR, and vibrational spectroscopic techniques such as Fourier-transformed infrared (FT-IR) spectroscopy and Raman spectroscopy (and to a lesser extent optical techniques such as fluorescence spectroscopy, and electrochemical measurements).

The advent of 'soft-ionisation' methods such as ESI and MALDI has enabled the application of MS to the analyses of proteins. The arrival of analysers like quadrupole ion-traps, time-of-flight (TOF) and Fourier-transformed ion cyclotron resonance (FT-ICR) improved mass accuracy, resolution and sensitivity of measurements. Direct-infusion and analysis of whole cells or cell extracts using ESI-MS (Goodacre *et al.*, 1999; Vaidyanathan *et al.*, 2002) shows promise for the rapid characterisation of organisms, cells or tissues for fingerprinting purposes. The capability for tandem mass spectrometry allows identification and characterisation of the detected species (Vaidyanathan *et al.*, 2002). It is possible to detect several metabolites and macromolecular types, including proteins, phospholipids and glycolipids. Improved mass accuracy is possible with the application of FT-ICR MS in conjunction with ESI or MALDI.

NMR is another technique that has been shown to have the potential for generating rapid metabolic fingerprints (Nicholson *et al.*, 2002; Raamsdonk *et al.*, 2001). It is less sensitive than MS, but can be used non-invasively, permitting *in vivo* measurements. The use of magic angle spinning (MAS) for high resolution enables minimisation of sample inhomogeneities, making NMR more applicable to the analysis of biological samples. ^1H , ^{13}C , ^{31}P NMR can all be used to trace metabolites along pathways.

Vibrational spectroscopic techniques, which are also non-invasive (albeit in the spectral sense), but which are of lesser sensitivity and resolution, can be used. These techniques comprise those that are based on molecular bond vibrations to quantify chemical species, including near-infrared (NIR), mid-infrared (MIR) and Raman spectroscopic techniques. The major advantage of these techniques is the rapidity and ease of spectral acquisition, enabling non-invasive measurements to be made with little or no sample preparation, and the potential for acquiring fingerprints spatially on the sample surface. However, sufficient signal resolution

is to be ascertained for the desired effect to be monitored, in order to use spectral information as protein or metabolic fingerprints.

For extraction of relevant information from metabolic fingerprinting and footprinting techniques, spectral pre-processing and chemometrics using unsupervised (such as principal component analysis) or supervised learning (such as artificial neural networks) techniques are usually resorted to (Goodacre *et al.*, 2004; Nicholson *et al.*, 2002; Raamsdonk *et al.*, 2001).

The availability of data from the application of the above technologies will undoubtedly require efforts towards large-scale maintenance of data torrents and mining of information from them (Goodacre *et al.*, 2004). As with genomics and transcriptomics, bioinformatics efforts towards these are at different stages of development for proteomics and are beginning to appear for metabolomic data.

6. Implications in monitoring food quality and authenticity

The application and utility of the above technologies in monitoring food quality and authenticity can be appreciated from the issues that concern this area of food science. Food, processed or packaged, is generally of plant or animal origin. Reliable authentication of the species of origin, authentication of geographical origin (provenance), monitoring adulteration of food for economic gains, detecting and monitoring food safety are some of the issue concerning food quality and authentication. In addition, with the increased use of genetically modified (GM) produce, an increasing concern is monitoring the traceability of GM food, within the context of 'substantial equivalence'. Identification of unique biomarkers or groups of markers to characterise food is therefore a broad objective in monitoring food quality and authenticity.

Although DNA based methodologies (Luthy, 1999; Popping, 2002) are in use, proteomic and metabolomic fingerprints have the potential to be useful for rapid screening on a routine basis and can complement more elaborate DNA based assessments. Proteins form a substantial fraction of organic matter in meat, fish or their products. Protein-fingerprinting approaches would therefore be suitable in the authentication of these foodstuffs. For processed foods, metabolic and protein-fingerprinting approaches can be useful in authenticating quality of produce at different stages of production. Metabolites and proteins confer toxicity or allergenicity to food and are therefore suitable candidates as

markers for monitoring nutritional quality. In addition, aspects such as taste and nutrition may be traced at the phenotypic level by monitoring the metabolic and protein profiles of food, and can be used for authenticating the quality of raw materials that can in turn be useful in assessing the potential quality of the end products.

The potential for applying mass spectrometric and vibrational spectroscopic techniques in food authentication is illustrated in Figures 4 and 5. Metabolic information obtained from direct analysis of vegetable oils using ESI-MS (Goodacre *et al.*, 2002) (Figure 4 B) and Raman spectroscopy (Lopez-Diez *et al.*, 2003) (Figure 4 C) can be analysed using PCA (Figure 4 A and D) to discriminate the oils based on type or cultivar. Techniques such as these and MALDI-MS that

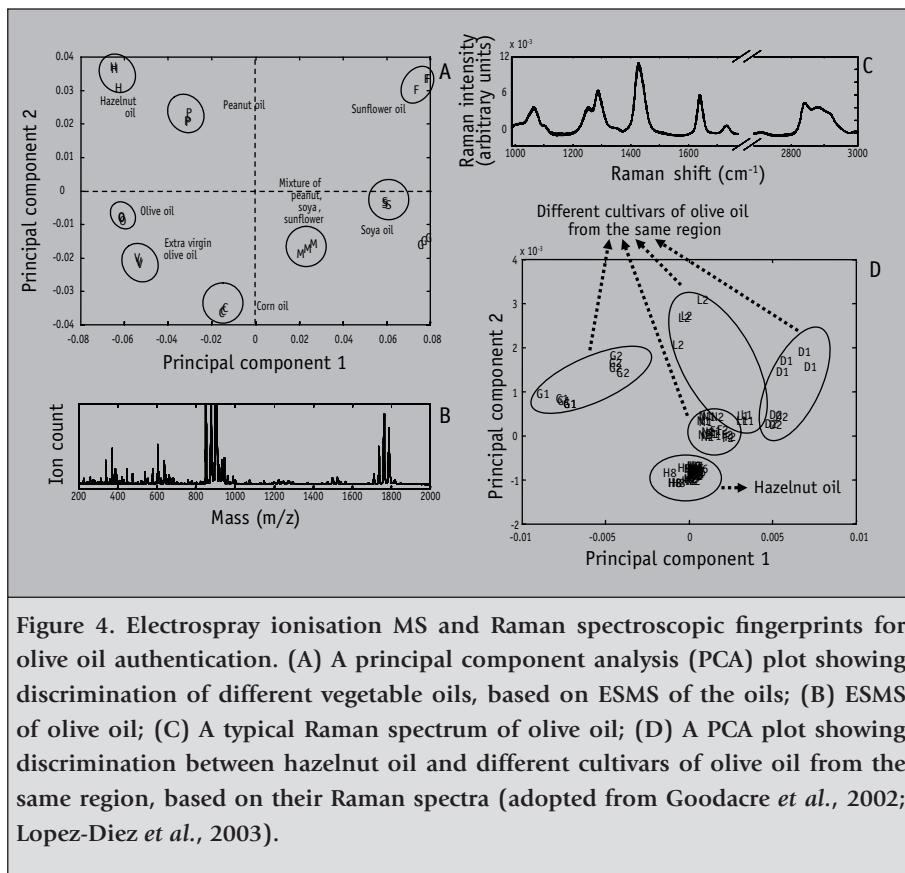
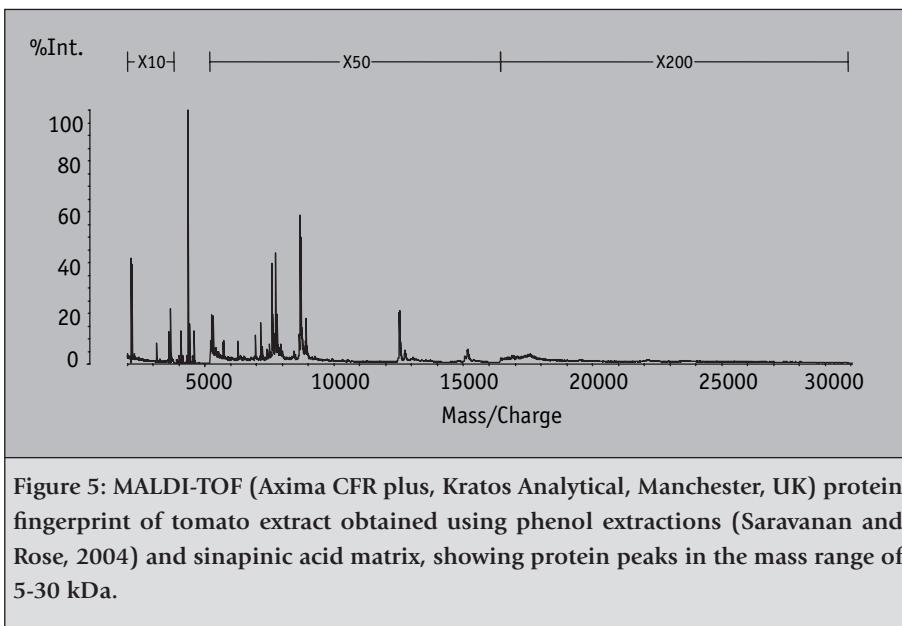


Figure 4. Electrospray ionisation MS and Raman spectroscopic fingerprints for olive oil authentication. (A) A principal component analysis (PCA) plot showing discrimination of different vegetable oils, based on ESMS of the oils; (B) ESMS of olive oil; (C) A typical Raman spectrum of olive oil; (D) A PCA plot showing discrimination between hazelnut oil and different cultivars of olive oil from the same region, based on their Raman spectra (adopted from Goodacre *et al.*, 2002; Lopez-Diez *et al.*, 2003).



can be useful in generating rapid protein fingerprints, as illustrated in Figure 5, will be useful in providing information on the functional status of food and for the routine assessment of food quality and authenticity.

A major issue in food traceability is that of genetically engineered crops. It has been a subject of intense debate ever since field trials were introduced in the 1980s. There has been extensive experience of GM crops in commercial agriculture over the past decade and there are scientific and economic concerns with respect to transgenic crops (Smyth *et al.*, 2002), especially with respect to gene flow resulting in undesirable transfer of traits from crop to wild species, and the importance of environmental and toxicological studies on the raw foodstuff (Schubert, 2002; Smyth *et al.*, 2002; Snow, 2002). It has been proposed that an operational definition of food safety assessment include a minimum list of macro- and micronutrients, anti-nutrients, inherent plant toxins, secondary metabolites, and allergens to be analysed for each GM crop species and of their baseline concentrations in conventional varieties (Schenkelaars, 2002). In this regard, it may be essential to generate metabolomic and proteomic databases and use these in making food safety assessments.

7. Conclusions

Following from the genomic revolution, post-genome science is currently evolving towards characterisations at the proteomic and metabolomic levels. Subsequently, monitoring food quality, authenticity and traceability in the post-genome era will be increasingly driven by proteomic and metabolomic database-based assessments. However, challenges with respect to standardisation of protocols for reproducible comparisons between research groups and the structuring of proteomic and metabolomic databases, in addition to the development of bioinformatics tools for effective mining of data from these technologies will have to be addressed before proteomic and metabolomic strategies find wider practical application in food science and other areas. Nevertheless, rapid methods capable of generating reliable, reproducible proteomic and/or metabolomic snapshots of systems can still be used in combination with chemometric approaches for screening of large samples, where possible. In future, stringent regulations driven by public awareness of genetically modified foods will influence to a greater extent the application of 'omic' technologies, especially those of proteomes and metabolomes, to food authentication and traceability.

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Rapid identification of plant and animal species in foods

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Abstract

The European Commission funded the project 'Development of quantitative and qualitative molecular biological methods to identify plant and animal species in foods', which was designed to develop and to validate new analytical methods to support emerging legislative instruments, ultimately contributing to food safety and consumer reassurance. Qualitative and quantitative polymerase chain reaction and also protein-based methods, were developed, tested and/or optimised and validated for the detection of the most relevant meat and plant species in foods. Some hidden potential allergenic compounds, for example nuts, peanuts, soybeans or crabmeat were also included. Four methods were validated in an interlaboratory study. The potential for an extended degree of automation and enhanced sample throughput was investigated by multiplex polymerase chain reaction, polymerase chain reaction-enzyme-linked immunosorbent assay and array chip technology, respectively. The range of methods developed in this project should cover most of the frequently used animal and vegetable species used in the European Union food market place and be well suited to support existing and emerging legislative instruments.

Keywords: PCR, validation, food, traceability, authenticity

1. Introduction

An enormous stream of goods intended for the food and nutritional industries passes the frontiers of European Member States every month. From January to April 2005, the European Union (EU) exported and imported food products worth of 15.8×10^9 and 19.4×10^9 Euros, respectively (source: Eurostat, <http://epp.eurostat.ec.eu.int>). Apart from basic foodstuffs, high quality products of special composition as well as a great variety of local specialities prepared under recognised knowledge characterise the European food marketplace. However, profitable products as well as agricultural production aids provoke

misuse and imitation. Fraudulent replacement of food components by cheaper ones, false or misleading ingredient lists and falsified custom documents are rather common problems in the EU which national food control and customs offices have to face daily. In a recent report on fraud prevention published by the European Commission (EC, 2005) irregularities, especially in the areas of olive oil production, butter fat, cheese, beef/veal and poultry meat exports, are highlighted. Apart from significant economic damage, issues of potential health risks for the consumer, e.g., the presence of allergens in food or ruminant meat in the feed chain are of increasing importance. This is leading to the need for, and emergence of, new food labelling directives in Europe. Against this background robust, quick and reliable qualitative as well as quantitative methods for the identification of plant or animal species in foodstuffs are urgently needed in EU Member States in order to:

- enable effective food control;
- shield products from imitation and adulteration;
- ensure law enforcement; and
- inform and protect the consumer.

This need described above provides the rationale and context for the EU-funded project 'Development of quantitative and qualitative molecular biological methods to identify plant and animal species in foods' (MolSpec-ID).

Up to now the vast amount of official methods for the detection of plant and animal species in food are exclusively based on protein analysis. Predominantly immunological approaches are applied. Although immunoassays or comparative analysis of protein patterns (e.g. isoelectric focussing, etc.) are easily carried out and do not require cost intensive equipment, there are some decisive disadvantages. Firstly, the availability of relevant antibodies might be limited or restricted to only a few selected species of the broadest commercial interest. Secondly, the production of antibody sera is to a certain degree sophisticated, needing specific equipment and can be very time-consuming. Proteins might be degraded during manufacture and processing of foodstuffs and, therefore, might no longer be detectable either by immunological methods or by electrophoretic separation. In the latter case, the evaluation of complex patterns calls for a high degree of experience and reliable reference patterns, which are difficult to standardise.

On the other hand, the analysis of DNA, as demonstrated by its application in, e.g. forensic medicine, has proved to be an ideal alternative tool to develop highly specific and sensitive detection systems for all kind of organisms in

different matrices even after rough processing. The most successfully applied DNA analytical tool is the polymerase chain reaction (PCR). By using this technique a specific target sequence is amplified exponentially using a pair of particularly designed short oligonucleotides (the primer). The accumulation of PCR products can be followed either in real-time by using a fluorescent detection probe or can be made visible by ethidium bromide staining as end products after separation on an agarose gel. Advantages of the technique are obvious. In general, DNA is much more stable under normal food processing conditions than protein. Detection systems based on DNA analysis can be performed by any experienced laboratory at any time, since the species-specific reagents (primers and probes) and all necessary chemicals can be purchased from different independent suppliers. Primer pairs can be synthesised quickly without limitation for any species of interest and identical composition. An important advantage over protein-based methods is the great potential for standardisation of PCR methods. Moreover, the potential for high throughput by analysing many samples in parallel is evident. Encouraged by the progress and experience in the field of detection of genetically modified organisms in food, first DNA-analytical standards for the purpose of species analysis in food and feed have recently been developed by a German national standardisation board and included in the catalogue of the Official Collection of German Methods for Food Analysis (2000, 2002). Nevertheless, the introduction of molecular biological methods for species identification in food and feed is just at the starting point and European standards in this field are not yet available.

The project MolSpec-ID was initiated to develop analytical methods using DNA-based approaches for the qualitative and quantitative identification of plant and animal species in foods to monitor product safety and traceability. The project was co-ordinated by the Federal Institute for Risk Assessment (BfR), Berlin, Germany. Fourteen partners, representing national authorities, universities, institutes, and small and medium sized enterprises (SMEs) from eleven European countries took part in the project. The budget was 3.1×10^6 Euros, including 1.4×10^6 Euros provided by the EC. The workload was separated into five work packages (WPs) (Figure 1). WP1 members delivered reference materials, evaluated DNA extraction methods and established qualitative as well as quantitative DNA-based methods for the analysis of species posing potential health risks or species relevant for potential fraud reasons. WP2 was devoted to aspects of throughput enhancement using multiplex polymerase chain reaction (PCR) and PCR-enzyme-linked immunosorbent assay (ELISA) as basic steps on the way to chip and array-technologies. WP3 focused on the adaptation of

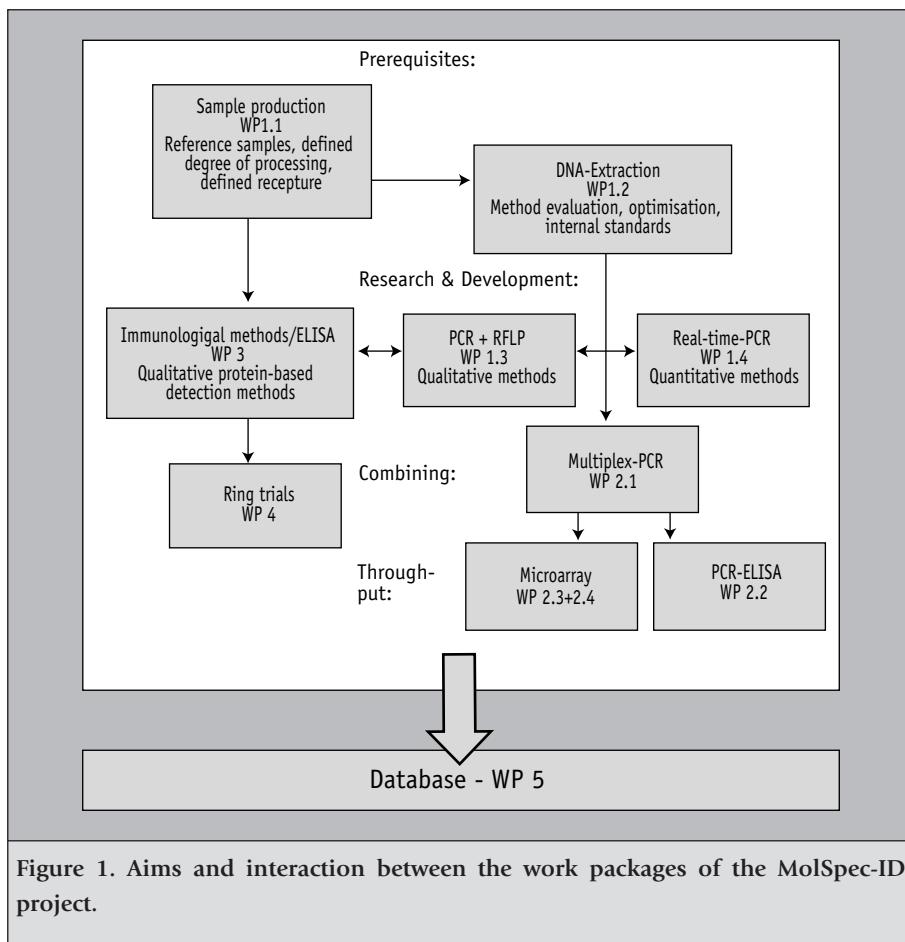


Figure 1. Aims and interaction between the work packages of the MolSpec-ID project.

protein-based methods to processed foods. The issue to be addressed was to clarify the applicability and limitations of protein-based systems compared with those based on nucleic acid detection. The aim of WP4 was to perform interlaboratory comparative and validation studies with four selected methods developed in the frame of the MolSpec-ID project. In addition to qualitative ring trials it was decided to conduct at least one quantitative ring trial to allow direct comparison of one PCR-based method with one protein-based assay. Finally, all information and results gained were to be incorporated into a user-friendly internet accessible database to be developed under WP5. The database is linked to a MolSpec-ID project website (<http://www.molspec.org>), which contains in addition detailed information on the project's membership, structure and work packages, meetings, milestones and deliverables.

2. Materials and methods

2.1. Reference materials

Authentic reference materials from over 50 animal species and subspecies were collected, including species of regional relevance such as reindeer or exotic species like kangaroo, ostrich, special beef breeds, etc.

Additionally, forty-eight different basic meat product samples were prepared containing varying amounts of seven animal species (cattle, pig, chicken, turkey, sheep, duck and horse) in varying ranges from 0, 0.05, 0.1, 0.25, 1, 2.5 to 45.7% in the final product. Recipes for all the processed samples enabled the product's shelf-life conditions to be met. To produce emulsion-type sausages the meat was minced and mixed, e.g. with vegetable oil, ice, salt, spices (pepper, nutmeg, cardamom, cinnamon) as necessary. Four different categories of canned products were prepared from the basic '7-species' mixture with Fc-values of 0.8 (equivalent to home canned cans), 3.4 (normal cans), 12.2 (tropical cans) and 31 (ultra-high heat treatment). One type of raw sausage was produced from cattle/pig/pig fat (corresponding to 38/30/28% of the final product). Liver pie was produced from four species (pig, cattle, chicken, turkey) with different amounts between 0, 0.6, 1.5, 2.5, 48, 51.4% of the respective species in the final product. In addition, cans heated to a core temperature of 72°C were prepared from these mixtures. Model type Hamburger-type products contained cattle, pig, lamb, chicken, horse, turkey in varying amounts between 0 and 90% of the final composition. Four types of surimi were produced and heated in cans to a core temperature of 70°C. The paste contained two fish species (Pacific whiting/hake and Alaskan pollock) and crab meat in varying percentages fish/crab of 19/51, 65/5, 70/0 and 60/10, respectively, and contained ice, phosphate, sodium chloride, sorbitol and saccharose. Sixteen samples from cereals as a source of gluten were prepared in a background matrix of infant formula made from rice powder. Rice flour was mixed with 0.01, 0.1, 0.5, 1 and 5% barley, rye and wheat flour, respectively. Wheat, barley, rye as well as pea, soybean, peanut and celery were further used as sausage adulterating ingredients to yield low amounts between 0.01 and 1.3% in the final product.

The various reference materials as well as meat product samples described above were used by the appropriate MolSpec-ID Partners for method development, in-house validation and interlaboratory studies.

2.2. DNA extraction

DNA extraction was performed using kits and/or individually adapted systems based on the following principles:

- Cetyltrimethylammonium bromide (CTAB)-precipitation and liquid phase extraction (e.g. Official Collection of German Methods for Food Analysis, 1998).
- Chaotropic solid phase extraction, (e.g. GeneSpin™ genomic DNA purification kit, Whatman International Ltd.; Wizard®, Promega; NucleoSpin®, Macherey Nagel GmbH, etc.).
- Non-chaotropic solid phase extraction (e.g. InviSorb® Spin Tissue Kits, Invitec GmbH).

2.3. PCR method development and validation

PCR and real-time PCR system development was performed by the project partners according to an agreed common scheme consisting of (1) strategy (search for suitable target sequences, search for published systems, design of new primer systems, adaptation and optimisation of published systems), (2) pre-testing/optimisation with reference material, (3) in-house validation of the method with appropriate reference material, and (4) ring trial (optional, for selected methods only). To approach steps (1) and (2) partners used their individual in-house procedures (data base research mode, primer design software, chemicals, thermocycler and other devices, etc.). Detailed method descriptions are compiled in the project's final technical progress report, available from the EC or the authors of this publication, on the MolSpec-ID project's online database, and in a number of scientific publications (see references). Additional information can be found in paragraph 3 at the respective method. Real-time PCR was carried out by most partners using an ABI PRISM® 7700, GeneAmp® 9700 (Applied Biosystems), or LightCycler® (Roche Diagnostic Corp.) device.

Concerning steps (3) and (4) (in-house validation, ring trials), a common procedure, provided by the co-ordinating partner, had to be used. All terms and definitions for performance parameters listed below are in line with international standards, for example, ISO 5725 (ISO 1994, 1998) or the IUPAC harmonised guidelines (Thompson *et al.*, 2002). The appropriate partners therefore accepted that systems had to be in-house validated according to the scheme summarised in Tables 1 and 2.

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Table 1. Rules for in-house validation parameters for qualitative and quantitative PCR methods.

Parameter	Objective	Method	Result
Sensitivity	Determination of the limit of detection (LOD).	Dilute extracted DNA in water to reach low copy numbers. Dilution steps should cover 4 order of magnitudes (e.g. 1,000/100/10/1/0.1).	Lowest number of genome copies to be detected, but not necessarily be quantified. Positive signal must be reached with at least 95% of the samples.
Specificity	Determination of taxons to give a positive result (species/subspecies) and respective negative controls avoiding the influence of other food ingredients.	Select species of major relevance in foods and close taxonomic relationship. All methods should be tested at least with chicken, turkey, cattle, sheep and pig. Include subspecies/breeds, etc. that have to result in positive signal.	List of species, which have been tested positive or negative.
Selectivity	Determination of any species interference resulting in ambiguous signals not due to the expected specific signal for the analyte under investigation.	Test method with mixed samples (e.g. '7-species'-samples), at least a mixture of pig, cattle, chicken, turkey and sheep. For plants include soya, potato, wheat and nuts.	List of species, which have been tested positive or negative concomitantly.
Applicability on food matrices	Demonstration of the applicability of the method on processed foods. Defining the scope of the method.	Use reference material with defined degree of processing. For each individual processing step, at least 3 replicates should be analysed.	List of food matrices (including a description of the processing steps) where all replicates result in a positive signal.

Table 2. Additional rules for in-house validation parameters for quantitative methods.

Parameter	Objective	Method	Result
Sensitivity	Determination of the limit of quantitation (LOQ).	The LOQ is determined as described for LOD determination.	Lowest amount of genome copies, which can be measured quantitatively in a sample with acceptable accuracy and precision. The LOQ must be reached with 95% of the samples. Smallest and highest number of genome copies, which are within the linear standard curve.
Range (linearity)	Determination of the linear range at which quantitation can be carried out.	Proceed as described for sensitivity determination; relate results to standard curve. Quantify three samples with known concentration (in five-fold replicates). Use dilution steps from the curve.	Mean value (\bar{x}) = mean value of the replicates of each sample: $\bar{x} = \frac{1}{n} \sum_{k=1}^n x_k.$
Trueness	Determination of the mean value and subsequent calculation of the bias between the mean and true value of the analyte under investigation.		x = replicate value; k = replicate number; n = total number of replicates. Bias = difference between the mean value of each sample and the true (assigned) value of this sample: bias = $\bar{x} - x_{true}$ Standard deviation (s , random samples): $s_r = \sqrt{\frac{1}{n-1} \sum_{k=1}^n (x_k - \bar{x})^2}.$
Precision	Determination of the standard deviation of results achieved with repeated measurements of the same sample (repeatability) or with measurement of the same sample in different laboratories (reproducibility, determined in ring trials)		These parameters will be calculated on the basis of the results derived from trueness determination.

2.4. Calibration curves and copy numbers

For quantitative analysis at least 5 standard dilutions (each standard in three replicates) had to be used. The lowest standard had to be below 1 genome equivalent, the highest standard equivalent to the number of copies, which can be found in a commonly used DNA-isolate from 100% raw material for this method (concentration and volume). Three samples with known concentration (in five-fold replicates) had to be investigated in parallel. Determination of the DNA-concentration extracted from the matrix prior to dilution was possible either fluorometrically or by agarose gel-based methods as described (Sambrook and Russell, 2001). Adjustment of copy numbers was based on data published for plant DNA C-values at <http://www.rbгkew.org.uk/cval/homepage.html>; for animal species genome size information was taken from the Animal Genome Size Database (<http://www.genomesize.com>).

2.5. Standard Operating Procedures

Standard operating procedures (SOPs) had to be laid down according to the general outline below:

1. Head: name of the method; scope; developer; date of release.
2. Materials:
 - 2.1. devices;
 - 2.2. chemicals.
3. Procedure:
 - 3.1. DNA/Protein extraction/purification and determination of quality/ yield;
 - 3.2. detection;
 - 3.3. confirmation.
4. Results:
 - 4.1. evaluation;
 - 4.2. calculation;
 - 4.3. validation parameter.
5. General remarks and literature.

2.6. Interlaboratory studies

Interlaboratory studies were carried out and evaluated under consideration of international guidelines as laid down by ISO 5725 (ISO, 1994, 1998) or IUPAC (Horwitz, 1995). Minimum requirements were: in-house validated method

available as SOP, at least 8 (preferably 12-16) participants, samples composed of at least five levels of each target organism to be tested, for qualitative DNA-based methods inclusion of processed cans ($F_c=3.2$ or higher), for quantitative DNA-based methods a lower degree of processing (sausage) could be tested if higher degrees of processing (e.g. heat treated cans, $F_c=3.2$) gave no quantifiable results. Outlier tests were calculated in accordance with ISO 5725 (Cochran, Grubbs, Double Grubbs test).

2.7. ELISA-PCR, hybridisation array and chip technology

For improvement of high-throughput hybridisation array systems, ELISA-PCR and chip technology were considered as appropriate tools in order to analyse huge amount of samples in parallel.

2.8. Protein-based methods

Commercial kits have been investigated with processed samples of poultry, beef and pig meat (e.g. Tepnel BioSystems Ltd.; Immuno-Tek ELISA, Zeptometrix Corp., USA) and gluten-containing cereal-mixtures (Ridascreen® Gliadin, R-Biopharm AG). Polyclonal rabbit-antibodies for in-house systems tracing the crab meat protein arginin-kinase (Åkerström, 2002) and tropomyosin (Malmheden-Yman, 2003) have been prepared as published.

2.9. Database

The online database and the MolSpec-ID project website were developed and implemented by the Centre for Biosafety Research and Assessment of Technology (BATS) and is available at <http://www.bats.ch/molspec> or <http://www.molspec.org>.

3. Results

The most crucial step for the successful application of PCR is the extraction of amplifiable DNA. Three types of extraction principles were evaluated and adopted for different species or processed food samples using CTAB protocols, commercial kits or combinations thereof. Optimised CTAB protocols were identified as being the method of choice for most applications as they achieved high yields of good-quality DNA. This was confirmed independently for animal tissues (Binke *et al.*, 2002) as well as materials of plant origin (Olexová *et al.*,

2004). As a faster alternative, commercially available kits based on chaotropic solid-phase extraction also performed well, e.g. with fine ground materials such as flours or biscuits. However, CTAB solubilisation combined with liquid-phase extraction improved performance especially with difficult food products, for example chocolate containing biscuits (Olexová *et al.*, 2004). DNA concentrations were measured for different types of tissues. Variations were found for, e.g. muscle tissue between 0.27 g/kg and 0.49 g/kg, which is in line with data previously published (Herbel and Montag, 1987). Significant variation of 30-50 % regarding the DNA content, e.g. between muscle and fatty tissue were found, which has to be considered if quantitative methods are applied (Binke *et al.*, 2004).

Not necessarily the quantity, but also the quality of the extracted DNA is the most important factor to conduct a successful PCR experiment to identify a certain component. Therefore, an internal amplification control system was attempted with the aim to monitor DNA isolation and recovery rates of different extraction methods in repeated reactions. The initial system based on PCR-derived fragments between 63 and 212 base-pair (bp) size of a sequence of the *fimC* gene of *Salmonella typhimurium* proved to be not stable in solution and after storage. The method was thus found not to be suitable for routine analysis. To overcome this problem a *fimC* fragment of 455 bp size was cloned in a pUC19 plasmid. Three different internal standards (IS) were derived from the linearised or cleaved plasmid of 470 bp and 3,156 bp (two IS). IS were added to selected food matrices of animal and plant origin at three concentration levels, DNA was isolated with different methods as mentioned above and the IS was quantified using real-time PCR to recognise a *fimC*-specific 96 bp fragment. The result was plotted against the logarithm of the initial amount of DNA copy numbers of a serial dilution of IS to calculate the respective recovery rates. These turned out to vary considerably for individual isolation methods between 4.6 to 37.6%. The results of this study have been published (Piknová *et al.*, 2004).

Based on target sequencing or in a few cases on published methods (e.g. cereals, horse) about twenty PCR based systems have been developed and optimised and/or adapted to certain matrices for the qualitative analysis of the following species: pig, cattle, goat (two systems), sheep, five bovine and five deer species, ruminants, ostrich, kangaroo, hazelnut, almond, fish species/crab, peanut, wheat/barley/rye, celery, fowl (chicken, turkey, duck breeds, goose). At least ten quantitative PCR based systems for the analysis of the following species have been developed: cattle, chicken, goat, horse, almond, hazelnut, pig, turkey/

duck/chicken, pea, and soybean. All methods had to be validated according to a common scheme (see Tables 1 and 2) laid down by the partner responsible for co-ordination of the MolSpec-ID project.

Table 3 lists the most important outcomes of the MolSpec-ID project: the qualitative or quantitative methods developed and/or validated, the partner primarily responsible for the development and information where additional documentation can be sourced. It should be mentioned that Table 3 does not cover all the outputs of the MolSpec-ID project as some results are potentially the basis of commercial test-kits and as such are the subject of ongoing confidential development. Any interested parties should refer to the co-ordinating partner for further information.

In addition to species-specific detection systems reference systems to be used in quantitative analysis were also established as well as systems to detect the presence of animal or plant or eukaryotic DNA relative to the total amount of plant and animal DNA, respectively. New primer sets for the primer binding region (border primers) of a myostatin PCR reference (meat) system (Laube *et al.*, 2003) were developed by BfR, Germany. A qualitative PCR system for the detection of eukaryotic DNA based on a highly conserved part of an 18S rRNA sequence and universal primers for the detection of animal, birds and fish based on the conserved part of the mt 16S rRNA sequence, was developed by Graz University of Technology (TU Graz), Austria.

Most of the qualitative PCR systems are based on the detection of multicopy mitochondrial genome sequences (e.g. cyt b gene, D-loop). The LOD for these systems measured with differently processed samples mainly lies in a range of 0.1-0.5% (w/w) of the respective ingredient (or 2-10 genome equivalents, depending on species). LODs for allergenic compounds were determined to be 5-10 mg protein/kg (5-10 ppm) using the qualitative peanut system and 2 ppm (LOQ = 10 ppm) for the quantitative soybean approach. For the qualitative nut PCR systems (almond/hazelnut) no suitable reference material was available. The LOD was determined to be 0.1% (w/w) in biscuits. Real-time PCR systems used for relative quantification were based on single copy genes. For these systems a LOQ of about 10-20 genome equivalents was measured with serial diluted DNA.

About 10 multiplex systems have been developed by different partners to serve as a basis for further automation by, e.g. chip and array technology, respectively.

Table 3. Qualitative and quantitative PCR systems, which have been developed and in-house validated in the frame of the MolSpec-ID, project. Availability of standard operation procedures (SOP)/collaborative studies (COL) and documentation/literature is indicated.

Species	Qualitative	Quantitative	Developed by	SOP	COL	Documentation/Publication
Almond	+	+	INIA, Madrid	Validation report		Final report ¹ ; validated by VUP (Kuchta, 2004)
Cattle		+	BfR, Berlin	+		Laube <i>et al.</i> (2003)
Cattle		+	GeneScan, Freiburg	+		Final report ²
Celery	+		VUP, Bratislava	+		Dovičovičová <i>et al.</i> (2004)
Cereals	+		VUP, Bratislava	+	+	Kuchta, T. (2004), system based on Dahinden <i>et al.</i> , (2001). Ring trial report ¹
Chicken		+	BfR, Berlin	+		Final report ¹
Chicken	+	+	TU Graz	+		Remler <i>et al.</i> (2004a,b)
Duck	+	+	TU Graz	+		Remler <i>et al.</i> (2004a,b)
Fish, crab	+		IMR, Bergen			Final report ¹
Goat	+		BfR, Berlin	+		Final report ¹
Goat	+	(semi)	BAFF (BFEL), Kulmbach	+	+	Binke (2004a). Ring trial report ¹
Hazelnut	+	+	INIA, Madrid	Validation report		Final report ¹ ; validated by VUP (Kuchta, 2004)
Horse		+	Eurofins, Nantes	+	+	Final report ¹ ; ring trial report ¹
Horse	+		BAFF, Kulmbach	Validation report		Binke (2004b), system based on commercial primer (Cibus, Germany)
Pea		+	Nestlé, Lausanne	+		Final report ¹
Peanut	+		Nestlé, Lausanne	+		Final report ¹

Table 3. Continued.

Species	Qualitative	Quantitative	Developed by	SOP	COL	Documentation/Publication
Pig	+	+	GeneScan, Freiburg	+		Final report ²
Ruminants	+	+	Utrecht University			Final report ¹
Ruminants		+	Nestlé, Lausanne	+		Final report ¹
Sheep	+		BfR, Berlin	+		Final report ¹
Soybean		+	Nestlé, Lausanne	+		Final report ¹
Turkey	+	+	TU Graz	+		Remler <i>et al.</i> (2004c)

¹Publication in MolSpec-ID on-line database.
²Preliminary protected confidential knowledge (laboratory prototype).

An ELISA-PCR system was developed (TIB MOLBIOL, Genoa, Italy) to speed-up throughput for the concomitant detection of cattle, pig, sheep and horse using biotin-coupled consensus PCR primers based on cyt b gene and the myostatin reference gene for potential quantitation. PCR products are bound to a streptavidin-coated microtiter plate and hybridised to specific fluorescein labelled probes. The LOD for cattle was determined to be 2 genome equivalents, for pig, sheep and horse 20 genome equivalents. Specificity testing gave no false positive result with 9 tested species. Selectivity testing with processed samples revealed a LOD for cattle, sheep, horse of 0.5% (w/w) and for pig of 0.1% (w/w). No false positive or false-negative results occurred. Using PCR products from different multiplex systems, an array system was developed (GeneScan, Freiburg, Germany). PCR products are directly incubated on a chip in hybridisation buffer resulting in a specific binding to the complementary probes, which are covalently bonded to the surface of the chip. Prior to the hybridisation the double stranded DNA is stained with a fluorescence dye (Cy5). Signals are detected and analysed in a biochip reader. The chip was tested with pure genomic DNA and samples

delivered by BFEL (Stuttgart, Germany). The chip works well for the detection of pig and chicken in pure and even in complex samples, but with a high limit of detection (e.g. $\geq 2.5\%$ of pork in meat mixtures). Signals for horse, cattle, fish were too weak to be detected. A soy sample tested as a control showed weak false positive signals for horse, but no signal for soy as expected. By application of complex samples or high amounts of amplicons unspecific signals were generated. Testing with this type of chip is not as sensitive as real-time PCR and yields ambiguous results. As a conclusion the chip needs further optimisation.

The second format, which was developed (TIB MOLBIOL, Berlin, Germany) is based on universal 16S-rRNA sequence-targeted primers amplifying DNA from cattle, horse, goat, pig, sheep, kangaroo, rabbit, dog, cat, human, chicken, duck, turkey, ostrich and goose. A subsequent detection was carried out with different capture probes in an ELISA format. The applicability and selectivity of the system was tested with meat mixtures containing all frequently occurring species, including ostrich and kangaroo, as adulterants in meat in different amounts. The data obtained show that the model array recognises all species from the samples above a concentration of 0.25% (w/w), except for pork with a detection limit of 0.5% (w/w). Very weak cross-hybridisation was observed for duck and goose, and for beef and chicken. Since both systems are laboratory prototypes subject to further development, they are regarded as being confidential and, as such, will not be published in the MolSpec-ID online database. However, summaries and contact addresses for all single results, including methods, are linked to the database or in the official technical implementation plan, which will be disseminated by the EC after approval of the final report.

Regarding protein-based methods, commercial test kits to detect pig, cattle and poultry were evaluated on processed samples (BFEL, Stuttgart, Germany; FRIP, Prague, Czech Republic). In some cases kits had to be further optimised to obtain better performance with the reference materials. LODs of $\leq 1\%$ (w/w) for pig, cattle, turkey, chicken, sheep and goat in reference samples ('7-species' mixture) were obtained. Evaluation reports are available at the MolSpec-ID website. Antibodies were raised against the shellfish (crab) proteins arginine kinase and tropomyosin (NFA, Uppsala, Sweden) to identify shellfish in mixed products (e.g. surimi) as an example for a 'potentially hidden' allergenic component. In Western-blot analysis the rabbit anti-arginine kinase antibody displays a dominating protein band of 40 kDa in SDS polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme can be used as a tracer to monitor the quality of surimi products or to help identifying the presence of shellfish as a potentially allergenic

compound. The 40 kDa protein band is also detectable in electrophoretic patterns of crabmeat or surimi containing crabmeat. The antiserum was characterised and used for the development of an ELISA format. Other tested immunological methods - immunodiffusion (ID), rocket immunoelectrophoresis (RIE) or the commercial Biacore system (Biacore, Uppsala, Sweden) - were less useful. Immunoblotting using the antibody showed that arginine kinase could be detected at levels of 8 ppm (Åkerström, 2002). Antibodies to tropomyosin were raised both in rabbits and hen's egg (IgY). The antisera were characterised by immunoblotting, ELISA, RIE, a surface plasmon resonance (SPR) technique and SDS-PAGE. Furthermore a sandwich ELISA format was developed utilising the IgY antibodies coated onto the surface of microtiter plate wells (Malmheden-Yman, 2003).

Four interlaboratory studies were carried out with selected methods. Detailed ring trial reports are available in the MolSpec-ID database. The results can be summarised as follows:

- Qualitative PCR system (optimised protocol based on Dahinden et al. (2001), see Table 3) for the detection of gluten containing cereals (wheat, rye, barley). Ten partners took part in this study. Seventeen unknown samples containing wheat, rye or barley (0, 0.01, 0.1, 0.5, 1 and 5%) in an infant formula in a rice matrix were investigated; 8 results were evaluated. The LOD was determined to be 0.5% in 100% of the examined samples. To improve the LOD, the extraction protocol was further optimised by using TaqBead™ Hot Start Polymerase (Promega, USA) and tested in a supplementary ring trial repeating the previous cereal ring trial, but only with 5 partners. Four results were returned: LOD of 0.01% (three laboratories) and LOD 0.5% (one laboratory).
- Detection of gluten in food using a commercial kit. The ELISA test Ridascreen® Gliadin R7001 for determination of gliadin/gluten from R-Biopharm (Germany) was used in this interlaboratory study, which was organised by partner NFA, Uppsala (Sweden). Twelve partners took part, 16 different samples of rice flour containing different amounts of wheat, barley and rye were investigated (each species 0, 0.01, 0.1, 0.5, 1 and 5%, respectively, with cereals in the background). High values of the relative standard deviation (RSD) were observed for all contaminated samples, reaching the limit of quantification for the method about 0.01%. For all other concentration levels a RSD around 20-30 % was reached, except for the barley samples, where higher values occurred. In general lower RSD values are obtained when ethanol was used for the extraction compared to the 'cocktail' (protein extraction buffer of

unknown composition delivered with the kit). A significant influence of the choice of extraction method was observed. The 'cocktail' gave twice the values of gliadin when compared to the use of ethanol extraction. In conclusion, the test kit seems to be suitable for the measurement of gliadin between 5 to 2,000 µg/g food sample.

- Relative quantification of horse DNA using the LightCycler® system (see Table 3, Eurofins). Eight laboratories took part and delivered meaningful results. Six unknown samples (two degrees of processing) with 0.1, 0.5 and 1% horsemeat had to be investigated. One false negative result has been observed at the 0.1% level; no false positive results at the 0% level. In conclusion, the method is fit for quantification of low horse DNA contents in the range between 0.1-1%.
- Relative quantification of goat DNA in sausages by real-time PCR using the TaqMan™ technology (see Table 3, BFEL). Nine partners took part. Eight sausage-type samples containing 0.1, 0.5, 1.0, 2.0, 2.8 and 20% goat in meat mixtures were investigated as blind duplicates by each partner. Five results were achieved. A LOD and LOQ of 0.1% goat in meat or 0.05% (w/w) for goat in the processed product were determined.

All information will be entered into the MolSpec-ID online database. Version 1.0 of the database contains the following numbers of records: 31 PCR sets, 99 primers and probes, 68 taxons, 29 publications, SOPs and validation data sets, 47 reference samples, 48 sequences, 15 genetic elements, 17 organisations, 41 persons. The database is hosted by the Swiss partner BATS (<http://bats.ch/molspec>) who developed the prototypes and the final online version.

4. Discussion

As laid down by the EC in the European Whitepaper on Food Safety (EC, 2000a), it is the declared aim to create the highest quality standards for food and feed throughout the EU. To reach this goal, common European laws have recently been introduced. Commission Directive 2002/86/EC (EC, 2002) requires, with effect from 1 July 2003, labelling of the ingredient 'meat'. Moreover, animal species and their quantities used must be declared on the food label according to the 'quantitative ingredient declaration' (QUID) (EC, 1997). The range of methods developed under the MolSpec-ID programme cover all relevant and frequently used animal species in the EU food markets and are perfectly suited to support the new legislative instruments. With sensitivities of about 0.1% (w/w) of the respective species the methods are fit for purpose to detect adulterations

even in processed products like sausages or canned products. Furthermore, the validation scheme applied was performed according to internationally accepted guidelines. This will pave the way for the conversion of selected methods into future European standards.

Two DNA analytical standards for the purpose of species analysis in food and feed have recently been developed by a German National Standardisation Board, soybean in frankfurter, and differentiation of fish species (Official Collection of German Methods for Food Analysis 2000, 2002). Nevertheless, the introduction of molecular biological methods for species identification in food and feed is just beginning and European standards in this field are not yet available.

Regarding allergens the food labelling directive (EC, 2000b) was amended to ensure that consumers will be informed of the complete contents of foodstuffs. In particular the '25%' rule is abolished, which allowed non-declaration of components under this threshold. Furthermore, ingredients of known allergenic potential or potentially causing food intolerance are listed in the annex to the amending directive (EC, 2003). This list includes the following commodities and products thereof: gluten-containing cereals, crabmeat, eggs, fish, peanut, milk (including lactose), nuts (e.g. almond, hazelnut, walnut and other nuts), celery, mustard, sesame seed, and sulphites and sulphur dioxide above 10 mg/kg or 10 mg/l (expressed as SO₂). The amending directive took effect on the day of publication in the Official European Journal, 25 November 2003. Foods produced as of 25 November 2005, which are not labelled according to the law will be definitely banned from the market from this date.

The MolSpec-ID PCR methods developed include commodities listed in the annex of the amending directive cited above. However, testing potential allergens requires the production of reference materials, which contain only traces of the analyte in the lower ppm range. Such materials could be produced only, e.g. for some of the most relevant allergens such as soybean, peanut or gluten-containing cereals. It could be shown that detection and quantification of contaminating traces of a material was possible by real-time PCR-based methods. However, in the case of the cereal detection assay, the PCR methods need further optimisation to reach the sensitivity achieved with a commercial antibody-based assay.

At this point the following question is justified. Can protein-based assays realistically be replaced at all by DNA-based methods in the case of allergen detection, since evidently DNA-based methods are not targeted to the

physiologically active protein? However, a food matrix containing detectable protein not accompanied by traces of DNA, is a rather hypothetical case. Thus, DNA is well suited as a marker for the presence of an allergen. Keeping in mind that persons sensitised to food allergens are reacting not only to one type of major allergenic protein, but highly individually and possibly to minute amounts of a broad and divergent range of proteins and glycoproteins, the detection of DNA can be used as a valuable and quick tool at least for screening purposes.

The European Committee for Standardisation (CEN) established a new working group (WG 12) on detection methods for food allergens within the Technical Committee 275 in Berlin on 30 May 2002. The experts of this panel came to the conclusion that PCR-based methods are acceptable as an indicative method in parallel to the specific (mostly antibody-based) detection of allergenic proteins or proteins characterising the commodity. Analogous considerations are also made concerning the introduction of indicative PCR methods in the field of identification of animal proteins in the feed chain as an alternative to microscopy (Von Holst *et al.*, 2004).

Considering the aspects of speed and throughput, at first glance both protein- and DNA-based methods can be performed in a comparable time frame. Parallel investigation of several samples and automation is possible by real-time PCR, PCR-ELISA, but also antibody-ELISA formats on 96-well microtiter plates. DNA extraction and PCR analysis can be performed in only 5 hours if necessary. Real-time PCR will speed up the analysis considerably by using detection probes during the amplification process. The time-consuming post-PCR confirmation step using an agarose gel electrophoresis to identify PCR products is no longer necessary. On average, for both methods one working day must be calculated from receiving a sample to the evaluation of analysis data. However, speed should not only be seen in the actual time needed for the analysis, but should also be judged in terms of the potential to react quickly to suddenly arising safety and traceability concerns touching food markets and legislative issues. In this regard PCR-based methods are a most valuable tool and supplementation to existing antibody-based methods. Whereas antibody production takes several months, a PCR method can be established and fully validated within a few weeks. Once the primer and PCR conditions are published, every laboratory can introduce the method in a short space of time.

5. Conclusions

In the frame of the European research project MolSpec-ID robust methods, which are suited for the analysis of plant and animal species in food have been developed and, in some cases, validated. The technologies applied were DNA extraction, PCR-RFLP, real-time PCR, multiplex-PCR, PCR-ELISA, DNA sequencing, SCAR, microarray technology, protein-based ELISA, western blot, immunoelectrophoresis, and others. From the results it can be concluded that molecular biological methods based on the specific amplification of trace amounts of DNA in certain foodstuffs by PCR techniques are an appropriate tool to improve the food control needed to preserve the high quality and safety standards of foods in Europe. Four methods were evaluated in a ring trial according to internationally accepted guidelines. A comparison of a protein-based and a PCR-based method for the detection of gluten in cereals was included in interlaboratory studies. Commercial and newly developed antibody assays were tested and recommendations for their applicability and limitations on processed food materials established.

Transparency and broad dissemination of results was, and is, a major feature and objective of the project. Therefore, in addition to several existing publications, posters and lectures, about 80% of the overall results (approx. 55 single results) are published in the MolSpec-ID database. Only a minor share (approximately 20%) of the project outcomes will have an initially protected status because of individual exploitation plans including patents. All outcomes are described at least in outline in the technology implementation plan (eTIP), available from the EC or the co-ordinating partner.

Based on the results of the MolSpec-ID project it can be concluded that molecular biological methods based on the specific amplification of trace amounts of DNA still present in processed foodstuffs, by PCR techniques are likely to become an additional, important and appropriate tool contributing to improved food control, and preservation of high standards of quality and safety of food and feed in Europe. This, in turn, will underpin greater security of, and increased consumer confidence in, our food supply.

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Rapid detection method for microbial spoilage using FT-IR and machine learning

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Abstract

The requirement for real-time monitoring in the modern and highly automated food processing environment has stimulated research into rapid microbiological testing. The conventional microbiological approach to food sampling has changed little over the last half century. It has been estimated that there are currently in excess of 40 methods to measure and detect bacterial spoilage in meats. The ideal method for the on-line microbiological analysis of meat would be rapid, non-destructive, reagentless and relatively inexpensive. These requirements can be met via the application of a spectroscopic approach. Fourier transform infrared spectroscopy is a rapid, non-destructive technique, which can be used to generate biochemical fingerprints. It is shown that this technique can be used directly on the surface of food and, with appropriate machine learning, can produce accurate estimates of microbial loads on chicken and beef. It is, therefore, believed this method has considerable potential for application in the food industry.

Keywords: FT-IR, microbial spoilage, metabolic fingerprinting, evolutionary-based algorithms

1. Introduction

There would seem to be an obvious requirement within the food processing sector from 'farm to fork' for real-time microbial monitoring in what has become a high-throughput and highly-automated industry, and this has stimulated research into rapid microbiological testing procedures. At present, no technology within the food industry exists for a rapid (minutes as opposed to hours) and accurate detection system for microbiologically spoiled or contaminated meat or poultry. Meat and poultry are generally described as spoiled due to undesirable changes making them unacceptable to the consumer (Jackson *et al.*, 1997). These organoleptic changes can be visual changes (such as discolouration),

malodours or slime formation, or any other characteristic which makes the food undesirable for human consumption that leads to the foodstuff being described as 'spoiled' (Jackson *et al.*, 1997; Jay, 1996). Typical estimates for sensory nasal detection are around 10^7 to 10^8 bacteria per cm². Whilst it has been established that endogenous post-mortem enzymatic activity within muscle tissue, such as the activity of Ca²⁺ proteases (calpains), can contribute to some biochemical changes during storage (Alomirah *et al.*, 1998; Koohmaraie, 1992, 1994, 1996; Schreurs, 2000), it is accepted that detectable organoleptic spoilage of muscle foods is a result of the decomposition and the subsequent metabolite formation caused by the growth and catabolic activity of microorganisms (Braun *et al.*, 1999; Kakouri and Nychas, 1994; Nychas and Tassou, 1997; Schmitt and Schmidtloenz, 1992a,b; Stutz *et al.*, 1991).

The current approach to food sampling in microbiological terms has regrettably remained markedly similar over the last 50 years. A review of the literature shows that at present there are in excess of 40 methods to detect and enumerate bacterial spoilage in meats and poultry (Ellis and Goodacre, 2001). These include enumeration methods based on microscopy, ATP bioluminescence and the measurement of electrical phenomena (Champiat *et al.*, 2001; Seymour *et al.*, 1994), as well as detection methods based on either immunological or nucleic acid-based procedures (Jabbar and Joishy, 1999; Scheu *et al.*, 1998; Yost and Nattress, 2000). Whilst improvements in terms of rapidity have been demonstrated in some of the more recently proposed technologies (Kumudavally *et al.*, 2001; Mayr *et al.*, 2003; Suzuki *et al.*, 2001), the fact remains that of those methods currently employed in the food industry, the recurring theme is that they are time-consuming, labor-intensive and give retrospective information (Ellis *et al.*, 2002). However, in a modern food-processing environment monitoring procedures need to give results in real time in order that corrective action can be taken as soon as possible.

Fourier transform infrared (FT-IR) spectroscopy is a rapid, high-throughput, non-destructive analytical technique, which is continuously being developed for an ever-increasing range of applications in both scientific and commercial settings. FT-IR involves the observation of molecules that are excited by an infrared beam, resulting in an infrared absorbance spectrum, which represents a 'fingerprint' characteristic of any chemical or biochemical substance. Its major advantages when compared to other techniques, are its rapidity, as an infrared spectrum can be collected in seconds, and the fact that it requires minimum sample preparation and background training (Dunn and Ellis, 2005). Initial interest within the food

industry was mainly reserved to near infrared spectroscopy (NIR) (Monin, 1998; Murray *et al.*, 2001; Ru and Glatz, 2000), because of its ability to provide rapid bulk measurements of lipid and protein. However, it is becoming increasingly evident that mid-infrared spectroscopy has much promise for future industrial applications in the food and related industries (Johnson *et al.*, 2003; Mura *et al.*, 2001; Riquet *et al.*, 1998; Safa and Abbes, 2002; Yang *et al.*, 2001) as it provides for a more information-rich analysis, since the fundamental vibrational modes are measured rather than broad overtones or harmonics (Stuart, 1997).

2. Materials and methods

2.1. Meat sample preparation for FT-IR

In general for our poultry and beef analyses no pre-preparation of these muscle foods, such as washing, inoculation with bacteria or removal of fat or connective tissue was undertaken. To accelerate the microbial spoilage process, meat was comminuted in a coffee mill. Once prepared, samples were removed and placed into the upturned lid of a sterile 90 mm Petri dish and, using the sterile Petri dish base, pressed manually to a thickness of ~5 mm. A sterile Petri dish base was used to cover each sample so that the meat did not dry out. Again, in order to speed up the spoilage process meat samples were stored at ambient room temperature (typically 22±1 °C) for the duration of the experiment.

Classical microbiological plating methods were used for enumeration of the bacteria. These measurements took 48 hours culturing on LabM blood agar base at 25 °C.

2.2. Horizontal attenuated total reflectance (HATR) FT-IR spectroscopy

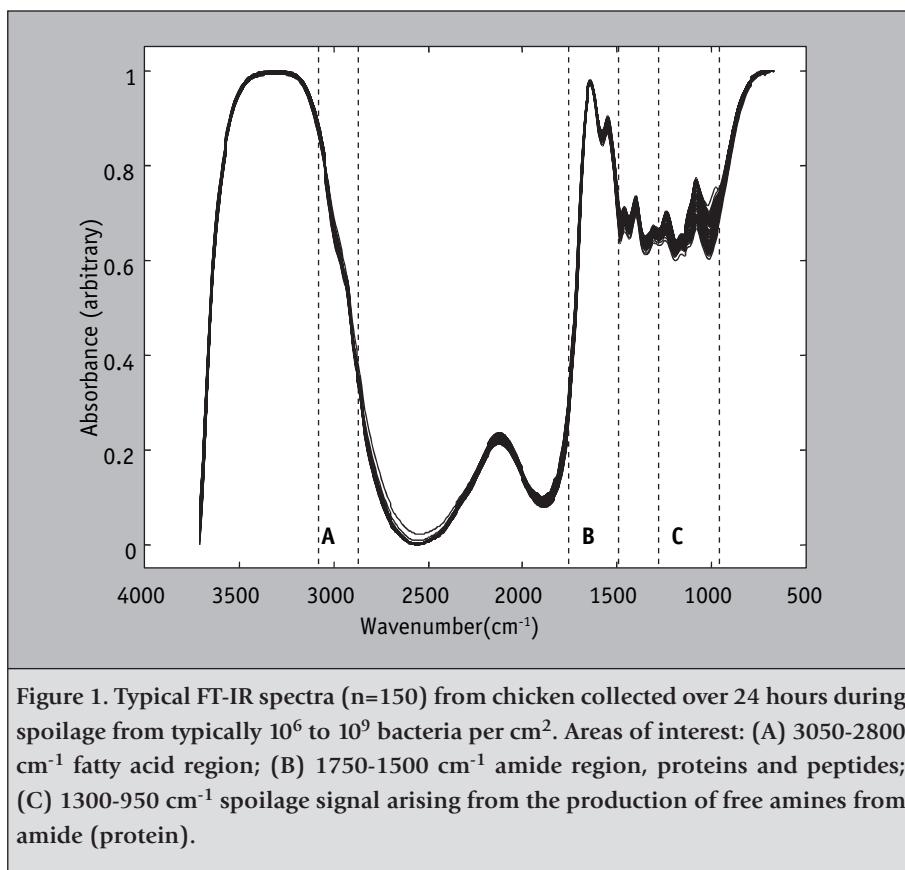
In general all FT-IR analyses were undertaken using a ZnSe HATR accessory (Spectroscopy Central Ltd., Green Lane, Warrington, UK) on a Bruker IFS28 infrared spectrometer (Bruker Optics Ltd., Banner Lane, Coventry, UK) equipped with a deuterated triglycine sulphate (DTGS) detector. The ZnSe HATR crystal was capable of 10 external reflections with the evanescent field (Banwell and McCash, 1994) effecting a depth of 1.01 µm (Spectroscopy Central Ltd.) and its working dimensions were 60 mm x 10 mm.

At hourly intervals, replicates were measured and the aerobic upper surface of the meat sample was placed in intimate contact with the ZnSe crystal. After

analysis the crystal was cleaned as detailed elsewhere (Ellis *et al.*, 2002, 2004). FT-IR spectra were collected over the wavenumber range 4000-600 cm⁻¹. Typical spectra are shown in Figure 1. All spectra were collected in reflectance mode with a resolution of 16 cm⁻¹ and to improve the signal-to-noise ratio 256 scans were co-added and averaged. Collection time for each sample spectrum was 60 seconds.

3. Analysis of FT-IR data

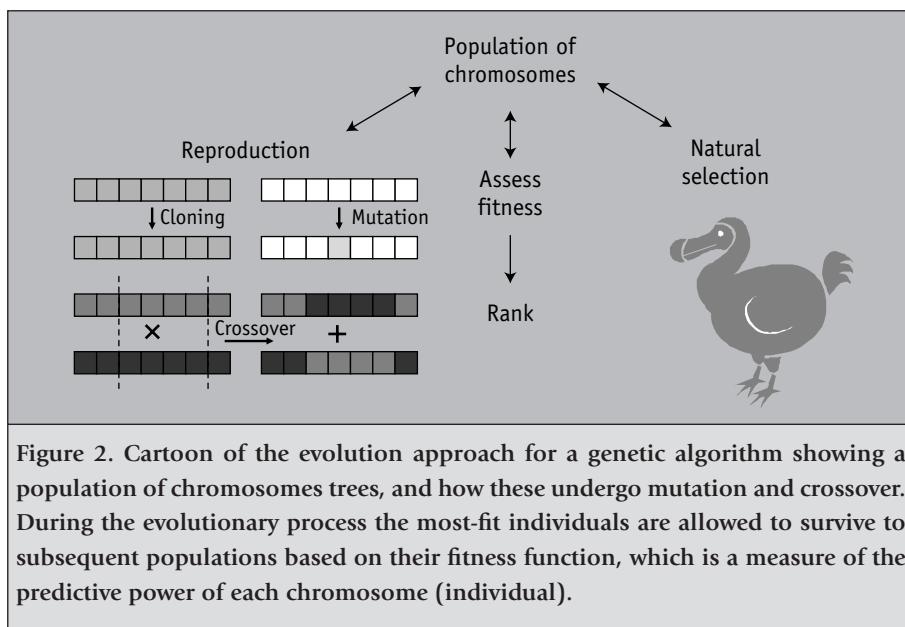
For the quantitative analysis of FT-IR spectra the general idea is to 'correlate' the FT-IR fingerprints with the level of bacteria. That is to say transform the IR data into the bacterial total viable counts (TVCs) per cm². For this purpose we



generally employed the supervised learning algorithm partial least squares (PLS) regression (Duda *et al.*, 2001; Martens and Næs, 1989; Massart *et al.*, 1997).

Whilst PLS is recognised as an excellent method for the quantitative analysis of biological systems (Holland, 1992), the information as to which wave numbers in the infrared spectra are important is often hard to determine. This is especially true when the loading matrices are complex, which we found generally to be the case. Therefore, alternative strategies need to be adopted and we have based this on our pioneering work in evolutionary computational methods based on genetic algorithms and/or genetic programming (Broadhurst *et al.*, 1997; Brown *et al.*, 2005; Gilbert *et al.*, 1997; Goodacre *et al.*, 2000, 2004; Jarvis and Goodacre, 2005; Kell, 2004; Kell *et al.*, 2001).

Genetic algorithm (GA) and genetic programming (GP) can be used to produce models, which allow the deconvolution of hyperspectral data in chemical terms. These methods use the concepts of Darwinian selection to generate and optimise a desired computational function or mathematical expression (Figure 2). The particular strength of these approaches is not only that it develops accurate models, but that those models may be made to be comparatively simple and



therefore allow robust variable selection to be performed. Once variables are selected these can be related to molecular vibrational modes and hence biochemical knowledge.

4. FT-IR investigations in chicken and beef spoilage

The comminution of samples for the series of experiments in order to accelerate the spoilage process were successful in that for poultry and beef the final $\log_{10}(\text{TVC})$ of bacteria were one and two orders of magnitude above the initial levels, respectively (Table 1). The resulting microbial spoilage within 24 hours at ambient temperature was anticipated, as it is known that comminution accelerates microbial spoilage due to several factors, such as the rupturing of cell walls, which releases a source of nutrients, increases the surface area to volume ratio, and distributes bacteria that would normally be restricted to the surface throughout the meat substrate. It was also observed that the use of pH alone as an indicator of microbial spoilage or remaining shelf-life would be insufficient. During the spoilage of poultry, for example, it was found that pH fluctuates

Table 1. Comparative pH, bacterial loads from the spoilage of chicken and beef (adapted from Ellis *et al.*, 2002, 2004).

Experiment	Initial pH	Final pH	Initial $\log_{10}(\text{TVC})$	Final $\log_{10}(\text{TVC})$	Room temp. (°C)	Spoilage (hours)
Poultry						
a	6.02	6.79	6.86	9.20	21.5	14
b	5.52	6.05	6.62	8.64	23.1	17
c	5.94	6.83	6.77	9.04	23.3	10
Mean	5.87	6.67	6.67	9.02	22.6	13.6
Beef						
a	5.33	5.33	5.18	6.94	21.6	n/a ¹
b	5.50	5.53	5.34	7.58	24.7	20
c	5.41	5.41	5.68	7.39	20.9	20
Mean	5.42	5.43	5.45	7.38	22.4	20

¹did not spoil.

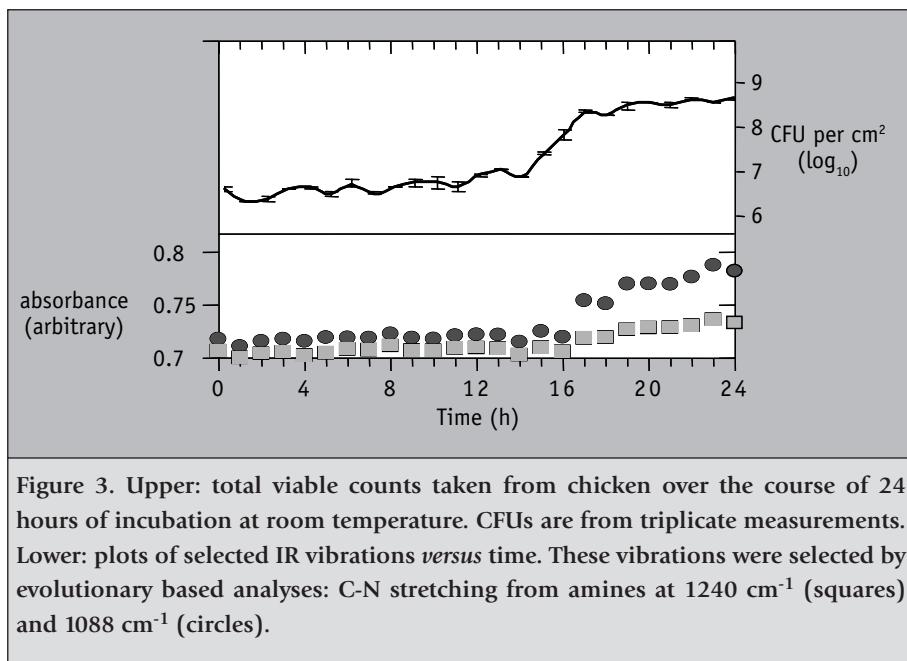
prior to spoilage and only increases significantly once levels of bacteria have reached $\sim 10^8$ colony forming units (CFU) per cm². Whilst for experiments on beef, it was observed that pH fluctuated within a very narrow range (5.32-5.53) throughout the course of bacterial spoilage, clearly demonstrating the strong buffering capacity of this particular muscle food (Madruga and Mottram, 1995; Puolanne and Kivistö, 2000).

A total of 150 infrared absorbance spectra from one of these experiments are shown in Figure 1 and illustrate the reproducibility of HATR and the robustness of the sample preparation method. In Figure 1 the regions attributable to vibrations from CH_x from fatty acids, and C=O, N-H from amide I and amide II vibrations from proteins and peptides are highlighted. In addition, the region in the infrared spectra where most variance occurs and, therefore, is most probably attributable to the microbial spoilage process, is also highlighted. Indeed this was the region, which was found to be most correlated with total viable counts using both GA and GP.

For the analysis of chicken spoilage we found that the most intense peaks, which appear in fresh chicken, are the Amide I (C=O vibration at 1640 cm⁻¹) and Amide II (N-H deformation at 1550 cm⁻¹) bands from proteins and peptides. It was found that the Amide II band was the only vibration that was negatively correlated with spoilage, which suggested that the protein content of the meat was decreasing during spoilage. By contrast, the peaks at 1240 cm⁻¹ and 1088 cm⁻¹, which are both C-N vibrations from amines from free amino acids, were positively correlated to spoilage.

For the analysis of beef spoilage similar results were found: GA and GP selected wavenumber vibrations from within the region 1420 cm⁻¹ to 1400 cm⁻¹ again from C-N attributable to amides. In this analysis more variables were selected, perhaps due to the complexity of the beef substrate. For example, other vibrations that were selected, were from free amines at 1112 cm⁻¹ and 1374 cm⁻¹.

For the spoilage of chicken, the infrared absorbance from vibrations of free amines was directly correlated with total viable counts of bacteria (Figure 3). This clearly demonstrates the hypothesis generating nature of the multivariate data analysis, which suggests that the onset of sensory spoilage occurs when free amines started to increase. That is to say, the most significant metabolic process, which occurs at spoilage is the start of proteolysis. Indeed, it is known that spoilage in meat is most frequently associated with the post-glucose utilisation



of amino acids by aerobic microorganisms such as pseudomonads, and the onset of the enzymatic degradation of proteins and peptides, leading to the production of free amino acids (Braun *et al.*, 1999; Dainty, 1996; Nychas and Tassou, 1997).

5. Conclusions

It has been clearly demonstrated that FT-IR spectroscopy is a powerful method, which can be used to estimate the level of bacterial spoilage in beef and chicken. The particular strength of FT-IR is that it is able to acquire a metabolic snapshot and can be calibrated to quantify, non-destructively, the microbial load of food samples accurately directly from the sample surface in only 60 seconds.

Related work has very recently shown that infrared and Raman spectroscopies can be used to differentiate between muscle foods (Ellis *et al.*, 2005). In particular the authors demonstrated the ability to discriminate clearly between closely related avian species and distinct muscle groups within those species. This shows the

generic application of infrared within food science and could also be adopted for determining the provenance of meat and meat products.

With further development it is suggested that it will be possible to calibrate the 'holistic' metabolic fingerprint generated by FT-IR to the levels of specific organisms including those pathogenic ones, which are implicated in food poisoning such as *Salmonella* species and *Campylobacter jejuni* from chicken, and *Escherichia coli* O157:H7 from beef. In addition, the emergence of field portable infrared technology will bring this method closer to *in situ* measurements and could therefore be implemented from farm to fork.

We therefore believe that this approach has considerable potential and will aid both the food safety regulatory bodies and the hazard analysis critical control points (HACCP) system as a real on-line microbial analysis technique.

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Flow cytometry as a rapid tool for microbiological analysis in the food industry: potentials and restrictions

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Abstract

Flow cytometry offers an alternative towards standard microbiological methods. It is a fast and sensitive method, which can also provide useful quantitative and qualitative information concerning microbial populations. The wide range of available fluorescence probes allows the analysis of many different characteristics of micro-organisms. Therefore, the use of flow cytometry can provide new applications in the quality and process control of food and drink products, and in the control of fermentation processes. Recent developments in flow cytometry, particularly compact devices using a laser diode, are discussed regarding their effectiveness to determine microbiological quality, process and hygiene parameters in different food sectors such as dairy, vegetables, meat and beer, and to assess the viability and activity of yeast and starter cultures.

Keywords: flow cytometry, food microbiology, fluorescence, viability, micro-organisms

1. Introduction

The introduction of good manufacturing practices (GMP), hazard analysis critical control points (HACCP) and quantitative risk analysis (QRA) has made food production better controlled in a cost-efficient way. Food microbiological analyses are still needed within HACCP programmes for hazard analysis, control of raw materials, process lines and environment, and for validation and verification. The function of food microbiological analyses is to confirm the quality and safety of food, often determined by detecting colonies of indicator micro-organisms in suitable media. Conventional testing methods make

use of specific media to isolate and enumerate viable bacterial cells in food. They require at least one day to produce definitive results, because they rely on the ability of micro-organisms to multiply resulting in visible colonies. It is well known that conventional culture methods for detecting indicator and pathogenic bacteria in food and water may underestimate numbers due to sublethal injury, inability of target bacteria to take up nutrient components in the medium, and other physiological factors reducing culturability. Moreover, culture medium preparation, inoculation of plates, colony counting and biochemical characterisation make these methods labour intensive. Therefore, more sensitive, rapid microbial detection methods are needed to complement or replace these conventional culture procedures. Rapid detection (24 hours or less) and identification of micro-organisms or contamination sources is a help in making decisions concerning production lots, minimising the expenses due to unnecessary storage and raw material losses.

Numerous technologies have been developed to provide faster results than conventional cultural methods, flow cytometry (FCM) being one of them. Several FCM techniques have been developed and are routinely applied in many areas of microbiology including public health, drinking water, food, pharma and biotechnology. The technique permits analysis of single cells in a suspension, e.g., for DNA content, immunogenic properties, protein content, intracellular pH and enzyme activities. FCM is a rapid technique originally developed for analysing mammalian cells and it is a well-established technology in medical diagnostics. Despite the many advantages of FCM, implementation of the technique in microbiological laboratories is still limited. In this paper the technique and its applications in microbiology are described, various possible methods of microbiological labelling for flow cytometric studies are discussed, and some results of a research project on the evaluation of a compact flow cytometer and its application possibilities as a microbiological quality, process and hygiene control device in the food industry are presented.

2. Flow cytometry

2.1. Technical basis

FCM is a technique for quantifying components or structural features of cells primarily by optical means. It may be considered as a form of automated (fluorescence) microscopy in which, instead of being fixed to a slide, the sample is injected into a fluid passing through a sensing region of the flow cell. The

internal workings of all modern optical flow cytometers are fundamentally similar. A schematic configuration of a typical flow cytometer is given in Figure 1. FCM systems are composed of four basic components: (1) a fluidic system, (2) a focused light source, (3) filters and photodetectors, and (4) a computer with software. Flow cytometers utilise a fluid flow system to deliver particles, such as cells, from the sample tube into a flow cell containing a sheath fluid, to an illumination zone where light scattering and fluorescence emission is recorded by the cytometer's sensors and converted into analogue electronic signals. The computer collects, stores and analyses the data.

Flow cytometers are able to record data at a rate of thousands of events per second. From two up to more than ten different parameters can be collected for each particle passing through the interrogation point, i.e. where the light source is focused on the sample stream, and the light collection and detection optics are arrayed. Together, these parameters provide an optical characterisation of each particle, which typically includes measures of light scattering and fluorescence. The amount and direction of the light scattered by a particle are largely related to its properties such as size, surface and internal structure, and refractive index.

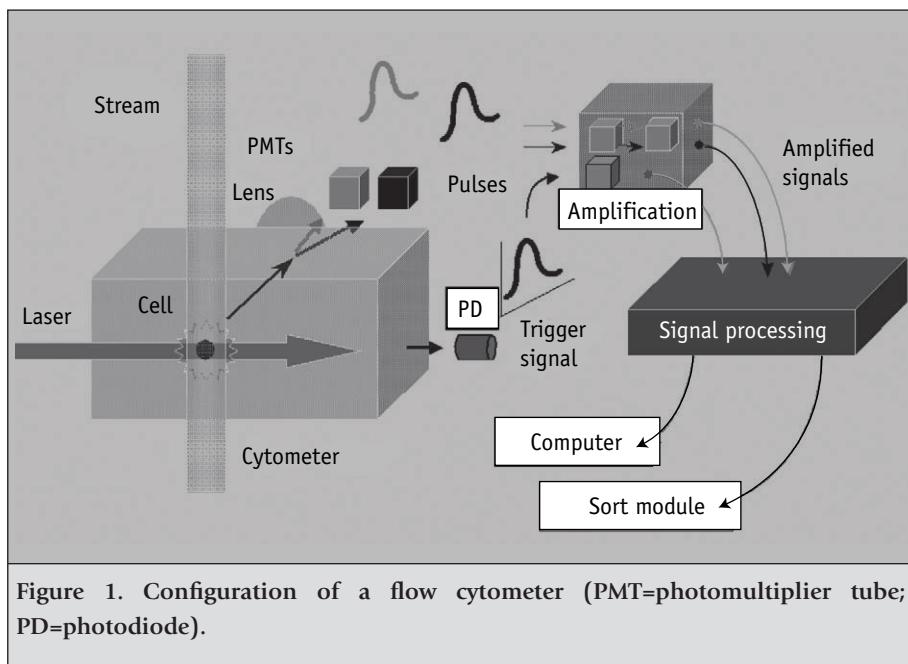


Figure 1. Configuration of a flow cytometer (PMT=photomultiplier tube; PD=photodiode).

Scattered light collected in the same direction as the incident light (forward scatter, FSC or forward angle scatter, FALS) is related to cell size. Scattered light collected at an angle of 90° (side scatter, SSC or right angle scatter, RALS) gives an idea of the particle complexity, which is related to cell surface roughness and number of organelles present in the cell. Size and complexity are considered intrinsic parameters, since they can be obtained without staining the sample. To obtain additional information samples can be stained using different fluorochromes. Some flow cytometers are able to physically separate or 'sort' particles of interest based on their cytometric characteristics (cell sorters), offering the opportunity to collect and to reanalyse them by other methods.

2.2. Fluorescent probe technology

While a number of useful measurements can be made on cells by using light-scattering measurements alone, the ability of the flow cytometer to quantify particle-associated fluorescence makes the technique of special usefulness.

A fluorescent probe is a fluorophore designed to localise a specific region of a biological specimen or to respond to a specific stimulus. Fluorescent probes can be divided into two broad categories: (1) those that are used to label other probes such as antibodies and oligonucleotides, and (2) those whose fluorescence is related to particular properties of a cell. The number of fluorescent probes is continuously increasing. Commonly used probes and their cellular target sites are shown in Table 1 and Figure 2. Their availability has led to new techniques for assessing viability of micro-organisms based on different properties of the cell such as cytoplasmic membrane integrity, membrane potential, enzyme activity, respiratory activity, relative DNA content, and pH gradients (see paragraph 2.3).

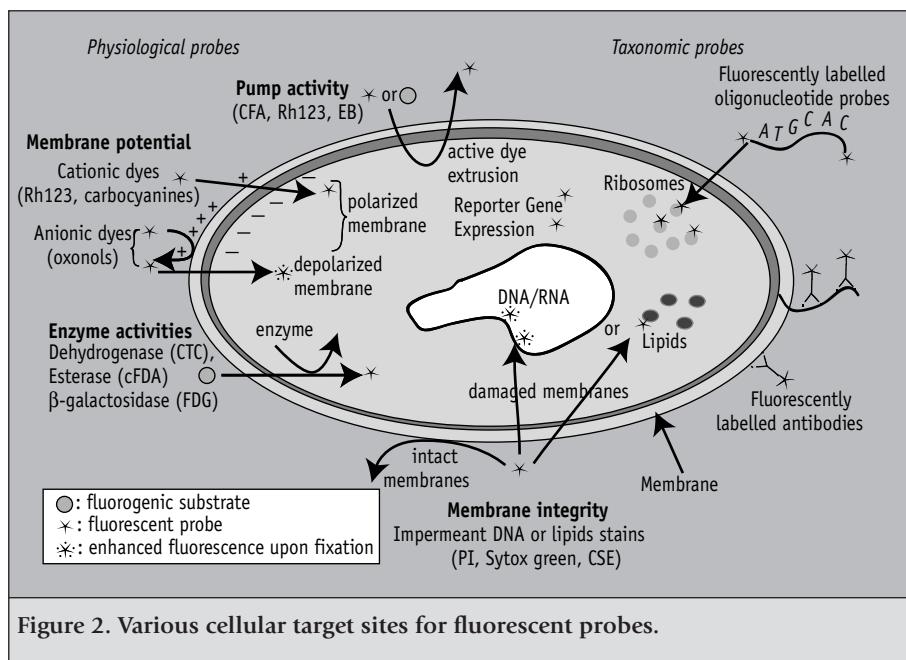
2.3. Assessment of viability

Conventional methods used most frequently for assessing the viability of micro-organisms rely on specific microbiological media to isolate and enumerate viable cells. The major disadvantages of such methods are the time needed to produce results and the failure to isolate 'viable, but non-culturable' organisms. Immunological and molecular techniques can be performed in a shorter timespan, but they provide only semi-quantitative information, which is not satisfactory for various applications, and they do not discriminate between viable and dead cells. FCM in combination with fluorescent probe technology

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Table 1. Commonly used fluorescent probes in flow cytometry.

Category of staining	Dye	Extinction _{max} (nm)	Emission _{max} (nm)	Application
DNA staining	Ethidium bromide	510	595	DNA, RNA
	Propidium iodide	536	623	DNA, RNA, dead cell stain, viability, DNA cell cycle
	Hoechst 33342	340	450	DNA, cell cycle
	DAPI	350	470	DNA, cell cycle, detection, enumeration
	Chromomycin	440	555	
	Mithramycin	440	575	
	TOTO-series	514	533	detection, enumeration
	SYTO-series	530-567	544-583	viability, Gram staining
	SYBR Green I	494	521	DNA quantification, viability
	TOPRO-3	642	661	viability
Protein/ antibody	SYTOX Green / Orange	504/547	523/570	live/dead, cell cycle
	Hexidium iodide	518	600	Gram staining
	Fluorescein isothiocyanate	494	520	
Membrane potential	Cy3 / Cy5	550/651	570/674	nucleotide sequence identification, reporter gene
	CFDA-SE	519	542	cell tracking
	Rhodamine 123	507	529	viability of Gram-positive bacteria
	Dihexylocarbocyanine	482	500	
Enzyme activity	Oxonol dye	493	516	viability, antibiotic susceptibility
	Fluorescein derivatives	494	520	
	C12-resazurin	563	587	
pH indicators	CTC (dehydrogenases)	530-550	varies	respiratory activity; viability
	CFDA (esterases)	492	517	viability
	BCECF	460-510	520-610	cell internal pH, viability
	SNARF-1	490-540	587-635	cell internal pH



is an appealing technique for fast viability assessment based on the separate analysis of thousands of individual cells in a short time and providing additional information on live and dead cells and their physiology.

Loss of membrane integrity represents significant damage for a cell due to multiple membrane-linked functions, e.g. selective permeability, active transport and motility. Therefore, integrity of the cytoplasmic membrane has been exploited as an indicator of cell viability in both prokaryotes and eukaryotes. Membrane integrity can be detected by dye exclusion or dye retention. Dye exclusion probes are used extensively, especially DNA binding compounds (e.g. propidium iodide, SYTOX Green, TOTO and TOPRO series). Exclusion of these impermeant probes by cells with intact membranes is considered as an indicator of viability. Membrane-permeant dyes such as the SYTO-series and SYBR I Green, which cross the cytoplasmic membranes of both dead and living cells, can be used in combination with the above mentioned dyes for a better distinction between live, injured and dead cells.

Membrane potential is the electrical potential caused by an ionic concentration gradient across the microbial cell membrane, originating from selective permeability and active transport of charged molecules through the membrane. Bacteria normally maintain an electrical potential gradient of over 100 mV across the cytoplasmic membrane, with the interior negative with respect to the exterior. Changes in membrane potential, caused by depletion of energy sources or perturbation by physical or chemical agents, also provide a means of assessing the viability of a cell. This is usually measured by 'distributional probes', i.e. lipophilic dyes that can readily pass the cell membrane and accumulate according to their charge. Cells with a membrane potential actively take up lipophilic cationic dyes such as Rhodamine 123 and 3,3'-dihexyloxacarbocyanine ($\text{DiOC}_6(3)$) or actively exclude lipophilic anionic dyes such as the negatively charged $\text{DiBAC}_4(3)$ known as oxonol.

Monitoring cellular esterase activity is another approach to determine the viability of cell populations by means of FCM. Esters of fluorescein (e.g. FDA, CFDA, BCECF-AM) are non-fluorescent molecules, which diffuse into cells where they are hydrolysed by intracellular non-specific esterases to polar fluorescent products retained by cells with intact membranes. Dead or dying cells with compromised membranes rapidly leak the dye, even if they retain some residual esterase activity. The intracellular accumulation and efflux of fluorescein in living cells has been reported to be dependent on changes in cellular metabolism, membrane potential and membrane integrity. BCECF-AM and CFSE are also convenient for measuring the intracellular pH. The application of these types of viability markers in industrial quality assurance has been commercialised by Chemunex (France), detecting yeasts and bacteria in food and pharmaceutical products.

Respiration activity in aerobic bacteria can be detected using the substrate 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC). CTC acts as an electron acceptor in the electron transport system and can be reduced by a variety of dehydrogenases to an insoluble fluorescent formazan, which accumulates inside the cell (Sieracki *et al.*, 1999). Since electron transport is directly related to cellular energy metabolism in respiring cells, the ability of cells to reduce tetrazolium compounds can be considered an indicator of bacterial activity.

3. Advantages and limitations of flow cytometry

For many purposes FCM seems to be superior to conventional culture methods for microbiological analysis. FCM is able to make rapid, quantitative

measurements of multiple parameters of each cell within a large number of cells. This makes it possible to define the properties of an overall population as well of its subpopulations. The large amount of data on individual cells is useful in the detection of small changes, which are not normally detected by conventional methods. Light scattering from the cells gives information on size, shape and structure, cell mass and growth. FCM provides a highly effective means for rapid analysis of individual cells at rates up to 1,000 cells per second. By labelling the cells with specific fluorochromes or fluorescent conjugates, which bind with high specificity to one particular cellular constituent, it is possible to measure a wide variety of cell constituents such as proteins, carbohydrates, DNA, RNA and enzymes. When compared to conventional culturing techniques, there is also the advantage of being able to detect viable, but non-culturable cells. Flow cytometers can be equipped with a flow-sorting unit, which can be used for isolation and subsequent characterisation of subpopulations of cells in mixed populations. Another key characteristic of FCM is its versatility: the same apparatus can be used to perform different analyses, i.e. specific staining with fluorescent antibody or nucleic acid.

Compared to other rapid techniques, such as enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), FCM is a very sensitive technique: as few as 10 yeast cells and about 10^2 bacterial cells per ml can be detected. Moreover, FCM can be made even more sensitive, if combined with other techniques such as immunomagnetic separation. However, the enumeration of rare events (i.e. one target cell in 10^6 non-target cells) is extremely difficult. The small size of bacteria and the low number of DNA molecules present to be stained (typically three orders of magnitude less than a mammalian cell) require instruments with high sensitivity. The main experimental difficulty in analysing bacteria using FCM is that many of their biological characteristics, including size, shape and DNA content, vary depending upon the growth conditions used or the source from which the organisms were obtained. Therefore, strict reproducibility of these conditions is required to produce consistent data.

Despite the rapid and automatic analysis provided by FCM, the introduction of this technique in microbiology laboratories could be limited by the cost of the instrumentation and the need for well-trained operators. However, the continuing rise in computer power and in the installed base of flow cytometers is leading to a fall in costs in real terms. FCM devices have become more user friendly; the number of applications has been expanded and much cheaper instruments, more directly tailored to the microbiologist, using laser diodes as

sources and photodiodes as detectors of photons have been developed and are commercially available. Nevertheless, flow cytometers are not simple instruments and basic knowledge of the underlying principles is needed to enable correct interpretation of the results. The quality of the sample, the staining procedure, the specific probe and antibodies used, and the settings for the measurements are all important for the precision and accuracy of the measurements. The technique requires the cells to be in an aqueous suspension as single cells, which makes it only applicable as an *in situ* process, e.g. in water samples and some food samples. The application of FCM for the quantification of micro-organisms in solid food, or in aqueous samples, where the cells are present as aggregates or bound to particulate matter, may require pre-extraction to get the cells in a single cell (monodisperse) suspension and free of particles, which disturb the flow or block the narrow tubings. Other disadvantages for use in food microbiology are in some cases the lack of distinction between living and dead cells and interference with the food matrix. Finally, the detection limit of the instrument often requires pre-enrichment of the sample, thereby making quantitative detection of a particular organism present in low numbers difficult.

4. Present status and perspectives of flow cytometry

There is a continuing rise in both the number and percentage of flow cytometric studies, which use micro-organisms as subject. Improvements in the design of the flow chamber of modern flow cytometers have already led to a reduction in the level of background noise, and this is expected to improve still further. Improved electronic hardware will continue to increase the speed, accuracy, and precision of flow cytometric analysis and sorting, while continuing developments in the methods of artificial intelligence and the continuing fall in computer price/performance ratios will ensure that the multivariate nature of the flow cytometric approach is exploited to the full. Furthermore, there is a continuous development of new fluorescent probes for microbial analysis. Numerous probes have been introduced to distinguish live and dead micro-organisms, including dyes based on membrane permeability and membrane potential (see e.g. paragraph 2.3). Moreover, it should be noted that flow cytometric techniques can be used for a wide range of target bacteria, including potentially pathogenic species. The tremendous interest in oligonucleotide probe technology will lead to the continuing development and exploitation of cocktails of such probes for the analysis of complex microbial samples. And there remains much room for improvement in the generation, characterisation, and specification of fluorescent antibodies against specific microbial cells.

Lasers are one of the largest component costs of cytometry instrumentation. The availability of visible-wavelength diode lasers (e.g. frequency-doubled, diode-pumped yttrium aluminium garnet or YAG lasers, emitting at 532 nm) of low price, high efficiency, low optical noise and long lifetime is expected to promote broader applications of FCM. When considering a solid-state laser, it is important to choose one that has a long life span and provides a consistent power output and stability. A solid-state system with low electrical and cooling requirements makes it much smaller and more robust, and thus portable for field analysis. In addition, solid-state lasers emitting in the blue and ultraviolet become commercially available for use in FCM.

Although flow cytometrists are more familiar with fluorescent stains that can be excited at 488 nm, excitation at higher wavelengths reduces the problem of background autofluorescence for most microbes. This will probably encourage the development and improvement of fluorescent stains for these longer wavelengths.

The development of microfluidic flow cytometers may combine the best features of flow and static cytometers. While their analysis rate is typically no more than a few hundred cells per second, they can incorporate not only sorting capability, but also the ability to add and mix reagents, to observe individual cells over time, and to collect much weaker fluorescence signals than can be measured in conventional flow cytometers (Fu *et al.*, 1999).

5. Applications of flow cytometry in microbial analysis

FCM is of great interest to microbiologists. The applications in general and food microbiology are widespread and still expanding and with the opportunity for multiparameter analysis of single cells, the technique rapidly provides the user with information not available with other techniques. The ability to use a single instrument for numerous rapid microbiological assay procedures has obvious advantages for a microbiology laboratory over the current situation, where culturing, microscopy, or several dedicated instruments are needed. The use of FCM in microbiology allows fast enumeration, physical and biochemical characterisation, and even identification of cells from a non-homogeneous population. Microbial species can be differentiated by immunolabelling of species-specific antigens. In such instances, light scattering properties and a cocktail of fluorescently labelled antibodies specific for particular bacterial

species can be used to provide a multiparameter identification for the organism of interest. The main disadvantage is still the limited availability of antibodies directed against particular microbes and the cross-reaction with other species. Monoclonal antibodies can exhibit far more specificity and are thus to be preferred if available. In addition, non-specific binding may be partly overcome by blocking agents and the multiparametric nature of the flow cytometric approach allows much better discrimination between true and adventitious binding in contrast to ELISA methods.

The use of different sized fluorescent microspheres coated with antibodies against microbes is a new application of FCM for direct diagnosis (Vignali, 2000). This method detects the binding of specific microbes to antibody-coated microspheres by measuring the decrease in fluorescence emission of the microspheres due to the shading effect of microbes on both the exciting and emitting light. With different sized fluorescent microspheres several pathogens can be detected simultaneously in the same sample (Fulton *et al.*, 1997). An alternative approach to identify bacterial species in mixed microbial populations uses fluorescently labelled DNA probes specific for 16S rRNAs (Aman *et al.*, 1995). Nucleic acid probes can be designed to specifically target taxonomic groups at different levels of specificity, from species to domain. Peptide nucleic acid (PNA)-based probes offer advantages over DNA-based probes, particularly for hard to permeabilise microbial forms such as spores, encysted parasites, and Gram-positive bacteria (Newsome, 2003). The unique chemical make-up of PNA confers a number of advantageous properties, including rapid hybridisation kinetics, resistance to nucleases and the ability to hybridise to positions on the ribosome, which are inaccessible to DNA-based probes.

In addition to using DNA probes or antibodies for species identification, fluorescent dyes can also be used to probe the viability and metabolic state of micro-organisms. The effect of environmental stress, starvation or antimicrobial compounds on the membrane potential and membrane permeability status of bacteria and yeasts has been studied using fluorochromes, which distinguish between non-viable, viable, dormant and sublethal cells. Flow cytometric analysis of yeasts has been used to study the viability and vitality during fermentation, for assessment of fermentation efficiency, to discriminate between wild and lager yeasts, and for on-line prediction of the shelf-life of beverages. FCM offers the possibility of near real-time monitoring of microbial populations in industrial fermentations. Different developed fluorochromes and kits such as Live/dead® Bacterial viability and counting, Cell Vitality assay C12-resazurin/SYTOX Green,

Bacterial Gram Stain, Yeast Viability kit (Molecular Probes, Eugene, OR, USA) have been used for the FCM-based counting of live and dead bacteria and yeasts, simplifying staining protocols and making data interpretation easier.

FCM also offers the possibility of studying gene expression using reporter genes in yeasts and bacteria. The development of gene expression systems based on green fluorescent proteins facilitates this kind of study due to the simplicity of the technique. A FCM-based technique for bacterial genome fingerprinting analysis has also been described (Kim *et al.*, 1999).

While FCM has been frequently used to analyse bacteria in pure cultures, more limited use is seen with heterogeneous natural samples such as those encountered in food microbiology. Bacteria are small as compared to mammalian cells, and in FCM analysis the signal-to-noise ratio is of greater concern for bacterial cell samples than for mammalian cell samples, especially in food and environmental samples. The application of FCM to such samples is complicated by difficulties of distinguishing the organisms of interest from noise and debris, releasing bacteria from flocs or aggregates, and the low signal often obtained from natural bacterial samples. It is possible to circumvent these problems by using a multiparameter approach. When performing flow cytometric studies on samples, there is the facility to 'gate out' or exclude particles with particular properties from the analysis. Forward angle light scatter is frequently used as a gating parameter to exclude cell aggregates and debris from further analysis, however, a combination of light scatter and fluorescence is sometimes better for this purpose. Special care has to be taken for the presence of autofluorescent particles and debris stained by non-specific binding of the fluorescent dye. Double labelling with two fluorochromes of different membrane permeability allows more stringent bacterial discrimination of 'DNA surrounded by an intact cell membrane'. By defining regions of interest, exact numbers of cells/particles present in that region can be calculated and target cells can be adequately distinguished from the background. Sensitivity can be improved by reducing the background, i.e. all the light coming from other sources than the cells. In many flow cytometers forward scatter signals from bacteria are near or below the noise level and any mismatch of refractive index between the sheath fluid and the fluid in which cells are suspended, adds noise to the measurement. Therefore, it is desirable to correct such index mismatches to maximise the chance of obtaining usable signals.

One of the most challenging problems is sample preparation. Preconcentration of samples may relieve volume limitations and improve the detection of less

abundant particles, but will still be subject to other constraints. The detection sensitivity of optical systems is limited by the statistical abundance of an event and the signal intensity separating the event from background noise. Although it is possible to ignore non-target particles to some extent by excluding them from the data that are recorded, physical and electronic limitations on the rate at which flow cytometers can examine particles, are not as easily circumvented. Concentrating a sample to increase the abundance of rare particles only makes the analysis easier if most other particles can also be selectively removed. The precise limit for any given application will be influenced by many factors, including volume and electronic throughput limitations and the signal-to-noise ratio achieved for the target organism. More research is needed on techniques for separating micro-organisms from the food matrix and concentrating them before detection by immunological or nucleic acid-based assays.

However, the separation of bacteria from food particles without causing cell damage is a limiting factor and difficult task. Although FCM offers considerable advantages over microscopy or plate growth methods, it may still not be sensitive enough to detect bacterial cell concentrations less than 100 per ml. In these situations a short pre-incubation step prior to flow cytometric analysis may be envisaged increasing the bacterial load of the sample to a level at which it may be detected.

Some applications have been developed for FCM microbial analysis of food products such as meat, fruit juices, eggs and milk, e.g. detection of *Listeria monocytogenes* in milk, and *Salmonella typhimurium* in milk and eggs (McClelland and Pinder, 1994). Specific detection of *Escherichia coli* O157:H7 in food ingredients was described with the use of immunomagnetic separation and FCM (Seo *et al.*, 1998). There is also the ability of FCM to determine total bacterial numbers after clearing of milk and staining of bacteria with a readily available fluorescent stain. The sensitivity of this FCM procedure was 10^4 total bacteria per ml of milk. A complete FCM assay, including extraction and labelling, can be done in 1 hour, as no long incubation times are required. For routine analyses of milk quality an automated FCM instrument, the Bactoscan-FC (Foss Electric, Hillerød, Denmark), was developed. This instrument uses ethidium bromide to stain bacteria in milk; the disturbing milk components are reduced and dispersed by treatment with detergent and enzyme at 50 °C, providing results after 8 minutes (Suhren and Reichmuth, 1997).

Any flow cytometry viability assay method, which compares flow cytometric counts with plate counts (the 'golden' standard) must not only assume that the cells counted in the sample are viable, but also that they will grow on the plate medium used. In an attempt to partially address this problem, Chemunex (France) has developed a simple flow cytometer and a set of reagents, which could be reliably used in an industrial environment for the detection of viable micro-organisms. In the so-called D-Count and Bactiflow system, viable cells are detected by their ability to cleave a non-fluorescent precursor to release a fluorochrome (intracellularly).

6. Case studies

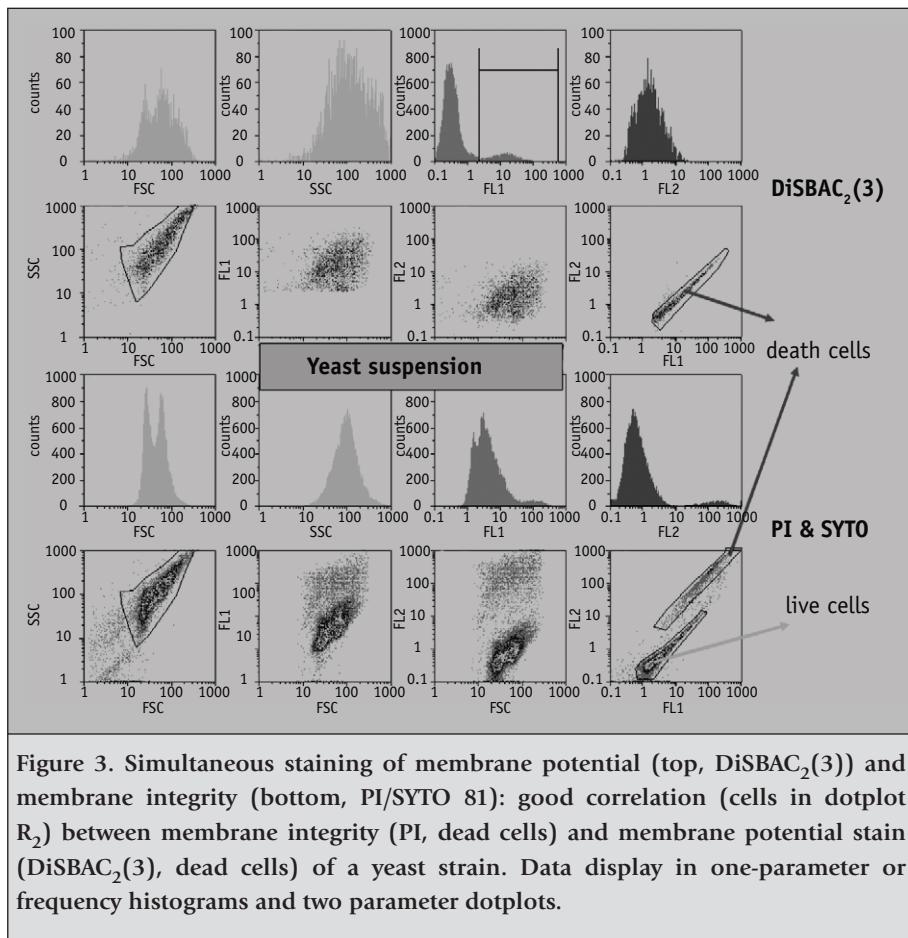
In a research project at our laboratory a compact flow cytometer was evaluated and investigated for its possible application as a microbiological quality, process and hygiene control device in the food industry (see e.g. http://webs.hogent.be/~trn987/index_uk.html).

The aims of this study were (1) evaluating the performance of the compact flow cytometer and the fluorochromes with regard to microbial cell activity and physiology, (2) creating protocols for sample treatment of different food products for flow cytometry analysis, (3) tracing cellular damage caused by processing factors through flow cytometry, and (4) developing reliable protocols for application of the compact flow cytometer in different food sectors (e.g. dairy products, beer, vegetables, and meat). FCM analyses were performed with a Bactiflow GL100 (Partec-Chemunex) equipped with a 100 mW YAG green laser diode operating at 532 nm, and with 4 parameters (2 scatters, FSC and SCC, and 2 fluorescence, orange FL1 and red FL2). Data acquisition, analysis and real time display were performed with Partec Flomax software. The flow cytometry methods were evaluated and compared to standard methods. The fluorescent stains used were the nucleic acid stains SYTO 81 orange (membrane permeable), propidium iodide (PI, membrane impermeable), SYTOX Orange (membrane impermeable), JOJO-1 (membrane impermeable), and hexidium iodide (Gram reaction), DiSBAC₂(3) (membrane potential, anionic dye), and C12-resazurin (metabolic activity).

The PI-SYTO 81 combination enables distinction of live/dead bacterial and yeast cells. This assay includes two proprietary nucleic acid stains, which differ in their ability to permeate the microbial cell membrane. Cells with intact membranes allow only SYTO 81 to enter the cell and fluorescence orange, whereas cells

with damaged membranes allows the PI dye to enter and fluoresce red. Viability studies after treatment of microbes with disinfectants, heat, high osmotic pressure, antibiotics and other antimicrobial compounds are possible based on the individual staining pattern. DiSBAC₂(3) is a negatively charged dye whose binding and fluorescence are decreased upon membrane energisation (live and active cells) and increased in non-active and death cells. Figure 3 shows the FCM results of these two viability assays applied on the same sample.

Before flow cytometric analysis can be carried out in milk and dairy products, milk fat and protein must be 'cleared' to avoid optical interference. A range of clearing agents such as enzymes, detergents and chelating agents was tested.



The different milk treatment steps and combinations thereof were followed by PI-SYTO 81 staining and FCM analysis. This resulted in high background counts and, therefore, it was not possible to improve the bacterial FCM count in milk $< 10^5$ cfu per ml. Based on this procedure a somatic cell assay for raw milk with a sensitivity of 10^5 cow cells per ml, which is below the standard level for good quality raw milk, and a total yeast count assay for fermented milk products with a detection limit of 5×10^4 cells per ml were developed. The flow cytometric PI-SYTO 81 assay was also applied to dairy starter cultures (freeze-dried and concentrated frozen) and probiotic products (freeze-dried and commercial probiotic drinks). In each of these products the bacteria were heterogeneous with respect to membrane integrity. The FCM viability assay with PI and SYTO 81 provided a clear discrimination between intact cells, injured or damaged cells, and dead cells. The proportions of these populations in the products tested differed. The FCM assay appeared to be very accurate and highly sensitive, providing tools to assess the functionality of different populations in fermentation starters and probiotic products.

The major obstacle with frozen vegetables is to differentiate bacteria from other debris in the samples because of the interference by the food matrix, i.e. suspended particles and autofluorescence. With samples of carrot, leek and spinach, the background particle counts were too high to allow reliable measurements to be made (detection limit $> 10^5$ bacterial cells per g).

FCM offers a rapid yeast viability assay, being in fact more rapid, objective and accurate than the methylene blue staining microscopy assay. A DNA staining protocol for yeast cells with propidium iodide (cell cycle analysis) was applied. The application of FCM for the determination of microbial contaminants in beer was also investigated. The minimal detection limits of yeast and lactic acid bacteria in beer were 10^2 - 10^4 and 10^3 - 10^4 cfu/ml, respectively, depending on the type of beer and sample treatment (centrifuging and washing).

7. Conclusions

In view of its versatility FCM is expected to become widely used in many fields soon. FCM offers opportunities for rapid and diverse analyses of microbial samples. The availability of low price, visible-wavelength diode lasers with a high efficiency and long lifetime is expected to promote broader applications of FCM in food microbiology. In addition, the advances in the available range of fluorescent dyes (physiological and molecular probes) make it possible to

determine the physiology of microbes and open the way to highly accurate and sensitive detection of micro-organisms in food.

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A novel PCR method for the detection of live micro-organisms

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Abstract

The spoilage of beer by bacteria is of great concern to the brewer as it can lead to turbidity and/or abnormal flavours. Detection methods based on the polymerase chain reaction are highly specific and provide results much faster than traditional microbiology techniques. However, one of the drawbacks is the inability to differentiate between live and dead cells. A novel method for live/dead cell determination using ethidium monoazide bromide is presented. Ethidium monoazide bromide can traverse dead cell membranes and bind covalently to DNA under exposure to light. The bound DNA cannot be amplified by the polymerase chain reaction, thus preventing the detection of dead cells.

Keywords: live/dead cell discrimination, ethidium monoazide bromide, real-time PCR

1. Introduction

The boiling step in the brewing process inactivates most of the organisms present and the final beer is a relatively poor medium for bacterial growth. It has a pH of about 4.5 to 4.0 and has a low concentration of sugars. Additionally, the typical ethanol content of 4-5% w/v and the bitter compounds, the iso- α -acids, have antimicrobial properties. Therefore, the range of bacteria that commonly contaminate is small. However, an infection by micro-organisms often leads to turbidity and abnormal flavours developing over time. Commonly, these can be traced back to either bacteria or non-brewing yeast (wild yeast). The lactic acid bacteria, of which *Lactobacillus* and *Pediococcus* are the two most commonly encountered species, is a group of bacteria, which can cause serious problems in breweries. *Megasphaera* and *Pectinatus* are two other types of spoilage bacteria mainly found in canned or bottled beers, as they only grow anaerobically.

In the brewery, measures are taken to minimise the risk of contamination by micro-organisms. For example, vessels can be washed with caustic soda killing any microbes effectively. Before they leave the brewery some beers are pasteurised, again reducing the number of live cells dramatically. Despite many precautions beers occasionally get infected. Low level contamination, although not a problem while the product is still fresh, might become problematic as micro-organisms proliferate during the beer's shelf-life. This can lead to consumer complaints and damage the image of the product, leading to a decline in sales.

Therefore the brewer requires a rapid sensitive method as a quality control tool for screening samples before release onto the marketplace. This tool should allow the detection of low numbers of cells and be quick for a rapid product release. The polymerase chain reaction (PCR) potentially fulfils these requirements. To test this and optimise the technique for beer a 3-year EU-funded project was set up (QLK1-CT-200-01251:BREWPROC). Brewing Research International and the Technical University of Munich were two of the participating research partners. PCR is a rapid method; with real-time PCR, allowing DNA amplification and detection simultaneously, sample analysis is performed within a few hours. On the other hand, the traditional microbiological methods of growing samples on agar plates require up to a week of incubation. PCR is also a very sensitive method, allowing detection of cells at low levels. Additionally, with the right choice of primers (oligonucleotides detecting the DNA of interest), the method can be either used as a 'screening' tool detecting a group of bacteria or as an 'identification' tool detecting one type of bacterium specifically. Despite all these advantages PCR also has a major disadvantage. The technology cannot discriminate between live and dead cells. The first step in a PCR analysis is extraction and purification of DNA from cells present in the sample. It is possible, for example after pasteurisation, to find dead bacteria or yeast in the beer. Therefore, extracted DNA can be from live or inactivated cells and during PCR amplification all target DNA will be amplified equally. This will lead to false-positive results from the dead cells.

A number of molecular methods have been tested to circumvent this problem (Keer and Birch, 2003). However, none of these are completely satisfactory. We attempted to analyse RNA (using reverse transcription PCR) rather than DNA, as RNA is known to enzymatically degrade more rapidly than DNA once a cell is killed. However, we found that after cell death the RNA signal persisted for days or weeks depending on the microorganism tested. Others (McKillip *et al.*, 1999) have also reported the persistence of DNA and RNA following cell death.

Another possible method exploits the density difference between live and dead cells, separating these on a density gradient before DNA extraction (Wolffs *et al.*, 2004). The simplest way to overcome false-positive results from non-viable cells is to incubate the sample in a suitable medium for several days, which allows the viable micro-organisms to outgrow the dead ones. But the addition of several days to the detection time defeats the objective of rapid analysis. Here, we tested a new technique using a dye molecule, ethidium monoazide bromide (EMA), which is usually used to study cell viability by selectively fluorescently labelling DNA from dead cells. EMA is only able to enter dead cells and binds covalently to DNA under light exposure, thereby blocking the PCR amplification of the bound DNA from inactivated micro-organisms (Nogva *et al.*, 2003).

2. Materials and methods

Saccharomyces cerevisiae cultures (NCYC 1324) were grown overnight in YM broth at 25 °C. *Lactobacillus brevis*, *L. lindneri*, *L. casei*, *Pediococcus damnosus*, *Megasphaera* and *Pectinatus* cultures were grown in MRS broth in anaerobic jars at 25 °C for 5 days. 2 ml of the cell culture was retained as a control (no EMA added) and the remaining culture treated for cell inactivation. Heat inactivation was achieved by 1 hour incubation in a 60 °C waterbath or 5 minutes in a 76 °C waterbath (here 0.5 ml sample volume was used). Alternatively, cell death was obtained by resuspending pelleted cells in diluted alkaline beer line cleaner (Savol) with gentle stirring for 30 minutes as indicated in the instructions. To determine the cell concentration before treatment and to confirm cell death following inactivation each sample was serially diluted. Then aliquots of the diluted samples were plated on either YM or MRS agar plates, which were grown either aerobically or anaerobically at 25 °C for 3 or 7 days for yeast or bacteria, respectively. 1 ml of inactivated cell culture was pipetted into a clear Eppendorf tube. EMA (Molecular Probes Europe BV) was first dissolved in water and then concentrated solution was added to samples at final concentrations of 10, 25 or 50 µg/ml. EMA is potentially carcinogenic and was handled accordingly. The sample tubes were wrapped in aluminium foil and incubated on ice away from light for 2 or 4 minutes. The tubes were then unwrapped, put on the side of a glass dish filled with ice and exposed to light from a 600W halogen lamp for 3 minutes or 300W for 2 minutes. DNA was extracted from untreated and inactivated samples with or without EMA addition. Cells were recovered by centrifugation and DNA was extracted using either the traditional phenol-chloroform method or by vortexing with glass beads to mechanically rupture cells. DNA amplification for the standard PCR was performed on a Hybaid

thermocycler. The primer pair for yeast was specific for a gene coding for a protein synthesis elongation factor (Eft- α). For the detection of *L. brevis* another specific primer pair was used based on the 16S rDNA sequence. After amplification in the thermocycler products were visualised on a 1.3% agarose gel stained with ethidium bromide. A 100 base pairs (bp) molecular weight marker was added at both sides of the gel and after electrophoresis bands were inspected in an ImageMaster (Pharmacia). Real-time PCR was either performed on an ABI Prism 7000 (Applied Biosystems) or an iCycler (BioRad). The TaqMan primers and probe system used for *S. cerevisiae* were from a yeast and mould screening kit (Pika, Weihenstephan). For the real-time PCR detection of bacteria TaqMan screening systems for either *Lactobacillus/Pediococcus* or *Pectinatus/Megasphaera*, both developed at the Technical University of Munich, were employed.

3. Results and discussion

When EMA is exposed to light it forms a reactive nitrene, which then reacts with water and loses its reactivity. EMA has also the ability to intercalate into nucleic acids. If it is photoactivated while tightly bound to DNA, a covalent complex is generated (Hixon *et al.*, 1975). When a cell's wall is damaged the cell dies. Its DNA can escape by permeating through the leaky cell wall where it will eventually be degraded by enzymes. If the DNA remains inside the damaged cell, it might be better protected from enzymatic attack. If EMA is added it intercalates into accessible DNA, which is either free or in damaged cells that EMA can permeate into. However, EMA cannot access DNA from live cells with undamaged non-permeable cell walls. When the EMA+cell mixtures are exposed to light, EMA is photoactivated and either becomes inactivated by reaction with water if unbound or forms a covalent bond with nucleic acid it is bound to. DNA with covalently bound EMA cannot be amplified by PCR as the polymerase cannot pass the point of attachment on the DNA. The result is that DNA from dead cells will not be detected, but the signal from live cells will persist. This should avoid false-positives from dead cells. The mechanism is presented schematically in Figure 1. Some good results have been obtained using this technique with bacteria in pure cultures or food systems (Nogva *et al.*, 2003; Brandl *et al.*, 2003; Rudi *et al.*, 2005a,b).

For our trials, initially yeast was used as the test organism. *S. cerevisiae* cultures (about 1.75×10^7 cells/ml) were either pasteurised by holding them at 60 °C for 1 hour or resuspended and gently stirred in alkaline beer line cleaner for 30 minutes. Both treatments resulted in cell death as confirmed by the absence of growth on

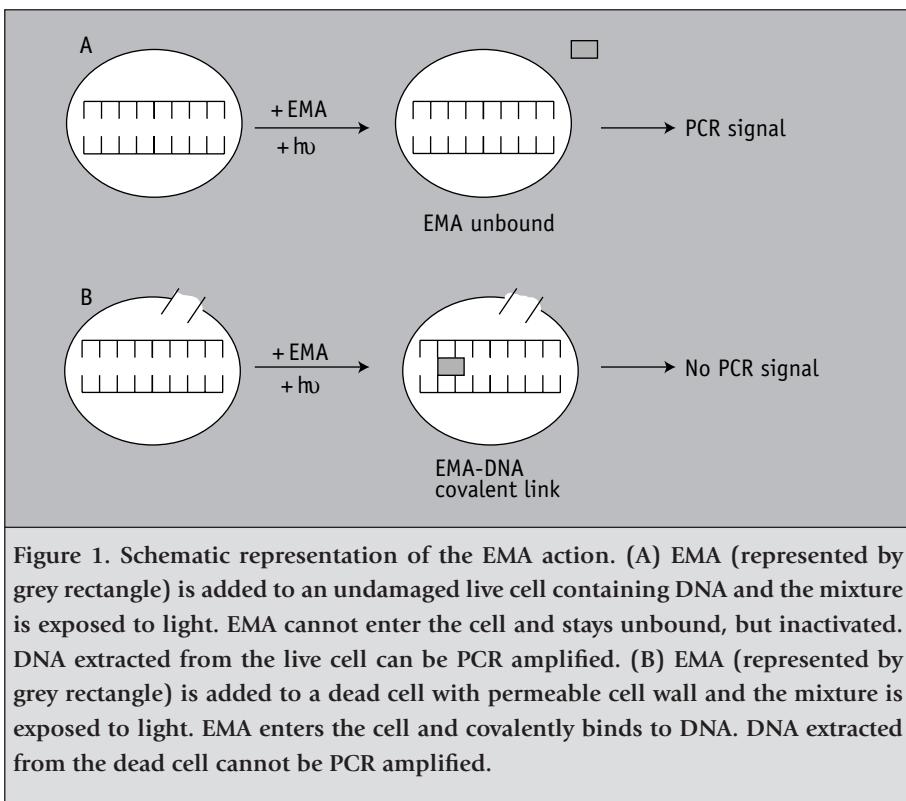


Figure 1. Schematic representation of the EMA action. (A) EMA (represented by grey rectangle) is added to an undamaged live cell containing DNA and the mixture is exposed to light. EMA cannot enter the cell and stays unbound, but inactivated. DNA extracted from the live cell can be PCR amplified. (B) EMA (represented by grey rectangle) is added to a dead cell with permeable cell wall and the mixture is exposed to light. EMA enters the cell and covalently binds to DNA. DNA extracted from the dead cell cannot be PCR amplified.

agar plates. One aliquot of each treated sample and a part of the untreated sample were mixed with EMA and exposed to light for photoactivation. DNA was extracted from all samples and amplified by PCR. Resulting PCR product was visualised by gel electrophoresis. As can be seen in Figure 2 live yeast cells give strong bands, whether EMA is added or not. This is to be expected, as EMA does not bind to DNA from live cells because it is not accessible when located in an undamaged cell. On the other hand, the pasteurised yeast sample exhibited a clear band with no EMA present, indicating that dead cells are detected. On addition of EMA, however, the signal disappears. The false-negative result has been suppressed. In the case of treatment with beer line cleaner there is no band detectable at all. Even the samples not containing EMA are negative. It is suspected that this harsh treatment disrupts cells completely and degrades DNA substantially, so that the target DNA for PCR is not intact anymore. It seems that pasteurisation is milder and DNA is less degraded, possibly by remaining inside the damaged cell wall.

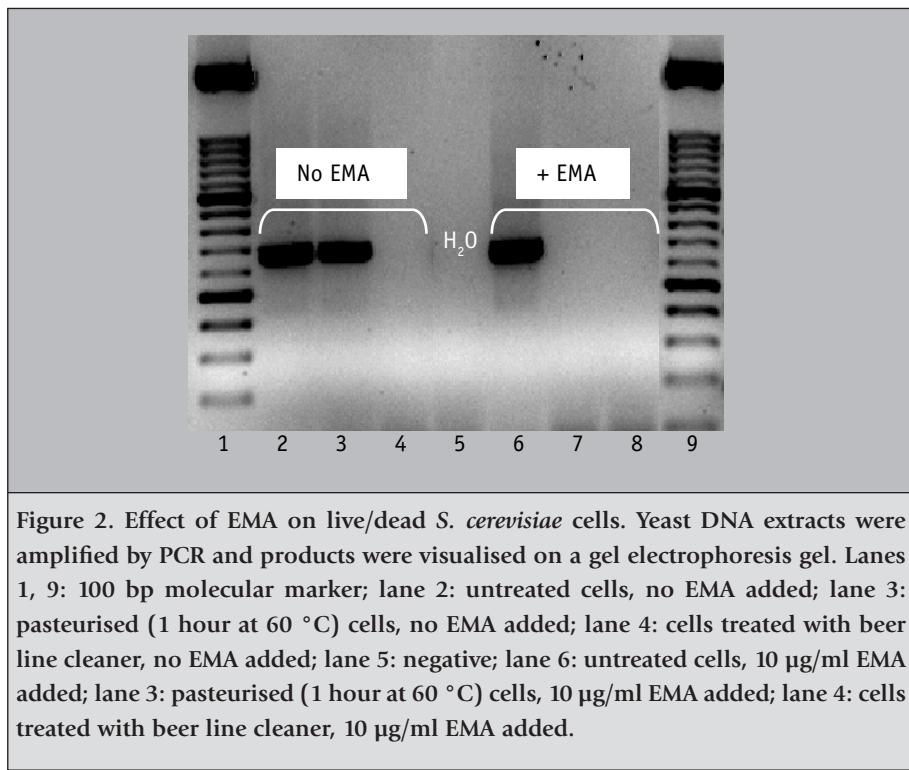


Figure 2. Effect of EMA on live/dead *S. cerevisiae* cells. Yeast DNA extracts were amplified by PCR and products were visualised on a gel electrophoresis gel. Lanes 1, 9: 100 bp molecular marker; lane 2: untreated cells, no EMA added; lane 3: pasteurised (1 hour at 60 °C) cells, no EMA added; lane 4: cells treated with beer line cleaner, no EMA added; lane 5: negative; lane 6: untreated cells, 10 µg/ml EMA added; lane 7: pasteurised (1 hour at 60 °C) cells, 10 µg/ml EMA added; lane 8: cells treated with beer line cleaner, 10 µg/ml EMA added.

A similar persistence of DNA was observed by McKillip *et al.* (1999) after mild inactivation treatment (3 hours at 60 °C) of *Escherichia coli*.

We also tested the applicability of the EMA-PCR mechanism to real-time PCR. Several mixes of live and dead yeast cells were prepared from an untreated and a pasteurised culture. The live to dead cell ratios were 0:1, 3:4, 1:2, 4:3 and 1:0. Parts of the solutions were supplemented with EMA and photoactivated as described above. DNA was extracted from all samples and real-time PCR carried out using a commercial yeast and mould screening kit. Real-time amplification plots are displayed as fluorescence versus PCR cycle number (40 cycles in our case) (see Figure 3). Results were analysed on-screen and threshold cycle values, the so-called C_t values, were determined. These are defined as the cycle at which the sample curves cross the threshold line. Positive samples exhibit an increase in fluorescence during the PCR run, whereas negative samples do not show any significant rise before the 35th cycle. The earlier in the run the curve rises, i.e. the

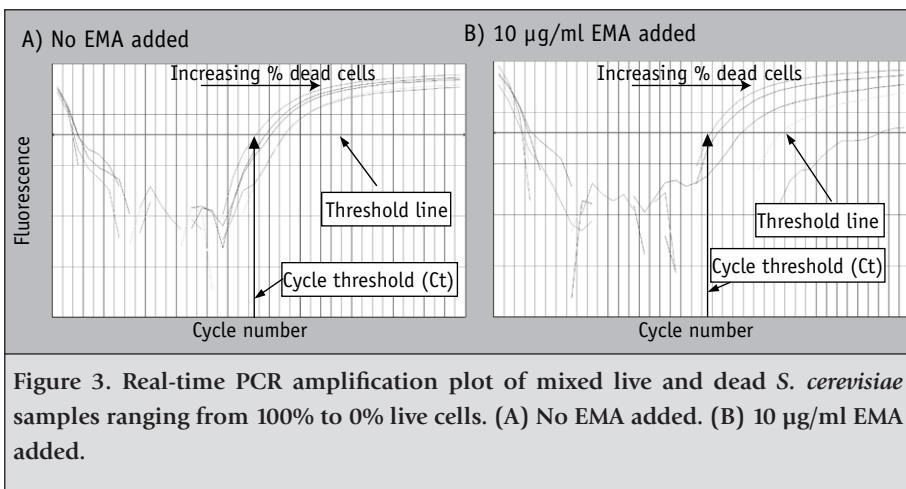


Figure 3. Real-time PCR amplification plot of mixed live and dead *S. cerevisiae* samples ranging from 100% to 0% live cells. (A) No EMA added. (B) 10 µg/ml EMA added.

lower the C_t value, the higher the target DNA concentration and the stronger the PCR signal is. Figure 3A shows the curves obtained for the samples with no EMA added. All of them lie close together with only slight differences in C_t values. Again we see that dead cells produced false-positive results. Even the sample consisting of 100% dead cells had a relatively low C_t value, i.e. a strong PCR signal. On addition of EMA the curves become more dispersed (Figure 3B). The samples with more dead cells exhibited a lower signal due to the contribution from dead cells being suppressed by the action of EMA.

The C_t values of this experiment are summarised in Table 1. The samples without EMA have values between 22 and 25, only showing a slight decrease in signal from live to dead samples. A regular real-time PCR experiment would therefore give a strong positive result for the sample containing 100% dead yeast, clearly a problem when dead cells are of no interest. The addition of EMA leads to increased C_t values for all samples containing dead yeast cells, corresponding to a weakening of the PCR signal. Any values above 35 are considered negative. The 100% dead cell sample is therefore negative, meaning that dead cells are not detected anymore. As the percentage of live cells in the sample increases, the difference between C_t values with and without EMA diminishes until identical results are obtained with the 100% live sample. Clearly, EMA has worked very successfully in this experiment. Thus, it can be employed for live/dead cell discrimination with standard as well as real-time PCR.

Table 1. Real-time PCR results of mixed live and dead *S. cerevisiae* samples.

Dead cells (%)	C _t value – no EMA	C _t value – 10 µg/ml EMA
100	25	39
75	24	30
50	23	25
25	22	23
0	22	22

In addition to yeast, we also wanted to test the effectiveness of EMA to differentiate live and dead bacteria. In the first instance *L. brevis*, a common beer spoiler, was employed. A bacterial culture containing about 9.1×10^8 cells/ml was divided in two and one part was pasteurised as described above for yeast. Cell death was assured by no growth on agar plates. One aliquot of each, untreated and pasteurised, was subjected to EMA treatment and then DNA extracts of all samples PCR were amplified. The resulting gel was as promising as for yeast. Figure 4 shows that the untreated control sample not exposed to heat had a strong band, whether EMA is present or not. The pasteurised bacteria sample has a similarly strong signal before addition of EMA, indicating again the detection of dead cells. The band, however, practically disappeared after EMA treatment. Again PCR amplification of dead cell DNA is inhibited.

Next the effect of EMA with a wider range of beer spoilage bacteria in beer was investigated. Beer was used since it was possible that beer ingredients might interfere with the EMA mechanism, making the use of this methodology unsuitable for direct detection in the drink. Bacterial cultures of *L. lindneri*, *L. brevis*, *L. casei*, *P. damnosus*, *Megasphaera* and *Pectinatus* were spiked into beer giving cell concentrations of 5×10^6 cells/ml (lactic acid bacteria) or 7×10^6 cells/ml (*Megasphaera* and *Pectinatus*). 0.5 ml of each sample was heat treated at 76 °C for 5 minutes and EMA was added at 50 µg/ml, followed by incubation in the dark and subsequent photoactivation. DNA was then isolated from the samples by centrifugation and vortexing with glass beads to rupture the cells. The real-time PCR result is shown in Table 2. All bacteria were clearly detected in the untreated samples as indicated by the low C_t values. After pasteurisation, cell death was assured by the lack of colonies on agar plates. The use of EMA had a variable effect for different bacterial species. In most cases, the signal is

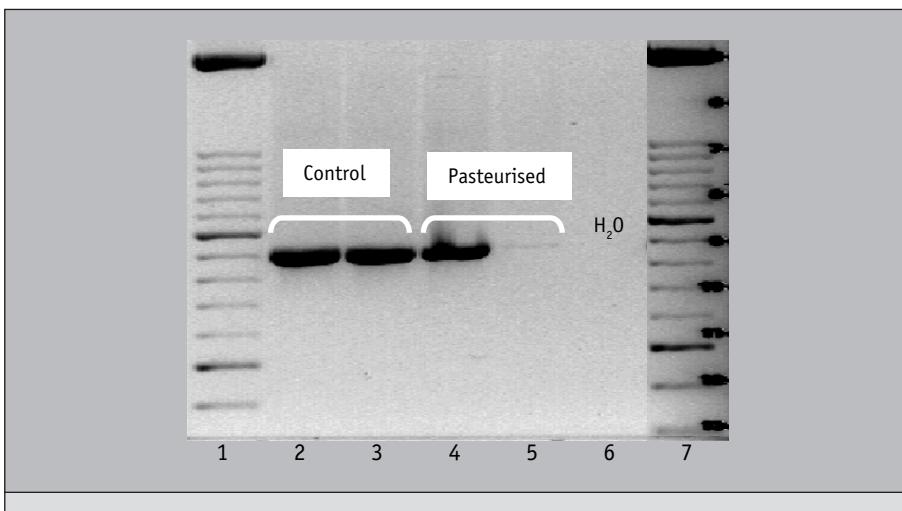


Figure 4. Effect of EMA on live/dead *L. brevis* cells. Bacterial DNA extracts were amplified by PCR and products were visualised on a gel electrophoresis gel. Lanes 1, 7: 100 bp molecular marker; lane 2: untreated cells, no EMA added; lane 3: untreated cells, 10 µg/ml EMA added; lane 4: pasteurised (1 hour at 60 °C) cells, no EMA added; lane 5: pasteurised (1 hour at 60 °C) cells, 10 µg/ml EMA added; lane 6: negative.

Table 2. Real-time PCR results for untreated and pasteurised bacterial samples.

Species of bacteria	C _t value	
	Untreated sample, no EMA	Heat treated, 50 µg/ml EMA
<i>L. lindneri</i>	24	not detected
<i>L. brevis</i>	22	38
<i>L. casei</i>	20	30
<i>P. damnosus</i>	22	27
<i>Megasphaera</i>	20	27
<i>Pectinatus</i>	23	22

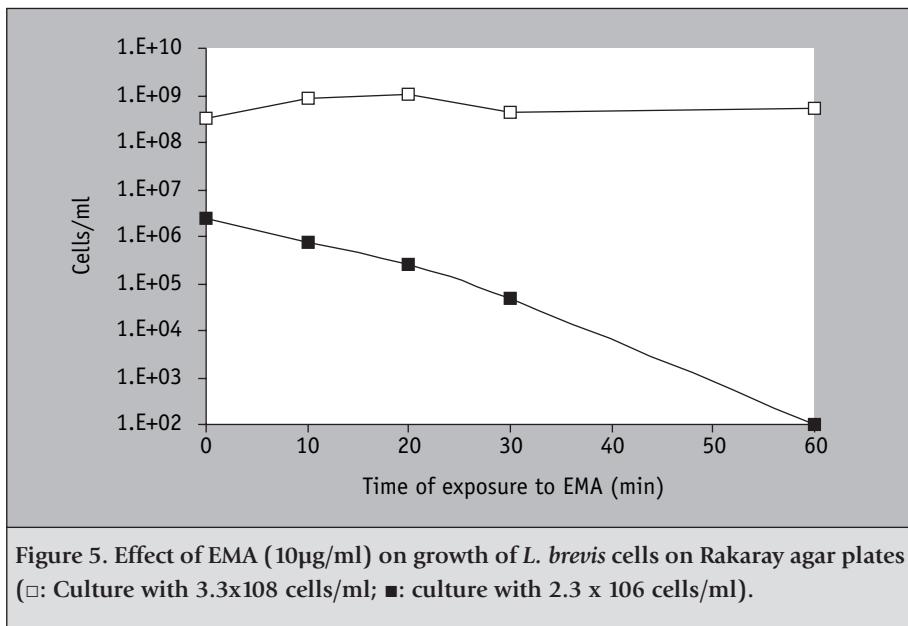
reduced compared to that from untreated sample. For *L. lindneri* and *L. brevis* the EMA treatment worked successfully, inhibiting the dead cell signal. This suggests that the EMA mechanism can perform without any problem in beer. However, with the other bacterial species the results were less convincing. The dead cell signal was not completely inhibited, increasing the C_t values only to 27 or 30, which is still considered a clear positive. The result for *Pectinatus* was particularly disappointing with no suppression of the dead cell signal. *Pectinatus* and *Megasphaera* belong to the group of Gram-negative bacteria. These bacteria have a cell wall structure different from the Gram-positive lactic acid bacteria. It can be speculated that EMA is not able to traverse the Gram-negative cell wall as easily as the Gram-positive one, influencing its effect for this group of bacteria, but EMA addition to some of the inactivated Gram-positive species (*Pediococcus*, *L. casei*) did not result in total reduction of the dead cell signal either. Additionally, several publications (Nogva *et al.*, 2003; Rudi *et al.*, 2005a) have shown EMA to work successfully for Gram-negative bacteria like *E. coli*, *Salmonella* and *Campylobacter*. Therefore, the difference in bacterial cell wall structure cannot account for all the differences in the PCR results. The common technique, also used in this study, to test for cells being dead after inactivation is their inability to form colonies on agar plates. However, in addition to dead and alive, cellular organisms can also exist as 'viable but not culturable' (VBNC) (Keer and Birch, 2003; Rudi *et al.*, 2005a). In this state they cannot grow on an agar plate, but are still alive; thus they would not be detected as live on plate culture, but would be detected with the EMA-PCR method. Such VBNC cells might have been produced during our inactivation treatment and, as their cell walls are intact, EMA will not enter them resulting in a discrepancy between plate and EMA-PCR signal.

The effect of EMA on untreated cells not exposed to heat, was also studied. Bacterial cultures were spiked into beer to a cell concentration of 5×10^5 cells/ml. EMA was added at a level of 25 µg/ml and the samples were illuminated after a 2 minutes incubation. DNA was extracted using the bead-beating method and real-time PCR was carried out. The results are tabulated in Table 3. It appears that the signal for all samples was reduced with EMA. This phenomenon has been observed previously (Nogva *et al.*, 2003; Brandl *et al.*, 2003) and various explanations can be put forward. Any untreated cell culture could be expected to consist of live cells only. However, there are commonly some dead cells present. These would be picked up by the EMA mechanism leading to a signal reduction on addition of the dye. Also, it is possible that EMA itself inactivates the live cells. This was supported by an experiment in which EMA (10 µg/ml) was added to

Table 3. Effect of EMA on untreated bacterial cells.

Species of bacteria	C_t value	
	Untreated sample, no EMA	Untreated sample, 25 µg/ml EMA
<i>L. lindneri</i>	29	34
<i>L. brevis</i>	28	30
<i>L. casei</i>	26	32
<i>P. damnosus</i>	25	30

a culture of *L. brevis* (2.3×10^6 cells/ml). The cell+EMA solutions were incubated for up to an hour and aliquots plated out. A gradual decrease in the number of colonies was observed down to 100 cells/ml at the end of the test (see Figure 5). The dye is somehow able to inactivate the cells by an unknown mechanism. In our experiments EMA incubations were a maximum of 4 minutes. However, during this time half of the cells might be inactivated according to these results.



Therefore, incubation times should be kept to a minimum, but still sufficiently long for EMA binding to accessible DNA. Interestingly, the cell culture being 100 times more concentrated did not show the same effect. These cells seem to grow well on plate, maybe because of the EMA concentration not being high enough for cell inactivation at this high cell concentration. Thus, for EMA treatment to successfully detect live cells, the EMA incubation time should be minimised as well as the EMA concentration should be carefully dosed depending on cell concentration. It is possible that different cell species exhibit different sensitivities to EMA with some being resistant to inactivation.

Finally, EMA-PCR was tested in a 'real life' setting. For this purpose a wheat beer was obtained from a commercial German brewery. Before packaging it was 'naturally' contaminated with *L. brevis* and pasteurised. The bacteria in the beer were therefore all dead and no growth was detected on plates. The C_t value for the sample prior to EMA addition was 28, but with 20 µg/ml EMA and 4 minutes incubation followed by photoactivation the real-time PCR signal vanished. This shows that the EMA-PCR method is applicable to real brewing samples.

4. Conclusions

Some inactivated cells are detected with conventional PCR, as no differentiation is made between DNA from live or inactivated cells. This is a considerable weakness of the PCR methodology as it can lead to false-positive results. Here we have found that EMA treatment prior to PCR generally reduces the signal from pasteurised cells. EMA is able to enter cells with compromised cell walls and intercalate into dead cell DNA. On light exposure a covalent DNA-EMA complex is formed. This bound DNA cannot be PCR amplified. It was shown that the EMA-PCR system is applicable to standard PCR (detection of PCR product on gel) as well as real-time PCR. It appears that the mechanism functions well with some micro-organisms like *L. brevis*, *L. lindneri* and *S. cerevisiae*, but not so well with others. The reason for this might have to do with VBNC cells, which have been damaged sublethally. Such cells have an intact cell wall, but do not grow on agar plates. They are therefore detected as live with EMA-PCR, but are considered dead on plating. EMA itself seems to inactivate bacterial cells when these are incubated with the dye. It is therefore recommended to keep the EMA exposure time to a minimum. Finally, it was proved that EMA-PCR can be successfully applied to commercial beer samples. In summary, the treatment of beer samples containing micro-organisms with EMA prior to PCR has great potential for reducing the false-positive signal from inactive cells. However, care

has to be taken when employing this method to minimise cell inactivation by limiting EMA incubation times.

Acknowledgements

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A rapid analytical technique for determining oxidation stability of food products

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Abstract

The food industry is continually developing new products to explore new market opportunities. The success of any new development is based on getting the right product to the right market area at the right price. The determination of product shelf life is fundamental to its quality and, therefore, a key factor to its final success. Many changes in formulas or technologies occur, so the importance of gaining the necessary information for determining product shelf life cannot be stressed enough. A rapid analytical technique was used to examine fat oxidation, which is a main factor affecting product shelf life. This approach provides valuable information on oxidation stability of raw materials and food products.

Keywords: fat oxidation, oxidation stability, oils, fats

1. Introduction

Chemical reactions occurring between atmospheric oxygen and food sensitive components, are some of the most important causes of product degradation. Specifically, fat oxidation is recognised as one of the main factors affecting food shelf life, even for low fat products, causing product rancidity by the formation of off-flavours due to aliphatic aldehydes or other volatile compounds. Therefore, oxidation stability is one of the most important product characteristics to be evaluated during food product development and control.

Typical analyses to evaluate oxidation stability include:

- organoleptic testing;
- evaluation of oxidation markers on raw material such as the peroxide value (titration method), anisidine value (colorimetric method), TOTOX value (calculated as anisidine value plus 2x peroxide value), aldehyde content (high performance liquid chromatography method);
- Rancimat method for oils and fats (conductivity measurement).

However, more accelerated tests predicting the stability of finished products are needed to shorten the time of shelf life studies. In this paper the use of a rapid analytical technique, which is able to provide information related to fat oxidation in samples of foods, oils and fats, is described in more detail.

2. Evaluation of oxidation stability

2.1. Rapid analytical technique

The evaluation of oxidation stability of food samples can be performed in an accelerated way, at comparatively high temperatures, in the presence of a measurable oxygen pressure. Useful information is obtained by recording the decrease in oxygen pressure, since oxygen is consumed during fat oxidation. This technique, based on ASTM standard D942-02 (ASTM International) proved to be very useful for this type of evaluation. In previous studies by Barilla in collaboration with TNO (the Netherlands), it appeared possible to study the relative oxidation stability in different formulas of pasta sauces (Bersellini, 2001).

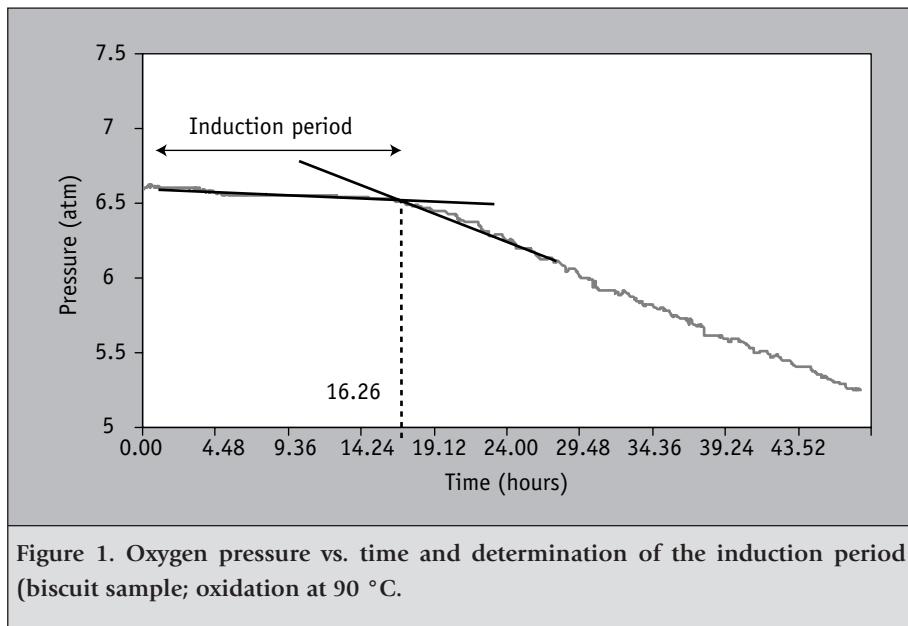
Unfortunately, these studies, using the oxidation test reactor designed according to ASTM D942, could be performed only on products having a high fat content (>10%). Products and raw materials with very low fat content could not be analysed. To tackle this problem Barilla started a collaboration with Velp Scientifica, taking into consideration the previous experiences. The original instrument was completely re-designed, exclusively with the aim to increase its sensitivity and, hence, its application field.

Particularly, to increase the signal of the instrument and to reach higher sensitivity levels, the importance of the following aspects was noticed:

- increasing the sample amount, which can be introduced;
- reducing the oxygen volume to make the pressure drop more evident;
- increasing the sample exposure surface and reducing thickness to make the oxidative process more homogeneous;
- wetting the sample to avoid burning, if needed;
- providing the management software with a smoothing system for the signal to counterbalance small temperature variations of the thermostat, which causes pressure oscillating variations;
- improving the stability of the temperature in the chambers to reduce oscillation in the pressure during data acquisition, which is important to get

a stable behaviour of oxygen pressure (low noise) and to establish properly the induction period.

With the new version of the oxidation test reactor, Oxitest (Velp Scientifica, Italy), it is now possible to introduce up to 80-100 g of sample (depending on its density) into the oxidation chamber. The surface area of the sample is increased and the available volume for oxygen has been strongly reduced. The sample is submitted to an oxygen pressure of ~6 bar and high temperature, usually in the range 90-110 °C, with 90 °C the most used. When oxygen consumption starts, the pressure drops. A particular useful parameter is the induction period, the time during which oxidation proceeds at a relatively slow rate (Figure 1). The longer the induction period, the higher the oxidation stability. The accompanying software controls the entire oxidation process of the sample and the induction period is calculated automatically. Further information is available on the amount of oxygen consumed and, more important, on the rate of this consumption. These improvements enable us to the measurement of oxidation stability of, for example, cereals with a fat content of ~3%. The instrument is equipped with 2 separate titanium oxidation chambers to analyse samples in duplicate or to analyse different samples simultaneously under the same conditions.



2.2. Fields of application

The new version of the oxidation test reactor is suitable to determine oxidation stability of raw materials (oils, fats), and all kinds of fat-containing food products without preliminary fat separation. Among the instrument's possible applications are:

- Rapid comparison of different formulas of a product by performing oxidation at a prefixed temperature, e.g., 90 °C. In less than two days an estimation of oxidation stability can be obtained (Figure 2a).
- Obtaining a prediction of oxidation stability during a shelf life study. Products are measured at defined time intervals and experimental curves are built (Figure 2b).
- Verifying the incidence of different lots of the same raw material(s) directly on the final product.
- Coupling to other types of analyses, e.g., gas chromatography, to evaluate formation of oxidation products and/or disappearance of flavour compounds. This application is important when a sharp drop in oxygen consumption is not visible (Figure 3).
- Evaluating the performance of different packaging materials. Figure 4 shows an example of the effect of different packaging materials on the oxidation stability of pasta sauce analysed after storage at defined temperatures for some months.
- Replacing, in some cases, the determination of peroxides, avoiding the use of organic solvents.

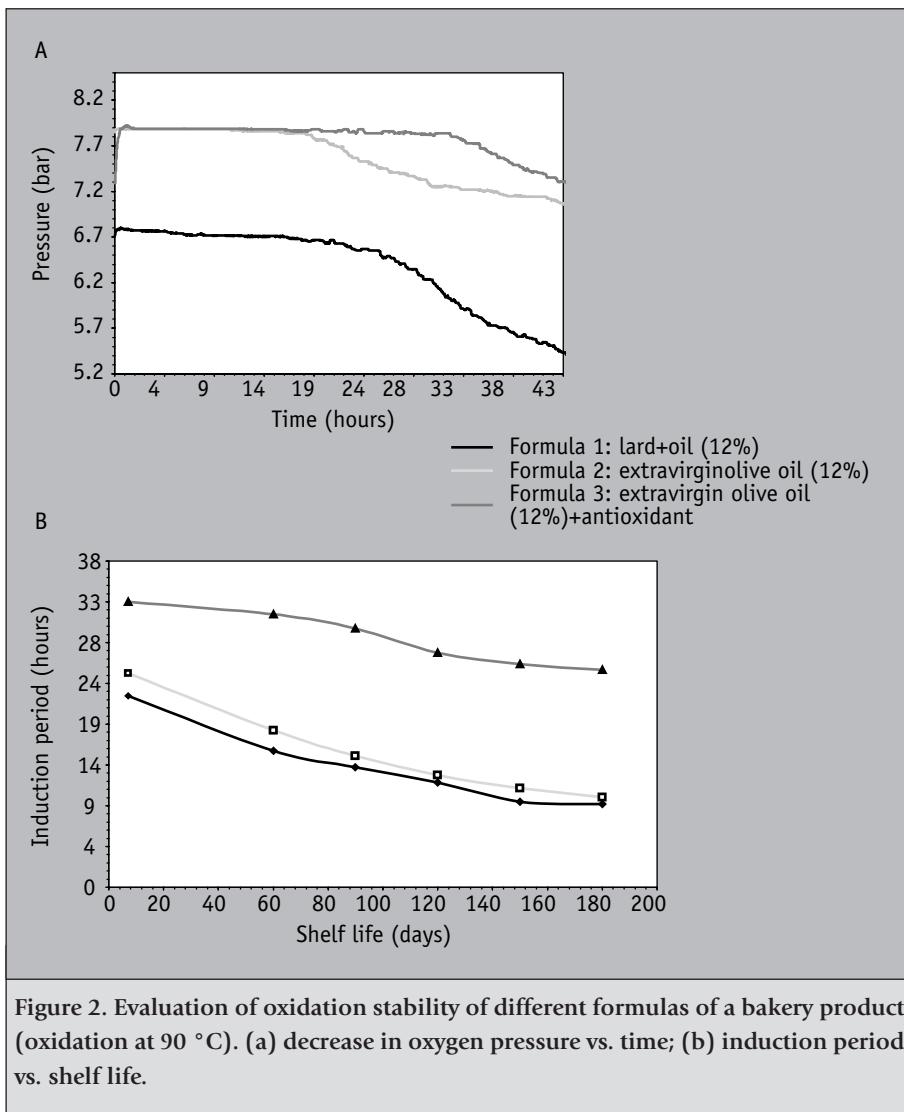


Figure 2. Evaluation of oxidation stability of different formulas of a bakery product (oxidation at 90 °C). (a) decrease in oxygen pressure vs. time; (b) induction period vs. shelf life.

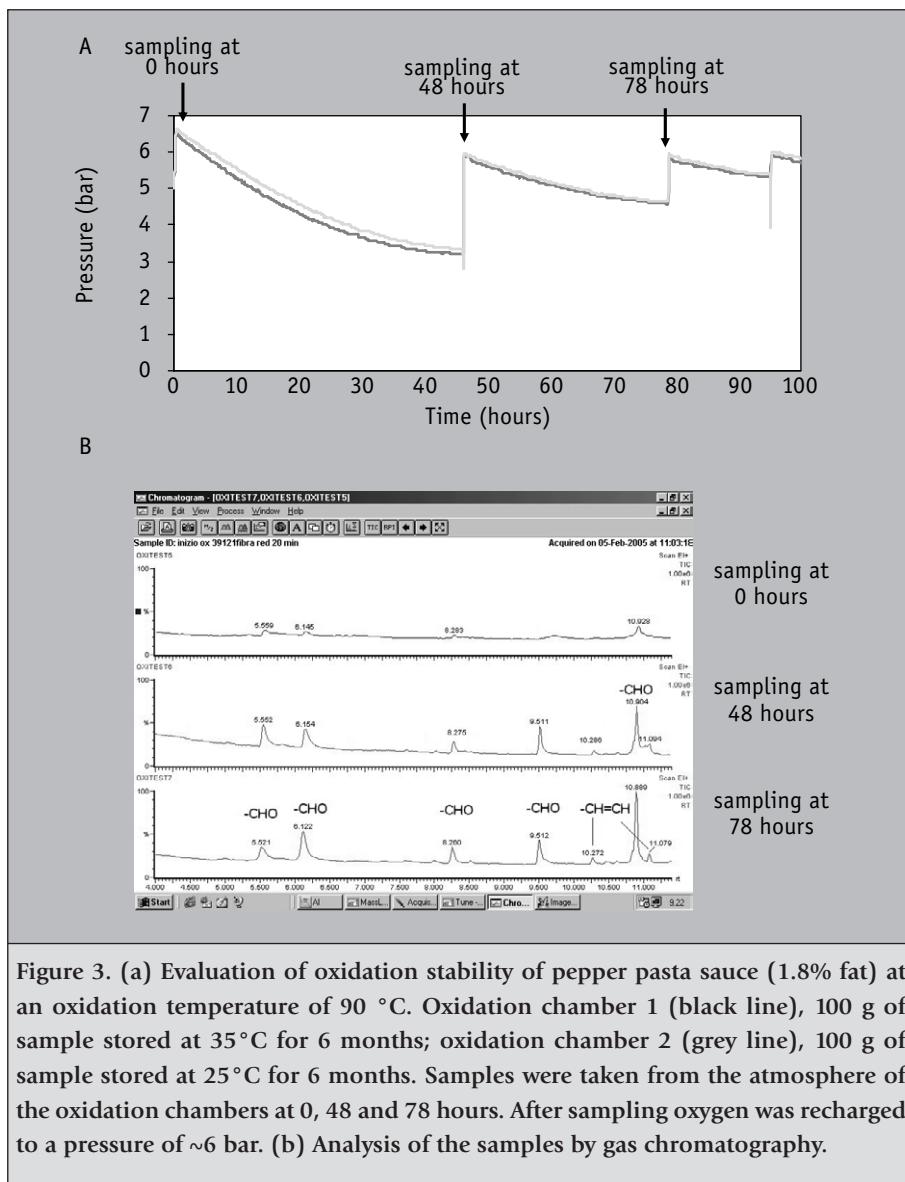


Figure 3. (a) Evaluation of oxidation stability of pepper pasta sauce (1.8% fat) at an oxidation temperature of 90 °C. Oxidation chamber 1 (black line), 100 g of sample stored at 35°C for 6 months; oxidation chamber 2 (grey line), 100 g of sample stored at 25°C for 6 months. Samples were taken from the atmosphere of the oxidation chambers at 0, 48 and 78 hours. After sampling oxygen was recharged to a pressure of ~6 bar. (b) Analysis of the samples by gas chromatography.

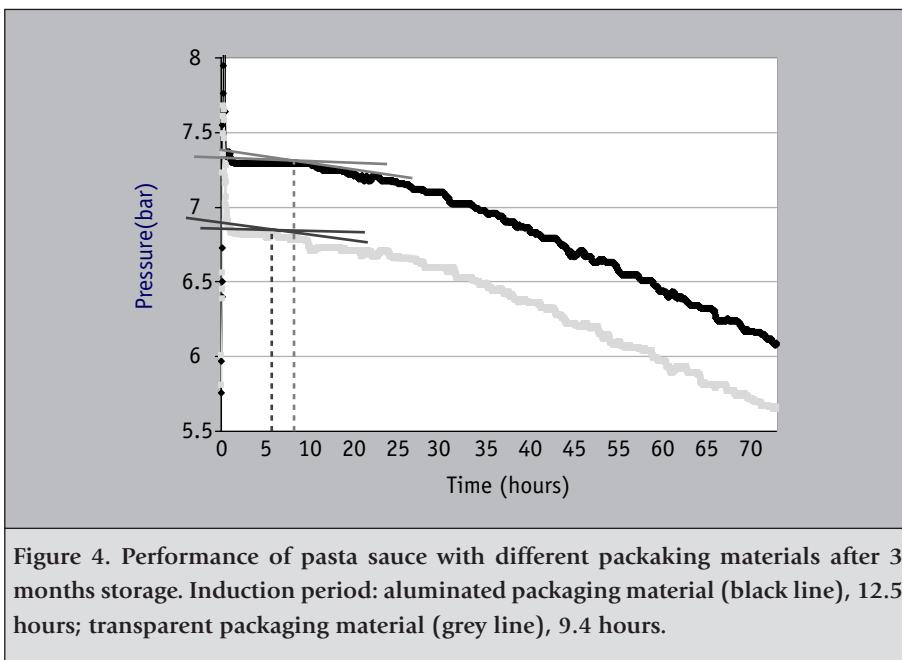


Figure 4. Performance of pasta sauce with different packaking materials after 3 months storage. Induction period: aluminized packaging material (black line), 12.5 hours; transparent packaging material (grey line), 9.4 hours.

3. Conclusions

The rapid analytical technique has been tested with satisfactory results. It appeared to be applicable to determine the oxidation stability of many food samples (liquid, semi-solid, solid), including raw materials and final products. The latter do not need separation of fat prior to the test. Some examples are bread, crispbread, biscuits, cakes, sauces, creams, meat, cheese, butter, lard, fish oil, dried fruit, vegetable oils, chocolate, cocoa, cereals, etc. It is concluded that the technique is suitable for providing useful information on fat oxidation in food development and quality control. This information would otherwise be obtained only by long-lasting and expensive instrumental and organoleptic tests.

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Considerations on labour- and cost-efficient immunoassay protocols and formats exemplified by the detection of food adulteration and fraud

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Abstract

Antibody-based analytical methods have been developed in a range of formats and have been shown to be effective for the rapid detection of adulteration. A review of the performance of the main species-specific immunoassays, which have been developed, has confirmed that formats such as ELISA still provide the best option for routine rapid analysis. Assays for novel adulterants that are rapid, specific and reliable can be easily developed using existing formats. Method developments that further increase sensitivity are less important for the food industry because of the frequently high concentrations of adulterants in foodstuffs. A demand for effective tests that can be operated outside of the laboratory environment has been identified. The lateral flow format appears to fulfil the demand for on-site testing, although the range of analytes to which this format can be applied needs to be extended. Recently developed nanoparticle antibody labelling methods will prove useful in achieving multi-analyte capability. The development of many additional commercial immunoassay tests is possible, however, matrix complexity warns that their use must only be recommended for foodstuffs for which they have been validated.

Keywords: food, adulteration, immunoassay, ELISA, lateral flow assay, transverse flow assay, dipstick assay

1. Introduction

Food adulteration is the process by which wholesome food is deliberately mixed with or replaced by lower quality or inappropriate materials. It has been known for centuries, with some of the earliest examples alluded to by the Romans over 2000 years ago (Collins, 1993). Despite the variety of legislation introduced

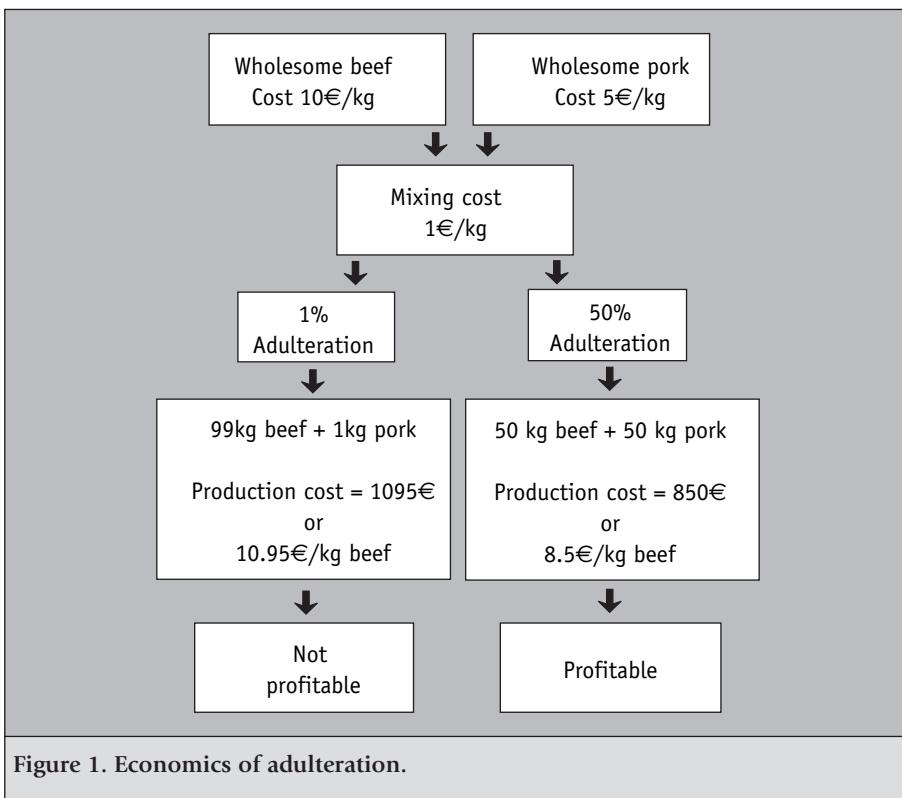
across the world, which is intended to end the practice of adulteration, this activity is believed to continue unabated. Hence it is the role of the analyst to detect adulteration in order for enforcement agencies to apply the legislation (FAO, 1987).

Adulteration is a deliberate act on the part of food manufacturers and/or food processors. It should not be confused with contamination, which is the accidental introduction of unwanted materials into the food (Watson, 2001). Adulteration does not normally result in microbial contamination of food materials. An exception would be those occasions when the adulterator mixes contaminated, usually old or condemned materials with the same type of fresh wholesome material in order to pass the entire consignment off as wholesome. Because adulteration involves the addition of inferior quality ingredients, attempts will often be made to disguise the adulterants, usually through the use of colourings and flavourings. These observations might provide additional circumstantial evidence of adulteration, however, they are not conclusive.

Adulteration is usually carried out to reduce costs and maximise profits, however, the process of adulterating food actually involves a cost to the adulterator. The adulterator is forced to adulterate the food with cheaper material on a large scale to recoup the costs of mixing and to achieve a profit from differential prices between the adulterant and the original food. Therefore, in discussing analytical techniques applicable to food adulteration, it may be generally assumed that the adulterant will be present in relatively large quantities when compared to contaminant levels. The economics of adulteration are a factor, which can be taken into account by the analyst when considering the type of assay to be used to detect the adulteration. On the basis that the adulteration process must be cost-effective to the adulterer, the analyst can assume that rapid analysis requires high specificity. A rapid assay for the detection of adulterants perhaps requires a detection limit of only 1%, as values below this are unlikely to be encountered (Figure 1). The analyst can therefore concentrate on ensuring that the assay is indeed rapid. For the purpose of this paper, a rapid assay is considered to be one in which the results are obtained in minutes rather than hours.

2. Assay formats

An initial consideration should be whether it is necessary to extract the analyte before carrying out the analysis, because this procedure reduces the rapidity of the assay. For rapid methods heterogeneous assays are typically preferable to



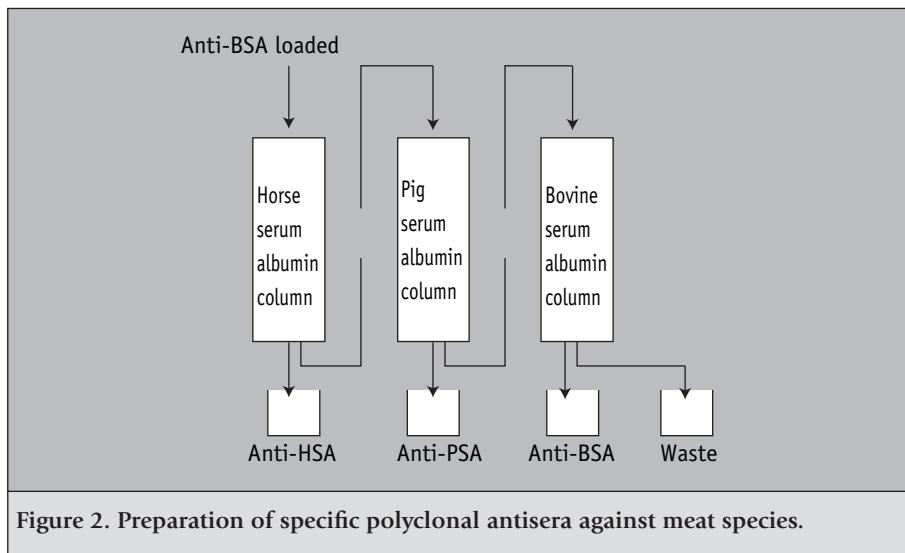
homogeneous assays (Gosling, 2000), however, it may be possible to combine extraction and analytical steps to produce a rapid format heterogeneous assay (Smith, 1992). Thus, in discussing immunoassays for the detection of food adulteration, the applications of different assay formats to the problem will be addressed (Smith, 1990).

Issues such as health and safety preclude the use of techniques such as radioimmunoassay in the food industry. Other immunoassay formats such as immunodiffusion do not provide a basis for rapid testing, whilst electrophoretic techniques may require detailed expert analysis following separation. The cost implications of certain assay formats, for example fluorescence polarisation immunoassay, also potentially preclude their use in the food industry if cheaper alternatives are available. Additionally, the analyst in the food industry is looking for procedures that are not simply rapid, but cheap, simple and safe (Allen and

Smith, 1987; Bonwick *et al.*, 2003; Shim *et al.*, 2004). Thus, in order to evaluate immunoassays for the detection of food adulteration, the implications of different assay formats will be reviewed. In particular, data from immunoassays for species adulteration will be examined.

2.1. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) exists in a range of formats, which are rapid, safe and inexpensive. For these reasons, ELISAs satisfy most of the requirements of the food analyst (Bonwick and Smith, 2004). A good illustration is provided by the ELISAs that have been developed to detect adulteration of meat species. The determination of species of origin of raw meat samples is generally based on the detection of specific differences in blood proteins, particularly serum albumin. The similarity of these proteins is an advantage for immunoassay development. This is because it is possible to immunise an animal with albumin from a single species and obtain a number of different useful antibodies. On a commercial scale anti-bovine, anti-porcine and anti-equine antibodies are obtained from the fractionation of rabbit serum containing antibodies raised specifically against bovine serum albumin (Figure 2).



This serum provides anti-bovine antibodies and additionally anti-porcine and anti-equine antibodies, each of which is used in highly sensitive and specific commercial assays for these individual species. Antibodies such as these can be used in a competitive ELISA format, providing a method that is both rapid and extremely sensitive, as well as being fully compatible with the requirements of the food industry. Indeed, in studies carried out over 15 years ago it was possible to demonstrate the detection of pork in mixtures with beef or buffalo meat at levels several orders of magnitude below the nominal 1% value (Ayob *et al.*, 1989; Ayob and Smith, 1990), which this paper has set for financially rewarding criminal adulteration (Table 1). Thus, the simple competitive ELISA for meat speciation has become a commercial success. Analysts are able to purchase pre-coated plates and pre-diluted reagents in kit form and are able to examine 20 samples simultaneously in 40 minutes, following preparation of the samples.

The reliability of such assays is now well established with approximately 14 species being detected using commercially available systems. The preparation time for this simple type of assay is approximately 10 minutes, involving homogenisation in an aqueous buffer to produce a liquid sample for the analysis. There are ways of automating the homogenisation and thereby minimising the time for the extraction, which may be considered if needed. The microtitre plate ELISA has the capacity for adaptation to a variety of automated formats for high throughput screening systems, but it has the disadvantage that it is not designed to handle single samples. For applications requiring analysis of a single sample there are two formats available, which can be applied on site. These are the dipstick and the immunochromatographic formats.

Table 1. Detection of pork in mixtures with beef by direct non-competitive ELISA. Data from Ayob *et al.* (1989).

Pork (%)	Beef (%)	Expected recovery (%)	Observed recovery (%)	Percentage recovery
40	60	40	40.6 ± 1.5	101.5 ± 3.8
20	80	20	21.6 ± 0.5	108.2 ± 2.6
10	90	10	9.9 ± 0.3	99.5 ± 3.0
5	95	5	5.2 ± 0.2	104 ± 4.6
1	99	1	1.1 ± 0.1	110 ± 5.0

2.2. Single sample tests

Dipstick systems have been developed which consist of a paddle, pre-coated with antibody, which can be dipped into a liquid sample where reactions with antigen occur (Gabaldon *et al.*, 2003). The paddle is removed and transferred to a series of bottles containing sequentially wash buffer, second antibody with enzyme label, wash buffer and finally enzyme substrate. The result is a visible colour change without quantitation. For a single sample this assay can take approximately five minutes and can be carried out on-site. Despite their simplicity, dipstick systems have not found a great deal of favour with users because they do not incorporate a method of recording the result.

Immunochromatographic methods are also applicable to single samples. The transverse flow format requires operator intervention at several points, whilst the lateral flow systems are independent once the sample is loaded. The transverse flow format is usually found in the form of a credit card-sized unit containing highly absorbent materials accessed via a pad of filter paper on to which a dot or dots of reagents have been dried. The prepared sample in buffer is poured onto the filter paper through which it passes, leaving specific reactants bound to the reagent dots. Addition of a further solution containing labelled second antibody results in the formation of an immunological sandwich and visualisation of the result. Two reagent dots are usually present, one being the control thus providing positive and negative confirmation of assay function. Visualisation is usually achieved by means of reagents consisting of coloured nanoparticles bound to the final immunological component of the assay.

Lateral flow immunochemical devices (Figure 3) are much more common than transverse flow systems, although they consist of essentially the same components. In this format all the reagents have been incorporated into the analytical device so that all the reactions take place in the device without operator intervention following addition of the liquid sample. Briefly the liquid sample is added to an adsorbent pad into which the reagents have been (freeze-)dried. The pad acts as both a filter and a reagent reservoir. The liquid sample rehydrates the reagents and the reactions begin as the liquid flows along a nitrocellulose strip attached to the pad. On the nitrocellulose strip lines of reagent have been drawn and dried. The approaching sample may be able to react with these whilst non-reactant species flow past leaving a visible line at the reaction site if the sample is positive. This technology is capable of extensive development with the possibility of several different lines on the test strip allowing simultaneous

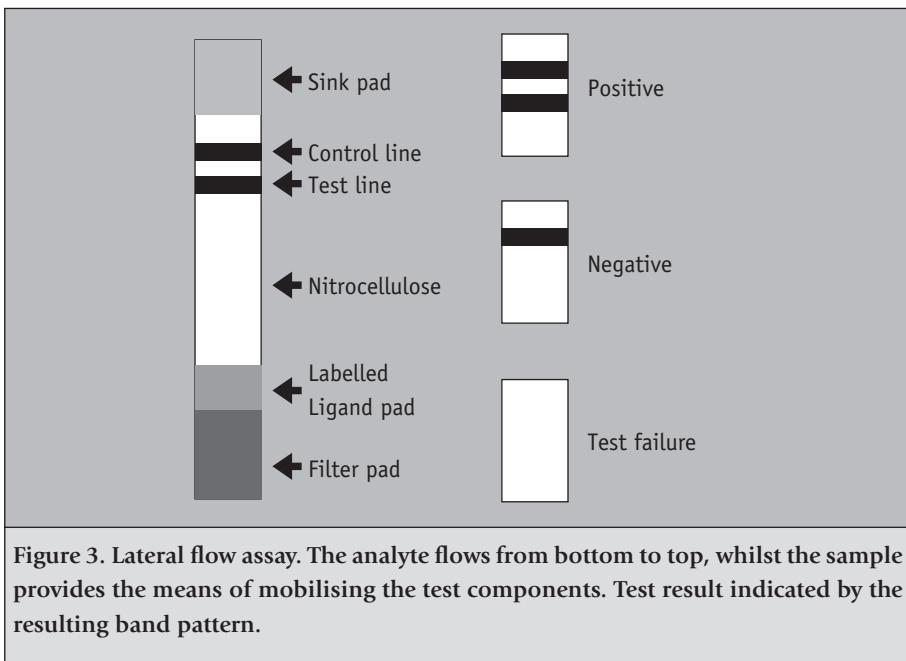


Figure 3. Lateral flow assay. The analyte flows from bottom to top, whilst the sample provides the means of mobilising the test components. Test result indicated by the resulting band pattern.

detection of several analytes in one sample, and multiple coloured colloidal particles available for the clear differentiation of the results (Figure 4).

The applications of the various immunoassay methods are different and therefore analysts should consider their needs and select the system appropriately. The ELISA system has been applied to the detection of a wide range of materials used

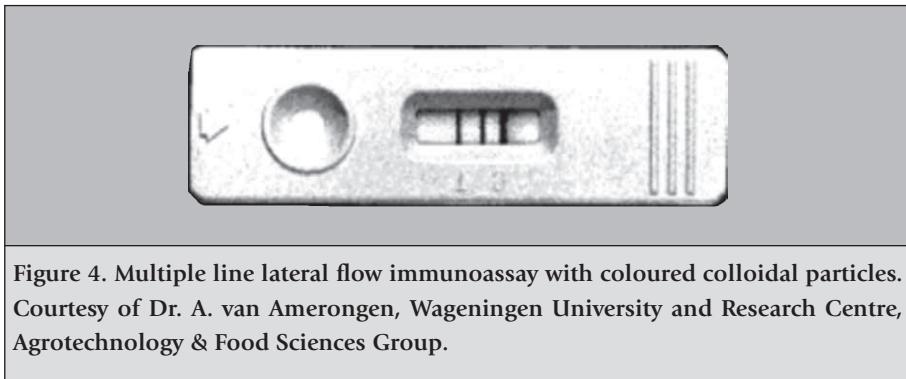


Figure 4. Multiple line lateral flow immunoassay with coloured colloidal particles. Courtesy of Dr. A. van Amerongen, Wageningen University and Research Centre, Agrotechnology & Food Sciences Group.

in food adulteration and indeed it might be claimed that the development of an application normally arises using ELISA technology from which the reagents can be taken and the other assay formats developed.

3. Assay performance

The original food ELISA was probably developed by Hitchcock *et al.* (1981) for the detection of soybean protein. This method involved a protracted extraction step, which took 5 days and thus could not be considered a rapid method. By altering the basic premise of the assay, Rittenburg *et al.* (1987) were able to reduce the assay time to less than four hours, of which 3 hours were required for the extraction, denaturation and renaturation of the analyte. A feature of this soya protein assay is that the detection is of renatured soya protein, which is produced during the extraction process. The reason for following this route is that soya protein has only a limited solubility in aqueous buffers whilst renatured soya is highly soluble. This approach has a significant built-in advantage in that the extraction process very simply removes any potential cross-reactants prior to the immunoassay, thereby increasing the sensitivity and specificity of the assay. Thus a first premise for rapid methods is that the optimum method may not always be direct measurement of the selected analyte. Specific derivatives may be equally useful in determining the presence of other suspected adulterants.

Recently an alternative to the AOAC method (Rittenburg *et al.*, 1987) of soya detection was published (Castro-Rubio *et al.*, 2005). This method involves defatting the meat samples with acetone, solubilisation of soybean proteins in buffer and the subsequent identification of soybean proteins in the chromatogram obtained by perfusion reversed-phase chromatography with ultraviolet (UV) detection. Detection limits were 0.07% and 0.25% (w/w) respectively, however, it is uncertain whether this technique has significantly advanced the capabilities of the food analyst to detect adulteration of meat with soybean protein.

Milk is an interesting material for the food industry. It has been used as an adulterant to increase the bulk of meat products, however, it has also been adulterated (Perez *et al.*, 1992). This adulteration is most frequently seen when cow's milk is added to goat or sheep's milk, which is going to be used to make cheese. In this situation, the costs of mixing the adulterant with the pure material are minimal and therefore the possibilities for low levels of adulteration are greater. Milk contains a range of species-specific substances, which can be used as the basis for its detection. Those which have been used include casein, whey

proteins and immunoglobulins (Rittenburg *et al.*, 1984; Hurley *et al.*, 2004). Original milk protein assays were able to detect 1.0% cow's milk in sheep or goat's milk (Perez *et al.*, 1992). These assays have continued to develop and recent assays based on the measurement of IgG show that 0.1% cow's milk can be detected (Figure 5).

A similar trend can be observed for soya assays in which the development of ELISAs using different analytes and detection systems has allowed an improvement in sensitivity. Percentage adulteration detectable by ELISA has increased from 0.01% (Allen and Smith, 1987; Hewedi and Smith, 1990) to 4×10^{-5} % (Kuhlhoff and Diehl, 2004). It is interesting to note that in the case of meat species detection, the recently developed techniques such as quantitative PCR (Dooley *et al.*, 2004) have yet to significantly exceed that of the original ELISA reported by Ayob *et al.* (1989).

Although the sensitivity of immunoassays for adulterants has progressively increased, a key issue remains the rapidity of the procedures. Recently developed techniques such as surface plasmon resonance initially appear to be more rapid than assays such as lateral flow or ELISA. However, when factors such as the number of samples that can be assayed simultaneously are taken into consideration, the differences between the procedures are reduced. These observations suggest that since the introduction of the ELISA, only a limited improvement in the rapidity of food sample analysis has occurred (Table 2).

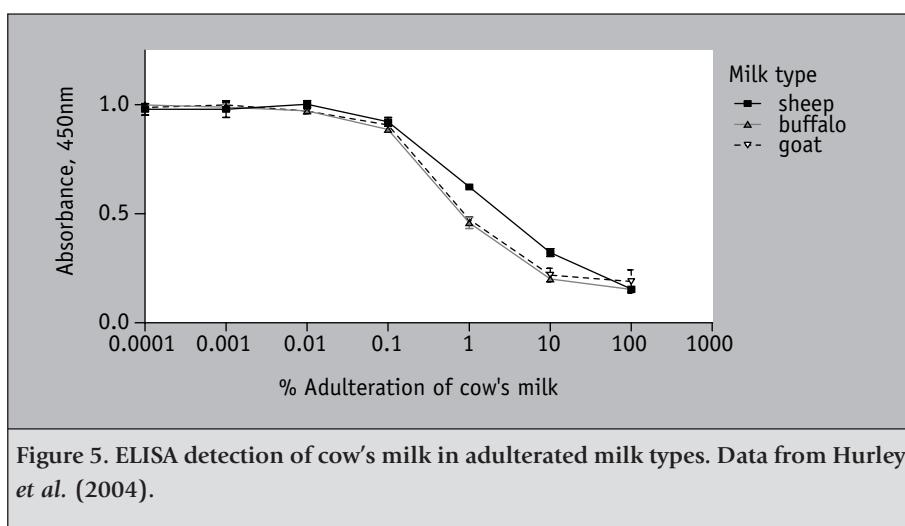


Table 2. Typical performance of rapid assays in the authors' laboratory.

Type of assay	Number of simultaneous samples	Assay duration (minutes)	Individual sample analysis time (minutes)	Controls measured simultaneously
ELISA	20	40	2	Yes
Dipstick	1	5	5	No
Transverse flow	1	2	2	No
Lateral flow	1	2	2	No
Surface plasmon resonance	1	2	2	No

4. Discussion

Based on these observations, immunoassays still appear to provide the best option for routine rapid assays. Future developmental requirements still include assays suitable for on-site studies away from the laboratory. The lateral flow format appears to fulfil the on-site option, particularly if the range of analytes to which it can be applied can be increased by introduction of multi-analyte formats. Whilst the development of high throughput analytical methods are a worthy goal, an important consideration guiding future research and development of assays for adulterants should be the requirements of the food industry itself. Despite the generally acceptable performance of immunoassays as a means of detecting adulterants, the rate-limiting step, and thus time-consuming element of many of the assays is sample preparation and the need to extract analytes from the various matrices. This is often an area that has been neglected, however, recent developments such as the use of labelled liposomes, offer promise for the analysis of some foodstuffs and are clearly worthy of further study (Wen *et al.*, 2005).

Recent events, such as the detection of Sudan 1 in a range of food materials in the UK, have shown that adulteration is a continuing problem. The use of new substances as adulterants is likely, however, it is difficult to anticipate what will arise. It is tempting to speculate that tests for both adulterants and permitted ingredients might need to be developed for food products.

5. Conclusions

Immunoassays are rapid and continue to be sufficient for the current analytical needs of the food industry. Assays, which are rapid also need to be specific so that the results are reliable. The quantities of adulterants are usually large so sensitivity may not be as important. Existing assay formats are applicable to novel analytes, although it is important to take account of the requirements of end users such as the food industry.

Acknowledgements

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Considerations on labour- and cost-efficient immunoassay protocols and formats

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Molecular biological methods in authenticity testing

B. Popping

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Abstract

The loss of revenue for industry through counterfeiting brand name products is significant. Not only does this apply to common products like music CDs or clothes, but also and in particular to brand products in the food industry. While modern chemical and physico-chemical techniques can identify certain types of fraud, there are a great number of cases where only DNA-based technologies can reveal the adulteration. Several years ago, DNA technologies were considered as methods used only in universities, primarily for research purpose with no 'real-life' applications. However, this has changed and a number of laboratories have specialised in offering such services to industry. This article will review DNA-based techniques commonly used for authenticity testing.

Key words: DNA-based methods, authenticity, fraud, counterfeit, traceability

1. Introduction

Fraud not only affects the revenue of food industry, it also leads to mistrust of the consumers, which ultimately effects the food industry again. As a recent example, standard-quality rice has been sold as the premium-priced Basmati rice. But how can a consumer tell if the product is what the label declares? What makes Basmati rice different from other rice? Here, a combination of DNA-based methods and physico-chemical methods (stable-isotope methods) can help. Another example is BSE: buying meat in the supermarket, how can the consumer or the enforcement authorities check if the animal has been tested for BSE and is therefore safe for food-use? Again, a combination of two methods, protein detection and genetic fingerprinting allows tracing animals from abattoir to the fork. In future it is likely that all animals which have economical importance will have taken their unique genetic fingerprint to allow farm to fork traceability. And the same methodology is used in forensics and paternity analysis: parents can unambiguously be identified and offenders traced and prosecuted. Molecular biological tools are nowadays common tools to help authenticity testing in a wide range of fields.

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2. Pathogen identification

Bacteria are certainly the first that springs to mind when the application of molecular biological methods is considered. The identification of spoilage pathogens like *Salmonella* and *Listeria* using polymerase chain reaction (PCR) methods is very common these days. It has for certain matrices the advantage to be faster than conventional microbiological approaches. As an example, fresh meat can be tested using PCR methods after 6-12 hours (including pre-enrichment) instead of 3 days. For dried herbs and spices, where the bacterial cells are likely damaged and require more time to recover, DNA methods do not provide a significant advantage over conventional microbiological methods.

Apart from testing bacterial strains, it can be useful to test for subtypes, which are more common in some parts of the world than in others. As an example, outbreaks of specific *Salmonella* strains in Asia have been identified and traced using pulse field gel electrophoresis (PFGE) and riboprinting. In the former, the whole bacterial genome is isolated and run across a gel using electrical pulse fields. This gives a strain-specific pattern and allows determination of subtypes. For riboprinting, specific DNA of the 18S-gene (which is part of the ribosome gene-group) is amplified by PCR and subsequently digested using sequence-specific restriction enzymes. The restricted DNA is then applied on an agarose gel, which is exposed to an electric field. DNA-fragments migrate according to size through the gel and the result is a specific band pattern. This allows distinguishing between different subtypes of bacteria.

Another field of application where also PFGE and riboprinting is used is the outbreak of foodborne diseases in an area. Using the above methods, an outbreak of *Salmonella agona*, which contaminated herbal tea given to children, was identified and contained.

3. Varietal identification

3.1. Coffee

Certain varieties of coffee, especially wild coffee, are more expensive than farmed coffee. This applies especially to Ethiopian coffee from the Bonga forest. The coffee varieties which grow there are endemic to the region. Since the genomic differences to other coffee varieties are widely unknown, commonly used methods like PCR, which targets variety-specific, sequences (e.g. *trnL*, other chloroplast

sequences, etc.) cannot be used. An alternative technique was published by Zietkiewicz *et al.* (1994): simple sequence repeat-anchored PCR. However, this was not used for the authenticity of coffee. Blair *et al.* (1999) used it for rice and Zeltz *et al.* (2005) used it for the identification of wild coffee, which grows only in Ethiopia. In short, the method, also called inter short sequence repeat (ISSR), amplifies regions between the frequently in the genome occurring short sequence repeats by PCR. The amplified products on a gel result in a varietal-specific pattern. Therefore, varietal-specific sequence is not required, only the sequence for the common regions before and after the short sequence repeats. This technique can in principle, be applied to any organism where varietal identification is relevant.

3.2. Potatoes

Consumers and manufacturers look for specific characteristics in potatoes for different purposes: the salad potato should withstand cooking, French fries should be crisp after frying, and potatoes should be soft for making puree. A number of potato varieties are known to have the desired characteristics. But once the potato is processed into more or less regular shapes, it is impossible to tell the varieties apart. Here, a DNA-technology based on micro-satellite analysis can resolve the problem (Ashkenazi *et al.*, 2001; Veilleux *et al.*, 1995). Using five different primer sets allows the identification of more than 50 commercially important potato varieties, including common varieties such as Bintje, Solana, Desiree and Red Rascal.

3.3. Pasta

Pasta is made from durum wheat (*Triticum durum*). However, in the European Union (EU) 3% of non-durum wheat, e.g. *T. aestivum*, in pasta is allowed. To determine the quantity of non-durum wheat, real-time PCR is used, which targets D-genome sequences present only in *T. aestivum* (Bryan *et al.*, 1998).

3.4. Fruits

In the fruit-juice industry, juices from higher-price fruits (e.g. oranges) are sometimes adulterated by lower-price fruits (e.g. mandarins). Here, instead of the cyt b gene in animals, the maternally inherited multicopy chloroplast gene rbcL is used to detect adulteration. The rbcL gene codes for the L-subunit of the ribulose-bisphosphate carboxylase and the variability between species

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is sufficiently high to allow species-specific primer systems. A qualitative PCR allows the identification of a particular species. This still works in processed products like yoghurts, jams, jelly and drinks. The presence of 150 pg DNA per mg wet weight is sufficient for detection.

3.5. Genetically modified foods

Since 1996, foods produced by modern biotechnology can be found on the shelves of retailers. The first product was the transgenic tomato puree from the Flavr Savr™ tomato. Less than a year later, products containing transgenic maize and soya could be found. Consumer concern and green-rights activists' pressure led to legislation to label transgenic products accordingly. Maize and soya are main-staple crops and the segregation systems are not always effective, so that unbeknown to the producer, retailer or consumer genetically modified material can be present in the product. To test for the presence, primer systems have been designed which target elements not normally present in the conventional counterparts, e.g. promoters and terminators of viral or bacterial origin. In addition, a EU regulation demands labelling of products if more than 1% transgenic material is present. To comply with labelling, quantitative real-time analysis is performed to analyse the amount (Popping, 2001; Lipp *et al.*, 1999).

3.6. Allergens

There are three main reasons why allergens may not be declared on products. One is accidental contamination of a product produced on the same manufacturing line (e.g. plain dark chocolate, which is produced subsequently to hazelnut chocolate and might therefore be contaminated with traces of hazelnuts). Another reason is that manufacturers assume a heat-treatment may destroy allergens. However, some, like milk proteins are very stable. The third reason is the replacement of higher priced with lower priced products. Two examples are the replacement of pine-kernels in pesto with much cheaper peanut, and the blend of hazelnut nougat with roasted soya, which has a similar taste. Peanut- or soya-allergic consumers would not be aware of or even suspect the presence of peanut or soya in these products and unsuspectingly consume it, which might trigger a severe allergic reaction. Here, apart from commonly used enzyme-linked immunosorbent assays (ELISAs), species-specific PCR methods are being applied. While for most matrices both methods are equally sensitive, PCR is considerably less expensive if testing for several allergens in the same sample is required. Here, a novel approach is taken applying a similar modular PCR

approach as for the screening of genetically modified organisms (Popping *et al.*, 2005). The screening using real-time PCR allows the identification of up to twelve allergens in a single assay, which is impossible to do by ELISA.

3.7. Meat speciation

Meat speciation is important for more than one reason: (1) to identify the presence (or absence) of high price species, and (2) to allow ethnic groups a choice (hallal, kosher) where the consumption of pork meat (Arab countries) or beef meat (India) is forbidden by religion. Here, several detection methods (Patterson and Jones, 1990; Calvo *et al.*, 2001; Lahiff *et al.*, 2001; Meyer *et al.*, 1995) are being used, which range from qualitative PCR over RFLP to quantitative real-time PCR. These techniques are applied depending on the analytical question asked. If, for example, the question of presence or absence of pork is asked, species-specific qualitative PCR is used. If however, the question is which species are present, RFLP is more applicable. And, if quantification is required, real-time PCR is the method of choice. There are a number of pitfalls with these techniques. If the absence of beef meat is tested for, the presence of bovine milk or cheese products can lead to a false-positive result. Here quantitative real-time PCR can help to determine the quantity of bovine DNA. And if certain quantities of a specific species are to be ascertained, quantification might lead to an inaccurate result since the DNA yield from different tissues is different.

3.8. Fish

Once morphological characteristics are removed the premium-priced Atlantic salmon (*Salmon salar*) cannot be distinguished from the lower-priced rainbow trout (*Oncorhynchus mykiss*). However, a technique called restriction fragment length polymorphism (RFLP) allows the identification of 10 different salmon-like species (Russell *et al.*, 2000; Carrera *et al.*, 2000). Here, a cyt b fragment with conserved and variable regions is amplified by PCR. The primers are selected to anneal in the conserved regions to amplify DNA from all species. The DNA between the primers has a higher degree of variability. This characteristic is exploited by the use of sequence-specific restriction enzymes. The enzymes digest the PCR amplicon and produce a species-specific pattern, often called 'fingerprint'.

In a similar way, different tuna-species are identified. While yellowfin and bluefin tuna are high-price species, sardines (*Sarda sarda*) are a low-price species. Here again, PCR is used to amplify a conserved region of cyt b. However, since there

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are too few differences in the sequence between the tuna species, RFLP cannot be used. Instead the fact that single nucleotide chains depending on their nucleotide sequence, form different structures in a denaturing gel, is exploited. Even a single nucleotide difference causes the single DNA strands to have different shapes (polymorphisms) and migrate differently in a gel. This allows to distinguish the tuna varieties. The method is termed single strand conformation polymorphism (SSCP) (Rehbein *et al.*, 1998; Hold *et al.*, 2001). However, the method is somewhat more laborious than RFLP and always requires reference DNA to be run on the same gel for comparison. A recent project funded by the European Commission successfully trialled this method in an interlaboratory study with good success using tuna mixtures, but no finished products (like tuna sandwich, etc.).

4. Fraud and counterfeiting

4.1. Wine and olive oil

It is extremely difficult to establish the origins of wine and olive oil using DNA technologies. One approach has been to target yeasts associated with olives, grapes, oil and wine. Target for characterisation was the LTR retrotransposon (Ty element) (Lenoir *et al.*, 1997) using amplified fragment length polymorphism (AFLP) or similar techniques. However, in wine little DNA is found and recovery of DNA from wine is very unreliable. This method has been more successful for olives, where different yeast strains are associated with olives and olive oil. The yeast strains in olive oil appear to be associated with the production site (fattoria) where the olive oil was produced. And since the number of production sites is limited, the olive oil can be traced back to the fattoria. However, this technique is not yet applicable for routine analysis.

For the identification of the origin of olive oil, a second, non-DNA-based technology has proven very useful. The technology is called site-specific natural isotope fractionation nuclear magnetic resonance (or SNIF-NMR for short) (Gonzales *et al.*, 1999; Martin *et al.*, 1996). The basis of this technology is that certain elements have naturally occurring stable isotopes (^{16}O and ^{18}O , ^1H and ^2H , ^{12}C and ^{13}C). The ratios of the different stable isotopes vary from one geographic location to another. These ratios are maintained in the organic material from that region, e.g., plants, animals, etc. The SNIF-NMR technology allows to measure these stable isotope ratios at individual positions in a given molecule. With an appropriate database listing the location and typical stable-isotope distribution, the origin of olive oil (and wine) can be identified.

4.2. Rice

There are two cultivated rice species, *Oryza sativa* and *O. glaberrima*, and 20 wild species, as well as many different cultivars. Recognised types are long-grain Basmati and Camargue. Basmati is the highest-price type (Verma *et al.*, 1999). It originates from the foothills of Himalayas and has a strong popcorn aroma, caused by 2-acetyl-1-pyrroline. The rice elongates by more than 75% during cooking and has a distinct chalky grain with low transparency. The type of rice is identified by the use of microsatellite markers, where the ratio of two alleles is determined. But how can the origin be determined? Again, SNIF-NMR is used to identify if the rice originates from the foothills of Himalayas.

4.3. Offal

The use of offal in meat products reduces the quality of that product. But how can offal be identified (Al-Jowder *et al.*, 1999; Harris *et al.*, 1997)? All previous examples exploited the differences of DNA sequence between species. But if offal from the same species is used, how can this be identified? The answer lies in the methylation pattern of inactive genes and a chemical process. Genes that are not used in certain tissues are inactivated by methylation of specific cytosine (C) residues within the promoter. Detection of a gene in unmethylated state in a tissue that does not express this gene (i.e. the gene has a methylated promoter region) indicates other tissue origin. As a suitable target, genes that are expressed in target tissue but not in muscle tissue are used. For the determination of the methylation status the DNA is treated with sodium bisulphite. All unmethylated C become uracil (U), which is amplified as thymine in PCR, while methylated C remains unchanged. Therefore, primers are designed that amplify only unmethylated sequences from internal organ-specific genes.

4.4. Package counterfeiting

It is well known that low quality products sell better in brand name wrapping. But this damages the reputation of brand names and causes financial losses to the industry and the consumer. But how can counterfeits be identified? One recent development is the incorporation of beads in the packing material that contain synthetic DNA. This synthetic DNA works as a barcode and can be amplified or sequenced. If a complaint about the quality of a product is received, the company only needs to analyse the DNA in the package material to find out if the product is authentic. As an example, the Australian wine industry was rocked in 1998

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when fake bottles of the country's most prestigious wine, Penfolds Grange, were uncovered, sparking concern of a new racket in fraudulent premium wines. Most recently, the Australian winemaker BRL Hardy used specific DNA from 100 year-old wine to code the neck-wrapping of premium price wines.

5. Traceability

With the BSE crisis still fresh in the minds of many people, traceability from abattoir to fork, or ideally from farm to fork, has become an important issue. How does the consumer know if the animal whose meat he is eating has been BSE-tested? How does he know which farm the animal has come from? It is well known that paper-documentation is often insufficient and prone to fraud. If an animal loses his ear-tag, which sometimes happens, is it now anonymous? Again, DNA technology is here to help (Reed *et al.*, 2001; Schnabel *et al.*, 2000). By using a combination of protein-based BSE-test and DNA-test (microsatellite analysis), which uses the same tissue (Eurofins-TAG™), the meat purchased by the final consumer can still be traced back to the slaughtered and BSE-tested animal. To achieve this an aliquot of the tissue used for BSE testing is genotyped and the information is stored in a database. Using the stored information, profiles from meat samples taken at the butcher's shop can be compared to the existing profiles in the database. Ideally, this can be applied to all economically relevant animal species at the point of birth, so that the individual animal is traceable from farm to fork.

6. Conclusions

In summary, molecular biological techniques have become an every-day tool to solve a number of problems and questions in the areas of varietal/species identification, fraud, traceability and paternity analysis.

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Enforcement of food standards legislation using DNA-based techniques

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Abstract

The UK Food Standards Agency has a programme of work in support of enforcement of labelling and other food standards legislation by developing novel methods, and undertaking national surveys on the UK market. Many of the new methods take advantage of the rapidly developing area of DNA techniques, which have been applied amongst other things to meat and fish speciation, mislabelling of potatoes, adulteration of Basmati rice, adulteration of durum wheat pasta and detecting meat in vegetarian foods.

Keywords: authenticity, DNA-techniques, meat speciation, fish speciation, Basmati rice, durum pasta, vegetarian foods

1. Introduction

Advances in DNA technology in forensics have opened up new possibilities of solving criminal investigations. Similarly, applying DNA technology to determine the origin of foods or its ingredients has also assisted in checking or verifying labelling or description claims and enforcing food legislation.

The UK Food Standards Agency has a dedicated programme of research to check the authenticity of foods, and many of the approaches used are based on DNA technology. Methods are developed and validated, then used in national surveys to check a specific authenticity or misdescription issue. Follow-up action will depend on results of the survey, and may involve prosecution of individual companies by local government authorities. The outcome of surveys may reveal a more widespread problem that requires central action, which may be a co-ordinated enforcement exercise, or development of a code of practice or guidance. DNA techniques are slowly being introduced as routine methods in enforcement (Public Analyst) laboratories, and development of a portfolio of methods will assist the justification of the investment in these techniques.

2. Food authenticity programme

The UK Food Standards Agency's food authenticity programme is aimed at revealing where foods are misdescribed. There are a large number of legislative measures covering the description and composition of food that goes under the general umbrella of 'food standards'. Most of the legislation is European Community (EC) legislation, but the UK still has national rules covering labelling requirements of potatoes and meat products. The legislation covered is summarised in the Table 1.

Misdescription can occur when the food does not meet the requirements of a legal name, or substitution by cheaper ingredients occurs, or the food is extended with adulterants such as water or starch, or a process is not declared, or the correct geographic origin is not given, or an ingredient is over-declared in a quantitative declaration. Incorrect declaration of any one of the above may be done for profit and is misleading the consumer.

Table 1. Food standards legislation.

Labelling or description	Legislation
Name of the food - legal name	Food Labelling Directive Vertical (Breakfast) Directives, EC Marketing Regulations, national (UK) rules on potatoes and meat products
List of ingredients	Food Labelling Directive - generic names
Mention of a process	Food Labelling Directive Quick-frozen Food Directive
Geographic origin	Food Labelling Directive Protected Denomination of Origin (PDO) Protected Geographic Indication (PGI) EC Marketing Regulations Beef Labelling Regulations
Quantitative ingredient declaration (QUID)	Food Labelling Directive Vertical (Breakfast) Directives

The food authenticity programme is just one of many research programmes funded by the Agency to assist its policy development. The policy objectives of the programme are firstly to contribute to the objective of having honest and accurate labelling to help consumers make informed choices of the food they buy. Secondly, the programme assists enforcement authorities by providing the tools to determine compliance with the standards legislation, and identifying incidents of misdescription and fraud. It also underpins the Agency's labelling policy by revealing where problems are occurring. Table 2 gives a list of surveys and other surveillance activities undertaken in the programme over the past 5 years. Full details of each survey are published on the Agency's website (www.food.gov.uk).

The programme of work is now overseen by a working group made up mainly of local authority representatives. This is to ensure that the Agency's programme is integrated as much as possible into the national local government enforcement survey programmes of local authorities. There is also a working group on methodology, which is responsible for ensuring that methods used in surveys have been properly evaluated and are robust. The group develops protocols, identifies where there are gaps in methods, and where further work is required. These requirements feed back into the Agency's research programme, where

Table 2. Surveys undertaken by the UK Food Standards Agency.

Survey	Year
Meat species present in meat products	1999
Olive oil (checking for mislabelling and adulteration)	1999
Apple juice (checking for adulteration)	1999
Tuna products (checking whether tuna species)	2000
Water in chicken – retail (extraneous water) and catering (added water)	2000/2001
Irradiated foods (herbal supplements and prawns)	2002
Maize oil (checking for adulteration)	2002
Added water in scallops /scampi – chilled, frozen and products	2002
Potato varieties declared at retail (part 1 and 2)	2003
Horsemeat in imported meat products, e.g., salami	2003
Basmati rice adulteration with non-Basmati varieties	2004
Fat content in minced meat (checking claims such as lean, 'superlean')	2004

longer-term research projects are commissioned to develop and evaluate new methods. Generally, the methods use novel technology and most of the research has focussed on two main technologies: isotopic measurement for plant and geographic origin, and DNA for species origin.

3. Methods developed using DNA techniques

Table 3 summarises all the applications or projects using different DNA techniques that the UK Food Standards Agency has funded with different contractors as part of its food authenticity programme.

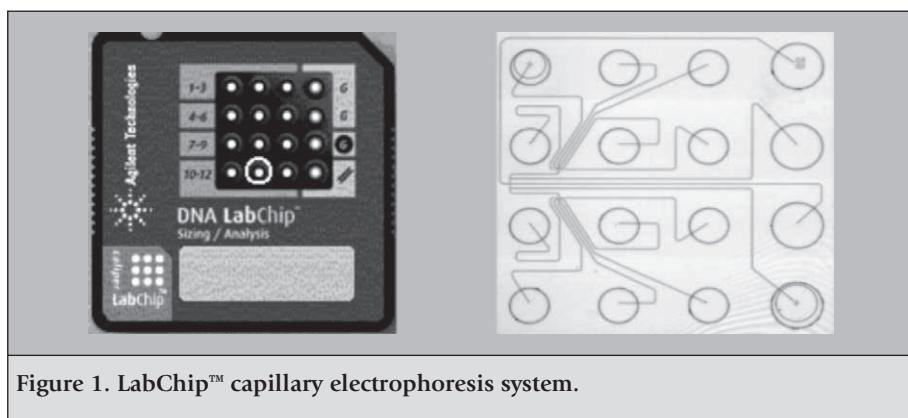
Table 3. Completed DNA-based projects.

Issue	DNA technique
Rice varieties	polymorphic microsatellites or simple-sequence length polymorphism (SSLP)
Potato varieties	
Origin of tea	
Olive varieties	
Fruit species in jams and pulps	single nucleotide polymorphisms (SNPs)
Rice varieties	
Olive varieties	
Durum wheat pasta	real-time polymerase chain reaction (PCR)
Quantitative meat and fish species	
GM soya	
Exotic meat species	
Meat species in vegetarian foods	
Differentiation of meat tissues, CNS, offal	methylation events - PCR
Fish species	
Meat species	restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP)

3.1. PCR-SSCP and PCR-RFLP

The first use of DNA methodology goes back to 1998, when a survey of smoked salmon and salmon products was carried out to check whether any substitution with trout, a cheaper fish, had occurred (FSA, 1998). Samples were screened using isoelectric focussing of the soluble proteins, but species confirmation used polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. The method of PCR-SSCP for salmon species was developed from an EU Project (AIR 2-CT94-1126 (1994-1997)), which originally applied the technique to canned tuna species. The Agency also used this method for tuna species in a survey on canned tuna and canned tuna products reported in April 2000 (FSA, 2000).

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique has recently proved to be more user-friendly for identifying a number of white fish species than the accepted method of isoelectric focussing of fish proteins. This method has been adapted to a simple lab-on-a-chip format (using the Agilent 2100 Bioanalyser capillary electrophoresis system) with Agency funding, and has proved to be simple, cheap, and yet robust. It has been collaboratively tested in several laboratories (Dooley *et al.*, 2005). On the basis of this trial, it has been adopted in several enforcement laboratories in the UK. It has an added advantage of not requiring authentic samples of fish to be run each time, once a database of fish DNA restriction fragments have been established. Figure 1 shows a picture of the lab-on-a-chip used for white fish species identification.



3.2. DNA microsatellites

DNA microsatellites are simple base repeat sequences, which normally occur in the non-transcribed section DNA between genes, and are often used to genotype closely related plant varieties.

3.2.1. Potatoes

Figure 2 shows schematically four plants with alleles of differing numbers of repeat units at one microsatellite location. A PCR of these alleles produces fragments of different sizes, subsequently separated and identified by gel electrophoresis. UK Food Standards Agency funded research (FSA, 2003) has developed methods to identify all commercially sold potato varieties using only five microsatellite markers (Table 4). The method for potatoes has been applied in two national surveys to check potato varieties in wholesale and retail sale. The survey revealed that for one specific variety (King Edward), 43% of the samples were mislabelled.

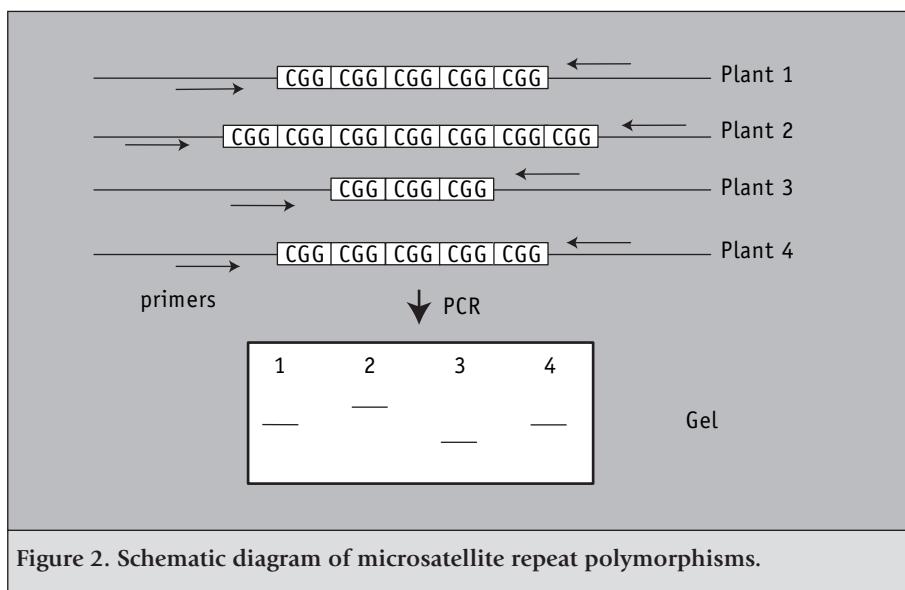
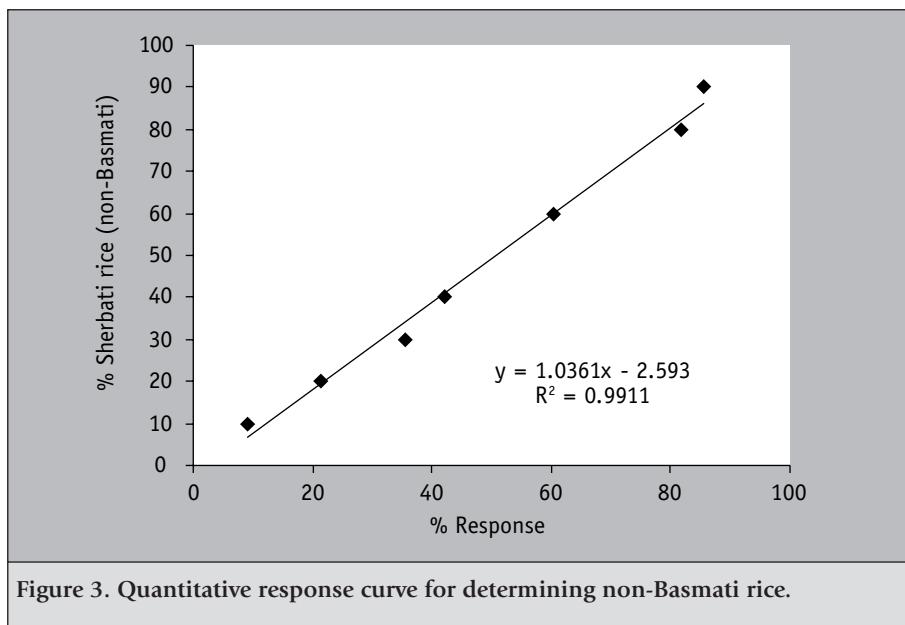


Table 4. Five microsatellite markers to identify potato varieties.

Repeat motif	Primer sequences	Predicted size (base pairs)	Annealing temperature
(TAA) ₆	CCTTCCCCCTAAATACAATAACCC CATGGAGAAGTGAAACCGTCTG	162	55 °C
(ACTC) ₆	TCTCTTGACACGTGTCAGTCAAAC TCACCGATTACAGTAGGCAAGAGA	249	60 °C
(CT) ₄ ... (CT) ₈	CAACTCAAACCAGAAGGCAAA GAGAAATGGGCACAAAAAAACA	193	55 °C
(CTGTTG) ₃	TTTAAGTCTCAGITCTGCAGGG GTCATAACCTTTACCATTGCTGGG	166	55 °C
(TAC) ₅ ... (TA) ₃ ... (CAT) ₃	TCTCACCAAGCCGGAACAT AAGCTGCCGAAGTGATTITG	188	55 °C

3.2.2. Basmati rice

Basmati rice is a premium rice grown in certain areas of North East India and the Pakistani Punjab. It is favoured by consumers for its strong aroma and unique cooking quality. These qualities stem from the plant's genetics, which is well adapted to the geographical area where it is grown. There are only certain varieties approved by the Indian (12 varieties) and Pakistani (5 varieties) authorities, which can be exported as Basmati. Because these specific Basmati varieties are two to three times the price of other long grain varieties, and Basmati has become the biggest selling type of rice on the UK market, the problem of adulteration has been an issue for some time. Methods to authenticate Basmati have focussed on identifying the approved varieties using microsatellite markers. The Agency has developed both a screening method followed by a quantitative method (see Figure 3) to determine which varieties are present and also to measure the amount of non-Basmati rice. This method was subsequently used in a national survey (FSA, 2004). Of the 363 retail samples collected, 46% contained non-Basmati, 23% of the samples had more than 10% non-Basmati, and 10% of the samples had more than 60% non-Basmati, which is the upper limit of accurate determination on non-Basmati. Rice being sold above this limit had little Basmati content. On the basis of the survey, formal follow-up samples



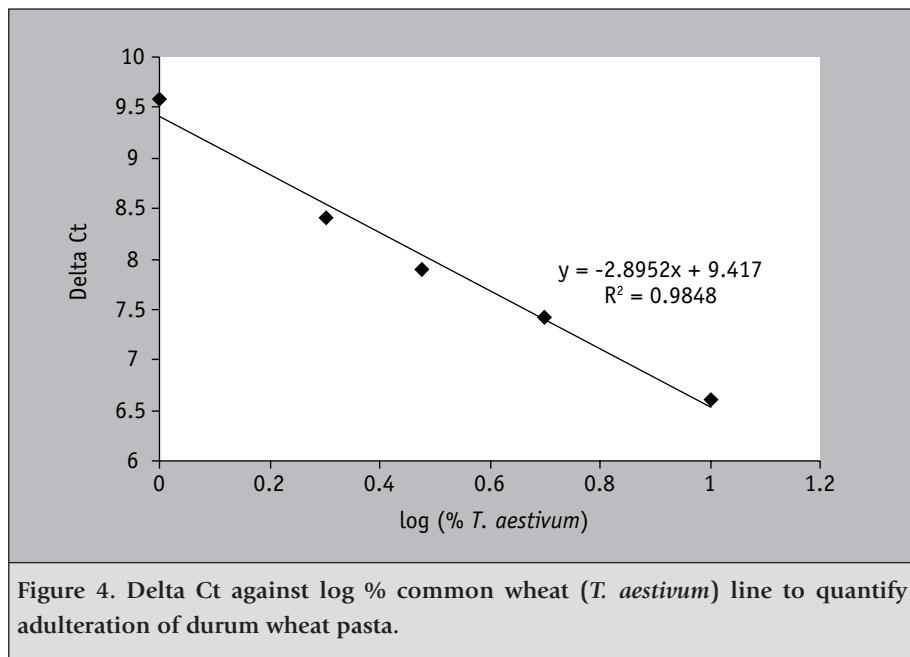
have been taken by a local government enforcement authority and prosecution against two companies is in progress.

3.3. Real-time PCR

Real-time PCR is a sensitive technique for measuring the copy number of a specific target gene. The threshold PCR cycle number of a target DNA sequence is compared to that of a 'normalising' or general gene. This permits the delta Ct to be compared to a set of standards and hence find the proportion of the target DNA component to the normalising gene sequence. Its main use has been the quantitative measurement of genetically modified (GM) soya or maize in raw flour-form samples. Real-time PCR application to composite and processed foods containing GM soya has proved more difficult, especially in a meat matrix, although the Agency carried out a limited survey on GM soya in baked products (FSA, 2002).

3.3.1. Durum wheat pasta adulteration

A real-time PCR assay has been developed to measure common wheat adulteration of durum wheat pasta, where the matrix and process are fairly consistent and hence reproducible results can be obtained. The method is based on the genetic differences between the two wheat varieties. Durum wheat (*Triticum durum*), usually the single ingredient of pasta, is tetraploid possessing 4 sets of chromosomes, 2 each of two genomes A and B. In contrast, common wheat (*T. aestivum*) is hexaploid, with 6 sets of chromosomes, with three genomes A, B and D. A 116 base pair (bp) single copy sequence of the PSR 128 gene in the D genome is used as the target sequence of the real-time PCR and a conserved region, which is present in both varieties, is used as an internal standard to normalise the data. Figure 4 shows the quantification of common wheat in durum wheat pasta using the difference in Ct value (delta Ct) between the target and normalising internal standard PCR curves.



3.3.2. Quantitative determination of meat species in meat products

Although PCR-RFLP was used initially for a meat speciation survey (FSA, 1999), more sensitive and reliable species-specific methods have been developed in real-time PCR format. The Agency has funded work to develop a real-time PCR assay for determining meat species quantitatively in mixed species meat products (Dooley *et al.*, 2004). This type of analysis is now necessary as a result of changes in the Food Labelling Directive and quantitative ingredient declaration (QUID). Evaluation of this method was carried out by challenging it with meat products (sausages) made with different species in different concentrations. Tables 5 and 6 show some of the results to evaluate the quantitative DNA determination for individual meat species using different calibrations, from standard meat mixes, DNA mixes, and a copy number dilution curve to creating a plasmid of meat species sequence. It is demonstrated that obtaining accurate measurements of meat species using real-time PCR for enforcement purposes is difficult, possibly because the amount of DNA varies with cut of meat. In addition, there are matrix and processing interferences with the assay. However, robust protocols have been developed for qualitative determination of meat species (Hird *et al.*, 2005).

3.3.3. Quantitative determination of fish species in fish products

Quantitative determination of named fish species in a fish product (e.g., cod in a mixed fish pie) in theory should be easier than meat. The flesh of fish is

Table 5. Comparison of three different calibration methods to determine meat species by real-time PCR.

Actual amount of turkey in turkey/lamb sausage % (w/w)	Determined % (w/w) turkey		
	meat admixture	DNA admixture	DNA dilution calibration curve
50	16.6 ± 2.2 (2) ^a	-----	14.0 ± 3.5 (2)
10	4.6 ± 1.0 (6)	3.6 ± 1.3 (3)	2.3 ± 1.2 (6)
1	0.5 ± 0.1 (2)	0.6 ± 0.2 (3)	0.2 ± 0.0 (2)

^aNumber of replicate determinations between brackets.

Table 6. Determination of % chicken in a chicken/pork sausage using a plasmid calibration curve and real-time PCR.

Actual amount of chicken in chicken/pork sausage % (w/w)	Determined % (w/w) chicken in chicken/pork sausage	
	Plasmid calibration curve 1	Plasmid calibration curve 2
10	6.5 (2) ^a	12.4 (2)
50	57.3 (2)	117.7 (2)
90	75.6 (2)	160.6 (2)

^aNumber of replicate determinations between brackets.

relatively uniform compared to cuts of meat. In most fish products the processing is light - cooking, flash frying (apart from canning). The existing method for the determination of a fish ingredient involves measurement of the nitrogen content, and using a factor to convert to fish content. The nitrogen content of haddock was measured from 3 different fishing grounds over different seasons, and compared to the DNA copy number. The measure of uncertainty of nitrogen content of the haddock was similar to the DNA copy number (Hird *et al.*, 2004). This indicated that the accuracy of fish content should be broadly similar if measured by nitrogen content or DNA copy number. The advantage of using the DNA copy number method is that it is specific to haddock. Haddock was added to several fish products and then determined using real-time PCR. Table 7 shows that results obtained are more promising than meat species determination (Hird *et al.*, 2004).

Table 7. Determination of haddock content in model fish products.

Sample	Actual % haddock (w/w)	Calculated % haddock (w/w) ± standard deviation
Cod in crispy coating	40	33 ± 8.8
Fish	25	29 ± 8.7
Fish	20	20 ± 6.5

3.3.4. Meat contamination of vegetarian foods

An innovative DNA method to check for the presence of meat in vegetarian foods has been developed under the Agency's authenticity programme in a real-time PCR format (Wiseman *et al.*, 2004). One issue, which had to be overcome, was that vegetarian foods, as distinct from vegan foods, may contain egg and dairy products (principally cheese). A genomic meat assay was developed based on the myostatin gene. This was a universal assay for meat species: beef, pork, lamb, chicken and turkey (see Figure 5A). Egg only contains one genome copy per egg, and hence chicken myostatin gene is unlikely to be detected at the levels used in composite foods. Dairy products, on the other hand, usually contain larger amounts of beef DNA from stomatic cells from the cow's udder, which enters the milk. This problem was overcome by developing an additional test, raising a probe to the Sry gene of the bovine Y chromosome, only present in male cattle (Figure 5B). Most industrial meat is a mixture from male and female bovine animals. Therefore, in the case of a product containing a dairy ingredient, this second test would indicate the presence of male bovine DNA, which could only be from meat.

4. Conclusions

DNA technology is developing rapidly and has been applied to support enforcement of labelling and standards legislation. In particular the identification of meat, fish and plant species even in composite processed foods has been successful. Use of DNA technology for quantitative determination of ingredients at this stage is only partially successful. On the one hand, application to measure durum pasta and Basmati rice adulteration has been successful, but on the other hand accurate determination of meat ingredients in meat products has proved more difficult. The Agency's authenticity programme is examining alternative approaches to DNA technology for quantitative meat species, e.g. proteomics with mass spectrometry.

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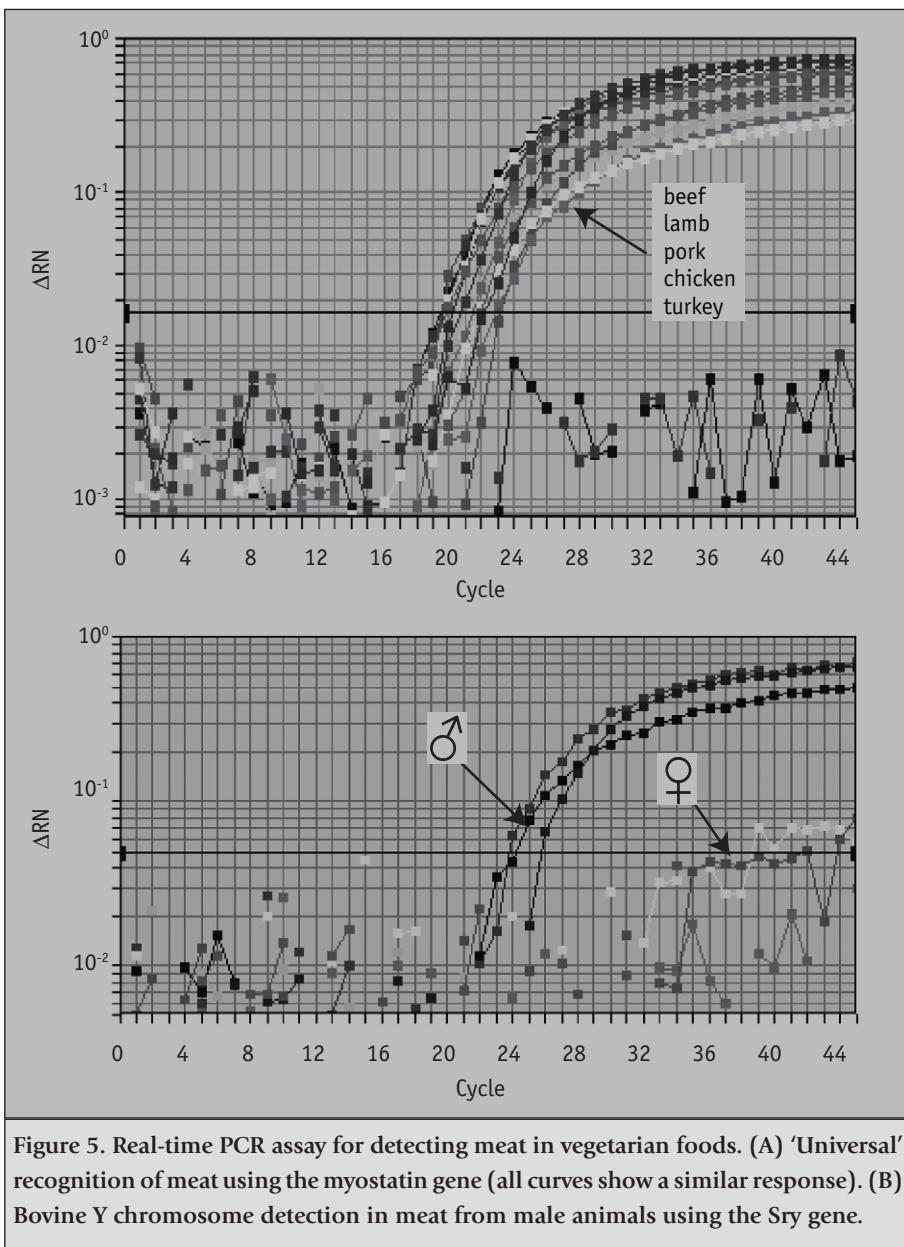


Figure 5. Real-time PCR assay for detecting meat in vegetarian foods. (A) 'Universal' recognition of meat using the myostatin gene (all curves show a similar response). (B) Bovine Y chromosome detection in meat from male animals using the Sry gene.

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Rapid methods for testing of oil authenticity: the case of olive oil

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Abstract

Food authenticity is of major concern to producers and consumers, evolving continuously in accordance with the growing global market. Analytical techniques have been developed delivering suitable solutions to sophisticated adulterations. Classical tests, most of which are based on chromatographic methods, are being replaced with newer techniques based on spectroscopy, isotope and trace element analyses, amongst other methods. The authenticity issues of the three classical edible oil groups - fish oil, vegetable oil and olive oil - have been used to cluster the information on the most remarkable classical and sophisticated analytical methods and techniques.

Keywords: authenticity, edible oils, olive oil, analytical techniques

1. Introduction

The growing global market together with the ever more stricter international regulations on food control have increased the number of samples to be analysed as well as their analytical variables. Consequently, there is an increasing demand for rapid methods, which allow a fast overall view of food traceability and safety, a demand widely claimed by producers, sellers and consumers. The progress in development of modern equipment and, in particular, the increasing availability of physical instrumental analysis have stimulated the development of rapid methods for the authentication of edible oils. However, there is not a uniform idea on a valid definition for the term 'rapid method' due to the lack of conceptual clarity of the adjective 'rapid' and the need of an explanation for the noun 'method'. Even though rapid methods are characterised by time, the adjective 'rapid' does not include the condition of a time limit. In fact, it is not only a matter of methodology and instrumentation, but also of organisation up to the level of automation with more exacting requirements regarding the variables to be determined in each scientific field. 'Method', on the other hand, should include sample pre-treatment or sample preparation, chemical

or physical analysis, data analysis, and evaluation of the results. Furthermore, the method can be of varied significance depending on the kind of problem (e.g. adulteration vs. production system), the applicability range (e.g. general vs. particular solution), and the execution level (e.g. screening vs. quantitative determination).

The authentication and characterisation of edible oils, two terms that overlap and sometimes confuse the analysts, have been widely studied. However, there is no equal interest in all edible oils, taking into account the papers published in Science Citation Index journals. The importance of authentication of edible oils seems to depend on the ratio between the shares of value and volume. Thus, olive oil, which only represents 2% of the world production, has the highest rank due to the fact that it represents 14% of the international trade, just after palm oil (30%) and soybean oil (19%), but before sunflower oil (12%), rapeseed oil (9%), lauric oil (9%) and corn oil (3%). The ratios are seven for olive oil and one or lower for the other oils, respectively.

The chemical composition of edible oil is rather wide, although more than 95% correspond to tri-esters of glycerol. Fortunately, the relative proportion of each fatty acid is by no means constant, and varies depending on species, subspecies (varieties), climate, geographical origin, etc. The unsaponifiable matter, which represents approx. 5%, clusters several groups of chemical compounds (sterols, alcohols, waxes, hydrocarbons, phenols, carotenes, chlorophylls, volatiles, etc.), some of them being specific of crude edible oils. With such a panoply of chemical compounds the possible armoury of techniques to authenticate any edible oil is wide enough. Table 1 shows the techniques clustered into four groups, defined by their most remarkable characteristic, plus the fifth miscellany group with a basic characteristic consisting of hyphenated techniques: gas chromatography (GC), reversed-phase liquid chromatography (RPLC) GC, high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), mass spectrometry (MS), near infrared spectroscopy (NIR), Fourier transform infrared spectroscopy (FTIR), Fourier transform Raman spectroscopy (FT-Raman), metal oxide semiconductor sensor (MOS), surface acoustic wave sensor (SAW), polymerase chain reaction (PCR), isotope ratio mass spectrometry (IRMS), inductively coupled plasma- atomic emission spectrometer (ICP-AES), atomic absorption spectrometry (AAS), flame atomic absorption spectroscopy (FAAS), electrothermal atomisation-AAS, headspace (HS) CG, HS-MS, GC-MS, HPLC-MS, ICP-MS, elemental analyser-pyrolysis-isotope ratio mass spectrometry ($\delta^2\text{H}$ -EA-Py-IRMS), $\delta^2\text{H}$ -GC-Py-IRMS, supercritical fluid chromatography (SFC).

Table 1. Main characteristic of the techniques used in the authentication of edible oils.

Characteristic	Technique
Separation	GC, RPLC-GC, HPLC
Structural and pattern recognition	NMR, MS, NIR, FTIR, FT-Raman, MOS and SAW sensors, PCR
Stable isotope analysis	IRMS
Trace element analysis	ICP-AES, AAS, FAAS, ETA-AAS
In-tandem	HS-GC, HS-MS, GC-MS, HPLC-MS, ICP-MS, $\delta^{2}\text{H}$ -EA-Py-IRMS, $\delta^{2}\text{H}$ -GC-Py-IRMS, SFC

Regulators and consumers have suggested numerous criteria for defining the authenticity of a food product. Thus, the purpose of authenticity testing is not solely to detect a possible adulteration or falsification, but also to determine whether a foodstuff is truly authentic. Adulteration, geographical origin, extraction system, production system and subspecies, or variety, are the main authenticity issues associated with edible oils, although their relative relevance depends on the global market trend. Table 2 shows the issues and their possible sub-issues, as well as current examples for olive oil, which has traditionally been the objective of adulterators due to its high price and increasing demand from consumers. Although olive oil is the most studied and regulated edible oil, its authentication is still an unsolved problem for some kinds of adulterations, e.g. the addition of deodorised virgin olive oil to virgin olive oil. This should not be a surprise, since as soon as a method is known to detect one particular kind of adulteration (i.e. addition of seed oils by the quantification of sterols), the adulterators switch to another one. Once the analytical 'guard' is dropped (i.e. in the case of desterolised seed oils), they return to the original adulteration. Tables 3-5 summarise the current techniques used in authenticity testing of vegetable oils, in particular olive oil, and fish oils, however, most of them have not been evaluated with blind samples and intercomparison studies (Horwitz, 1995). They vary from classical to very sophisticated techniques, which are capable of discriminating minor differences associated with authenticity issues: GC, dynamic headspace (DHS) GC, liquid chromatography (LC) GC, supercritical fluid extraction (SFE) GC, solid-phase microextraction (SPME) GC, reversed-phase liquid chromatography (RPLC) GC, HPLC, HPLC-MS, reversed-

Table 2. Main authenticity issues and sub-issues, and current examples for olive oil.

Issue	Sub-issue	Example
Adulteration	Addition of cheap oil to expensive oils	Detection of refined hazelnut in ROO
	Addition of refined oils to crude oils	Detection of seed oils in VOO
	Addition of low to high oil categories	Detection of deodorised VOO in VOO
Geographical origin	Inexact label	Detection of VOO from several origins
	Subsidy	Import between countries
Production system	Traceability	Characterisation of PDO
	Organic vs. conventional	Addition of conventional to organic OO
Extraction system	Centrifugation and percolation	Characterisation of VOO two-phase centrifugation
	Cold-press vs. solvent	
Type	Variety	Characterisation of European VOO
	Species	Characterisation of edible oils
OO, olive oil; PDO, protected designation of origin; ROO, refined olive oil; VOO, virgin olive oil.		

phase (RP) HPLC, NMR, HS MS, FT-Raman, attenuated total reflectance-mid-infrared spectroscopy (ATR-MIR), FT-MIR, fluorimetry, MOS sensors, PCR, PCR restriction fragment length polymorphism (RFLP), PCR single strand conformation polymorphism (SSCP), IRMS, ICP-AES, ETA-AES, ICP-MS, FAAS, $\delta^2\text{H}$ -EA-Py-IRMS, $\delta^2\text{H}$ -GC-Py-IRMS, DHS SFC, differential scanning calorimetry (DSC). Some of them are irrefutable analytical methods in particular issues, e.g. stigmastadienes in the case of addition of refined to crude oils, but only a few might be defined as rapid.

Table 3. Overview of methods used in fish oil authenticity testing.

Method	Analyte - Indicative information	Applicability
NMR	$^{16}\text{O}/^{18}\text{O}$; $^2\text{H}/^1\text{H}$ lipids by ^{13}C , ^1H and ^2H	species identification geographical origin
^{13}C NMR	positional distribution of the ω -3 FA in the triglyceride moiety. fingerprint includes FFA, fatty acids esterified to mono-, di- and tri-glycerides, and phospholipids	adulteration with vegetable oil
PCR	DNA by PCR-RFLP and PCR-SSCP	species identification
GC	FA profile	adulteration with vegetable oil
SFC	TAG distribution	species identification process identification

FA, fatty acids; FFA, free fatty acids; TAG, triacylglyceride.
References: Aparicio *et al.*, 1998; Martinez *et al.*, 2003; Woolfe and Primrose, 2005

This paper describes the most rapid methods used for different kinds of authenticity issues related to olive oil. When possible, they are compared with the current most powerful analytical standard. Particular attention is paid to the adulteration of olive oil with hazelnut (*Corylus avellana* L.) oil, which has been one of the most difficult challenges for the analysts until recently (Bowadt and Aparicio, 2003). These edible oils have a very similar chemical composition (Benitez-Sánchez *et al.*, 2003) and adulterations at percentages lower than 20% are not detected with the current official analytical methods. All methods have been grouped according to the time needed for the entire analysis (sample preparation, physical/chemical analysis and data analysis), the limits of detection and quantification, as well as their advantages and disadvantages.

Table 4. Overview of methods used in seed oils authenticity testing.

Method	Analyte - indicative information	Applicability
GC	Linolenic (C18:3) and FA composition at TAG 2-position	Presence of GO and SO in SOY and RO
GC	Brassicasterol	Presence of SO and GO in RO
HPLC	γ -tocopherol	Adulteration of SO with SOY
GC	C60/C58 ratio	Adulteration of SAF with SO
GC	C48 concentration \times palmitic acid enrichment factor	Detection of stearins or oleins in palm oil
GC	Palmitic (C16:0) C50 and C54	Presence of palm olein in cottonseed oil
GC	C33, C36, C38, C40	Detection of mixtures of palm kernel and coconut oil
GC	Oleic (C18:1)	Detection of palm kernel olein oil in palm kernel
IR/MS	$^{13}\text{C}/^{12}\text{C}$ ratios	Presence of maize oils in other commercial oils
GC	Linoleic (C18:2), erucic (C22:1)	Presence of borage oil in evening primrose oil
GC	Linoleic (C18:2), stearidonic (C18:4)	Presence of blackcurrant seed oils in evening primrose oil
GC	C18 (oleic, linoleic, linolenic) FA	Geographical origin of GO
RP-HPLC	Tocopherols, tocotrienols	Characterisation of GR, HOSO and SOY
SPME-GC	Volatile compounds	Edible oil quality
RP-HPLC	Carotenoid/carotene profiles	Authentication of linseed, olive, sesame and wheat germ oils
DSC	Information of the temperature curve	Characterisation of palm, palm kernel and coconut oil
ATR-MIR FT-Raman	Selected wave numbers	Characterisation of seed oils

C, carbon number triacylglyceride; FA, fatty acid; GO, groundnut oil; GR, grapeseed oil; HOSO, high oleic sunflower oil; RO, rapeseed oil; SAF, safflower oil; SO, sunflower oil; SOY, soybean oil.

References: Aparicio and Alonso, 1994; Aparicio and Aparicio-Ruiz, 2000; Aparicio *et al.*, 1998; Baeten *et al.*, 1998; Biswas *et al.*, 2004; Gan *et al.*, 2005; González Martín *et al.*, 2001; Guillén and Ruiz, 2001; Hidalgo and Zamora, 2003; Hourant *et al.*, 2001; Jelen *et al.*, 2000; Kelly and Rhodes, 2002; López-Díez *et al.*, 2003; Wang *et al.*, 2006; Yang *et al.*, 2005

Table 5. Overview of methods used in olive oil authenticity testing.

Method	Analyte - indicative information	Applicability
HPLC	TAGs	Olive oil categories
RP-HPLC	Tocopherols, tocotrienols	
FT-IR, fluorimetry	Selected wavenumbers	
GC	Several compounds	
DHS-GC	Volatiles	VOO European varieties
HPLC	Chlorophylls	VOO Spanish varieties; Non-natural products in VOO
¹³ C-NMR	Selected δ_{ppm}	Italian VOO: variety and origin
GC	Aliphatic alcohols	VOO Extraction systems
GC	Terpenic alcohols	Altitude of Spanish VOO cultivars
GC	Stigmastadienes	Presence of any refined oil in VOO
HPLC	TAGs	Presence of MO, CO, SO & RO in OO
RP-HPLC	TAGs	Presence of canola oil in ROO
NMR	Squalene, sterols	Presence of POO in ROO
GC	Ratio R1/R2	Presence of all the desterolised vegetable oils in OO
MOS sensors	Sensor response	VOO: varieties and categories
HPLC-MS	Tri- and diglycerides	Characterisation of several seed oils
SPE-GC, RPLC-GC	Filbertone	Presence of crude HO in VOO
SPME-GC/MS		
HS-MS	All the fragments	
¹³ C-, ¹ H-, ² H-NMR, FT-MIR, FT-Raman	Spectrum peaks	Presence of some seed oils in OO
HPLC and GC	Theoretical vs. empirical TAGs	

Table 5. Continued.

Method	Analyte - indicative information	Applicability
LC-GC	Sterols	
$\delta^{2\text{H}}$ -EA-Py-IRMS	Ion ratio	Presence of HO in OO
$\delta^{2\text{H}}$ -GC-Py-IRMS	Isotope ratios FA	
ICP-AES and ETA-AAS	Ca, Co, Cr, Cu, Fe, K, Mn, Na, Ni, Pb, Zn	Geographical origin
ICP-MS and IFAAS	Al, Mn, Cu, Pb, Sn, Bi, Ba, Na, Ca, Mg	
CO, cottonseed oil; FA, fatty acids; HO, hazelnut oil; HOSO, high oleic sunflower oil; MO, maize oil; OO, olive oil; POO, olive-pomace oil; RO, rapeseed oil; ROO, refined olive oil; SO, sunflower oil; TAG, triglyceride; VOO, virgin olive oil. References: Aparicio and Alonso, 1994; Aparicio and Aparicio-Ruiz, 2000; Aparicio <i>et al.</i> , 1998; Baeten <i>et al.</i> , 2000, 2001, 2005; Blanch <i>et al.</i> , 1998; Bowadt and Moreda, 2000; Dobarganes <i>et al.</i> , 1999; EC, 2002; García-González and Aparicio, 2003; García-González <i>et al.</i> , 2004a,b; Gordon <i>et al.</i> , 2001; Hidalgo and Zamora, 2003; Harwood and Aparicio, 2000; Jiménez <i>et al.</i> , 2004; Luna <i>et al.</i> , 2006; Luterotti <i>et al.</i> , 2002; Mariani <i>et al.</i> , 1999, 2006; Morales <i>et al.</i> , 2004; Peña <i>et al.</i> , 2005; Sayago <i>et al.</i> , 2004; Tay <i>et al.</i> , 2002; Woolfe and Primrose, 2005; Zabaras and Gordon, 2004; Zeiner <i>et al.</i> , 2005		

2. Materials and methods

2.1. Samples

Selection of samples is not easy if the research is focused on determining the feasibility to detect low percentages of hazelnut oil in olive oil with diverse kinds of instruments and methodologies. The samples, produced within the EU-funded project 'Development and assessment of methods for the detection of adulteration of olive oil with hazelnut oil' (MEDEO), were selected to let the techniques work near their minimum detection levels. Oils from diverse varietals of species and geographical origins, obtained with several extraction systems, constituted the set of samples, which were split into training and test samples. The number of training samples was 68, while 35 test samples were analysed (blind samples). The admixtures of the training samples were prepared with refined and lampante virgin olive oils from several geographical origins (Greece, Italy, Morocco, Spain, Tunisia and Turkey) and two kinds of hazelnut oils (refined and crude) from France, Italy, Spain, and Turkey. The percentage of hazelnut oil in olive oil varied from 2 to 20% (2, 5, 8, 11, 14, 15, 17, and 20%). The preparation of the training samples was as follows: 50% of the samples were prepared by mixing Turkish olive oils with Turkish hazelnut oils, 20% of the samples were prepared with oils from other geographical origins, and 30% of the samples were genuine olive oils. The selection of the test (blind) samples was based on three assumptions: (1) the adulteration of refined olive oils with refined hazelnut oils is the current major problem, (2) the most common blends are made by adding Turkish hazelnut oils to Turkish olive oils, because the cheapest hazelnut oil is produced in Turkey (its production equals 80% of the hazelnut world production), and (3) there are always possibilities of adulterated mixtures, whatever the variety or the geographical origin of the edible oils.

2.2. Methods

Methods were set up and then validated with the set of samples described in paragraph 2.1, to detect the presence of hazelnut oil in olive oil at low percentages. Nine methods (three chromatographic, five spectroscopic and one isotopic) out of a total of fifteen used during the MEDEO project, are described.

2.2.1. Methods based on chromatography

The first three methods are based on separation techniques and are used to quantify volatiles (filbertone), sterols (free and esterified), triacylglycerides and fatty acids. Filbertone is a marker of crude hazelnut oil, the profile of sterols distinguishes olive oil from some seed oils. Recent studies have stated that the difference between empirical and theoretical triglycerides (derived from the fatty acid composition) might detect the presence of any seed oil in olive oil due to the fact that the latter follows the biosynthetic 2-restricted 1,3 random theory for fatty acids in triglycerides, while seed oils do not. SPME was used as a concentration step of the volatiles prior to their quantification by GC-MS. One g of oil sample was incubated in a 20 ml vial fitted with a silicon septum for 30 minutes at 80 °C with agitation, followed by desorption of the SPME fibre (50/30 µm DVB/Carboxen/PDMS) for 10 minutes at 250 °C. The GC column was a SPB-1 SULFUR (Supelco, USA) (30 m × 0.32 mm × 4 µm). The oven programme was 50 °C (held 1 minute) to 160 °C at a rate of 6 °C per minute. The injector temperature was 250 °C. The ion trap mass-spectrometer operated in full scan mode over the mass range 30-150. The temperatures of the transfer line and source were both set to 250 °C. Filbertone (5-methylhept-2-en-4-one) and the internal standard (5-methyl-2-hexanone) were detected and measured by the reconstructed ion chromatograms at m/z 69, 111, 98, 126 (filbertone) and m/z 58, 71, 81, 114 (internal standard).

The sterols were quantified by gas chromatography. The method involved the separation of the apolar fraction (containing esterified sterols) from the polar fraction (containing free sterols) by silica gel column chromatography. Thus, 5 g of the oil sample was weighed in a 50 ml vial to which 10 ml of n-hexane together with 1 mL of an internal standard solution (α -cholestanol and 50 mg cholesteryl stearate in 10 ml of n-hexane) was added. The whole solution was transferred to a chromatography column (25 g of silica gel in 80 ml of n-hexane) letting the sample flow until it reached approx. 2 mm above the silica surface. Then 150 ml of n-hexane/diethyl ether mixture (87:13 v/v) was passed at a flow rate of 2 ml per minute. The eluate, which contains the apolar fraction, was recovered in a 250 ml flask and evaporated completely by means of a rotary evaporator. Diethyl ether (150 ml) was immediately passed through the column. The new eluate, also recovered in a 250 ml flask and completely evaporated, contains the polar fraction. The apolar fraction was saponified with an ethanolic potassium hydroxide solution and the unsaponifiable matter was extracted with diethyl ether. The sterol fraction was separated by thin layer chromatography (TLC)

and analysed by a GC capillary column (IOOC, 2004c). A simple mathematical procedure based on content of esterified sterols and the ratios between some esterified and free sterols, allowed to detect the presence of hazelnut oil in olive oil at low percentages.

HPLC analysis of the triglycerides requires a sample purification step prior to chromatographic analysis. Oil sample was purified by passing through a SPE silica gel cartridge (1 g, 6 ml) (Waters, USA). The cartridge was placed in a vacuum elution apparatus and washed under vacuum with 6 ml of hexane. The vacuum was released to prevent the column from drying and a conic flask was placed under the cartridge. A solution of the oil (approx. 0.12 g) in 0.5 ml of hexane was loaded onto the column, the solution was pulled through and then eluted with 10 ml of an hexane-diethylether (87:13 v/v) mixture under vacuum. The eluted solvent was homogenised and approx. half of the volume was poured into an other conic flask. Both solutions were separately evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. One residue was dissolved in 1 ml of n-heptane and poured into a 5 ml screw top glass tube for preparing fatty acid methyl esters (FAMEs). For triacylglycerol analysis, the other residue was dissolved in 1 ml of acetone and poured into a second 5 ml screw top glass tube. Determination of triacylglycerol composition was carried out in a RP-HPLC system composed of a Beckman Gold 126 pump unit, Beckman Gold 508 autosampler, Beckman-Mistral peltier column thermostat unit (Beckman-Coulter, USA), Gastorr 154 vacuum degasser (Flom, Japan), Perkin-Elmer PE-200 refractive index detector (Perkin-Elmer, USA), and Lichrosphere 100 RP-18 column (25 cm × 4 mm internal diameter, 4 µm particle size). Propionitrile was used as mobile phase at a flow rate of 0.6mL per minute. The column temperature was maintained at 20 °C. The injection volume was 10 µl.

The fatty acid composition was determined according to the official method (EC, 2002). The chromatographic characteristics were as follows: the GC system was an HP-5890 (Hewlett-Packard, USA) provided with a flame ionisation detector (FID) and a SP-2380 fused silica capillary column (50 m×0.25 mm internal diameter) (Supelco, USA) coated with cyanopropylpolysiloxane (0.25 µm film thickness). The oven temperature programme was isothermal at 165 °C for 10 minutes and then raised up to 200 °C at 1.5 °C per minute. Injector and detector temperatures were 220 °C and 250 °C, respectively. The carrier gas was hydrogen at 130 kPa.

2.2.2. Methods based on vibrational spectroscopy

FT-MIR spectra ($4,000\text{-}900\text{ cm}^{-1}$) were acquired with an Aegys MI2000 XS FT-IR spectrometer (Anadis Instruments, USA) equipped with a Michelson interferometer and an ATR crystal. A horizontal ATR crystal (ZnSe crystal with six internal reflections) plate was used to collect the spectral data. The ATR crystal was cleaned with hexane and wiped dry before putting the sample on its surface. For analysis of the unsaponifiable matter the samples were dissolved in hexane before being placed on the crystal, and the dissolvent was evaporated before spectral analysis. The reference spectrum was air collected before each sample analysis. Resolution was set at 4 cm^{-1} and 50 scans were collected per spectrum.

Raman spectra were acquired on a NIR-FT-Raman spectrophotometer 2000R (Perkin-Elmer, USA) equipped with a neodymium-doped yttrium aluminium garnet (Nd-YAG) laser source emitting at 1,064 nm. The 180° backscattering refractive geometry and an indium gallium arsenide (InGaAs) detector have been used. The spectral data were obtained with a resolution of 4 cm^{-1} and a laser power of 600 mW. For each spectrum, 50 scans were co-added and averaged to get a good signal to noise ratio. The unsaponifiable matter was diluted to 25% (w/w) with carbon tetrachloride and introduced in NMR tubes (75 mm, 5 mm i.d), while the entire oil was put into classical test tubes (75 mm, 12 mm i.d) and kept in a water bath at a temperature of $40\text{ }^\circ\text{C}$ prior to analysis. Analyses were performed using a thermostated sample holder at $45\text{ }^\circ\text{C}$.

2.2.3. Methods based on nuclear magnetic resonance

$^1\text{H-NMR}$ spectra were obtained from 50 μl edible oil samples dissolved in chloroform-d (700 μl) and DMSO-d (20 μl) and then placed into 5 mm NMR tubes. The spectra were recorded at 300K on a Bruker Avance AQS600 operating at 600.13 MHz (Bruker, Germany). $^1\text{H-NMR}$ free induction decays (FIDs) were recorded using the following acquisition parameters: acquired points, 32K; processed points, 32K; spectral width, 14 ppm; relaxation delay, 2 seconds; $\pi/2$ pulse, about 9 milliseconds; acquisition time, 1.5 seconds; number of scans, 4,000. The spectra were obtained by Fourier transformation. Baseline correction was done for a quantitative evaluation of all the peaks of interest. The intensities of the selected resonances were normalised by setting the intensity of the resonance at 1.55 ppm to 1,000. This normalisation gives an index proportional to the molar ratio between each compound and the total amount of fatty chains.

The selected proton resonances correspond to cycloartenol at 0.297 ppm, an unassigned signal at 0.982 ppm, β -sitosterol at 0.627 ppm, linolenic fatty chain at 0.921 ppm, and squalene at 1.626 ppm.

^{13}C -NMR spectra were collected from 100 μl oil samples dissolved in d-chloroform (600 μl) and then placed into 5 mm NMR tubes. ^{13}C spectra were recorded at 300K on a Bruker Avance AQS600 operating at 150.9 MHz using the following parameters: acquired points, 256K; processed points, 128K; spectral width, 195 ppm; digital resolution, 0.22 Hz per point; relaxation delay, 18 seconds; acquisition time, 1.5 seconds; $\pi/2$ pulse, approx. 7 milliseconds; number of scans, 256. The GARP sequence for proton decoupling was applied during the whole sequence. The intensities of the selected resonances were normalised by setting the resonance at 62.130 ppm to 100. The selected carbon resonance was due to six CO signals (sn-1,3 palmitic and stearic chains at 173.17 ppm; sn-1,3 eicosenoic and cis-vaccenic chains at 173.15 ppm; sn-1,3 and sn-2 oleic chains at 173.14 and 172.73 ppm, respectively; sn-1,3 and sn-2 linoleic chains at 173.12 and 172.72 ppm, respectively) and to three CH_3 signals at 14.13, 14.12 and 14.08 ppm due to saturated oleic and linoleic fatty chains, respectively.

The ^2H -NMR spectra of sample oils dissolved in chloroform (w/w) were recorded with a Bruker DPX 400, operating at 61.4MHz, fitted with a ^{19}F field-frequency-locking device. Relaxation times were determined using an inversion recovery sequence with twelve inversion time values ranging from 5 milliseconds to 6 seconds. The methyl peak proved to have the highest T1 and was therefore used to determine the repetition time according to $\text{AQ}+\text{D1}=5^*\text{T1}$. Acquisition parameters were the following: number of scans 3,300, acquisition time 3.7 seconds, pulse (90°) 11 microseconds, $T=313\text{K}$ and broadband decoupling. Each spectrum was considered as a matrix of 32,768 points. The standard normal variate method was used to correct for any variation in the signal to noise ratio between the spectra. Variations between the resonances of comparable lines were corrected by fitting clusters of these lines to the average spectra using a range of 1 Hz. The spectra provide information on hydrogen/deuterium isotope ratios at seven specific sites. Recent advances in isotope ratio mass spectrometry design and high temperature pyrolysis have made feasible the measurement of deuterium/hydrogen ratios by continuous flow devices.

2.2.4. Method based on isotopic studies

The $\delta^2\text{H}$ value of bulk oils was determined by a Eurovector EA3000 elemental analyser (Milano, Italy) coupled to a PDZ Europa 20-20 (Cheshire, UK) isotope ratio mass spectrometer. The elemental analyser was fitted with an alumina reactor tube (450mm×13mm i.d) made of high purity Al_2O_3 . The tube was plugged with a 10 mm layer of quartz wool at the lower end and filled with glassy carbon grit (300-800 μm ; Sercon, Cheshire, UK) to an overall height of 220 mm, at which point a further 10 mm layer of quartz wool was inserted. The temperature of the pyrolysis furnace was maintained at 1150 °C. A chemical trap containing magnesium perchlorate and carbosorb removed water vapour and carbon dioxide. The pyrolysis gasses passed through the GC column packed with molecular sieve held at 140 °C to separate H_2 , N_2 and CO before the effluent was split into a portion, which entered the ion source of the IRMS at approximately 0.05 ml per minute. Total run time was 400 seconds. The signal on m/z 2 produced by 1 μL of oil, was typically within the range 1×10^{-8} to 2×10^{-8} amps. Hydrogen isotope ratios were calculated with reference to the international standard NBS22 (International Atomic Energy Agency (Vienna, Austria) and reported as $\delta^2\text{H}$ ‰ vs. Standard Mean Ocean Water (SMOW). NBS22 has an assigned $\delta^2\text{H}$ value of $-118.5 \pm 2.8\text{‰}$ vs. SMOW. Liquid sample capsules (tin, 4.5 mm×2 mm diameter) were obtained from Exeter Analytical, Inc. (North Chelmsford, MA, USA). Aliquots of each oil sample were dispensed with a clean syringe into the tin capsules, which were then crimp sealed.

2.3. Statistical procedures

In-house mathematical procedures, i.e., Global and Exempla, as designed by their authors (IOOC, 2002, 2004a-b), were used for the methods based on the differences between theoretical and empirical TAGs. Multivariate statistical procedures (e.g. principal component analysis, stepwise discriminant analysis, partial least square analysis, etc.) or artificial neural networks (ANN) were applied to the methods based on spectroscopic techniques or the methods using more than one variable to characterise the oils. Univariate statistical analyses (ANOVA, Cochran, Brown-Forsythe, etc.) have been used for all the methods based on the information from one chemical variable. The statistical libraries containing univariate and multivariate procedures are diverse, and their kind (e.g. interactive vs. programmed) depends on the mathematical background of the authors.

3. Results and discussion

The efficiency of official food control is dependent on the ability to proceed with rapid tests for particular ingredients. The availability of physical methods for food analysis has contributed to the development of rapid methods, however, they are sometimes confused with screening methods and, consequently, applicable only for very specific authenticity subissues, sometimes being less specific or accurate. Because there is not a method qualified as official, rapid and global for all the authenticity issues of edible oil, numerous proposals have been designed to opt for that qualification or to compete with the current official methods. This is, however, not an easy task as the proposal has to fulfil three basic conditions: the samples for training purposes and to test the method have to represent the whole spectrum, the method has to improve the current standard in any aspect, and the method has to be validated with blind samples, carrying out an additional intercomparison study as well. Furthermore, the method should attempt to select those variables, which allow an extendable application.

Where a significant price difference exists between the product and its potential adulterant, adulteration levels as low as 5% may still be economically viable, e.g. in the case of olive oil. It is the target of adulterators, as it commands the edible oils in price and as it has an increasing reputability for its health and nutritional benefits, delicious taste and aroma. Currently, there is a proliferation of proposals suggesting that adulterants in virgin olive oil can be easily detected. However, most of them can only detect adulterations greater than 20%, sometimes with samples particularly chosen to produce fine results. The current analytical challenges lie in the protection of olive oil in any of the authenticity issues described in Table 2. Thus, it has been reported that quantities of hazelnut oil are being imported into the European Community seemingly not being declared to Customs and Excise. It is suspected that it is being used to adulterate olive oils bottled within the Community. The addition of refined hazelnut oil to virgin olive oil can be detected by the standard methods based on quantification of stigmastadienes, even at very low percentages of the adulterant (EC, 1991). However, the standard methods do not detect the presence of cold pressed hazelnut oil in virgin olive oil, if the resulting blend does not smell of hazelnut. Furthermore, the presence of refined hazelnut oil in refined olive oil or in blends of refined and virgin olive oils cannot be detected with the standard methods at percentages lower than 20%.

Advances in knowledge and technology have undoubtedly led to greater success in the fight against adulteration. However, it is equally true that the same techniques and knowledge are used by the adulterators to invalidate the usefulness of some standard methods in the near future. Such a competition requires not only a considerable investment to improve or develop techniques, but also to ensure that the pace of research in the detection of malpractice is rapid enough. Table 6 shows the most reputed methods for the current analytical challenges in the case of olive oil's most important authenticity issues. The information has been organised according to the sub-issues given in Table 2 with the aim of offering the reader a comparison of these methods by their basic characteristics. The techniques, and many of the methods, might be applied mimetically to the authenticity issues of other edible oils as well.

Concerning the addition of crude seed oils to olive oil the analytical challenge was, until recently, the detection of the presence of hazelnut oil. Analysts have proposed numerous methods, almost all based on the presence of filbertone. However, the methods based on enantiomeric analysis of filbertone, have problems to detect some adulterations at levels as high as 20% and show a high percentage of false positives. Only the method based on its quantification by SPME GC-MS (Aparicio, 2004) presents good limits of detection (LOD) and limits of quantification (LOQ).

The addition of any refined oil to a crude edible oil can be detected by the official method based on the quantification of stigmastadienes (EC, 2002). Today, there is not any other analytical method, which can improve its LOD, LOQ and accuracy. The current challenge, however, is to have only one method, which allows the detection of the presence of any edible oil - either refined or crude - in olive oil. This will avoid the bunch of official standard methods that have to be implemented to certify the authenticity of an olive oil sample. Two promising methods, based on the quantification of sterols and the difference between theoretical and empirical TAGs, have been investigated in the EU-funded project MEDEO (Aparicio, 2004) and they are currently being validated by intercomparison studies co-ordinated by the International Olive Oil Council (IOOC). Alternative methods based on spectroscopy (NMR, FT-MIR and FT-Raman) and isotopic analysis ($\delta^2\text{H}$ -EA-Py-IRMS and $\delta^2\text{H}$ -GC-Py-IRMS) were also investigated in this project. These are promising methods, which need even some improvements. A common need is a large database with information of edible oils of diverse agronomic and physicochemical characteristics. The other

Table 6. Basic characteristics of the methods proposed for the current analytical challenges to authenticity issues of olive oil.

Addition of crude oils to virgin olive oil	
Variable and technique:	Filbertone by SPME GC-MS.
Official method:	No, but the method has been validated with a blind trial.
Time of analysis:	Pre-treatment, 25 minutes; analysis, 20 minutes; data analysis, nil.
Limit of detection:	8% as LOD and 10 ppm of filbertone as LOQ.
Advantages:	Excellent LOQ and LOD with an easy and rapid method.
Disadvantages:	Restricted to adulterations with crude hazelnut oils. Filbertone quantification can be interfered by 2-octanone. Very oxidised VOO can mask the chromatogram too much.
References:	Bowadt and Aparicio, 2003; Aparicio, 2004; www.cica.es/aliens/igmedeo
Variable and technique:	Volatile fragments by HS-MS.
Official method:	No, and the method has not been validated with a blind trial.
Time of analysis:	Pre-treatment: nil; analysis, 30 minutes; data analysis, 45 minutes.
Limit of detection:	~10%, but not validated
Advantages:	Fingerprint of all the volatiles. Not restricted to particular crude edible oils.
Disadvantages:	Restricted to adulterations with crude hazelnut oils only. Ninety-nine percent of the qualitative information is common to both oils. Possible interference from the volatile profiles of varietal oils. Method requires multivariate statistical procedures or ANN.
References:	Peña <i>et al.</i> , 2005
Variable and technique:	Tocopherols or polar compounds by RP-HPLC.
Official method:	No, and the method has not been validated with blind samples.
Time of analysis:	Tocopherols: pretreatment, 5 minutes; analysis, 30 minutes; data analysis, 25 minutes. Polar compounds: pretreatment, 60 minutes; analysis, 40 minutes; data analysis, 25 minutes.
Limit of detection:	Tocopherols: not reported. Polar compounds: ~10%, but not validated.

Table 6. Continued.

Advantages:	Tocopherols: promising with deodorised oils.
Disadvantages:	The method is restricted to the adulteration with hazelnut oil and needs databases of varietal hazelnut and olive oils. Poor reproducibility of the polar compounds.
References:	Morchio <i>et al.</i> , 1999; Mariani <i>et al.</i> , 1999; Gordon <i>et al.</i> , 2001
Variable and technique:	Pyro-pheophytins by HPLC.
Official method:	No, and the method has not been validated with a blind trial.
Time of analysis:	Pre-treatment, 20 minutes; analysis, 25 minutes; data analysis, 10 minutes.
Limit of detection:	Not described.
Advantages:	Compounds are markers of a deodorisation (and refining) process.
Disadvantages:	The method needs a database with information of varietal virgin olive oils. Data should be analysed by pattern recognition algorithms.
References:	Serani and Piacenti, 2001
Geographical origin of virgin olive oils	
Variable and technique:	$\delta^{2\text{H}}$ by EA-Py-IRMS.
Official method:	No, but the method has been partially validated with blind samples.
Time of analysis:	Pre-treatment, nil; analysis, few minutes; data analysis, 5minutes.
Limit of detection:	Not reported.
Advantages:	Rapid method.
Disadvantages:	The method needs an olive oil database of producing regions. Harmonisation calibration procedure
References:	Rhodes <i>et al.</i> , 2003; Bowadt and Aparicio, 2003; www.cica.es/aliens/igmedeo
Variable and technique:	Multi-elements by ICP-MS or FAAS
Official method:	No, and the method has not been validated with blind trials.
Time of analysis:	Pre-treatment, 4 minutes; analysis, few minutes; data analysis, not reported.
Limit of detection:	Not described.
Advantages:	Rapid method. Promising, if the disadvantages are overcome.

Table 6. Continued.

Disadvantages:	Currently not applicable. The method needs olive oil and soil databases.
References:	Jimenez <i>et al.</i> , 2004; Zeiner <i>et al.</i> , 2005
Variable and technique:	Several chemical compounds (alcohols, sterols, hydrocarbons, etc.) by GC.
Official method:	No, but validated with hundreds of samples for several years.
Time of analysis:	Pre-treatment, 180 minutes; analysis, 300 minutes; data analysis, 30 minutes by expert system.
Limit of detection:	Certainty factors, 92% for Andalusian denominations of origin (DOPs), 95% for Spanish regions, 96% for the identification of major EU producing countries/varieties, etc.
Advantages:	High certainty factor. The largest VOO database. Fine chemical profile. Useful for the authentication of many monovarietal European and North African virgin olive oils.
Disadvantages:	Time-consuming. Many chemical analyses. Need to be updated.
References:	Aparicio and Alonso, 1994; Harwood and Aparicio, 2000; Aparicio and Luna, 2002
Addition of refined oils to virgin olive oil	
Variable and technique:	Stigmastadienes by GC.
Official method:	Yes, and validated with blind trials and intercomparison studies.
Time of analysis:	Pre-treatment, 90 minutes; analysis, 20 minutes; data analysis, no.
Limit of detection:	2%
Advantages:	Global method. Excellent LOQ (0.1 ppm) and LOD (2%).
Disadvantages:	The method does not detect refined oils obtained under soft decolouration and desodoration temperature <175°C.
References:	EC, 1991, 2002
Variable and technique:	cis/trans FAMEs by FT-Raman.
Official method:	No, but the method has been validated with blind samples.
Time of analysis:	Pre-treatment, nil; analysis, 10 minutes; data analysis, 10 minutes.
Limit of detection:	Higher than that for stigmastadienes.

Table 6. Continued.

Advantages:	Very rapid method.
Disadvantages:	Limit of detection. The method does not work properly with less unsaturated oils. No individual information from FAMEs.
References:	Baeten <i>et al.</i> , 2000
Addition of crude oils to virgin olive oil and addition of refined oils to refined olive oil	
Variable and technique:	Free and esterified sterols by LC-GC.
Official method:	No, but it is being validated with blind trials by IOOC.
Time of analysis:	Pre-treatment: 120 minutes; analysis, 45 minutes; data analysis, 15 minutes.
Limit of detection:	<8%, depending on the kind of edible oil.
Advantages:	Global method.
Disadvantages:	Time-consuming. Fine repeatability, but poor reproducibility. The application is restricted to a reduced number of vegetable oils.
References:	Mariani <i>et al.</i> , 1999, 2006; Bowadt and Aparicio, 2003
Variable and technique:	Difference between empirical and theoretical triglycerides by HPLC and GC for the detection of TAGs and FAMEs, respectively.
Official method:	No, but the method is being validated with blind trials by IOOC.
Time of analysis:	Pre-treatment, 60 minutes; analysis, 90 minutes; data analysis, 5 minutes by in-house algorithms.
Limit of detection:	~10%, but depending on the kind of edible oil.
Advantages:	Global and rapid method with individual information from FAMEs and TAGs.
Disadvantages:	Time-consuming. Remote, but possible false positives in the analyses.
References:	Cert and Moreda, 2000; Aparicio, 2004; www.cica.es/aliens/igmedeo

Table 6. Continued.

Variable and technique:	Selected ^{13}C - and ^1H -NMR peaks by ^{13}C -NMR and ^1H -NMR
Official method:	No, but the method has been validated by a blind trial
Time of analysis:	Pre-treatment: no; analysis, 4 hours for ^1H -NMR and 1.45 hour for ^{13}C -NMR, 5.45 hours in total; data analysis, 20 minutes applying ANN.
Limit of detection:	>10%, using ANN with set of data from ^{13}C -and ^1H -NMR. >15% only with ^{13}C -NMR data, and ~15% only with ^1H -NMR data
Advantages:	Great amount of information. The best results with refined oils only.
Disadvantages:	Time-consuming. Excellent repeatability but poor reproducibility. The method requires multivariate statistical procedures.
References:	García-González <i>et al.</i> , 2004b; www.cica.es/aliens/igmedeo
Variable and technique:	Bands of oil and unsaponifiable matter by FT-MIR or FT-Raman.
Official method:	No, but the methods have been validated by blind trials.
Time of analysis:	FT-MIR: pre-treatment, 5 minutes with oil, 65 minutes with its unsaponifiable matter; analysis, 5 minutes; data analysis, 25 minutes using ANN. FT-Raman: pre-treatment, nil with oil, 60 minutes with its unsaponifiable matter; analysis, 10 minutes; data analysis, 25 minutes using ANN.
Limit of detection:	>10%
Advantages:	Great amount of information.
Disadvantages:	Time-consuming, if working with the unsaponifiable matter. Restricted to hazelnut oils. Database needs to be extended to other oils. Unstable mathematical equations.
References:	Baeten <i>et al.</i> , 2000, 2005. Other proposals: Tay <i>et al.</i> , 2002; López-Díez <i>et al.</i> , 2003

Table 6. Continued.

Authentication of single varietal virgin olive oils

Variable and technique:	Volatiles by DHS-GC
Official method:	No, but the method has been checked with dozens of blind samples.
Time of analysis:	Pre-treatment, 15 minutes; analysis, 65 minutes; data analysis, 300 minutes.
Limit of detection:	Described in terms of major European varietal olive oils.
Advantages:	Great amount of information.
Disadvantages:	External interference factors (climate, ripeness, shelf-life).
References:	Harwood and Aparicio, 2000; Luna <i>et al.</i> , 2006

basic improvement concerns the design of a mathematical algorithm, which can be easily implemented by all analysts.

The increase in the number of protected designations of origin (PDOs) of virgin olive oil has also increased the number of proposals based on the detection of elements related to the soils or the quantification of minor compounds characterising the oils produced in the protected zones. International organisations should co-ordinate the building of a public database with physicochemical information on olive oils produced within the PDOs.

The addition of deodorised virgin olive oil to virgin olive oil is the current analytical challenge. The quantification of pyro-pheophytins seems to enlighten the control of this adulteration. However, a study on the kinetics of these compounds should be done before suggesting partially validated solutions. A database representing the edible oils involved in the authenticity issue, a good mathematical model to manage the data and a validation process with blind samples are the basic requirements to get a successful method.

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Species-specific detection of poultry in meat model mixtures and commercial sausage products by polymerase chain reaction-restriction fragment length polymorphism analysis

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Abstract

Polymerase chain reaction-restriction fragment length polymorphism was applied to species-specific detection of poultry meat in commercial sausage products. The sequence selected for amplification was a 359 base pair fragment of the mitochondrial cytochrome b gene as part of the template DNA. DNA was extracted from raw pork, beef, chicken, turkey, heat-treated model meat mixtures and from commercial sausage products using a DNA-binding silica resin, and subjected to polymerase chain reaction-restriction fragment length polymorphism analysis. Polymerase chain reaction analysis demonstrated a 359 base pair fragment for all meat samples examined. Rsa1 enzyme restriction profiles showed that poultry produced two different fragment patterns: two fragments (210 and 149 base pairs) for chicken and three fragments (149, 109, 101 base pairs) for turkey. The detection limit for chicken in meat model mixtures was 0.5%. Three positive commercial sausage samples were found to contain non-declared poultry.

Keywords: PCR-RFLP, species detection, chicken, sausage

1. Introduction

Species-specific identification of livestock and game meat is important for medical, ecological, financial and religious reasons. It is also important in the light of government regulations, because consumer protection and food safety have received greater attention in recent years. In most cases, cheaper or less

valuable meat of other species is added to the products. Many different analytical techniques have been developed and tested for species identification of meat, fish and dairy products. Most of these techniques like isoelectric focussing (IEF) and immunoassays (immunostick, enyzme-linked immunosorbent assays), are based on protein detection (Berger *et al.*, 1988; Bauer and Hofmann, 1989; Martin *et al.*, 1991; Hofmann and Blüchel, 1992; Hernández *et al.*, 1994). It has been reported that IEF is not suitable for identification of processed meat, because the soluble species-specific sarcoplasmic proteins (albumins, myoglobin) degrade rapidly at about 60 °C (Okayama *et al.*, 1991). Immunoassays are suitable in heat-treated products, but the antibodies often show cross-reactivity, e.g. in the case of domestic and wild boar, chicken and turkey, and beef, deer and mutton.

In general, DNA is more stable than proteins. However, Ebbehøj and Thomsen (1991) observed a shift in chain length from 30,000 base pairs (bp) into 300-400 bp in heat-treated meat products. Therefore, in these cases a smaller target sequence can be used for detection. Polymerase chain reaction (PCR) analysis of the mitochondrial cytochrome b or rRNA sequences with subsequent restriction fragment length polymorphism (RFLP) is often used to identify livestock (pork, beef, mutton), poultry, game meat, and fish species. (Meyer *et al.*, 1995; Matsugana *et al.*, 1998; Céspedes *et al.*, 1998; Branciari *et al.*, 2000). This paper describes an adaptation and application of a PCR-RFLP analysis for detection of poultry meat in commercial sausage products.

2. Materials and methods

2.1. Sample collection

Raw meat (pork, beef) and poultry (chicken, turkey) samples were obtained from a slaughterhouse (Bicske, Debrecen) and stored -18 °C until use. Heat-treated meat model mixtures contained water (40%), meat (total mass 30%), fat (24%), soy protein isolate (4%) and pickle salt (2%). These meat mixtures were prepared containing 0.5, 1, 5, 10, 50 and 100% chicken in pork meat and were heat-treated (core temperature 72 °C) under laboratory conditions. Commercial sausage products were purchased at a local market.

2.2. DNA extraction

DNA extraction was carried out with the Wizard method (Meyer *et al.*, 1994). This method is based on using DNA-binding silica resin to purify DNA from a

solution obtained after enzymatic (proteinase K) and chemical (SDS) treatment. All samples were cut with a sterile scalpel and 300 mg was digested in 430 µL TRIS-EDTA-SDS extraction buffer (pH 8.0) containing 20 µL proteinase-K (20 mg/ml) and 50 µL 5 M guanidine-hydrochloride. After 3 hours of incubation at 55 °C the suspension was centrifuged (10 minutes, 12,400 rpm, Jouan BR4i centrifuge). Supernatant (450 µL) was purified following the instructions of the manufacturer (catalogue no. A7280, Promega). Purity and concentration of the isolated DNA were assessed by determining the optical density ratio A260/280 (UV-1601 spectrophotometer, Shimadzu).

2.3. PCR and RFLP analyses

Amplification was performed in a reaction volume of 50 µl, which contained 25 µl Sigma PCR ReadyMix (P4600, Sigma; 1.5 U Taq polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.2 mM each dNTP), 0.5 µM cytochrome b1 (cytb1): 5'- CCA TCC AAC ATC TCA GCA TGA TGA AA-3' and cytochrome b2 (cytb2): 5'- GCC CCT CAG AAT GAT ATT TGT CCT CA-3' primers (Meyer, 1995.) and 250 ng DNA. PCR was carried out using a thermocycler (PDR-91, BLSA Ltd.) as follows: 1 cycle of 94 °C for 3 minutes, and 32 cycles of 94 °C for 5 seconds, 52 °C for 30 seconds, and 72 °C for 40 seconds, and a final extension of 72 °C for 3 minutes. A DNA free sample was used as a contamination control (blank). 8µl Amplified DNA products were further digested with 10 U Rsa1 enzyme (R4756, Sigma) at 37 °C for 2 hours. The DNA products (10 µl) were separated on 10% polyacrylamide gels (50 minutes, 200V), visualised by staining with ethidium bromide (Maniatis *et al.*, 1989) and documented with the Kodak EDAS 290 system.

3. Results and discussion

3.1. Meat and poultry

All DNA isolated from the meat and poultry samples had a A260/280 ratio between 1.7 and 2.0, which indicated that the DNA solutions were sufficiently pure for PCR. The DNA content of the solutions was between 7.18-9.25 µg/100 mg. In addition to the appropriate-quality DNA as measured spectrophotometrically, the absence of PCR inhibitors was confirmed with the vertebrate specific primer pair (cytb1/cytb2). In all cases 359 bp products were obtained from the DNA samples amplified with the cytb1/cytb2 primers (Figure 1A, lanes 3-6), indicating that they were of appropriate quality for PCR.

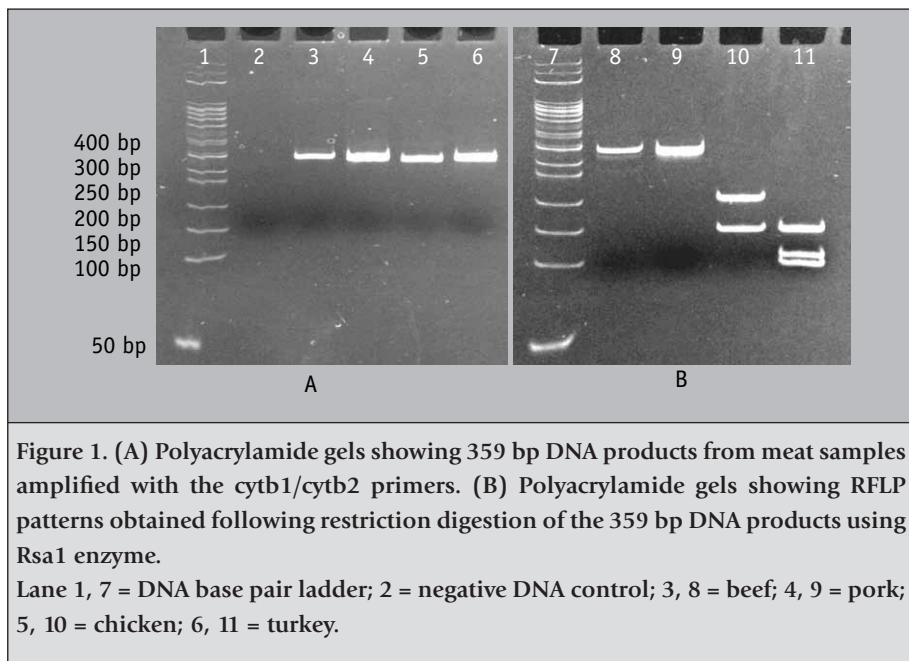


Figure 1. (A) Polyacrylamide gels showing 359 bp DNA products from meat samples amplified with the cytb1/cytb2 primers. (B) Polyacrylamide gels showing RFLP patterns obtained following restriction digestion of the 359 bp DNA products using Rsa1 enzyme.

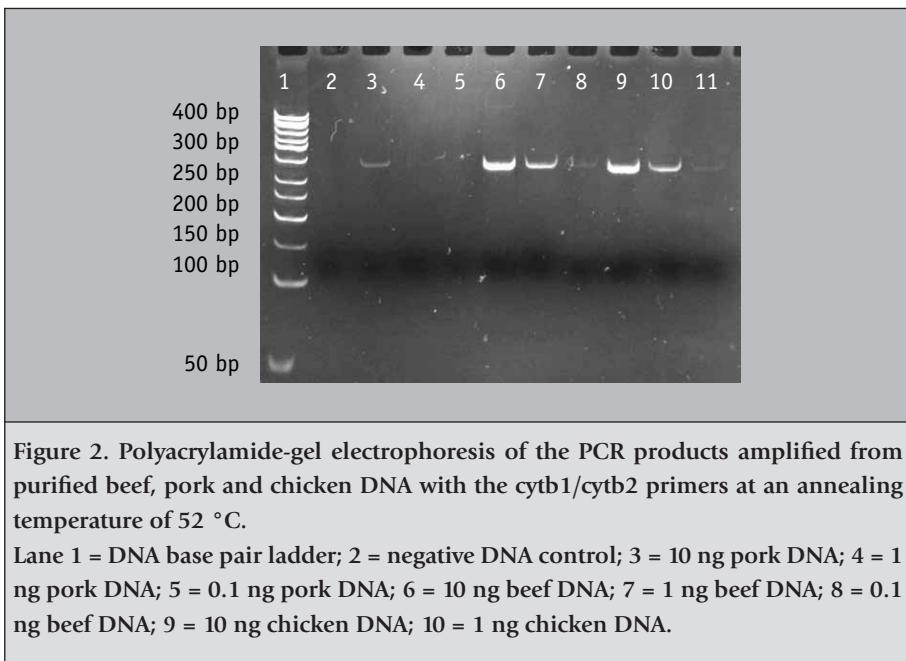
Lane 1, 7 = DNA base pair ladder; 2 = negative DNA control; 3, 8 = beef; 4, 9 = pork; 5, 10 = chicken; 6, 11 = turkey.

Following PCR the Rsa1 restriction enzyme was used for the digestion of the PCR amplicons. Poultry produced two different fragment patterns: two fragments (210 and 149 bp) for chicken and three fragments (149, 109, 101 bp) for turkey (Figure 1B).

It has been published for the cytb based PCR-RFLP assay (359 bp target sequence) that pork DNA can mask DNA from other species (Partis *et al.*, 2000). Preceding sausage analysis the sensitivity of the PCR was optimised by adjusting the primer annealing temperature for a better amplification of chicken DNA. Figure 2 shows the best amplification results, obtained at an annealing temperature of 52 °C. At this point beef and chicken DNA products gave a sharper PCR signal than pork DNA.

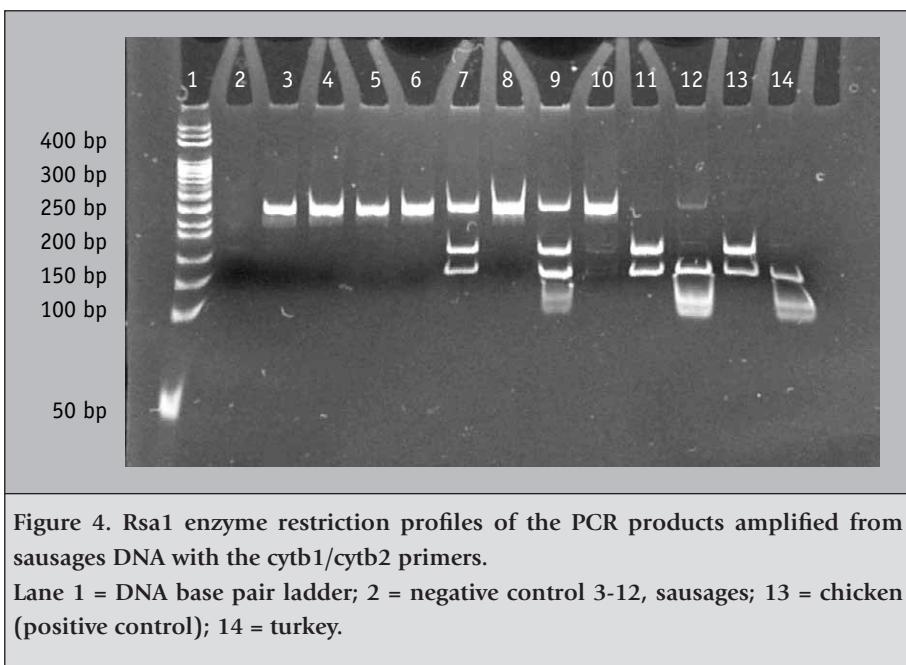
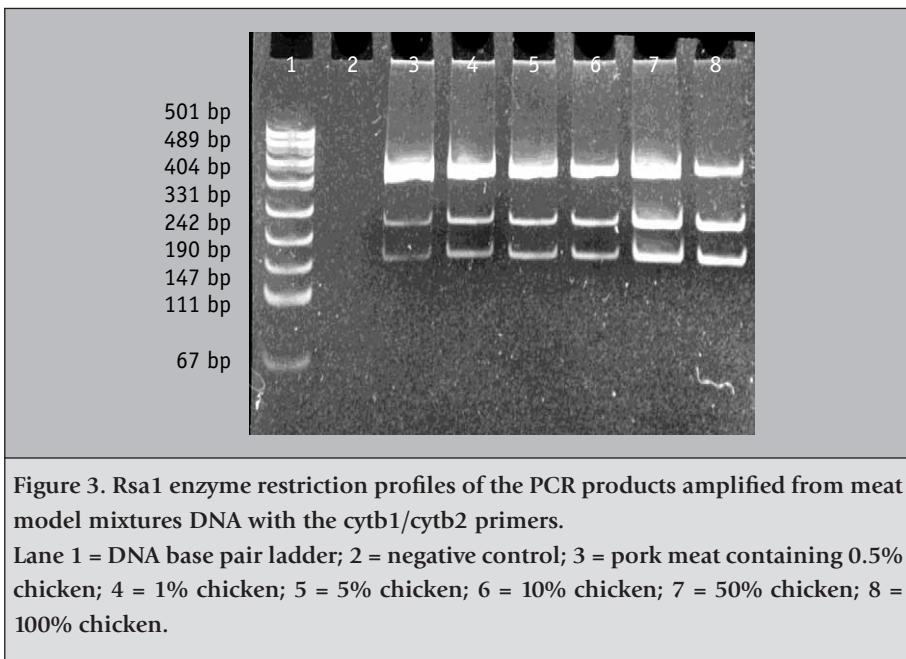
3.2. Meat model mixtures and commercial sausages

Meat model mixtures containing different levels (0.5-100%) of chicken in pork meat were used to study the effect of the food matrix on DNA purification and on the detection limit of the PCR-RFLP analysis. The chicken meat used for the



preparation of the meat model mixtures was applied as a positive control in sausage PCR-RFLP analysis. Concentrations of the isolated DNA solutions were between 4.66 and 7.10 µg /100 mg for the meat model mixtures, and between 5.05 and 14.60 µg /100 mg for the sausage samples. The substantial differences in DNA concentration of the sausage samples may be due to matrix effects, because these samples contained a high number of other components than meat, such as soy and spices.

With the meat model mixtures a detection level of 0.5% chicken meat was achieved (Figure 3). The results of the PCR-RFLP analysis of 10 randomly selected sausage products are shown in Figure 4. Five poultry meat positive samples were found. One pattern signal was very weak (lane 10), two other sausage products contained chicken (lane 7 and 11), one contained turkey (lane 12), and one contained DNA from both poultry species (lane 9). Three positive samples (lane 7, 9 and 10) were found to contain non-declared poultry.



4. Conclusion

In conclusion, PCR-RFLP analysis based on the 359 bp mitochondrial cytochrome b target and Rsa1 restriction enzyme is a useful method to identify chicken in raw and processed meat products. In principle, the sensitivity of mitochondrial sequence based PCR and PCR-RFLP will be higher than that of one-copy genome DNA-based assays, because the target sequences are present in higher copy numbers (up to 1,000) in each cell. Future experiments should also be carried out under factory circumstances to establish the effects of sampling methods, species-specific DNA fragments and product inhomogeneity on the reproducibility and detection limit of the assay. The method can be taken a step further by the design of new sets of primers within the 359 bp target sequence. With these primers species-specific PCR and real-time detection analysis with TaqMan assays will be feasible.

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Analytical methods for the profiling of intrinsic quality of green coffee: varietal and geographical characterisation

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Abstract

Coffee green beans of the two commercial coffee varieties, *Coffea arabica* and *C. canephora*, from the most important regions producing coffee in America, Africa and Asia were studied. The polyphenolic and methylxanthine contents analysed by liquid chromatography coupled with a UV-visible spectrometry and mass spectrometry, and the isotope ratios of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{11}\text{B}$ determined by elemental analysis isotope ratio mass spectrometry, were measured in coffee green beans. The two analytical approaches proposed were evaluated in order to characterise coffee according to the varietal and geographical origin. Quantitation of chlorogenic acids, cinnamate conjugates and caffeine allowed the varietal characterisation of coffee green beans unequivocally. Polyphenolic profiles and the methylxanthine contents, as well as the isotope values, enable classification by pattern recognition techniques for identifying coffee geographical origin at the continent level. Polyphenols and methylxanthines achieved 95% of correct classification for African and Asian Robusta coffees, and 94% for American Arabica samples. On the other hand, isotopic measurements achieved the 95% level of hits for American coffees, and 88% for African ones. Collection of higher number of samples should improve these results.

Keywords: coffee, polyphenol, caffeine, stable isotopes, geographical origin

1. Introduction

Coffee is one of the most important agricultural products from an economic point of view, being the second main commodity within the international trade, only next to oil. Moreover, coffee is the most important foreign exchange supplier for many agricultural oriented countries, and an attractive source for tax yield. Due to its large diffusion and high price, it is not unusual that

coffee is subject to adulterations throughout the production chain. The most common form of fraud involves the mislabelling to conceal the true origin of the product or special treatments. This affects the 'intrinsic quality' of coffee, since its organoleptic and nutritional properties are concerned. In other cases, coffee is adulterated with cheaper products, which represents a fraud related to the 'basic quality' of coffee. Consumers, coffee processors and farmers are principal victims of these fraudulent actions. Therefore, although there are established methods to determine the presence of external ingredients (ISO standards), and to assess some qualitative parameters of green coffee, a validated analytical method to confirm a coffee's appellation of origin is still lacking and needed. On the other hand, the importance of the authentication of the two commercial coffee bean varieties *Coffea arabica* (Arabica) and *C. canephora* (Robusta) lies on the fact that Arabica coffee is considered superior to Robusta and is, therefore, more expensive. Thus, the identification of adulteration and mislabelling at the varietal level is very important with special regard to consumer protection.

Several attempts have been made to develop analytical tools for coffee authentication. These approaches consist in the analysis of coffee tocopherols and triglycerides by high-performance liquid chromatography (HPLC) (González *et al.*, 2000), the determination of more than 50 elements in coffee by semi-quantitative scan inductively coupled plasma mass spectrometry (ICP-MS) (Prodolliet *et al.*, 2001), the analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ coupled with the site specific (D/H) isotope ratio by ^2H -nuclear magnetic resonance (NMR) on caffeine extracted from green coffee (Prodolliet *et al.*, 1997), or the characterisation of green coffee by $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{11}\text{B}$ analysis (Serra *et al.*, 2005). Total polyphenols together with other chemical descriptors (metals, amino-acids, furfurals, caffeine) have been used to perform varietal classification of coffee or to discriminate among different kinds of roasted coffees (Martin *et al.*, 1998). Chlorogenic acid content might also be useful as geographical origin indicator of Robusta coffee beans (Clifford and Jarvis, 1988). Caffeine, theobromine and theophylline determined by HPLC-MS or near infrared spectroscopy (NIRS) were used for varietal coffee authentication (Huck *et al.*, 2005). In this paper, two chemical approaches based on the analysis of the polyphenolic and methylxanthine contents by liquid chromatography coupled with UV-visible spectrometry, and on the measurement of the isotope values of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{11}\text{B}$ in the bulk green bean, are proposed. Our choice of selecting these chemical descriptors was driven by their relationship with varietal, environmental and climatic factors.

The main class of polyphenols present in green coffee beans are hydroxycinnamic acids, in particular the esters of quinic acid, commonly named chlorogenic acids (CGA). Six subgroups of CGA have been found in coffees: caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA), feruloylquinic acids (FQA), p-coumaroylquinic acids (CoQA), caffeoylferuloylquinic acids (CFQA), and feruloylcaffeoylquinic acids (FCQA) (Clifford *et al.*, 2003). CGA can also appear in green coffee conjugated with amino acids, such as tryptophan, tyrosine, and phenylalanine (Clifford and Knight, 2004). Phenolic acids play an important role in coffee quality, being implicated in its organoleptic properties, such as astringency, bitterness and aroma. In this sense, it seems that the quality of the beverage increases when the CGA content decreases, a fact that largely explains taste differences between Robusta and Arabica (Clifford, 1985). The methylxanthines caffeine, theobromine and theophylline are alkaloids regularly consumed from a wide variety of foods and beverages, coffee and tea being one of the most common sources (López-Martínez *et al.*, 2003). These compounds also contribute to coffee bitterness. The polyphenolic profiles and methylxanthine contents of coffees depend on coffee variety, climatology and the different methods of harvesting and processing (De Menezes, 1994).

Carbon and nitrogen, which are the main elements of living organisms, are here associated with boron, which plays an important role in coffee tree cultivation. The isotopic compositions of these elements are used as proxy for environmental parameters, which characterise a certain crop or area (Meinzer *et al.*, 1989). Since different elements represent different characteristics of the environment (water cycle, plant physiology, agricultural practices, hydric stress, soil geology, etc.), the combination of these indicators may be used to unequivocally identify a certain ecosystem of origin. A noticeable feature of using stable isotope abundance data in a multivariate approach to discriminate the origin of a product is that each single isotope is, in fact, the result of more than one unique phenomenon, and thus, very difficult to counterfeit for fraud purposes. The $^{13}\text{C}/^{12}\text{C}$ isotope ratio has become a classical means to distinguish between different photosynthetic pathways, and to study geographic and ecological distribution of plants. After the definition of models accounting for the fractionation observed in C-3 and C-4 plants, investigations have focused on the relationship between carbon isotope fractionation and water-use efficiency within the same type of plants, especially in the C-3 group. The enormous success of these studies has allowed the application of stable isotopes in genetic and agronomic studies, and in food analysis.

The $^{15}\text{N}/^{14}\text{N}$ isotope ratio in plants depends primarily on the source of nitrogen produced by symbiotic bacterial fixation or absorbed directly from soil nitrates. Nitrogen isotope ratios can also vary according to rainfall, altitude and other environmental factors that may hamper or enhance the vegetative activity of the plant. Boron is an essential microelement for plant growth. The behaviour of boron may be attributed to the influence of regional sources of boron used in agricultural practices (Marentes *et al.*, 1997). It seems that isotope composition of plants reflects that of the B supply with small fractionations dependent on plant species. Thus, significant isotopic $^{11}\text{B}/^{10}\text{B}$ variations due to the different origins of the samples have been found.

In these studies, a large sample set of coffee green beans from all the regions producing coffee in America, Africa and Asia is considered. The two data sets, gathering the polyphenolic and methylxanthine contents and the measurement of the stable isotope ratios, were evaluated separately by pattern recognition techniques, achieving complementary results, and affording useful analytical tools in order to perform varietal and geographical classifications of green coffees.

2. Materials and methods

2.1. Solvents and standard phenolics

Methanol (Carlo Erba, Milan, Italy) was of HPLC grade. Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Glacial acetic acid (Carlo Erba, Milan, Italy) and ascorbic acid (Merck, Darmstadt, Germany) were of analytical quality. All solvents used were previously filtered through 0.2 µm nylon membranes (Lida, Kenosha, WI, USA). Polyphenol standards were supplied as follows: 5-caffeooylquinic acid, p-coumaric acid, caffeic acid and ferulic acid by Sigma-Aldrich Chemie (Steinheim, Germany); caffeine by Merck (Darmstadt, Germany). Stock standard solutions of 5-caffeooylquinic acid, p-coumaric, caffeic acid, ferulic acid and caffeine at a concentration of 1 mg/ml were prepared in methanol and stored at 4 °C in darkness.

2.2. Plant material

Samples of green coffee of the Arabica (*C. arabica*) and Robusta (*C. canephora*) varieties were collected from roasters or coffee dealers able to supply samples with the assurance of the true origin of the coffees at least at the country level, and for several years (1998-2002). In few cases we were notified of the organic

agricultural practices used to cultivate the coffee (e.g. organic farming), but it cannot be excluded that other plantations used the same or similar techniques without indicating it on the package. Moreover, organic farming is still a controversial issue because different practices are gathered under the same denomination, even if quite different in practice. The 107 samples considered in this study came from 23 different countries representing the three continent producing coffees, America, Asia and Africa (Table 1). The beans of green coffee were ground and subsequently freeze-dried, and stored at room temperature in a dry chamber until analysis.

2.3. Analytical procedures

2.3.1. Direct solvent extraction and reversed-phase HPLC analysis

Freeze-dried coffee beans (0.1 g) were extracted with 10 ml of methanol-water-acetic acid (30: 67.5: 2.5, v/v/v) with ascorbic acid (2 g/l) in an ultrasonic bath during 15 min. Then, the crude solvent extract was filtered through a 0.45 µm PTFE filter (Waters, Milford, CA, USA) prior to injection into the HPLC system.

Table 1. Origin of coffee green bean samples.

America		Africa		Asia	
		Arabica	Robusta	Arabica	Robusta
Brazil	6			Cameroon	10
Colombia	1			Congo	1
Costa Rica	5			Ethiopia	7
Guatemala	9	1		Kenya	1
Honduras	1			Rwanda	1
Mexico	1			Uganda	6
Nicaragua	8			Zimbabwe	1
Panama	1				
Salvador	1				
Venezuela	2				
				India	2
				Indonesia	16
				Java	3
				Papua New Guinea	2
				Timor	1
				Vietnam	19

Chromatographic analysis was performed on a Hewlett-Packard Series 1100 system, equipped with a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a diode array detector (DAD), connected to a HP ChemStation software. A reversed phase Symmetry C18 (250×4.6 mm I.D., $5 \mu\text{m}$) column and a Symmetry C18 (10×3.9 mm I.D., $5 \mu\text{m}$) guard column (Waters, Milford, CA, USA) were used. The mobile phase consisted of solvent A (0.2% of acetic acid in water (v/v)) and solvent B (methanol). The elution conditions applied were: 0-30 minutes, linear gradient from 10 to 30% B; 40-45 minutes, 40% B isocratic; 45-50 minutes, linear gradient from 40 to 50% B; 50-55 minutes, 50% B isocratic; 55-60 minutes, linear gradient from 50 to 60 %B; and finally, washing and reconditioning of the column. The flow rate was 1 ml/min and the injection volume was $50 \mu\text{L}$. The system operated at 25°C . Methylxanthines were monitored and quantified at 280 nm, and hydroxycinnamic acids at 320 nm. Mass spectra were performed on a Finnigan MAT (San Jose, CA) LCQ ion trap mass spectrometer equipped with an APCI interface, in the positive ion mode, and using the following APCI experimental conditions: capillary temperature, 150°C ; vaporiser temperature, 450°C ; sheath gas flow rate, 80 arbitrary units; auxiliary gas flow rate, 26 arbitrary units; source voltage, 5 kV; source current, $80 \mu\text{A}$; capillary voltage, 46 V. Ion monitoring mode was full scan in the range 50-600 amu, which provides a complete mass spectrum for each analyte detected. MS^2 was carried out with a collision energy of 20%.

Polyphenol identification for which standards were available was carried out by comparison of their retention times, and their UV-visible and mass spectra with those of the standards. Some other chromatographic peaks were assigned to a particular polyphenol class according to their UV-visible and mass spectra and bibliographic sources. Quantification was performed by reporting the measured integration areas (DAD signal) in the calibration equation of the corresponding standards. Thus, chromatographic peaks that exhibit the UV-visible spectrum of caffeic acid, p-coumaric acid, ferulic acid or caffeine were quantified with the standard that corresponded to its spectrum.

2.3.2. Isotope measurements

For the determination of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values in continuous flow a Carlo Erba Elemental Analyser EA 1108 CHN coupled to a DeltaPlus mass spectrometer (ThermoQuest) was used. The analytical procedure was described in detail by Brescia *et al.* (2002). For $\delta^{11}\text{B}$ measurements coffee bean samples were dissolved

by microwave digestion, and the isotope abundance ratios were measured by negative thermal ionisation mass spectrometry using an extensively modified AVCO thermal ionisation mass spectrometer according to the method developed by Wieser *et al.* (2001). All the results were calculated according the following equation:

$$\delta \text{ ‰} = [(\text{Sample ratio} - \text{Reference ratio})/\text{Reference ratio}] \text{ ‰}$$

The results of carbon and nitrogen isotope ratio analyses are reported in per mil (‰) relative to V-PDB and Atmospheric Air, respectively, both defined as 0‰ point in the two different scales. The precision (standard deviation) for analysis of laboratory standard (glutamic acid) for $\delta^{13}\text{C}$ is $\pm 0.046\text{ ‰}$ ($n=10$) and for $\delta^{15}\text{N}$ is $\pm 0.063\text{ ‰}$ ($n=10$). To evaluate the precision of coffee sample analyses, nine samples have been repeatedly measured ($n=9$) with a calculated standard deviation of $\pm 0.12\text{ ‰}$ for $\delta^{13}\text{C}$ and $\pm 0.10\text{ ‰}$ for $\delta^{15}\text{N}$. The remaining samples were analysed in triplicate. Differences between laboratory standard and coffee sample performances are probably due to the slight non-homogeneity of the samples, more evident in measurements where the amount of the target element in the sample is higher, and thus, the amount of weighted sample introduced in the instrument lower. Boron isotope abundance ratios are reported as $\delta^{11}\text{B}$ values relative to the boric acid standard reference material SRM951. The external reproducibility of the measurement of the standard was $\pm 2\text{ ‰}$ (1s). Replicate analyses of coffee samples, including microwave-assisted acid digestion and isotope abundance measurement yielded data consistent with the external reproducibility of the measurement of the standard.

2.3.3. Data analysis and chemometric procedures

Data sets consisted of a matrix in which rows represented the coffee green beans analysed (objects), and columns, the concentration of individual polyphenols determined by HPLC-DAD or the isotope ratios (variables or features). Each coffee sample was represented in the n-dimensional space by a data vector, which is an assembly of the n features. Data vectors belonging to the same class or category (geographical origin) were analysed using chemometric procedures that have been described in literature (Latorre *et al.*, 2000; Padín *et al.*, 2001), namely cluster analysis (CA), principal component analysis (PCA), linear discriminant analysis (LDA), K-nearest neighbours (KNN), and soft independent modelling of class analogy (SIMCA). Statistic and chemometric data analysis was performed by means of the statistical software packages Statistica 6.1 (StatSoft Inc., Tulsa,

OK, USA, 1984-2004), Statgraphics Plus 5.0 (Statistical Graphics Corporation, Rockville MD, USA, 1994-2000), Parvus (Forina *et al.*, 2000), and SPSS for Windows 11.5.1 (SPSS Inc., Chicago, IL, USA, 1989-2002).

Cluster analysis is a preliminary way to study data sets in the search for natural groupings among the samples characterised by the values of a set of measured features. Owing to its unsupervised character, CA is a pattern recognition technique, which can be used to reveal the structure residing in a data set (Massart and Kaufman, 1983). CA was performed on autoscaled data, sample similarities were calculated on the basis of the squared Euclidean distance and the Ward hierarchical agglomerative method was used to establish clusters. Principal component analysis, performed on the autoscaled data matrix, allows to reduce the number of variables retaining the maximum amount of variability present in data, in order to provide a partial visualisation of data structure in a reduced dimension.

The supervised pattern recognition techniques LDA, KNN, and SIMCA were used in order to attain classification rule. These techniques were applied to the autoscaled data matrix of the polyphenolic profiles and the methylxanthines of coffees. The classification rules achieved were validated by means of a cross-validation procedure, which was performed by dividing the complete data set into a training set and an evaluation set. Samples were assigned randomly to a training set consisting of 75% of them, and the test set was composed of the remaining 25% of the samples. Such a division allows for a sufficient number of samples in the training set and a representative number of members among the test set. The same process was repeated four times with different constitutions of both sets to ensure that all the samples had the possibility to be included in the evaluation set at least once. The different pattern recognition techniques were applied to the four training-test sets obtained. The reliability of the classification models achieved was studied in terms of recognition ability (percentage of the members of the training set correctly classified) and prediction ability (percentage of the members of the test set correctly classified by using the rules developed in the training step).

In KNN, the inverse square of the Euclidean distance was used as the criterion for calculating the distance between samples, and the number of neighbours (K) was selected after studying the success in classification with different K values, applying this technique to a training set with all the samples.

3. Results and discussion

3.1. Polyphenolic profiles and methylxanthine contents of *C. arabica* and *C. canephora*

Polyphenol and methylxanthine concentrations in coffee green beans determined by HPLC-DAD are summarised in Table 2. Phenolic compounds detected were caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA), feruloylquinic acids (FQA), di-p-coumaroylquinic acids (diCoQA), caffeoyl-p-coumaroylquinic acids (CCoQA), feruloyl-p-coumaroylquinic acids (FCoQA), feruloylcaffeoylquinic acids (FCQA), ferulic acid (FER) and cinnamoyl-amino acid conjugates. p-Coumaroylquinic acids (CoQA) were also detected under the limit of quantitation. Some chromatographic peaks exhibited UV-visible spectra similar to caffeic acid or ferulic acid, showing maximum absorbance at 325 nm, or p-coumaric acid, at 311 nm, so, they were identified as hydroxycinnamic acids (HXC). Since no further information of their mass spectra was available, due to their presence at concentration levels under the MS limit of detection, it was not possible to know the chlorogenic acids (CGA) subgroup that they belong to. Robusta (*C. canephora*) green beans presented higher concentrations of most polyphenols than Arabica (*C. arabica*), which agree with the results obtained in other works (Clifford, 1985; Correia *et al.*, 1995). 5-CQA (1) was the polyphenol present in higher concentrations in both coffee varieties, 21-39 g/kg of dried coffee green beans. Contents of CQA (1-4), diCQA (5), FQA (6-8) and FCQA (15-16) were comparable to those obtained in green coffee in previous studies (Clifford, 2000). However, diCoQA (13), CCoQA (10-12) and FCoQA (15-16) are reported here for the first time in coffee green beans. In addition, three cinnamate conjugates were determined: caffeoyl-N-tryptophan (26), caffeoyl-N-tyrosine (27), and p-coumaroyl-N-tryptophan (28) in Robusta green beans, whereas only caffeoyl-N-tryptophan was detected in Arabica, and in lower concentrations. Two methylxanthines were found in Robusta coffee, caffeine (29) and theobromine (30). The latter was not detected in Arabica green beans. Caffeine concentrations were completely discriminant between both coffee varieties, since Arabica contained 12-19 g/kg dry weight (DW), and Robusta, 22-35 g/kg DW. As well, caffeoyl-N-tryptophan (26), CCoQA-2 (11), FQA-2 (7), CCoQA-3 (12), FCoQA-3 and FCQA-2 (16), diCQA (5), FQA-3 (8) and FQA-1 (6) are present in different concentration ranges in both coffee varieties, thus, they are also totally discriminant between them. Moreover, FCoQA-2 and FCQA-1 (15), p-coumaroyl-N-tryptophan (28) and CQA-3 (3) are only detected in Robusta green beans, whereas the hydroxycinnamic acid

Table 2. Concentrations of polyphenols and methylxanthines (mg/kg DW) in coffee green beans (Arabica and Robusta).

Ref.	Compound	Arabica				Robusta			
		mean	SD	min	max	mean	SD	min	max
1	5-CQA	29148	3607	21528	38710	29837	3828	20958	37574
2	CQA-1	2690	685	1609	43777	4327	757	2919	6248
3	CQA-3	nd				711	260	366	1554
4	CQA-4	3657	676	2314	5302	5405	779	4033	7480
5	dicCQA	1175	305	675	1941	3163	524	1789	4128
6	FQA-1	196	55	91	324	675	159	402	1094
7	FQA-2	2203	303	1641	2892	6538	815	4557	9089
8	FQA-3	251	72	87	407	923	195	639	1486
9	FER	36	16	17	88	184	59	51	324
10	CCoQA-1	2812	723	1608	4277	3188	600	2218	4846
11	CCoQA-2	1109	290	692	1848	3670	425	2685	4477
12	CCoQA-3	171	62	66	304	655	87	442	827
13	diCoQA	174	55	80	294	74	21	27	128
14	FCoQA-1	66	23	25	133	48	15	10	81
15	FCoQA-2 + FCQA-1	nd				753	84	564	954
16	FCoQA-3 + FCQA-2	14	6	7	31	488	119	278	808
17	HXC-1	10	3	4	20	20	6	7	31
18	HXC-2	15	5	8	34	39	29	13	196
19	HXC-3	nd				23	17		52
20	HXC-4	1124	291	696	1796	nd			
21	HXC-5	nd				5	26		
22	HXC-6	nd				29	85		
23	HXC-7	34	14	73	nd	7	22		
24	HXC-8	nd				19	64		80
25	HXC-9	nd				1711	284	1213	279
26	Caffeoyl-N-triptophan	187	71	73	373	62	181		2625
27	Caffeoyl-N-tyrosine	nd				33	13	13	662
28	p-Coumaroyl-N-triptophan	16563	1789	11575	19134	29324	2639	22224	79
29	Caffeine	nd				97	138	34706	
30	Theobromine	nd							915

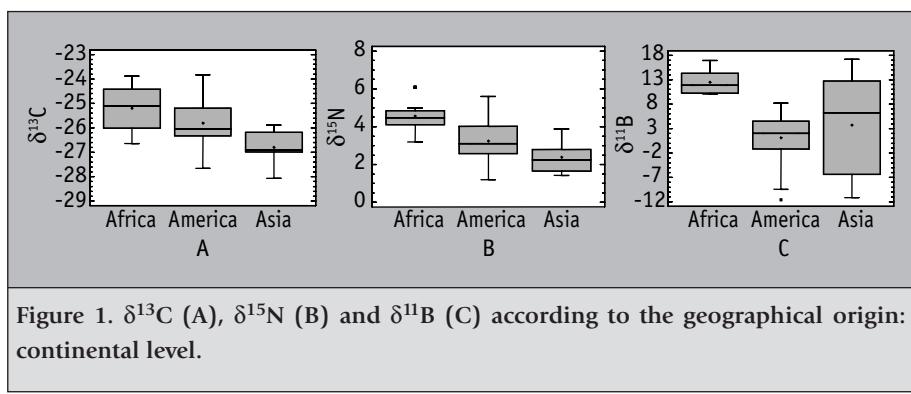
SD, standard deviation; max, maximum; min, minimum; nd, not detected.

HXC-4 (20) is found exclusively in Arabica samples, these compounds being, so, useful for varietal differentiation.

3.2. Isotope ratios in *C. arabica* and *C. canephora*

Figure 1 (A-C) summarises the results for the three isotope ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{11}\text{B}$), measured in 46 coffee samples (29 American, 12 African, and 5 Asian; the isotopic study of the rest of the samples is still in progress).

The mean $\delta^{13}\text{C}$ value of coffee is $-25.76\text{\textperthousand}$ (from -23.83 to $-28.06\text{\textperthousand}$, with a range of $4.23\text{\textperthousand}$) and the standard deviation is $1.0\text{\textperthousand}$. The high variability in the American samples (from -27.66 to $-23.83\text{\textperthousand}$) accounts for a good part of the variability. Asian and African samples, on the other hand, show values that are further apart from one another (from -26.64 to $-23.89\text{\textperthousand}$ for Africa, and from -28.06 to $-25.88\text{\textperthousand}$ for Asia). For these two continents, a first preliminary separation can be noticed on the basis of $\delta^{13}\text{C}$, even though, the overlapping does not allow a complete discrimination of the two provenances. $\delta^{15}\text{N}$ variability is in the same order of magnitude as that of $\delta^{13}\text{C}$, with a range of $4.88\text{\textperthousand}$ (with values going from 6.08 to $1.20\text{\textperthousand}$), while the mean value and standard deviation are 3.50 and $1.17\text{\textperthousand}$, respectively. American samples, again, show the highest variability (from 1.20 to $5.60\text{\textperthousand}$), while there is an important difference between Asian and African samples, which are quasi completely distinguishable on the basis of the $\delta^{15}\text{N}$ (Asia ranging from 1.42 to 3.88 , while Africa from 3.19 to 6.08). $\delta^{11}\text{B}$ shows a spread an order of magnitude higher than $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. The values vary from 17.20 to $-11.60\text{\textperthousand}$ with a range of $28.8\text{\textperthousand}$, a mean value of $4.23\text{\textperthousand}$, and a standard deviation of $7.57\text{\textperthousand}$. The three major boron inputs



are plant-available boron in the soil, boron in the water supply, and boron added in the form of fertiliser. It is likely that local growing conditions have the most significant impact on the plants' boron isotope composition. The most ^{10}B -depleted coffee samples were generally grown in America, and the most ^{11}B -enriched were from Asia and Africa.

3.3. Statistical data analysis

3.3.1. Polyphenols and methylxanthines

Varietal classification: *C. arabica* and *C. canephora*

The data set consisted of a 107×30 matrix, in which rows represented the coffee samples analysed (107 objects), and columns the concentration of 28 individual polyphenols, and two methylxanthines determined by HPLC-DAD (30 variables). The analysis of variance (ANOVA) performed on this matrix disclosed that caffeine and some polyphenols contents were significantly different in the green beans of both coffee varieties, *C. arabica* (Arabica) and *C. canephora* (Robusta). The Fisher index was calculated to establish the discriminant capacity of the variables one by one (Sharaf *et al.*, 1986). The total discriminant variables were those that presented the highest Fisher weights ($p < 10.35$): some hydroxycinnamic acids (15, 26, 11, 7, 12, 20, 16, 5, 8, 6, and 3) and caffeine (29). The box-and-whisker plots showed a complete separation of the concentration ranges of these compounds in the two coffee varieties. On the other hand, the total polyphenol content of the coffee green beans was not totally discriminant between the two coffee varieties.

Geographical origin of *C. arabica*

The analysis of variance (ANOVA) performed on data set of the green beans of *C. arabica*, which contained 20 variables (individual polyphenols and caffeine concentrations) and 50 coffee samples, revealed that there were significant differences for most of the variables among the three continents producing coffee (America, Africa and Asia). A least significant difference (LSD) test ($p < 0.05$) was also carried out on the data matrix, in order to check that there were not significant differences among years. Fisher test allowed to detect the most discriminant variables ($p < 0.001$) among the three continents (Sharaf *et al.*, 1986), which were the hydroxycinnamic acids 20 and 16. However, their box-and-whisker plots showed an overlap between the concentration ranges of the three categories (Figure 2A). Thus, none of the variables was able to discriminate among the green beans of Arabica coffee from the three continents by itself.

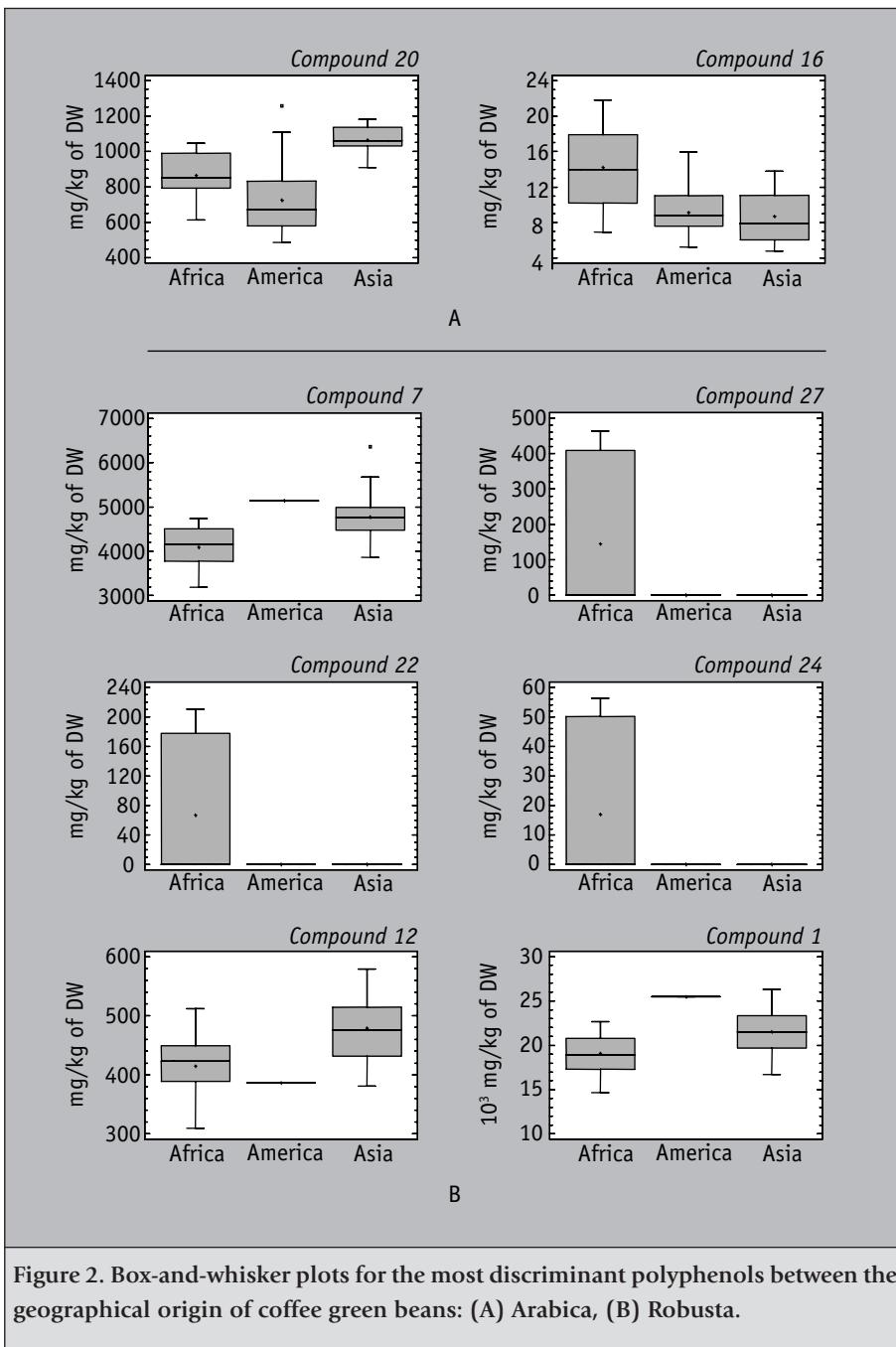


Figure 2. Box-and-whisker plots for the most discriminant polyphenols between the geographical origin of coffee green beans: (A) Arabica, (B) Robusta.

Hence, it was necessary to move on to multivariate data analysis in order to achieve a differentiation.

Cluster analysis (CA) result for Arabica coffee is presented as a dendrogram in Figure 3A. At a similarity level of 0.45, five clusters were found: cluster A made up of African coffees, and clusters B, C, D and E, containing all American samples. Asian coffees and some African ones are in the clusters of American coffees. These results agree with those obtained by principal component analysis

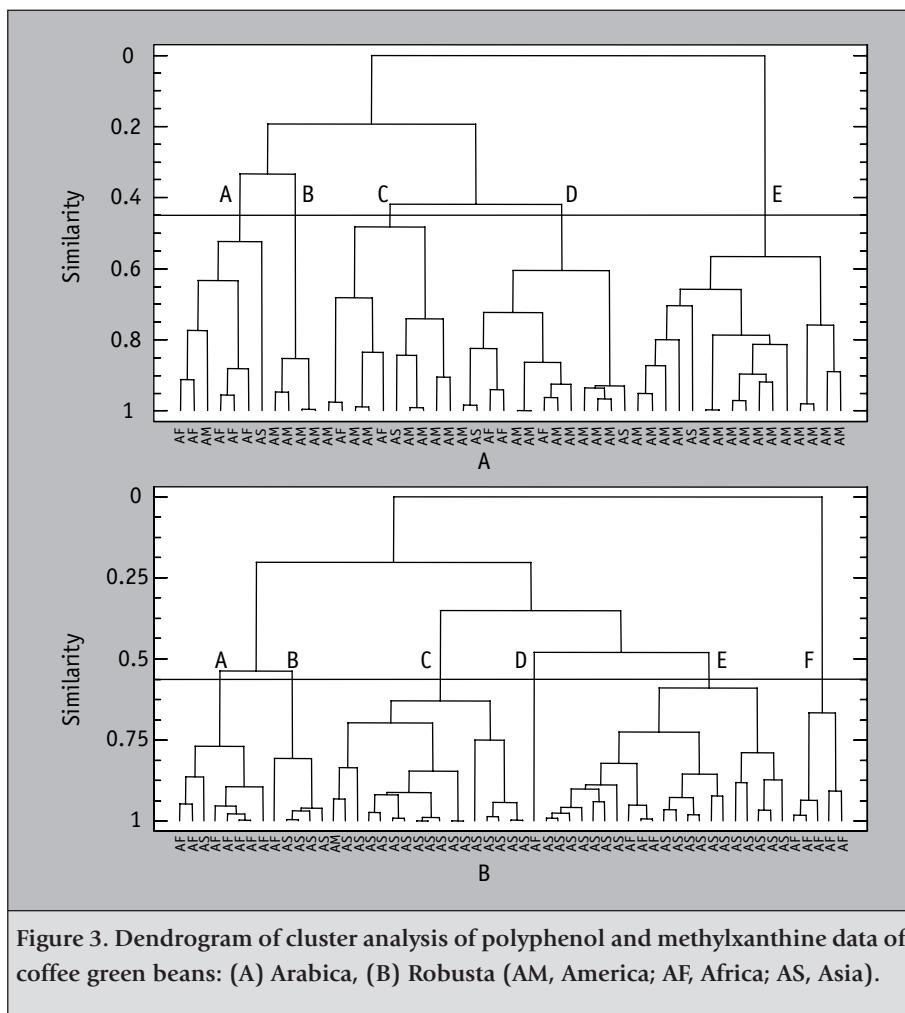


Figure 3. Dendrogram of cluster analysis of polyphenol and methylxanthine data of coffee green beans: (A) Arabica, (B) Robusta (AM, America; AF, Africa; AS, Asia).

(PCA), as evidenced the tridimensional plot of the sample scores in the space defined by the three first principal components (accounting for 67% of total system variability) (Figure 4A). So, a natural separation of African and Asian Arabica coffees is observed. Moreover, Asian and some African samples overlap with American samples. This is notably due to the fact that the number of Asian and African samples is very small comparing to American ones. However, these results disclosed that polyphenolic and methylxanthine contents of coffee green beans may be discriminant for the geographical origin of Arabica coffee. However, larger sample sets should be measured to achieve a classification model.

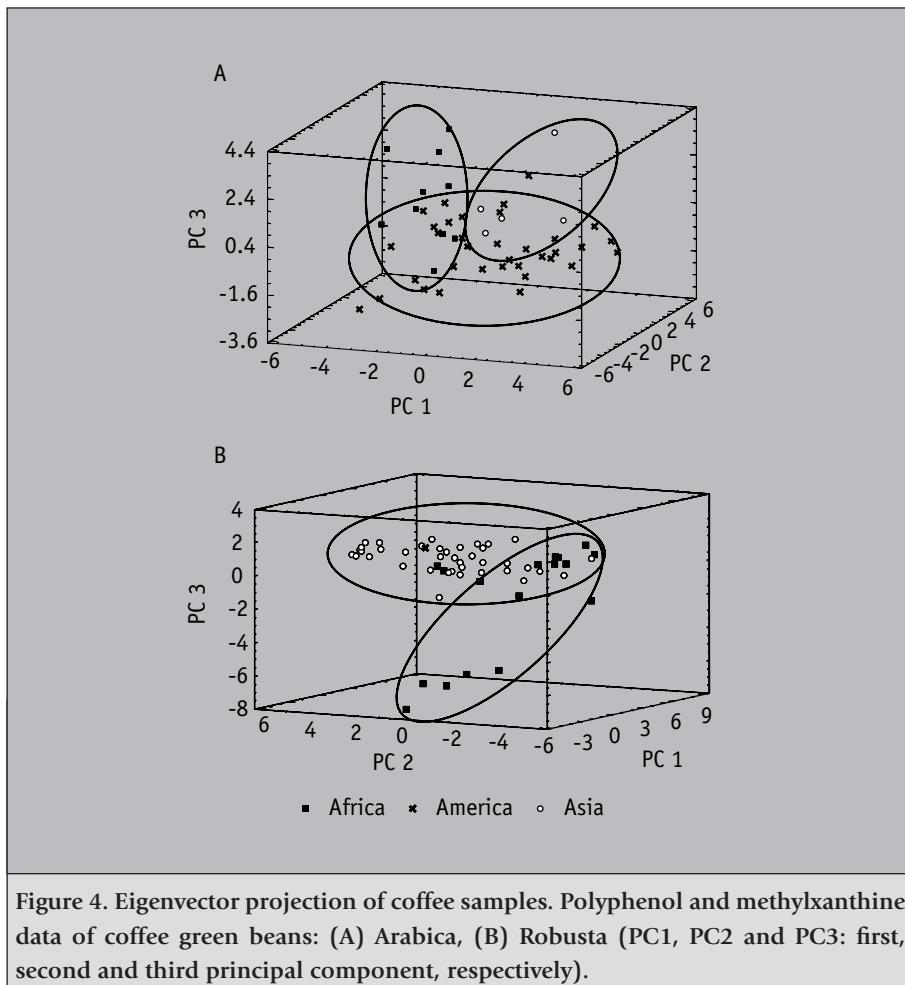


Figure 4. Eigenvector projection of coffee samples. Polyphenol and methylxanthine data of coffee green beans: (A) Arabica, (B) Robusta (PC1, PC2 and PC3: first, second and third principal component, respectively).

Linear discriminant analysis (LDA) (cross-validation: 5 sample sets with 80% of the samples in the training set and 20% of the samples in the test set) achieved satisfactory recognition abilities for the three geographical origins of Arabica coffees: 100% for American, 98% for African and 95% for Asian. The prediction ability for American coffees was also appropriate (94%). However, the prediction percentages for the African and Asian coffees were lower, 72% and 78% respectively, as a result of the reduced number of samples for both categories. In this case, the fact that recognition and prediction abilities are considerably different indicates that the results markedly depend on the samples that constituted the training and test sets, what is usually observed when the number of samples in each category is off-balance. Because of this fact, other supervised multivariate technique, such as KNN or SIMCA did not achieve satisfactory results.

Geographical origin of *C. canephora*

The Robusta coffee data set consisted of a 57×28 matrix (57 coffee samples and 28 variables (individual polyphenols and caffeine concentrations)). ANOVA showed that there were significant differences among the variables studied, regarding the three producing continents. The most discriminant variables were the hydroxycinnamic acids 7, 27, 22, 24, 12 and 1, which had the highest Fisher weights ($p < 0.001$), but their box-and-whisker plots showed overlaps between the concentration ranges in the three classes (Figure 2B). Thus, none of the variables measured was able to discriminate by itself between Robusta coffee green beans according to the geographical origin. Therefore, it was necessary to move on to multivariate data analysis.

The results achieved by CA are presented as a dendrogram in Figure 3B. Six clusters are observed at a similarity level of 0.55: cluster A contains African and one Asian sample; clusters B and E are made up with Asian coffees, and one or three African samples respectively; cluster C is formed by Asian samples; clusters D and F included only African samples. The only American Robusta coffee sample is in cluster C, what means that its polyphenolic composition was closer to Asian coffees than to African ones. In the tridimensional plot of the sample scores in the space defined by the three first principal components, a natural separation of the coffees is observed (Figure 4B). The three first principal components accounted for 70% of total system variability. PCA results agree with those obtained by CA, since the samples of the African and Asian categories are partially overlapped in the tridimensional plot of the three first principal components. These results disclosed that there exists notable differences among

Robusta coffee green beans from Africa and Asia, and that the polyphenolic profiles and methylxanthine contents of coffee green beans may contain useful information to attain coffee differentiation according to the established classes. These differences can be explained by the fact that polyphenolic profiles depend on agronomic (nitrogen fertilisation, pruning, chemical agents) and climatologic factors, which can change among regions and/or seasons (De Menezes, 1994; Awad and De Jager, 2002).

Table 3 shows recognition and prediction abilities afforded with each pattern recognition technique. LDA afforded excellent results for the Asian category, with recognition and prediction abilities ranging from 95% to 100%. LDA, SIMCA and KNN recognition abilities for the African category were also satisfactory (95–100%), but only KNN achieved appropriate prediction ability (95%). Thus, the pattern recognition techniques LDA and KNN led to complementary results. LDA allows the detection of almost all Asian coffees, and KNN achieves a level of hits of 95% for the African coffees. Thus, an authentication system, which permits the classification of Robusta coffees regarding their geographical origin, is achieved. The appropriate agreement between recognition and prediction abilities attained by each technique means that the decision rule derived is not dependent on the actual samples in the training set, hence the solution achieved is stable.

Table 3. Classification results for the supervised pattern recognition techniques applied to data of polyphenol and methylxanthine contents of Robusta coffee green beans.

Technique	Class	Recognition ability (%)	Prediction ability (%)
LDA	Africa	98.0	82.4
	Asia	97.4	94.9
SIMCA; normal range; $\alpha = 0.05$	Africa	100.0	75.0
	Asia	83.3	75.0
KNN (K = 3); inverse squared Euclidean distance	Africa	95.0	95.0
	Asia	88.3	81.3

*3.3.2. Isotope ratios: geographical origin of *C. arabica* and *C. canephora**

PCA applied to the isotopic data was used for grouping the different green coffee samples according to their continental origin. The two first principal components together explained 78% of the total variance of the data. Thus, a good discrimination appears to exist between African and American coffee green beans, and African and Asian beans. Correct classification of continental origin is achieved with a success rate of 88.2%. The 95.2% of the American beans and the 87.5% of the African ones were classified correctly. However, only 60% of the Asian samples were classified correctly, which can be attributed to the lower number of samples available.

4. Conclusions

The contents of certain chlorogenic acids and cinnamate conjugates, and caffeine in coffee green beans are characteristic for each of the two varieties studied, *C. arabica* (Arabica) and *C. canephora* (Robusta). This allows the varietal differentiation of coffee green beans unequivocally. Moreover, from this study, it is concluded that polyphenolic profiles and the methylxanthines contents of coffee green beans provide suitable information to enable classification rules to be developed by pattern recognition techniques for identifying coffees according to their geographical origin at the continent level. Thus, an authentication system was achieved, which allows the correct classification of Robusta coffees from Africa and Asia with a 95% of hits. Furthermore, American Arabica coffee green beans were satisfactorily predicted by LDA (94%), however, more than 20% of African and Asian beans were misclassified. On the other hand, stable isotope ratios of carbon, nitrogen and boron also showed to be good indicators of geographical-dependent parameters, and therefore, useful tools to infer the region of production of green coffee. Regarding the geographical origin, isotopic ratios afforded similar results to polyphenolic and methylxanthine contents for the American samples, however, better results were achieved for the African samples (88% of hits). So, both kinds of chemical descriptors afforded complementary results for the geographical classification of coffee green beans.

No satisfactory results were achieved for the Asian category when isotopic data were analysed or when the polyphenolic and methylxanthine contents of African and Asian Arabica coffees were considered. This was likely due to the low number of samples of these categories available for multivariate data analysis. In this sense, a further collection of samples should improve these promising results,

with the possibility to create data banks for the geographical characterisation of coffee. This is of great interest from several points of view: economical, honest producers, and consumer protection.

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The role of rapid methods in quality-oriented traceability

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Abstract

Product traceability is a prerequisite for modern food industry, imposed by legal regulations and commercial pressure from chain partners and society. In principle, however, the availability of traceability does not result in higher revenues. To still get a return on the investment for a traceability infrastructure, the traceability has to be made subservient to the business goals of a company. Consequently, the food industry looks for added value for the inevitable traceability infrastructure. One of the areas where this added value can be found is in improved quality management, of both physiological (intrinsic) product quality (taste, microbiological quality) as of less tangible (extrinsic) product quality (product origin, fair trade). Systematic collection and exploitation of product quality information throughout a chain can support many business functions (including logistics, quality management, process optimisation, and company image). Accurate and non-forgeable measurement of product quality attributes and environment conditions can benefit from developments in rapid method research. This paper introduces the need for detailed quality information in the chain, and describes how rapid methods can be deployed in collecting and processing this information.

Keywords: tracking and tracing, traceability, product quality, rapid methods

1. Introduction

Traceability on food chains is often seen as a means to ensure food safety, to minimise the impact of food incidents, and to manage liability issues. In this view, traceability is imposed onto food chains by external actors, which are not willing to pay additional prices for traceable products. Consequently, the implementation of traceability requires investments, which are not covered by improved revenues. As traceability is a basic requirement, it does not lead to

improved benefits. This is one of the reasons why traceability is only laboriously introduced in the food sector.

The broad introduction of traceability requires a clear view on the added value of traceability for food companies. FoodPrint (Vernède *et al.*, 2003; Verdenius, 2006) is a systematic approach for analysing and designing traceability systems. FoodPrint takes the business goals of food companies as starting point. In a strategic traceability analysis the goals of the organisation under study (company, production chain, or even an entire sector) are carefully studied and connected to potential benefits of traceability. Subsequently, the current and desired traceability information systems are modelled and traceability bottlenecks in relation to the business goals are surveyed. This forms the basis for the design of an improved traceability system.

Many of the added value opportunities depend on reliable, and fast available, quality information on products. Rapid methods provide a means to deliver that information, by accurately measuring product quality and environmental attributes. Important aspects remain measurement speed and timely input of measured values into the information systems.

The main goal of this paper is to introduce the role of rapid methods in tracking and tracing. It is not so much the description of an R&D process, as it is the description of another way of thinking about traceability. Section 2 introduces a number of main concepts in traceability. An important step is to envision traceability not only as an external requirement, but also as a vital business asset. Based on this realisation we describe in section 3 a specification and design approach for traceability systems that takes traceability as an instrument to realise business goals. In this section, different components of traceability are introduced. Then, section 4 describes the value of rapid methods for traceability systems, whereafter section 5 concludes.

2. Tracking and tracing, food safety and food quality

Traceability is the ability of a food producing company to follow and reconstruct the history of a food product through the production and distribution chain, from its initiation until its consumption. This presumes the ability to identify product entities, to register product flows, and if required, product attributes and process characteristics, and to document and analyse the registered information. A product entity in this respect is an identifiable individual product: not milk

The role of rapid methods in quality-oriented traceability

in general, but an individual tank at a certain time instance, or the actual pack of milk that is standing in front of me at this very moment. With the history we indicate the sequence of production and distribution phases that a product has gone through. What types of phases are of interest, however, depends on the purpose of operating the traceability system. There can be a number of different purposes to set up a traceability system, as shown in Table 1.

On the horizontal axis of Table 1 three orientations are listed: location, environment conditions and product attributes. The location orientation focuses on logistic aspects of product flows. The main issues here are time, place, and logistic phase of a product: where was the product at a certain time, and with what logistic status? The environment conditions orientation corresponds with process aspects of product flows. What conditions and process settings have been applied during production and distribution of products? The product attributes orientation relates to the product quality aspect of product flows. Here, the actual product quality is relevant, under the assumption that the quality has a dynamic aspect, which depends on the actual chain performance. This is typically the case for fresh and perishable products. Traceability systems

Table 1. A framework for traceability technology.

Function	Orientation		
	Location	Environment conditions	Product attributes
Information carrier	Bar codes, RFID tags, label, product	The product environment	Product
Registration	Manual and automatic optical readers, DNA analysis	Data logger, TTI, manual readers, data logger stations, remote readers	Analytical tools: manual readers, laboratory methods
Information processing	Storing data, real-time tracking, trace product history	ISO process quality, tracing process deficiencies	Food safety and quality applications

RFID = radio frequency identification; TTI = time temperature indicator.

with a quality orientation are typically focussing on the development of quality in the chain. The quality aspect needs to be defined, and a quality measurement technology needs to be selected.

The vertical axis of Table 1 enumerates the three main functions of traceability systems: information carrier, registration, and information processing. The information carrier realises the coupling between the physical product (lot) and the information, which belongs to that product or lot. Registration of a product entity entitles the scanning of the product's identity. By reading or scanning the information carrier the product identity is accessed, and can subsequently be used to access product (lot) data in a database. Registration information comprises at least the product identity, registration time, registering resource, and process phase. The detailed content of the information processing partially depends on the goals that are to be achieved by using a traceability system. The initial processing, storing the registered data in the database, is standard for all types of systems. In the functionality of additional processing modules, the specific goals of the traceability are reflected. In existing food chains, the most often encountered goal of a traceability system is to control the impact of food safety incidents. This implies a focus on assessing the potential scope of an incident by depicting the tree of involved products (the product genealogy) and on performing a product recall. With these kinds of applications, companies comply with government regulations on food safety, e.g. the European Union (EU) general food law regulation (EC, 2002) and hygiene regulations (EC, 2004a-d) and can avoid to be kept responsible for a food safety incident.

Traceability systems are, however, not limited to food safety and liability issues. They can serve other aspects of businesses as well, including logistics (e.g. reducing the vulnerability of a chain for external influences) and food quality management (e.g. better control understanding and control of quality development of products). Further in this paper, we will focus on the latter aspects of traceability: how it can help and support to improve quality management. In this case the condition or quality orientation of the traceability system becomes of vital importance.

3. Designing a traceability application: FoodPrint

With the recognition that traceability systems need to respond to business goals, an approach to designing traceability systems is required that takes these business goals as the starting point. FoodPrint is such a systematic approach for designing tracking and tracing systems. It integrates experiences from quality-

related tracking and tracing, and value traceability projects in food chains. It takes the business goals of an organisation as starting point, and uses these as guiding principle when translating legal and commercial traceability requirements into practical solutions.

Traceability systems influence different aspects of companies. In practice the focus often lies on technical aspects. Introducing traceability in a food company however also influences organisation, production process and information systems. Figure 1 depicts these aspects in the overall development process of traceability systems:

- *Organisation.* Although current legal requirements on traceability only require traceability at the company level, modern complex food chains and networks already go beyond company borders. At the organisation level, the arrangements are found that are necessary for obtaining traceability. Responsibilities for product and information handling, agreement on (the synchronisation of) procedures, definition of traceability information and the definition of information exchange standards in a chain, decision making and traceability goals are established at this level. Traceability goals can be focused on individual companies as well as on entire chains or networks.
- *Process.* The process design influences traceability and *vice versa*. Processes in food chains have been designed with the aim of efficiency and cost reduction, and not for traceability. Consequently many processes have inherent problems with detailed traceability (e.g., mixing raw materials in silos, lack of product registration in convergent and divergent product flows). Traceability may require changes in process design.
- *Information.* Traceability of product flows depends on the ability of processing information. Based on a process model, a specific traceability information model can be constructed. The information model specifies the structure of the information to be collected. This model can be based on a sector specific or generic reference model, when available. The model defines the information entities that are required for realising the traceability goals that have been defined for the company. Together with the process model, the information model constitutes the traceability model (Koenderink and Hulzebos, 2006).
- *Technology.* Traceability deploys a large variety of technologies. In many discussions on traceability, technology seems to be the primary point of discussion. In our view, technological choices form the final step of the design and implementation process. For a detailed overview of traceability technology we refer to Furness (2006), and Vernède and Wienk (2006).

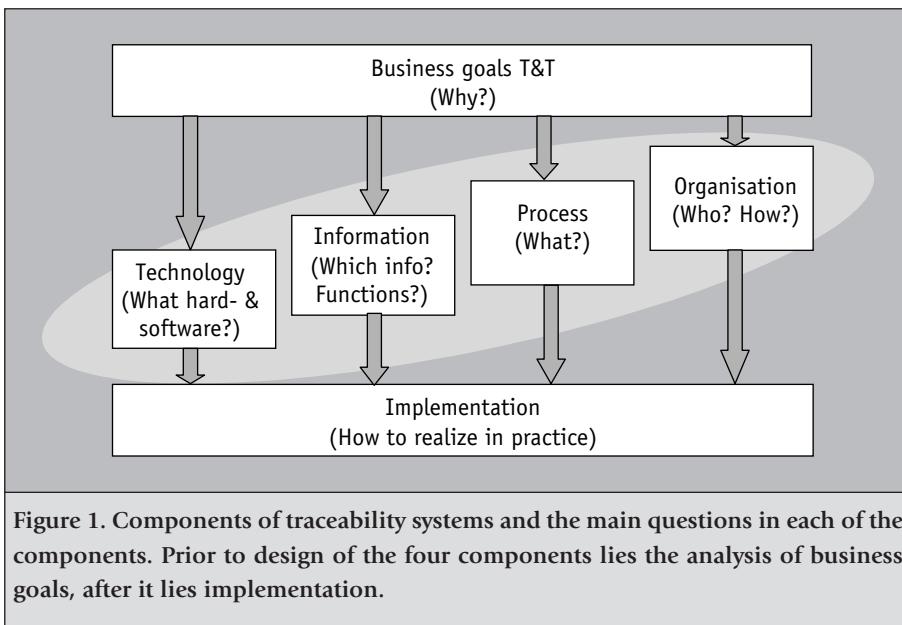


Figure 1. Components of traceability systems and the main questions in each of the components. Prior to design of the four components lies the analysis of business goals, after it lies implementation.

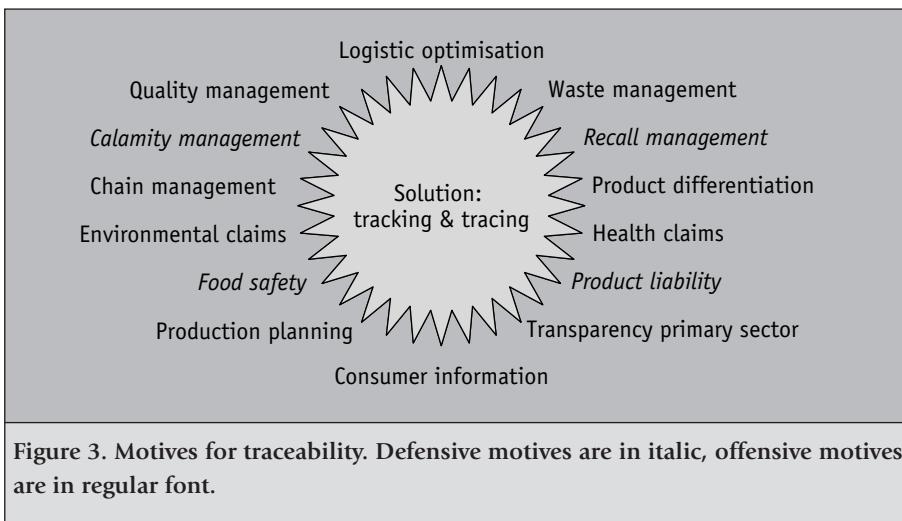
What are motives for the introduction of traceability concepts in food industries? Many actors are triggered in investments mainly when government regulations force them to do so. For traceability, this is not different. Experiences over the past few years with regulations in the EU on traceability show that the implementation of traceability concepts is slow, even when the availability of traceability facilities is mandatory. Apparently, the perception of traceability by industrialists is one of investments without clear benefits.

In terms of motives, the perception focuses on external or defensive motives (see Figures 2 and 3). When realising the objectives, which result from these motives, there is no clear benefit to obtain for the investing company. By doing the investment, the company defends its position on the market. The investments serve a 'license to produce', or a 'license to deliver', but they do not lead to a clear return in terms of an increased turnover, an increased margin or a better position on the market. Opposite to these defensive motives there may be offensive motives, which do translate in improving the position of the company. These offensive motives concentrate on cost reduction and return maximisation. Cost reducing motives include the reduction of product cost, by a better conversion of raw materials into products, product loss reduction and

The role of rapid methods in quality-oriented traceability

External motives	Cost reduction	Return maximization
Customer demand	Product cost reduction	Increase of market volume
Chain partner requirements		Broadening of the market
Government regulations		Added value

Figure 2. Grouping of motives for traceability.



improved control of quality development of raw produce. Process cost reduction may include reduction of resource cost (e.g., energy). Return maximisation can be realised by adding value to products, for instance by being able to substantiate

product claims such as organic, regional origin, allergen free and child labour free. Return maximisation also includes broadening of the market, for instance by positioning specific variants of the same product (taste variants, regional versus cheap variants). Finally, increasing market volume is an efficient way of maximising returns. This can be realised by using traceability information to pinpoint batches to the right location.

Consequently, our approach FoodPrint analyses the business goals of the food company or food chain of interest. These goals are linked to concrete and measurable tracking and tracing targets. Next, the current As-Is situation is analysed and by means of a bottleneck analysis, the To-Be situation is evaluated. This finally results in system-design, which can then be made operational by an ICT system integrator. During the process, FoodPrint differentiates among the following aspects of traceability: organisation, process, information and technology.

A traceability system is a coherent set of concepts, tools, working procedures, and equipment that enables the tracking and/or tracing of goods in a production and distribution environment. Typically, a traceability system supports a minimal set of functions. Three functions are required to establish record and store product and process data (Table 1):

- Product identification, the unique identification of production units. Product identification is realised by using a unique product characteristic (e.g. DNA, spore-elements, antibody markers) or a product number, attached to the product on an external tag (e.g. bar-code, see www.ean-int.org/barcodes.html), 2-dimensional dot matrix code, radio frequency identifier, see www.aimglobal.org/technologies/rfid/). The former is sometimes referred to as the primary identifier, and the latter as secondary identifier (www.euFoodTrace.org). When collecting individual products in logistic units (e.g. box/pallet/bin and container), a hierarchy of identification systems can be used, as can be observed in the EAN (European article number) system for coding products, EAN: GTIN (global trade item number), and for pallets and containers, EAN: SSCC (serial shipping container code).
- Product registration. At relevant positions in the process identification codes of products have to be registered. Relevant points are, at least, the points in the process where product flows converge and diverge. This allows the coupling between process parameters and product identity, as necessary to reconstruct product quality implications as encountered later in the distribution chain. The registered product code, the time stamp and the

identification code of the location/process phase identification, provisionally augmented with additional information on conditions, product state and product characteristics, are recorded in a data base.

- Information processing. The product registration information is stored, and when the storage of information in a data base system such that analysis and retrieval of relevant information is maximally supported.

On top of these three basic functions, additional analysis and retrieval functions can be developed:

- Localisation of product or product tracking, by indicating the physical location, process status (waiting, processing, in packaging station, finished, delivered), under whose custody a product resides, and in which process stage (if required).
- Reconstruction of the product genealogy, the record of converging and diverging product flows that document the product history.
- Reconstruction of product quality, the record of the development of product quality.
- Product recall, the support to withdraw a product from the market.

On top of an infrastructure that allows performing these activities, additional functions, such as condition monitoring, quality reconstruction, quality prediction, and various logistic, planning and administrative extensions can be realised.

4. Technologies for traceability and the role of rapid methods

As illustrated in Table 1, traceability technology can be seen along two axes: function and orientation. Three main functions are distinguished: information carrier, registration, and information processing. The information carrier can be attached onto a product, for instance by means of a barcode or an other label. This can be called the secondary identifier (FoodTrace, 2003). The information carrier can also be the product itself, for instance in the form of a unique DNA expression profile. This is often called the primary identifier.

Three main orientations that are distinguished are: (1) location, (2) environment conditions, and (3) product attributes. In a location (or logistic) orientation, the main interest is to establish a verifiable registration of locations where a product has been, including date-time stamps, and provisional additional information (production phase, responsible operator). The registration of a product identity is

the main issue here. This orientation is the basic prerequisite to enable the other two registrations. In the case of an orientation on environment conditions, the external conditions that influence the product have to be linked to the product identity. First, this requires the environmental conditions to be registered, either at a central facility (sensors) or at a local, product bound, facility (e.g. data logger, TTI). Next, in registering the product, environmental conditions and product identity need to be linked, by linking the centrally or locally registered information to the product ID registration. A similar set-up is required for the product attributes orientation. In this case, the internal product state (physical, (bio)chemical, biological), as carried within the product, is registered.

Rapid methods offer an opportunity for all three orientations. A key to success, in all these cases, is in accuracy, robustness and rapidness of the methods. Moreover, automatic data entry has to be facilitated, to allow for large-scale application. Finally, it is necessary that the costs of applying the technology be reduced to allow large scale commercially viable implementation.

In the case of a location orientation, rapid methods, by reading meaningful characteristics of a product, can serve as a unique product ID. For cattle, DNA can serve as such an information carrier; although rapid methods to read the DNA trace are not yet available. In pork chains, there have been experiments to mark animals with antibodies to synthetic peptides. Here, simple dipstick tests can be developed to read out ID codes. For both applications, commercial applications are available, or under negotiation. Other techniques, based on metabolite recognition, spore element detection can also be developed, if the market recovers.

For the environment conditions orientation, rapid methods can help to detect historical external conditions, related to growing conditions, growing location, and information on treatments and other growing circumstances.

For the orientation of product attributes (quality verification, authentication), rapid methods also offer promising possibilities. Various aspects of product quality can be included. In commercial terms, validating value added claims are of high importance. The question could be whether a product actually has the added value as claimed (regional origin, organic, etc.). Various rapid methods can be used to assess trace elements characteristic to a region, to detect extreme low amounts of pesticides, herbicides or fertilisers, may help to guarantee fair information. Moreover, they can help to assess detailed quality information,

physiological development, allergen content, and sensory and nutritional quality.

5. Conclusions

Traceability is a curative tool for managing food safety. In case of a food crisis, a traceability infrastructure allows accurate identification of high-risk products, and a dedicated recall action. The last decade, governments and food chain partners have issued commercial and legal regulations on traceability of food products. Complying with these regulations serves as a license to deliver, but does not generate better revenues. To generate additional value from a traceability system, the information content should change from a pure location orientation towards orientations that can support value-adding: environment conditions and product attributes. To enable this extension on traceability, existing technology (e.g. data logger and TTI technology for environment conditions) and new technologies can be used. For many of the required measurements, rapid methods can be made available. They may offer a robust and hard-to-forged solution for identification of food products. Moreover, they can support assessment of environment conditions. Their most promising contribution is in the measurement of product attributes, allowing accurate assessment of the product quality state.

An important research issue for application of rapid methods in traceability is how rapid methods can be used on large scale in routine applications. At various places, this is subject of research. Ease of use, cost reduction, and fast data entry, are typical research questions. This also implies the transfer from 'individual' tests towards 'in-line' or 'at-line' sensors. Currently, the application of rapid methods for traceability is an active field of research, amongst others in relation to EU research. Given current trends in rapid methods, such as miniaturisation, speed-up and reliability, routine application of rapid methods for traceability seems only a matter of time.

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An industrial vision on tracking, tracing and rapid methods in the food chain

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Abstract

Technological progress in tracking, tracing and rapid methods is eagerly expected by the industry to comply with increasingly stringent food safety regulations and to improve control over the supply chain. On the one hand, regulatory developments will indeed require increasingly complex, fast adapting and real-time methods for providing food safety related information to a wide range of food chain stakeholders. On the other hand, continuous change in food chain processes and in regulations will result in demand for improved control and management tools. Implementation of novel techniques by the industry faces two major challenges: providing value added traceability, not just compliance, and being cost competitive.

Keywords: tracking, tracing, traceability, food chain, supply chain management

1. Introduction

From an industry perspective, novel technologies need to be assessed not only on the ground of scientific interest or technical performance, but also, and primarily, by the value added provided to operators and consumers in the food chain. This applies in particular to tracking, tracing and rapid methods, whose potential added value has to match current and future industry needs, in particular supply chain management specifications. With a view to taking on board industry opinion and expectations, let us therefore consider in turn: (1) current issues in food chain industries, (2) supply chain developments, (3) traceability economics, and (4) the agenda for technology developments.

2. Current issues in food chain industries

In the European Union (EU) food chain industries are characterised by modest evolution prospects in terms of population and food budget per capita. With an annual turnover of over 1,000 billion Euros and some 3 million employed

manpower, the food chain is the single largest industry sector in EU25. However, this sheer size is not matched by increase rate: the average real sector growth rate is only about 2%, whereas the nominal rate is about 4%. This largely results from a combination of slow growing population and decreasing (in relative terms) household expenses in food. Hence, the food industry faces a severe challenge for achieving significant growth in turnover, profit and added value.

Before looking at specific supply chain issues, it is worth examining the overall industry structure in the EU. While the EU food market is still very national or regional oriented in terms of consumption patterns, tastes and preferences, the industry is rather concentrated. Although it consists of over 25,000 companies, the five largest firms account for over 40% of the market in most EU countries. In most countries, including the new EU Member States, these are large multinationals such as Danone, Nestlé, Unilever, Kraft or Sara-Lee. In most EU countries, large retail firms play an increasing role, not only in distributing food products, but also in having these produced by third party companies, to be sold under their private labels. In the UK, private labels account for about 40% of total food sales. To understand the situation completely, however, the food chain should not be looked at as a purely industrial structure, but as extending into agriculture, which mostly consists of small, family run units. Feed manufacturing is also an important agribusiness sector, whose annual production amounts to some 200 million tonnes (EU15), of which about 80 million are produced on the farm.

Food chain companies increasingly face two types of specific constraints: regulations and consumer expectations. Regulations, particularly at EU level, focus more and more on food safety, as being laid out in the EU general food law regulation (EC, 2002), which defines acceptable practices and standards, including traceability and/or hazard analysis and critical control points (HACCP) for food and feed ingredients, and products. This regulation also includes provisions for monitoring imports and exports to and from the EU. Since 2002, the creation of the European Food Safety Authority provides a platform for centralised and co-ordinated scientific assessment of health risks from food, including emerging risks, across the EU. It can be expected that regulatory provisions for food safety will continue to develop alongside with globalisation and consumer concerns, calling for increasingly complex and complete compliance practices from the industry.

In parallel with regulation requirements, responses to consumer expectations also increasingly determine industry practices and standards. While tastes and national preferences still significantly differ across the EU, some common consumption patterns emerge. Overall, the evolution of modern lifestyles results in consumers being more and more sensitive to service attributes linked to food products, such as time saved by using prepared meals and convenience obtained from packaging formats. While consumer organisations are increasingly vocal about obtaining detailed product information, most available studies paradoxically suggest that a majority of consumers remain little aware or little interested in labels. On the other hand, consumer perception of food safety crises such as BSE, dioxin, etc., in recent years has also resulted in lower risk tolerance or acceptance. A culture of zero risk is becoming more and more widespread.

3. Supply chain developments

Let us now examine specific supply chain aspects and trends in the food chain. While the food chain was traditionally driven by production, both for vegetal and animal products, it is now being increasingly driven by consumer demand. This shift of emphasis from push to pull extends from the retail stage onwards to upstream segments. This trend is supported by practices and systems such as Efficient Consumer Response (ECR) and Enterprise Resource Planning (ERP) systems. Since large retail companies control the majority of the food chain's relationship with the consumer, they are largely in the driving seat and exert considerable power on their suppliers by issuing demand-related information. They also increasingly request just-in-time delivery from their suppliers, which deeply influences the pattern of logistic flows, the location and functions of depots, etc. Moreover, retail chains lay down detailed standards (quality, information, traceability, safety, etc.), which they require their suppliers to adopt. The British Retailers Consortium (BRC) is just one example of such standards and TraceOne (by selected French retailers) is another. In this way, private traceability standards add to or combine with the ones defined by national or EU regulations.

Upstream in the food chain, agriculture is still very much driven by yearlong production cycles. Largely, both vegetal and animal products are produced in dispersed, family run, small size units. Post-harvest handling for crop products is primarily bulk logistics, with numerous mixing, sorting and transfer operations.

The above-described developments in the food supply chain raise specific control issues. Let us offer some illustrations:

- Effective implementation of the EU general food law regulation (EC, 2002) and the new Common Agricultural Policy (CAP) (EC, 2003) make authentication (product, origin, etc.) and fraud control increasingly necessary.
- Traceability becomes a general standard. It can indeed be expected that the provisions of the EU general food law regulation will progressively extend from the pioneering beef meat sector to many other sectors. Generalisation of traceability is not going to make substantial progress without addressing major difficulties such as bridging the information 'black holes' at the farm level, in bulk operations and in feed.
- Tracing/tracking from and to such places will require monitoring a large number of scattered units, bulk operations and remote production locations. In a number of cases, testing results will be relied upon to transfer seller to buyer responsibility from one stage to another in the food chain.
- Effective full chain traceability will indeed become a prerequisite to ensure collective responsibility in managing food chain safety, such as is being expressed in the EU general food law regulation or in the German regulation on co-existence between genetically modified (GM) and non-GM agriculture ('Gengesetz').
- In numerous cases, control will not only involve recording location and operations, but ensuring that selected process parameters, such as temperature, are maintained throughout the supply chain.

In conclusion, control requirements are becoming more complex and will demand more functional integration in traceability. This is illustrated by some examples in Table 1. The challenge is to move from regulatory compliance oriented schemes to value added ones. On the other hand, it can be expected that evolving food safety regulations will also pose additional demands on control and traceability systems.

With a view to identifying new or unfilled needs, it is interesting to examine current practices and trends. Various surveys in Logistics Europe (2005) suggest that:

- About 95% of food producers and 35% of retailers use some form of tracking and tracing system. This is not surprising, given that traceability is a legal obligation. However, in most cases this traceability is only internal to the company or to the production site, and does not extend to the entire supply chain or even to significant segments of it.
- Bar coding system is still the dominant recording device for lot identification. About 50% food producers indeed use EAN (European Article Number) bar

Table 1. Integrated traceability applications.

Specification	Main purpose	Typical applications
Full chain traceability, including production area, export, all production stages and logistics	Regulatory compliance	Beef chain in EU
Traceability integrated with EPR	Minimising cost of recall and control of suppliers	Retail (TraceOne)
Real-time quality testing	User/seller responsibility	Exports (port control); cold chain management
Traceability integrated with ECR and EPR	Maximising lot/stock value	Retail
Value added information to consumer at POS (Point of Sales)	Marketing	
Integration of HACCP and tracking and tracing, and rapid methods	Food safety	

codes (mostly the EAN-128 standard), which are particularly appropriate for packed products.

- There is currently a shift from bar codes towards radio frequency identification (RFID) which appears to be a very promising technique, allowing for a much larger data capacity and having the potential for carrying executable instructions. This transition is still hampered by the relatively high cost of RFID tags and readers (as compared to bar coding), but market and technology developments should rapidly bring these costs down.
- The trend toward integrating traceability and ERP systems is also accelerating. This reflects the growing complexity, internationalisation and sophistication of supply chain management in large food groups.
- The main purpose of most current traceability systems is still regulatory compliance and minimisation of recall cost. Value chain optimisation, quality control and safety management are in general not in their scope, with the result that they are mostly not integrated with quality testing or HACCP data. This fragmentation of systems does not reflect the actual imbedding

of corporate processes: traceability → quality management → operations management → market and consumer.

- There is a diversity of systems and of proprietary standards, which imposes a heavy administrative and IT burden on food/ingredient producers who supply several clients.

To comment on the last point, Meuwissen *et al.* (2003) describe three major families of traceability systems:

- System A, each link in the production chain gets relevant information about only the previous link, from that link.
- System B, each link gets relevant information about all previous links from the closest previous link.
- System C, each link of the production chain provides the relevant information to a separate organisation, which combines all the information for the whole production chain.

These three approaches reflect production requirements, power relationships along the supply chain and philosophies in IT architecture. It is likely that a large retail or producer group will be able to impose a type B solution, while a consortium of retailers will be able to impose a type C approach. Small companies tend to live with type A systems. It is clear that any company having to deal simultaneously with 2 or 3 different families of systems faces high administrative and information complexity.

4. Traceability economics

Cost considerations are of paramount importance when transferring technology from the laboratory to the industry. Excessive cost, be it investment or operating, will always be a limiting factor in technology adoption. Therefore, it is useful to fully understand the economics of traceability systems. From an economic perspective one cannot isolate traceability from the operations that are supported by it. Indeed, efficient implementation of a traceability system will usually imply some re-engineering of underlying operations (production and logistics) as well. Lot size optimisation is usually a major consideration in this re-engineering. Traceability will usually be more efficient (that is result is lower recall cost) under small production lots, whereas production cost will generally increase as production lots become smaller. Therefore, some trade off will have to be accepted between traceability effectiveness and production cost, which will result in an optimal lot size being defined at each stage in the production chain. This is illustrated in Figure 1.

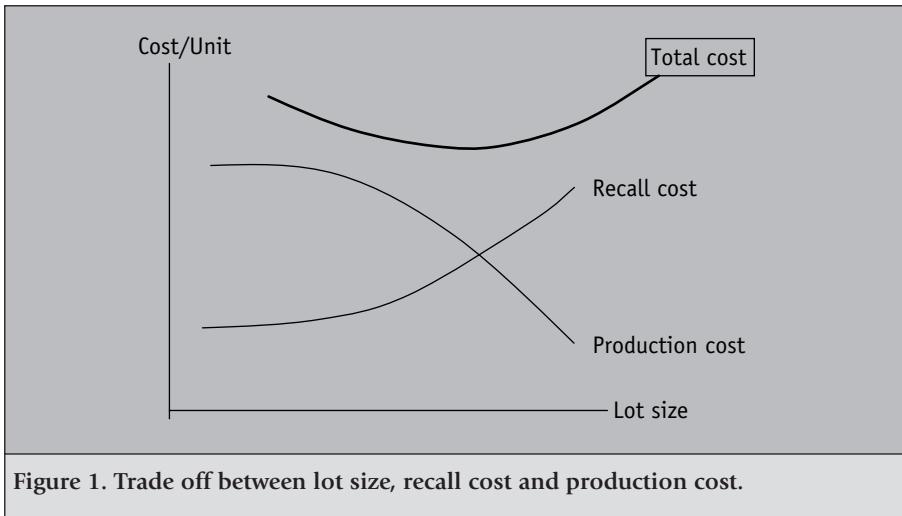
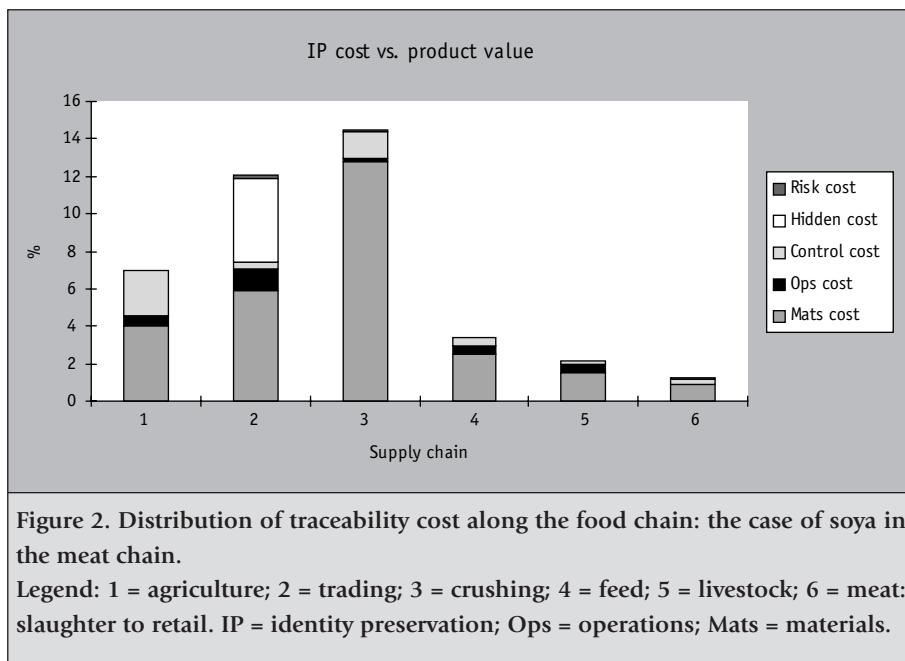


Figure 1. Trade off between lot size, recall cost and production cost.

Another major economic consideration is the allocation of total traceability cost in the supply chain. Several cases suggest that this (additional) cost is unequally distributed among operators. This deserves some explanation since it is not straightforward but can have significant consequences on technology adoption and diffusion. Traceability cost can indeed be broken down into components such as IT systems, operations re-engineering, organisation re-engineering, training, and supporting tests (e.g. polymerase chain reaction analysis in the case of GMO tracking and tracing). As one goes down the agrofood supply chain, traceability cost piles up until the stage where the final product is sold to the consumer. On the other hand, the product value also builds up. Because traceability cost and product value do not increase at the same rate throughout the supply chain, the relative traceability cost (i.e. traceability cost divided by product value) is not a constant, which means that the additional financial burden to trace a food product from field to fork is not equally shared by all operators in the supply chain.

This is illustrated in the case of the additional traceability and segregation costs for producing non-GM meat from non-GM feed. Figure 2 describes the additional cost for tracing and tracking non-GM soya in the meat chain. It is shown that, relative to product value at each stage, upstream stages bear a much larger cost weight than downstream stages, particularly retail. Sensitivity analyses further indicate that, whatever the changes in operational traceability design (accuracy, lot



size, etc.), practically all the cost impact will be supported by the upstream stages, and the retail stage will be relatively unaffected. This results from dilution of the traceability cost in the value chain as the latter builds up. Unless this unequal cost distribution is properly managed, it could distort the competitive position of selected segments of the food chain, such as agriculture. In turn, resistance to this distortion may limit the penetration of traceability in these segments, which would create blind spots in the supply chain, or even motivate some operators to adapt their operating base, for instance by delocalising their operations to low cost countries. Investments in Brazil by major EU poultry operators, such as Doux (France) suggests that this scenario is not just economics fiction.

5. Conclusions

Having set the scene of current supply chain management practices, it may be useful to conclude with requirements for the future including some agenda items for technology development. On the basis of current practices and sector trends, the following developments may be anticipated in the management of the food supply chain:

- An increasing emphasis on active management of quality and safety throughout the supply chain, as opposed to reactive management like crisis management and recall.
- Taking advantage of increased information storage capacity of active RFID.
- Integration of traceability systems and quality/safety control and testing.
- Continuous adaptation to changing and evolving regulation.
- Flexibility, that is interoperability between a variety of systems, standards and architectures.
- An ability to handle smaller items or lots.
- An ability to exert active and preventive quality control in small operations (e.g. farms) or in remote or ill-equipped production locations (e.g. plantations in third countries).

The above developments in supply chain management will result in new requirements for traceability as well as for rapid testing methods. In particular:

- Rapid testing methods will be more integrated in tracking and tracing systems;
- in certain instances, this integration should allow real-time testing and acting on the supply chain. This is the case of port operations, or deliveries of crops.
- Low cost RFID should be developed with both intelligence and active functions.
- Data generation and capture devices (sensing, recording) should be able to operate in rough or unsophisticated production conditions, such as farms in tropical climate, low educated manpower, etc.
- Diversity of standards is still a problem. On the one hand, common standards should be sought for similar supply chains, on the other hand, interoperability of system components should be aimed at.

This has definite implications for R&D developments. It is not straightforward that technologies developed in laboratories can effectively benefit operators and be readily implemented, therefore it should be taken care that:

- Not only technologies must be developed, but also organisations and the management around them have to adapt. This has implications for R&D strategies.
- Technologies must be developed for their own sake, but always in the context and understanding of operational processes and operating conditions.
- As far as possible, whole supply chain approaches must be taken, and not only company or operator oriented ones.

- The unsophistication of some operating environments or of some manpower must be taken on board.
- Developments in the regulatory environment must be monitored. On the other hand, information on technology development must also be fed back to regulators.

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Views on product traceability and rapid methods in food supply chain networks

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Abstract

Food product traceability ranks high on senior management agendas since the effectuation of the European Union general food law regulation. Since the functional requirements for rapid methods are determined by the strategies of actors and characteristics of processes in the food supply chain network, recent developments such as internationalisation, increased clock speed of processes and small lot size logistics put dynamic constraints on the analytical community to assist in fast, real-time and accurate food quality determination. Food processors should perform a risk analysis with specific focus on lot sizes, reaction times and maximum (allowed) recall volumes. This will identify new critical control points that may require new identification methods. Food product traceability is a multidisciplinary topic that is subject to developments in quality, marketing, information, logistics and technology management. The analytical community should therefore keep an open mind towards developments in these areas, as they will impact future requirements on rapid methods.

Keywords: product traceability, food industry, supply chain management, network

1. Introduction

Since the 1980s, management literature has stressed the need for collaboration among successive actors in the supply chain, from primary producers to final consumers, to better satisfy consumer demand at lower costs (Lambert *et al.*, 1998; Van der Vorst, 2000). This new way of managing the business within each link and the relationships with other members of the supply chain has been named supply chain management (SCM). A driving force behind SCM is the recognition that sub-optimisation occurs if each organisation in a supply chain attempts to optimise its own results rather than to co-ordinate its goals and activities with other organisations to optimise the results of the chain (Cooper

et al., 1997). This holds true especially in food supply chains because of shelf-life constraints of food products and increased consumer concern for safe and environment/animal-friendly production methods.

In recent years, the European consumer has become increasingly concerned about the safety of food and the negative effects of bio-industrial production. This concern has been strengthened by several sector-wide crises in the last decade (such as the BSE crisis, dioxin crisis, classical swine fever, and hoof and mouth disease in Europe). Governments, both national and international, respond by imposing new legislation and regulations on safety and quality of food products. Also retailers react by imposing new demands on their suppliers. To comply with the new demands, companies are forced to introduce sophisticated information systems that focus on identification and registration of food products and ingredients and product tracking and tracing capabilities.

This paper presents views on product traceability and implications for rapid methods in food supply chain networks. First, some main developments in food supply chain networks will be defined and discussed. Next, the issue of traceability of food products is elaborated upon resulting in a number of challenges and key decisions for traceability in food industry. Thereafter, the levels of traceability, traceability systems, and the trade-off between risk and control levels will be discussed. Finally, the implications of all this for rapid methods will be discussed, ending with some general conclusions.

2. Developments in food supply chain networks

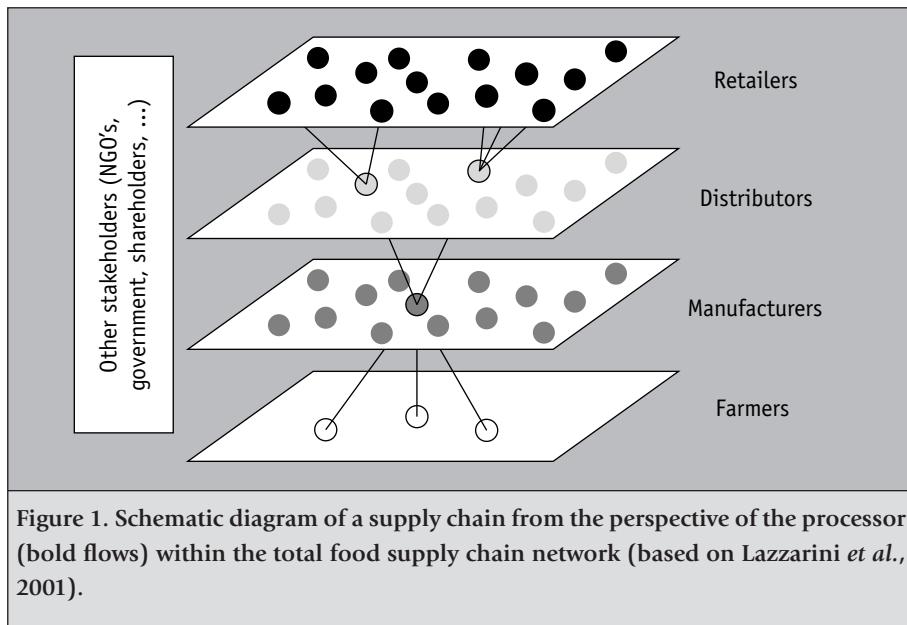
The modern consumer demands products of high and consistent quality, in broad assortments throughout the year and for competitive prices. Society imposes constraints on companies in order to economise on the use of resources, ensure animal-friendly and safe production and restrict pollution. The consumer has become increasingly concerned about the quality and safety of food and the negative effects of bio-industrial production. It is estimated that millions of Europeans get sick every year from food contamination. Important causes are *Salmonella*, *Campylobacter* and *E. coli* O157. Moreover, consumers can find recall announcements almost weekly in any newspaper (Trienekens and Van der Vorst, 2006).

Market demand is no longer confined to local or regional supply. This is changing the way food is brought to the market. Currently, even fresh produce shipped

from halfway around the world can be offered at competitive prices. This has spurred an enormous growth of product assortment in the supermarkets (in many European supermarkets in the 1990s the number of articles more than tripled from 10,000 to more than 30,000). Together with safety and quality demands of consumers, these developments have changed the production, trade, and distribution of food products beyond recognition. Governments, both national and international, are responding to this by imposing new legislation and regulations to ensure safe and animal friendly production, restricted pollution and to economise on the use of resources. Examples are the Codex Alimentarius standards (Food and Agriculture Organisation of the United Nations/World Health Organisation) and the European Union BSE regulations.

For food businesses this implies placing more emphasis on quality and safety control and environmental issues and, at the same time, shifting from bulk production towards production of specialities with high added value. Furthermore, because of their embeddedness in the network economy, collaboration with other parties becomes important for all businesses to achieve safe and high quality food products for the consumer. This means that business strategies must now move their focus from traditional economical and technological interests to topical issues such as the safety and healthfullness of food products, animal friendliness, the environment, etc. These processes are affecting the entire food chain from producer through to retailer. To effectively address (paradoxical) demands facing businesses, many problems and opportunities must be approached from a multi-disciplinary and farm-to-table perspective, and trade-offs must be made between different aspects of production, trade and the distribution of food (Trienekens and Van der Vorst, 2006).

The developments put dynamic requirements on the performance of the food system initiating a re-orientation of companies in the Dutch agriculture and food industry regarding their roles, activities and strategies (Van der Vorst *et al.*, 2005). The food industry is becoming an interconnected system with a large variety of complex relationships, reflected in the market place by the formation of food supply chain networks (FSCN, see Figure 1). In a FSCN different companies collaborate strategically in one or more areas, while preserving their own identity and autonomy. In a FSCN, organisations may play different roles in different chain settings and therefore collaborate with differing chain partners, who may be their competitors in other chain settings. In brief, chain actors may be involved in different supply chains in different networks, participate in a variety of business processes that change over time and in which dynamically



changing vertical and horizontal partnerships are required. To satisfy the increasing demands of consumers, government, business partners and non-governmental organisations (NGOs), and to obtain the 'license to produce and deliver', companies continuously have to work on innovations in products, processes and forms of co-operation in the FSCN.

Evaluating the developments for business practices and looking at their essence the following generic characteristics can be identified (Van der Vorst *et al.*, 2005):

- a focus on cost effectiveness with more and more attention to the combination of profit, people and planet;
- an increase in demand for guarantees relating to food quality and safety;
- an increase in (international) co-operation in supply chains whilst maintaining a high flexibility in partner selection;
- a decoupling of processes via modularisation and the creation of dynamic relationships between processes, which increases the complexity of traceability;
- a speeding-up of processes and reduction of batch sizes via rapid fulfilment techniques and parallel processing;

- a consolidation of product and information flows within organisations, supply chains and FSCNs;
- an introduction of new packaging and processing technologies that facilitate new logistics concepts with different transitions in form, place and time;
- possibilities to have a more detailed view on lot sizes with techniques to identify individual products (via radio frequency identification techniques and electronic product code standard); and
- an increased use of the potential of new rapid methods for product quality identification, information capturing and processing capabilities.

In short, a continuous change in the network structure, the processes, the management structures, and the resources used, can be seen.

3. Tracking and tracing of food products

Recently, the council of ministers of the European Union (EU) has adapted the general food law regulation (EC, 2002) serving as a steppingstone for future legislation on food safety. It aims to offer a high protection level to the European consumer and supports free trade between EU countries. Furthermore, it should lead to harmonisation of legislation within the EU by defining basic conditions and constraints for food legislation. One of the main instruments to guarantee food safety and reduce the size of a product recall is traceability. With regard to traceability, the general food law regulation states that companies must be able to identify the suppliers of its raw materials and the customer of its end-products on a transaction basis, to be implemented as of 1 January 2005. This general traceability requirement is non-prescriptive, but encompasses all food and feed business operators including primary producers. Retailers of goods to the final consumer are exempt from the requirements of forward traceability.

The basic idea of tracking and tracing is the possibility to determine where a certain item is located and to trace the history of that item. On the basis of that information, it should also be possible to determine the source of any (quality) problem of an item, and it should be possible to find out where the other items with the same problem are located in the supply chain. In literature the concept of traceability is often used as synonym to tracking and tracing (see e.g. Wilson and Clark, 1998; Van Twillert, 1999; Van Dorp, 2002). Tracking refers to the determination of the ongoing location of items during their way through the supply chain (see Figure 2). Tracing aims at defining the composition and the treatments an item has received during the various stages in the production

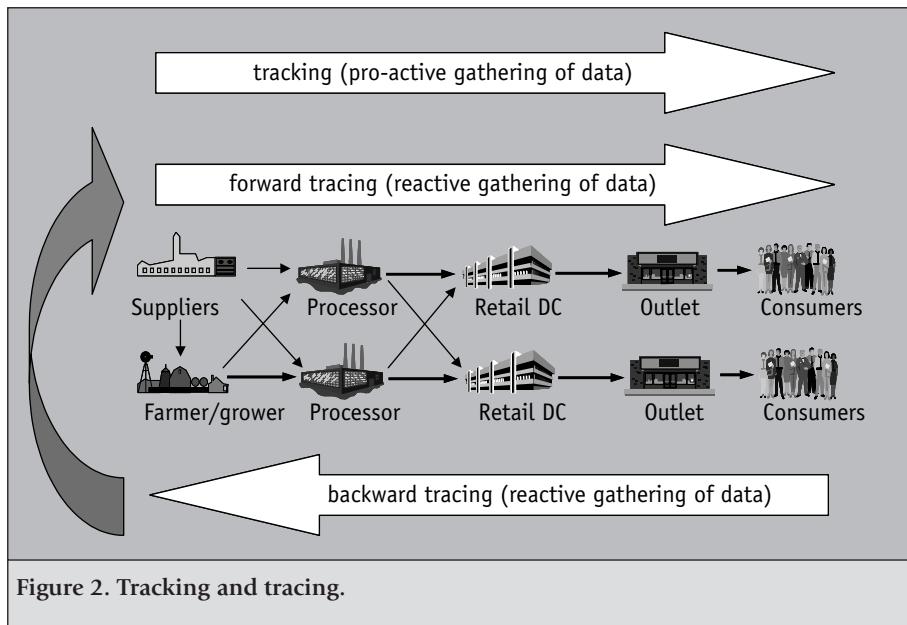
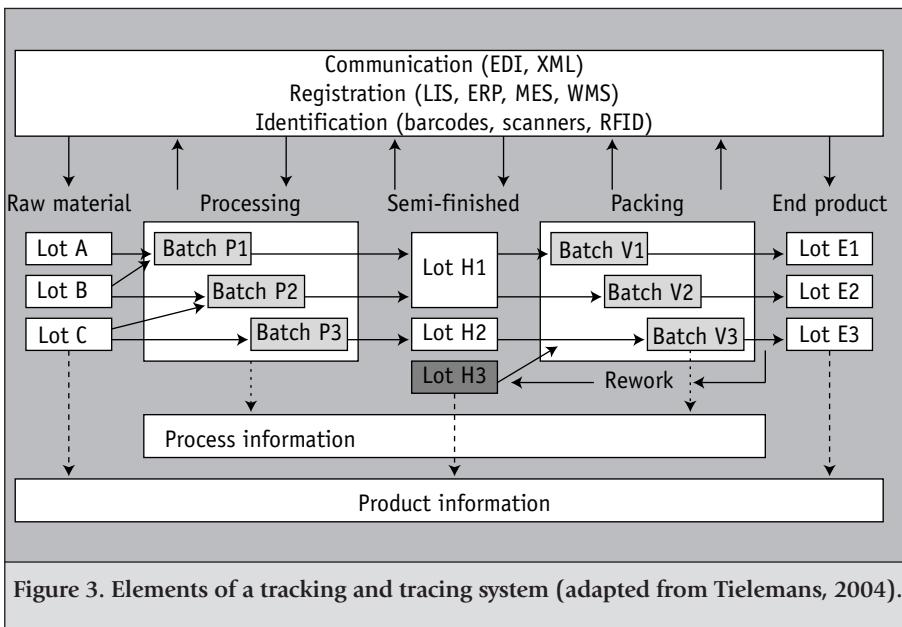


Figure 2. Tracking and tracing.

life cycle. Chain upstream (backward) tracing aims at determining the history of items and is used to determine the source of a problem of a defective item. Chain downstream (forward) tracing aims at the determination of the location of items that were produced using, for example, a contaminated batch of raw materials. In this paper traceability is defined as the ability to document and trace forward and backward a product (lot), and its history through the whole, or part, of a production chain from harvest through transport, storage, processing, distribution and sales. Traceability allows to closely monitor properties of objects as they are made and move through FSCN, thus allowing early warning for quality problems and hazards, to avoid these problems, and efficient recall and action when needed.

Traceability is of importance on chain level, as well as on company level. On company level a system should provide information on the location of the product and on the history of the product; this includes as well product information as process information (Figure 3). Product information relates to knowing the (static) characteristics of a product lot in each stock point, such as lot code, location, volume, and best-before-date. Process information relates to the (dynamic) process characteristics (such as temperature, humidity) and



characteristics of used and outgoing product lots in each process step (such as incoming lot numbers, volumes and rework lots). Especially rework complicates traceability since small process batches in a specific process step are re-used in another process batch. When the first batch was infected, as a consequence, also the batch in which it was re-used is infected. The aspect of dilution then becomes relevant; at what dilution degree becomes the infection risk-free?

The idea is to keep track of all lot input-process-output relations, i.e. which lot of raw materials has been used in which processing step and is consumed in which lot of end-products. Hereby, a lot is defined as a number of products (boxes, bags, pallets, etc.), which have unique and homogeneous characteristics with a common history in process conditions (Tielemans, 2004). On chain level, besides information on the location of products in the FSCN, also information on the history of the product is of importance. In this regard it is important to identify the current unique characteristics of lots (components) and the historical relationship between lots as they are transferred over the different supply chain stages.

Traceability must be managed by setting up a traceability system, which keeps track of product routes and of selected data. Traceability systems are mainly realised by linking existing information systems, which are in use for other purposes, such as invoicing, purchasing, production planning and scheduling, inventory management, etc. In general, each traceability system can be characterised by the following components (Van der Vorst *et al.*, 2003):

- Identification and registration of lots (e.g., animals, products) within companies in the supply chain. Most used are barcode printers and -readers, tags or radio frequency identification devices (RFIDs), ear tags and DNA-tracing.
- Administration and registration of traceability data in databases of enterprise resource planning (ERP) systems, warehouse management systems (WMS), laboratory information (management) systems (LIS) or manufacturing execution systems (MES).
- Communication of traceability data in the supply chain by EDI (electronic data interchange) or XML (extensible markup language).
- Supporting infrastructure in the FSCN for the capturing and exchange of information and the use of standards such as EAN (European article number) standard-128.

When chain information systems are in place to enable this information exchange, transparency can be created, which makes it possible to offer specific information to buyers and consumers. Transparency is defined as a shared understanding of, and access to, the product-related information, which they request, without loss, noise, delay and distortion (Hofstede *et al.*, 2004). This again can play a major part in (re)gaining the trust of the consumer. Moreover, by sharing information between partners information flows can be better managed resulting in lower costs and more flexibility throughout the chain. The development of such chain information systems is, however, not an easy task. Table 1 gives an overview of the benefits of traceability for different stakeholders in the chain.

Even though traceability systems are in place, companies can not always profit fully. When incidents occur, retailers often remove all articles from the shelves and not only the articles from the specific lot it concerns. Main reasons are that retailers have experienced in many cases that in time the number of products related to the incident grows and that consumer confidence in the product (group) decreases. Furthermore, incidents often lead to general import restrictions, thereby not doing justice to reliable traceability systems in place.

Table 1. Benefits of traceability for different stakeholders (based on Van Dorp, 2002 and Food Standards Agency, 2002).

Consumer	Business	Government
Protect food safety by effective product recall	Protect public health through the effective withdrawal of food products	Comply with relevant legislation
Enable avoidance of specific foods and food ingredients, whether because of allergy, food intolerance or lifestyle choice	Enable control with regard to human and animal health in emergencies	Be able to take prompt action to remove products from sale
	Losses are minimised by an efficient recall process	Be able to diagnose problems in production and pass on liability where relevant
	Information about raw materials and processes improves process and quality management	Assure food products and maintain market and consumer confidence
	Improved information retrieval simplifies audits	

4. Choosing the appropriate level of traceability

One of the basic questions companies have to deal with now is which traceability performance level to strive for in the near future; comply with minimum levels of traceability as requested by the government or follow an alternative strategy that might be more fruitful? And what are the consequences for the design and management of the supply chain? We can distinguish between three chain strategies of traceability (Van der Vorst, 2004):

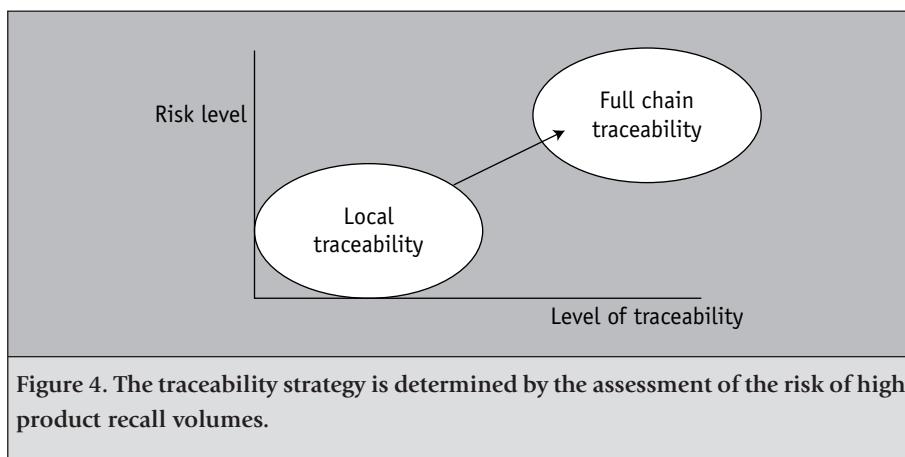
- *Compliance-oriented strategy.* Comply with rules and regulations with the help of end-of-pipe techniques. Companies that adhere to a compliance-oriented strategy focus on the registration of incoming and outgoing materials and leave the process as a black box.
- *Process improvement-oriented strategy.* Strive for control of the traceability of products within their own link by means of production integrated measures, which achieve both compliance with governmental rules and regulations, and an improvement of process performances (due to the increased information

availability). Examples of process-oriented measures are local ICT-systems that register all process data and the introduction of smaller lots.

- *Market-oriented (branding) strategy.* Aim for the establishment of full traceability within the supply chain to achieve competitive advantage (by creating added value in the market place). This requires the redesign of processes to separate small production lots, standardisation of information carriers, adjusted planning and control of production processes, etc. The traceability performance is the result of the joint effort to design and produce a product. This requires a chain structure in which the individual links work intensively together to open new markets.

Which traceability strategy is chosen depends on the risk a company is willing to take related to the maximum recall batch size (Figure 4). This is determined by a number of factors. First of all, the chance of an incident occurrence and the severity of the incident. This depends on a detailed risk analysis in line with the traditional hazard analysis critical control points (HACCP) approach. Secondly, the volume of an infected lot, which is determined by the lot segregation procedures – and adherence to those - in the company. Thirdly, the reaction time, or how fast can one identify the problem, isolate the infected lots and recall those lots? If this reaction time is long, infected lots may already have reached the market outlets. It is clear that rapid methods can alleviate this problem to a certain extent.

Companies have already spent a lot of time on the traditional elements of risk analysis, i.e. determining critical control points via the analysis of potential



occurrence and severity of incidents. However, most companies are not aware of the number of infected end products if a specific raw material was to be infected. Examples in practice have resulted in millions of end products that would have to be recalled, much to the surprise of the management team. So even if the chance of the incident occurrence is low, the volume of implications could be disastrous for the company. And therefore, it should imply to take appropriate actions to reduce this risk. Companies have to identify the optimal batch size in each stage of the production and distribution process. And based on the risk analyses they have to define the product sampling regime. This has a direct impact on the performance requirements of rapid identification methods.

5. Challenges for traceability and rapid methods

We have briefly discussed developments that are taking place in FSCN and zoomed in on the aspect of traceability of food products. From these developments we can distil the following challenges for traceability and rapid methods in food companies:

- How to guarantee composition, origin, safety, integrity and other properties of products at all stages in the FSCN to show due diligence in the control of food quality?
- How to minimise the risk on large recall volumes?
- How to trace origin and cause of problems when they occur as fast and accurate as possible and how to perform an effective recall?
- How to create transparency in the network by informing stakeholders of FSCN about properties of products, processes and production means?

These challenges can only be met by (joint or co-ordinated) development of business processes and products in FSCN, having necessary properties and associated quality and transparency systems. Because of the internationalisation, increase of clock speed of the FSCN and decrease of lot sizes, there is a need for:

- (quality) tracking and tracing and transparency systems, and potential new logistics concepts such as ripe-on-arrival, floating stock, etc.;
- fast, cheap, robust, sensitive, real-time, accurate and validated testing methods for food safety/quality identification methods;
- unambiguous relationships between samples and logistic lot sizes;
- identification methods at different traceability levels (e.g. identifying a flock of birds versus identifying an individual cow);

- fast and reliable communication systems with the principal (especially in case of a recall);
- international standards in identification and communication techniques;
- early warning systems for quality problems and hazards; and
- pro-active control systems to cope with deviations and reduce risks at lowest cost.

6. Conclusions

It may be concluded that developments in FSCN put stringent requirements on traceability and rapid methods to assist in fast and accurate food quality determination. More specific, the requirements for rapid methods are determined by the objectives and characteristics of the FSCN. Companies should perform risk analyses with specific focus on lot sizes and maximum (allowed) recall volumes. This determines the inspection/sampling regime and its requirements, hence the usefulness of rapid methods. Because of the internationalisation of FSCN there is a need for the development of international standards for ICT systems and rapid identification methods. Food product traceability is a multidisciplinary topic, which is subject to developments in quality, marketing, information, logistics and technology management. The analytical community should therefore keep an open mind towards developments in these areas. Future research should focus on the development of early warning systems and pro-active planning concepts using sophisticated rapid methods and ICT systems.

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