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Food Safety Testing: Rapid Molecular Methods for Chemical and Biological Hazards

by

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals (**I-V**):

I Virve Hagren, Steven R. H. Crooks, Christopher T. Elliott, Timo Lövgren, and Mika Tuomola (2004) An all-in-one dry chemistry immunoassay for the screening of coccidiostat nicarbazin in poultry eggs and liver. *J Agric Food Chem* **52:**2429-2433.

II Virve Hagren, Lisa Connolly, Christopher T. Elliott, Timo Lövgren, and Mika Tuomola (2005) Rapid screening method for halofuginone residues in poultry eggs and liver using time-resolved fluorometry combined with the all-in-one dry chemistry assay concept. *Anal Chim Acta* **529:**21-25.

III Virve Hagren, Pekka Peippo, Mika Tuomola, and Timo Lövgren (2006) Rapid time-resolved fluoroimmunoassay for the screening of monensin residues in eggs. *Anal Chim Acta* **557:**164-168.

IV Virve Hagren, Piia von Lode, Anniina Syrjälä, Tero Soukka, Timo Lövgren, Hannu Kojola, and Jussi Nurmi (2008) An automated PCR platform with homogeneous time-resolved fluorescence detection and dry chemistry assay kits. *Anal Biochem* **374:**411-416.

V Virve Hagren, Piia von Lode, Anniina Syrjälä, Teemu Korpimäki, Mika Tuomola, Otto Kauko, and Jussi Nurmi (2008) An 8-hour system for *Salmonella* detection with immunomagnetic separation and homogeneous time-resolved fluorescence PCR. *Int J Food Microbiol* **125:**158-161.

In addition, some unpublished data are included.

ABBREVIATIONS

B/B_0	value calculated by dividing the signal of the sample/standard by the signal of the zero sample/standard
BPW	buffered peptone water
BSA	bovine serum albumin
CCα	decision limit
CCβ	detection capability
CFU	colony forming unit
DCC	N,N'-dicyclohexylcarbodiimide
DELFIA	dissociation-enhanced lanthanide fluoroimmunoassay
DNA	deoxyribonucleic acid
DNC	4,4'-dinitrocarbanilide
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EU	European Union
EVIRA	Finnish Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
HACCP	Hazard Analysis and Critical Control Point
HPLC	high performance liquid chromatography
IAC	internal amplification control
IgG	immunoglobulin G
IMS	immunomagnetic separation
ISO	International Organization for Standardization
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MRL	maximum residue limit
mRNA	messenger RNA
MS	mass spectrometry
NASBA	nucleic acid sequence-based amplification
NHS	N-hydroxysuccinimide
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription PCR
S/N	signal-to-noise ratio
SPE	solid phase extraction
SPR	surface plasmon resonance
TRF	time-resolved fluorometry
WHO	World Health Organization

ABSTRACT

The central goal of food safety policy in the European Union (EU) is to protect consumer health by guaranteeing a high level of food safety throughout the food chain. This goal can in part be achieved by testing foodstuffs for the presence of various chemical and biological hazards. The aim of this study was to facilitate food safety testing by providing rapid and user-friendly methods for the detection of particular food-related hazards.

Heterogeneous competitive time-resolved fluoroimmunoassays were developed for the detection of selected veterinary residues, that is coccidiostat residues, in eggs and chicken liver. After a simplified sample preparation procedure, the immunoassays were performed either in manual format with dissociation-enhanced measurement or in automated format with pre-dried assay reagents and surface measurement. Although the assays were primarily designed for screening purposes providing only qualitative results, they could also be used in a quantitative mode. All the developed assays had good performance characteristics enabling reliable screening of samples at concentration levels required by the authorities.

A novel polymerase chain reaction (PCR)-based assay system was developed for the detection of *Salmonella* spp. in food. The sample preparation included a short non-selective pre-enrichment step, after which the target cells were collected with immunomagnetic beads and applied to PCR reaction vessels containing all the reagents required for the assay in dry form. The homogeneous PCR assay was performed with a novel instrument platform, GenomEra™, and the qualitative assay results were automatically interpreted based on end-point time-resolved fluorescence measurements and cut-off values. The assay was validated using various food matrices spiked with sub-lethally injured *Salmonella* cells at levels of 1-10 colony forming units (CFU)/25 g of food. The main advantage of the system was the exceptionally short time to result; the entire process starting from the pre-enrichment and ending with the PCR result could be completed in eight hours.

In conclusion, molecular methods using state-of-the-art assay techniques were developed for food safety testing. The combination of time-resolved fluorescence detection and ready-to-use reagents enabled sensitive assays easily amenable to automation. Consequently, together with the simplified sample preparation, these methods could prove to be applicable in routine testing.

1 INTRODUCTION

Food safety has a major impact on public health, and therefore, food safety issues have gained considerable attention. The recent high profile food-related crises such as bovine spongiform encephalopathy, dioxin contamination outbreaks, and epidemics caused by foodborne pathogens have shaken consumer confidence in the ability of the food industry and the authorities to guarantee food safety. To respond to consumers concerns, the EU introduced an integrated "from farm to fork" strategy, which became the cornerstone of the EU's food safety policy (Anonymous, 2000). The aim of this policy was to provide the same high level of health protection to consumers all over Europe. Food safety was designed to rest on the following pillars: reviewed and updated legislation, appropriate scientific advice for decision making, data collection and analysis, consumer information, and official enforcement and control measures covering all aspects of the food chain from raw materials to food consumption. The basis for food legislation was set in Regulation 178/2002, which presented the general principles and requirements of food law and provided the framework for other related legislation (Anonymous, 2002b). Following that, several steps were taken towards to improved food safety control. For example, the European Food Safety Authority (EFSA) was established, the aim of which is to scientifically assess and communicate on risks associated with the food chain (Anonymous, 2002b). Furthermore, the control of chemical and biological hazards in food, which was traditionally based on reactive and enforcement-orientated approach relying mainly on regulatory testing of end products, was updated with the introduction of the Hazard Analysis and Critical Control Point system (HACCP) (Anonymous, 2004a, 2004b). The application of HACCP provided a more effective and comprehensive control approach by relying on the prevention of hazards and it also placed more responsibility for food hygiene and safety on food operators. In addition, the introduction of a harmonized approach for official controls which are performed by the authorities to verify the compliance with feed and food law, animal health, and animal welfare rules, further strengthened the realization of the food safety policy (Anonymous, 2004c, 2004d).

Altogether, several important steps have been taken at the legislative level to improve food safety. Nevertheless, due to globalization of food production and distribution, food-related hazards can spread fast in the food chain. To confront this challenge, combined and continuous efforts, including the development and implementation of novel methods of analysis for food control, are required to achieve a high level of food safety now and in the future.

2 REVIEW OF THE LITERATURE

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) define food control as "… a mandatory regulatory activity of enforcement by national or local authorities to provide consumer protection and ensure that all foods during production, handling, storage, processing, and distribution are safe, wholesome and fit for human consumption; conform to quality and safety requirements; and are honestly and accurately labelled as prescribed by law." (FAO/WHO, 2003). Food is considered to be unsafe if it is injurious to health or unfit for human consumption (Anonymous, 2002b). The potential sources of hazards that can compromise food safety include biological, chemical, and physical agents. The hazards can enter the food chain, e.g. due to improper agricultural practices, poor hygiene, misuse of chemicals, lack of preventive controls in food processing and preparation, improper storage, and contaminated raw materials, ingredients, and water (FAO/WHO, 2003).

This literature review focuses on selected chemical and biological hazards present in food, namely a group of veterinary residues and a particular foodborne pathogen, respectively, and introduces control activities and methods that are used in food safety testing to detect these hazards in various foodstuffs.

2.1 VETERINARY RESIDUES AND FOOD SAFETY

There is a wide variety of potential chemical hazards such as veterinary drugs, feed additives, growth promoters, dioxins, heavy metals, and pesticides that can pose a risk to food safety. Anticoccidial feed additives represent one group of substances which are targeted within the national residue control programmes according to Directive 96/23/EC (Anonymous, 1996). The following sections describe measures and methods used in the residue control of a selected group of veterinary residues, namely anticoccidials, or coccidiostats.

2.1.1 Coccidiosis and anticoccidials

Coccidiosis is a protozoal disease affecting a number of domestic animals. The symptoms of coccidiosis in poultry vary, e.g. from diarrhoea, reduced growth rate, and loss of egg production to death. Thus, even a mild form of this disease has a negative impact on the poultry industry. Because most of the damage caused by the infection occurs before clinical signs become apparent, the prevention of this disease is considered to be even more important than the treatment.

The protozoa responsible for coccidiosis in chickens mainly belong to the genus *Eimeria*. Flocks are usually infected with several *Eimeria* species, of which *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*

are the most common. The protozoa have a very complex life cycle containing an exogenous and endogenous stage with asexual and sexual phases. In brief, the infectious process takes approximately four to seven days, and it starts when the host ingests sporulated oocysts, which contain four sporocysts each with two sporozoites. When these oocysts enter the intestines of the host, sporozoites are liberated and they penetrate the epithelial cells. Various *Eimeria* species infect different parts of the intestine. After a complex reproductive process, oocysts are formed and passed out in the droppings, and under proper environmental conditions the cycle can start again as new oocysts sporulate and become infectious. Due to intensive rearing of chickens, coccidiosis can spread rapidly throughout a flock. The disease is diagnosed by determining oocysts in faeces or intestinal scrapings and by monitoring, e.g. the flock appearance, feed intake, growth rate, mortality, and rate of lay. (Allen and Fetterer, 2002; Merck, 2005.)

Anticoccidials are used as feed additives in poultry production to provide prophylactic control of coccidiosis. A wide range of coccidiostats has been developed during the years, since continuous use can cause progressive loss of efficacy due to emerging drug resistance in the parasite population. Therefore, to diminish the resistance problem, coccidiostats can be switched during a single grow out or rotated with successive flocks. Currently, the group of polyether ionophores, including lasalocid, monensin, narasin, salinomycin, maduramicin, and semduramicin, form the mainstay of coccidiosis control. The amounts of selected ionophores used in Finland are presented in Table 1. Ionophores affect both the extra- and intracellular stages of the life cycle of *Eimeria*. They form complexes with alkaline cations, and thus, interfere with cell membrane function by affecting ion transport (for a review, see Pressman and Fahim, 1982). Because ionophores can affect the cardiovascular system (Pressman and Fahim, 1982, 1983), ionophore residues in food may pose a health risk for humans. In addition to ionophores, a class of chemical anticoccidials, including compounds such as diclazuril, halofuginone, nicarbazin, and robenidine are used to control coccidiosis in poultry.

Table 1. Consumption of selected ionophores in Finland 2000-2007.

Compound	Active substance, kg/year							
	2000	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
Monensin	--	1 475	1 969	4 422	5 808	8 669 [a]	9 788 [b]	5 560 [c]
Narasin	2 549	2 101	5 569	5 769	5 518	3 205	2 481 [d]	8 007 [e]
Salinomycin	2 829	3 272	28	3	10 [f]	374 [g]	1 328 [h]	35 [i]
Lasalocid	2 796	3 624	3 349	176	--	--	--	--

[a] For exported feed 13 kg; [b] For exported feed 43 kg; [c] For exported feed 5 kg; [d] For exported feed 2 kg; [e] For exported feed 22 kg; [f] For exported feed 10 kg; [g] For exported feed 190 kg; [h] For exported feed 317 kg; [i] For exported feed 35 kg. Based on EVIRA (2008).

Broilers are normally fed with anticoccidials almost throughout their lives because even subclinical infections are detrimental to the growth and feed conversion rate of the birds. If a correct level of medication is used, no cross-contamination and improper use of medicated feed occurs, and withdrawal periods prior to slaughter are observed, coccidiostat residues should not be found in excess in the food chain. Egg laying hens are not allowed to be fed with anticoccidials during the laying period at all because of the potential risk for accumulation of unwanted residues in eggs. However, studies have confirmed that eggs and poultry are still commonly contaminated with coccidiostat residues, although the awareness of the industry has increased and feed formulations have been improved (Kennedy *et al*., 1998; Cannavan *et al.*, 2000; Mortier *et al.*, 2005b; Danaher *et al.*, 2008). At the EU level, the most commonly found anticoccidials in 2006 in poultry and eggs were nicarbazin, lasalocid, robenidine, and diclazuril (Anonymous, 2008). The main reasons for the incidence of residues have been reported to be the improper use of anticoccidials and feed contamination (Kennedy *et al.*, 2000; McEvoy, 2002).

The use of coccidiostats in the EU will be phased out in the near future (Anonymous, 2003b), and therefore, other means of controlling coccidiosis are required. The first line of defence is the application of high hygiene standards in poultry farms to reduce the number of oocysts in the environment, but the actual control of coccidiosis requires chemoprophylaxis and/or vaccination strategies. Laying hens obtain immunity within the first few months of their lives either naturally (with the help of anticoccidials) or through vaccination (Chapman, 1999). Because protective immunity after natural infection takes several weeks to develop, it is not a feasible option for broilers with a short lifetime. Therefore, although vaccines have not yet been widely utilized in broiler production, early vaccination schemes may prove to be a noteworthy option (Williams, 2002). However, the antigenic variation of *Eimeria* strains and the cost issues involved in the development of various types of vaccines still pose challenges to coccidiosis control (Dalloul and Lillehoj, 2006).

2.1.2 Veterinary residue control in the EU

Veterinary medicinal products are substances used in animal husbandry for treating or preventing disease in animals (Anonymous, 2001). Improper use of veterinary medicinal products can lead to the occurrence of residues of these substances in foodstuffs, which has raised concerns among the public. To protect human health against possible harmful effects resulting from exposure to these residues, the EU has addressed food safety issues at several levels especially during the last decade. The key elements of legislation providing the framework for residue control in the EU are briefly presented here.

Prior to authorization for use in food-producing animals, the safety of veterinary medicinal products has to be assessed (Anonymous, 1990, 2001). After the safety evaluation, the necessity of establishing maximum residue limits (MRLs), i.e. the maximum concentration of residues legally permitted to be present in food, for that

particular substance is estimated. Feed additives have not been included in this MRL evaluation until recently (Anonymous, 2003b). In addition to the safety file with the proposed acceptable daily intake based on the no observed (adverse) effect level, the establishment of MRLs for a particular substance requires, e.g. the selection of a marker residue and consideration of other related issues such as the ratio of the marker residue with regard to total residues and the residue depletion kinetics. Moreover, the MRLs should cover each edible tissue (or product) of each target species for which the substance is intended based on the tissue residue distribution pattern. For example, the edible tissues for poultry consist of muscle, liver, kidney, and fat and skin in natural proportions. The evaluated substances are categorized under the Annexes of the Regulation 2377/90 (MRL Regulation) as follows: substances, for which final MRLs have been established (Annex I), substances, for which it is not considered necessary to establish MRLs (Annex II), substances with provisional MRLs (Annex III), substances, for which no MRL could be established because residues of these substances, at any concentration level, in foodstuffs of animal origin create a health hazard, and thus, their use is prohibited (Annex IV) (Anonymous, 1990). Currently, MRL Regulation is under review with the aim of simplifying the existing legislation and improving the availability of veterinary medicinal products (Anonymous, 2007). The MRLs provide the basis for the establishment of reference points for residue control purposes (Anonymous, 1996) and of withdrawal periods, which is the time required to pass between the last administration of the drug (or the feed additive) to animals and the production of foodstuffs from such animals ensuring that the residue levels in tissues or products have fallen below the stated MRL. Thus, assuming that the substance is used properly and according to regulations, after the withdrawal period the food should be safe to consume. (Anonymous, 2005b.)

Residue control is defined in Directive 96/23/EC, which describes the system for monitoring of substances and residues thereof in live animals and animal products (Anonymous, 1996). The Directive also determines specific sampling levels and frequencies, as well as the groups of substances (Table 2), to be monitored. The minimum number of samples to be analyzed for each food commodity is linked to the production figures for the preceding year. To fulfil the requirements of the Directive, the Member States have established national residue control programmes, the results of which are reported annually to the Commission. The Finnish Food Safety Authority (EVIRA) is responsible for implementing the programme in Finland. As an example, EVIRA tested more than 10 000 samples in 2005 for the presence of 80 different chemical compounds and 99.7% of the samples were negative or below the legally permitted concentrations or action levels (EVIRA, 2006a). The implementation of coccidiostat residue monitoring to the national residue control programmes has been complicated by the fact that although coccidiostats are included in the MRL evaluation, many of the compounds do not yet have official MRLs. Therefore, Member States have set national action levels to guide the monitoring task. For instance, in Finland the action level for ionophore residues in eggs has been 10 µg/kg and in poultry any positive result has resulted in an investigation (National Food Agency *et al.*, 2005).

Table 2. Substances to be monitored in residue control according to Directive 96/23/EC.

Group A - Substances having anabolic effect and unauthorized substances
– Stilbenes, stilbene derivatives, and their salts and esters
– Antithyroid agents
– Steroids
– Resorcylic acid lactones including zeranol
– β-agonists
– Compounds included in Annex IV to Council Regulation (EEC) No 2377/90
Group B - Veterinary drugs and contaminants
– Antibacterial substances including sulphonamides and quinolones
– Other veterinary drugs (anthelmintics, anticoccidials including nitroimidazoles, carbamates and pyrethroids, sedatives, non-steroidal anti-inflammatory drugs, and other pharmacologically active substances)
– Other substances and environmental contaminants (organochlorine compounds including PCBs, organophosphorus compounds, chemical elements, mycotoxins, dyes, and others)

Abbreviations: PCB, polychlorinated biphenyl.

In contrast to other areas of food safety testing, the methods used in residue control can be freely selected as long as they comply with the requirements set in the Decision 2002/657/EC (Anonymous, 2002a). The Decision relates to Directive 96/23/EC and outlines the criteria for the performance of screening and confirmatory methods and for the interpretation of results. It aims to guarantee the quality and comparability of test results, which are generated by laboratories approved for official residue control. Moreover, to ensure harmonised implementation of Directive 96/23/EC the concept of a minimum required performance limit was introduced for methods which are used to detect substances with no set permitted limit, substances with no authorization, or prohibited substances. The main advantage of Decision 2002/657/EC is its flexible adaptation to emerging problems and technical developments, which enables easier implementation of novel analytical methods in routine use (Stolker and Brinkman, 2005).

2.1.3 Methods for the detection of anticoccidial residues in food

The methods of analysis used in residue control are generally divided into two categories: screening and confirmatory methods. Screening methods are defined as "…methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results.", and confirmatory methods as "…methods that provide full or complementary information enabling the substance

to be unequivocally identified and if necessary quantified at the level of interest." (Anonymous, 2002a). In principle, by combining screening and confirmatory methods a cost-effective system for residue control can be achieved. The initial screening, which is performed with a rapid and inexpensive qualitative method, classifies samples as negative or potentially positive. The suspect samples have to be further analyzed with a confirmatory method to obtain unequivocal identification and quantification of the substance. Nevertheless, it seems that confirmatory methods are often used for both purposes and the development and validation of screening methods has received less attention. To be truly applicable for routine use, high throughput screening methods should be inexpensive, rapid, robust, and reliable, as well as being able to detect preferably multiple residues simultaneously at the required levels. Consequently, it can be very challenging to combine all these favourable characteristics in a single assay.

The first assays to detect the presence of coccidiostats were based on growth inhibition of *Eimeria* in cell culture and were mainly used to study the effects of anticoccidial activity (McDougald and Galloway, 1973; Strout and Ouellette, 1973). Moreover, various thin-layer chromatography methods combined with bioautography were developed for ionophores, which have antibiotic effects and can inhibit the growth of certain bacteria (Weiss and MacDonald, 1985). The following sections introduce current screening and confirmatory methods and sample preparation procedures for selected anticoccidials, mainly ionophores, halofuginone, and nicarbazin, in food samples.

2.1.3.1 General principles of sample preparation

Sample preparation forms an integral part of the method of analysis and its role is even more pronounced when more sensitive detection of residues is required. Typically a combination of sample preparation techniques is used for complex sample matrices such as food. Therefore, regardless of the analyte or method of analysis, sample preparation for residue control using food matrices tends to be slow, and thus, it is the limiting factor in the overall sample throughput. Consequently, solutions for simplifying, automating, and speeding up sample preparation without compromising the assay performance would be particularly beneficial because they could provide savings in time and money and eventually lead to an increased testing rate. In particular, screening methods aiming to minimize the cost and analysis time may settle for a simpler sample preparation, although sometimes at the expense of analyte recovery.

Foodstuffs of animal origin used in residue control commonly require extensive sample preparation. The sample preparation can include several steps: sampling, homogenization, extraction, clean-up, and concentration. The aim is to provide a homogeneous, representative sample for analysis where the analyte in question is recovered and concentrated to enable detection of residue concentrations typically at the level of µg/kg. In addition, interfering substances, which may co-extract with the analyte, should be excluded. The extraction procedure is dependent on the analyte and

its concentration, the sample matrix, and the method of analysis. Organic solvents such as acetonitrile, ethyl acetate or methanol are normally used in extraction, although some approaches using only aqueous extraction have been reported (Elissalde *et al.*, 1993; Beier *et al.*, 1998). After extraction, further purification steps, e.g. another liquid extraction step or solid phase extraction (SPE) may be necessary, if sample matrix effects still cause interference in the assay. A final evaporation step can be included in the procedure to concentrate the extract, and thus increase the sensitivity of the assay. Further information regarding sample preparation techniques can be found, for example, in an article by Ridgway *et al.* (2007).

2.1.3.2 Screening methods

Screening methods for coccidiostats mainly rely on antibody-based techniques. Coccidiostats form a heterogeneous group in terms of chemical structures and sensitivity requirements. Therefore, multi-analyte approaches using broad-specificity antibodies where the entire class of drugs sharing similar chemical structures and MRLs or action levels can be screened simultaneously (Korpimäki *et al.*, 2004), cannot be utilized in coccidiostat screening. However, two members of the ionophore group, salinomycin and narasin, provide a minor exception to that rule, as their structures differ only by one methyl group enabling 100% cross-reactivity (Kennedy *et al.*, 1995; Peippo *et al.*, 2004).

Coccidiostats are small molecular weight analytes, often referred to as haptens, and they have to be conjugated to a carrier protein such as apo-transferrin (Crooks *et al.*, 1997), human serum albumin (Connolly *et al.*, 2002), or keyhole limpet hemocyanin (Rowe *et al.*, 1994; Beier and Stanker, 2001) to evoke antibody production. In general, the challenge in antibody production for coccidiostats is to obtain antibodies that recognize hapten and not parts of the carrier protein. The antibodies may also detect the metabolites of the parent compound, which cannot usually be tested because of the lack of reference standards (Elliott *et al.*, 1998). Occasionally the hapten has to be modified to facilitate conjugation to carriers. For example, the structure of 4,4'-dinitrocarbanilide (DNC), the marker residue for nicarbazin, is not particularly suitable for conjugation, and therefore, a hydrazone derivative and a DNC mimic (4'-nitrosuccinanilic acid) containing a carboxyl group for conjugation have been suggested as alternatives to conjugation instead of the less reactive parent compound (Beier and Stanker, 1998). However, the monoclonal antibodies generated with the hydrazone derivative did not recognise free DNC (Beier and Stanker, 1998). The mimic, on the other hand, was successfully employed to create polyclonal antibodies for nicarbazin (Connolly *et al.*, 2002), but the corresponding monoclonal counterparts did not perform as well in terms of sensitivity (Beier and Stanker, 2001).

The selection of screening methods for coccidiostats is still limited. The reported antibody-based methods for coccidiostats are mainly based on a heterogeneous competitive immunoassay format with enzyme labels (enzyme-linked immunosorbent assay, ELISA) (Kennedy *et al.*, 1995; Crooks *et al.*, 1997; Watanabe *et al.*, 2001; Huet

et al., 2005). The assays are usually performed in a standard microtiter plate format allowing easy handling of samples and high assay throughput. In addition to capacity issues, immunoassays are easy to perform, relatively rapid, and amenable to automation. Immunoassays relying on detection technologies such as time-resolved fluorometry (TRF) (Crooks *et al.*, 1998; Peippo *et al.*, 2004) and other assay formats such as biosensors (McCarney *et al.*, 2003; Danaher *et al.*, 2008) have also been reported for use in coccidiostat analysis. Biosensors form a heterogeneous group of compact analytical devices comprising two distinct elements: a biological recognition element (e.g. enzyme, antibody, microbial cell) either integrated with or in close contact (at least in principle) with a signal transduction element (e.g. optical, electrochemical, piezoelectric), which converts the signal from the biological element to a quantifiable signal (for reviews, see Patel, 2002 and Baeumner, 2003). Immunosensors, which use antibodies as recognition elements, represent the form of biosensors commonly utilized in veterinary residue analysis (Ricci *et al*., 2007). Because biosensors also provide possibilities for portable systems, analysis of turbid samples, on-line monitoring, and sensitive detection, there is continuous interest in the development of biosensors not only for the food safety market, but also for the field of medical, military, and environmental applications (Alocilja and Radke, 2003). However, there are still some general technical barriers, cost considerations, and performance issues that need to be solved, e.g. by utilizing advances in transducers and recognition elements together with the possibilities offered by nanotechnology before large-scale commercialization of biosensors for food safety can occur (Luong *et al.*, 2008; Palchetti and Mascini, 2008).

Some examples of different types of screening assays for coccidiostat residues are presented here. Huet *et al.* (2005) described a competitive ELISA for nicarbazin and halofuginone residues in eggs and chicken muscle. The same sample preparation procedure using acetonitrile extraction and hexane wash could be applied to both analytes. The assay performance in terms of detection capability (CCβ) was good, but the practicability of the assay suffered from the overnight incubation step. McCarney *et al.* (2003) reported the development of a regenerable optical immunosensor based on surface plasmon resonance (SPR) for nicarbazin residues in poultry liver and eggs. The sample preparation included acetonitrile extraction and liver samples were further purified with hexane wash. The SPR assay cycle took only 7 min to complete, providing results in nearly real-time. The same SPR assay was later modified for poultry samples and used in a large-scale survey to investigate the incidence of DNC residues in Ireland (Danaher *et al.*, 2008).

In summary, the current role of antibody-based methods in official residue control is in the screening of samples and potentially positive samples have to be re-analyzed with a confirmatory method. At best, antibody-based assays can offer good sensitivity, robustness, inexpensive analysis, high throughput, and speed of analysis with generally less complicated sample preparation compared to confirmatory methods. To be applicable to routine testing, these methods, regardless of the chosen assay format, have to be validated with appropriate sample matrices and have adequate detection

limits for screening purposes. The remaining issues to be considered are the complexity of sample preparation and preferred assay time. The level of sample preparation depends on the analyte, sample matrix, and overall performance of the assay, which is partly affected by the choice of antibody, other assay parameters, and detection chemistry. Unfortunately, the development and application of either in-house or commercial antibody-based methods to the field of coccidiostat analysis is still limited. For instance, there are several commercial assays available for other residues such as antimicrobials and growth promoters, but the screening tests for coccidiostats are only now emerging on the market.

2.1.3.3 Confirmatory methods

The combination of liquid chromatography (LC) with mass spectrometry (MS) represents the method of choice for residue control for most classes of veterinary drugs (for reviews, see Balizs and Hewitt, 2003 and Stolker and Brinkman, 2005). Although LC-MS^n methods provide good sensitivity and specificity and enable quantification and confirmation, they often require more extensive sample preparation than antibody-based assays. Thus, the overall analysis time and assay throughput, which is also limited by the sequential nature of the analysis as opposed to the batch-mode commonly used in immunoassays, have been considered to be limiting factors in LC-MS^n. LC-MS^n equipment is also expensive to acquire and operate, and therefore, the cost of analysis is higher than with screening methods.

Previously, LC was used in combination with ultraviolet absorbance detection systems (Anderson *et al.*, 1981; Schenck *et al.*, 1992; Draisci *et al.*, 1995; Matabudul *et al.*, 1999), but currently most methods rely on the use of (tandem) MS with electrospray ionization interface. Recently published methods based on LC-MS-MS for coccidiostats are presented in Table 3. In general, these methods fulfil the sensitivity requirements, i.e. have a proper decision limit (CCα) and CCβ, and most have been validated according to Decision 2002/657/EC. Many methods employ sample preparation procedures, which are capable of recovering residues of multiple coccidiostats. The level of sample preparation varies and is partly dependent on the selected analytes and matrices. The use of organic solvents in sample preparation is still common practice, although the volumes have been reduced as the sample preparation methods have improved. An example of these improvements is the introduction of SPE, which has become a routine tool in sample preparation, although the selection of an optimal SPE column and extraction conditions can require several attempts (Rosén, 2001). The separation of coccidiostats in LC is mostly performed with non-polar C18 columns and the complexity of the mobile phase is dependent on the application. For example, the mobile phase can vary from a simple combination of acetonitrile and ammonium acetate solution (Rosén, 2001) to a complex mixture of acetonitrile, water, methanol, tetrahydrofuran, and trifluoroacetic acid (Matabudul *et al.*, 2002). Many of the LC-MS^n methods are used for both screening and confirmation purposes and the screening aspect can be strengthened, e.g. by pooling of the samples (Rosén, 2001).

Table 3. Recent LC-MS-MS methods and related sample preparation procedures for anticoccidials in chicken tissues and eggs.

Analyte	Sample matrix	Main steps in sample preparation	Reference
LAS, MON, NAR, and SAL	Muscle and egg	Weighing of the homogenized sample, mixing with anhydrous sodium sulfate, acetonitrile extraction with shaking, centrifugation, SPE, evaporation to dryness, reconstitution, and filtration	Rokka and Peltonen (2006)
LAS, MON, NAR, and SAL	Egg	Weighing of the homogenized sample, acetonitrile extraction with vortexing and sonication, centrifugation, evaporation to dryness, reconstitution, sonication, and filtration	Mortier *et al.* (2005a)
DIC, HAL, LAS, MAD, MON, NAR, NIC, ROB, and SAL	Muscle and egg	Weighing of the minced/mixed sample, mixing with anhydrous sodium sulfate, acetonitrile extraction with shaking, centrifugation, SPE, evaporation to dryness, and reconstitution	Dubois *et al.* (2004)
DIC, DIM, HAL, NIC, and ROB	Egg	Weighing of the homogenized sample, acetonitrile extraction with vortexing and sonication, centrifugation, evaporation, and filtration	Mortier *et al.* (2003)
HAL	Liver and egg	Weighing of the minced/homogenized sample, trypsin digestion o/n with shaking, 2x ethyl acetate extraction with shaking followed by centrifugation, 2x ammonium acetate extraction with shaking, hexane wash, SPE, evaporation to dryness, and reconstitution	Yakkundi *et al.* (2003)
LAS, MON, NAR, NIC, and SAL	Liver and egg	Weighing of the sample, mixing with anhydrous sodium sulfate, acetonitrile extraction with shaking, centrifugation, SPE, evaporation, and filtration	Matabudul *et al.* (2002)
MON, NAR, and SAL	Liver and egg	Weighing of the homogenized/mixed sample, homogenization + extraction with methanol, centrifugation, and SPE	Rosén (2001)
NIC	Liver and egg	Weighing of the minced/homogenized sample, homogenization + extraction with acetonitrile, centrifugation, evaporation to dryness, hexane wash, and centrifugation	Yakkundi *et al.* (2001)

Abbreviations: DIC, diclazuril; DIM, dimetridazole; HAL, halofuginone; LAS, lasalocid; MAD, maduramicin; MON, monensin; NAR, narasin; NIC, nicarbazin; o/n, overnight; ROB, robenidine; SAL, salinomycin; SPE, solid phase extraction.

In summary, the continuous development of MS techniques together with improved sample preparation procedures is expected to enhance the performance of these systems even more and enable truly multi-residue analysis (Stolker and Brinkman,

2005; Stolker *et al.*, 2007). Thus, LC-MS^n methods will continue to have a major role in residue control as confirmatory methods and they may also retain a permanent role in screening, if antibody-based methods do not respond to that challenge.

2.1.4 Future trends in residue control

Future developments in the methods used in residue control are likely to be comparable with those occurring in clinical diagnostics. The key requirements for testing methods include speed, simplicity, and robustness of analysis with adequate detection limits and minimal sample preparation. Currently, the complex sample preparation represents a bottleneck in residue analysis. Solutions for easier sample preparation could include the use of automated sample pre-treatment systems allowing multi-residue extractions, higher throughput, and reduced consumption of organic solvents. Sample preparation could also be simplified by using other sample matrices than foodstuffs. For example, liquid samples such as plasma and urine are homogeneous by nature and do not require extensive clean-up prior to analysis. However, correlation between residue levels in these samples and the corresponding tissue, as well as depletion kinetics, have to be established before this kind of approach (i.e. predictive indicators) would be feasible. As an example, the analysis of serum or plasma for monensin residues has been suggested to be an indicator of the residue levels in liver (Atef *et al*., 1993; Crooks *et al.*, 1998). Moreover, testing could be more directed to the beginning of the production chain, namely to feedstuffs, as feed contamination, due to, e.g. cross-contamination in feed mills or farms, is a common reason for anticoccidial residues in foods (Kennedy *et al.*, 1998; Cannavan *et al.*, 2000; Cannavan and Kennedy, 2000; Kennedy *et al.*, 2000; McEvoy, 2002; Yakkundi *et al.*, 2002). For example, Campbell *et al.* (2007) developed a portable and rapid lateral flow device for on-site testing of nicarbazin in feedstuffs where the sample was simply ground in methanol and diluted in assay buffer prior to analysis. The assay was sensitive enough for screening at concentrations at or above 2 mg/kg, which was adequate based on previous data (Cannavan and Kennedy, 2000). With regard to methods of analysis, multi-residue confirmatory methods based on LC-MS^n will remain at the core of residue control, but hopefully also rapid and user-friendly antibody-based screening methods in new assay formats will find their way into routine residue testing. In addition, techniques such as transcriptomics and proteomics based on the measurement of effect rather than of target compound concentrations may create entirely novel concepts for detecting residues in food. These techniques are currently under investigation in the EU-funded project "BioCop" (www.biocop.org).

In conclusion, because veterinary medicinal products are commonly used in animal husbandry, residue control plays a significant role in ensuring food safety. Therefore, advances in sample preparation and analytical methods used in residue control will in their part strengthen the overall level of food safety.

2.2 FOODBORNE PATHOGENS AND FOOD SAFETY

The biological safety of food can be compromised for several reasons such as bacteria, viruses, prions, toxins, and parasites. These agents can cause foodborne illnesses, typically either infectious or toxic in nature, through the ingestion of contaminated food or water. Foodborne illnesses caused by micro-organisms such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 are a large and growing public health problem, representing a significant financial burden to countries in terms of medical care and lost productivity. For example, the annual cost of all cases of salmonellosis in the USA has been rated to be over $2 500 million (in 2007 dollars) (Economic Research Service, 2008).

The following sections concentrate on one of the most commonly occurring foodborne pathogens, *Salmonella*, and the measures and methods used in *Salmonella* control.

2.2.1 *Salmonella* spp. as a foodborne pathogen

The genus *Salmonella*, one of the leading causes of foodborne illness (Tirado and Schmidt, 2001; EFSA, 2007), belongs to the *Enterobacteriaceae* family and consists of gram-negative, facultatively anaerobic, motile (except for rare non-motile serovars), rod-shaped bacteria. *Salmonella* taxonomy and nomenclature is complicated and various approaches, including biochemical and serological characteristics and deoxyribonucleic acid (DNA) homology, have been suggested as the basis for classification. The WHO Collaborating Centre for Reference and Research on *Salmonella* recommends a classification system according to the Kauffmann-White scheme where the genus is divided into two type species, *S. bongori* and *S. enterica*, and the latter is further divided into six subspecies designated by names (and Roman numerals) as follows, *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Grimont and Weill, 2007). Serovars belonging to subspecies I are named and others are only designated by formula. After preliminary identification of *Salmonella*, further confirmation is done with biochemical and serological tests where the latter comprises the agglutination of bacterial antigens such as somatic (O) lipopolysaccharides on the external surface of the bacterial outer membrane, flagellar (H) antigens associated with the peritrichous flagella, and capsular (Vi) antigen present in only a few serovars with *Salmonella*-specific antibodies. In addition, phage typing can be used for further subtyping of some serovars mainly for epidemiological purposes. Currently, the Kauffmann-White scheme contains over 2 500 serovars of *Salmonella* (Grimont and Weill, 2007). (D'Aoust *et al.*, 2001.)

The widespread occurrence of *Salmonella* spp. in the environment together with intensive animal husbandry creates a serious threat for food safety throughout the food chain. Moreover, the pathogen can adapt to extreme conditions, and thus, is very resilient to various stresses utilized in food processing. (D'Aoust *et al.*, 2001.) Mead *et al.* (1999) estimated that of all *Salmonella* infections the rate of foodborne transmission is 95%, although the organism may also be transmitted through direct contact with

infected animals or humans. *Salmonella* infection is most frequently acquired through foodstuffs contaminated with animal faeces, and poultry, pig meat, and eggs have been reported to be the major sources of human infections (EFSA, 2007). At later stages of food processing, contamination can occur through infected humans, unclean food preparation areas, or cross-contamination with other raw foodstuffs, if appropriate hygiene measures are not followed. Consequently, the risk of contracting *Salmonella* from foods can be reduced by proper storage of foods, thorough cooking and good hygiene throughout the food production chain.

The disease caused by *Salmonella* is called salmonellosis. The reported infectious dose has varied from a small number of cells (D'Aoust, 1985; Kapperud *et al.*, 1990) to over 10^5 cells (Kothary and Babu, 2001) depending on the particular *Salmonella* serovar, type of food, and physical condition of the infected individual (D'Aoust, 1985; Waterman and Small, 1998; Kothary and Babu, 2001). In general, the outcome from the exposure to foodborne pathogens depends on a number of host factors such as pre-existing immunity, state of nutrition, age, and ability to elicit immune response. Thus, the incidence, severity, and lethality of foodborne diseases are higher in certain vulnerable segments of the population. The incubation period for the most common form of salmonellosis, gastroenteritis, can vary from a few hours up to three days, and the acute symptoms, nausea, vomiting, diarrhoea, abdominal pain, and fever, usually last for four to ten days. The infection can also result in more serious conditions including enteric fever (caused by serovars Typhi and Paratyphi), bacteremia or reactive arthritis. After the onset of clinical symptoms, the diagnosis is based on the isolation of the bacteria from stool. The treatment of gastroenteritis caused by *Salmonella* is based on resting and prevention of dehydration, but in severe cases antibiotics may be required. However, the prophylactic use of antibiotics in animal farming has contributed to the emergence of multidrug-resistant *Salmonella* strains, which can complicate the treatment of salmonellosis (Rabsch *et al.*, 2001).

The Centers for Disease Control and Prevention has estimated that the annual incidence of foodborne illness caused by pathogens in the USA is approximately 76 million cases in total, resulting in 325 000 hospitalizations and 5 000 deaths (Mead *et al.*, 1999), and approximately 1.4 million cases with 15 000 hospitalizations and 400 deaths are caused by non-typhoidal salmonellosis (Voetsch *et al.*, 2004). In the EU, zoonoses (diseases that are naturally transmissible directly or indirectly between animals and humans) affected more than 350 000 citizens in 2006 (EFSA, 2007). *Salmonella* was the second most commonly reported zoonotic agent and the leading cause of foodborne outbreaks; in 2006 approximately 160 000 confirmed human cases were reported and over 3 100 outbreaks with serovar Enteritidis being the predominant cause were registered that affected almost 23 000 people and resulted in 23 deaths (EFSA, 2007). In Finland during the past ten years the number of reported cases of *Salmonella* infections has approximately been between 2 000 and 3 000 annually (National Public Health Institute, 2008), and the majority of the cases are acquired from abroad (EVIRA, 2006b; EFSA, 2007). Nevertheless, because many mild cases

are not reported, the actual number of *Salmonella* infections is likely to be much higher.

2.2.2 *Salmonella* control in the EU

Food industry and authorities share the responsibility for controlling the microbiological safety of food. While the industry follows legislation and self-control plans according to HACCP, the authorities ensure industry compliance with the legislation and monitor food safety through national control programmes. As discussed earlier, traditional food safety control has relied on general sanitation inspections and end product testing, thus being a reactive rather than a preventive approach. Together with the EU's integrated "from farm to fork" policy and good manufacturing and hygiene practices, HACCP aims to create a prevention-based food safety system reaching throughout the food chain. HACCP relies on the identification of risks, integration of appropriate control measures into the design of the process, and implementation and monitoring of these controls. Therefore, the testing focus can, in principle, be gradually shifted from the end products towards the raw materials combined with adequate in-process monitoring.

In the EU, the microbiological safety of food has been thoroughly addressed at legislative level. The measures include, for instance, general microbiological criteria, which function as tools for assessing the safety and quality of foods and that of the processes for manufacturing, handling, and distribution (Anonymous, 2005a), the obligation of the Member States to annually monitor and collect data on zoonoses, zoonotic agents, antimicrobial resistance, and foodborne outbreaks (Anonymous, 2003a), and the specific control of *Salmonella* (Anonymous, 2003c). In Finland, the National *Salmonella* Control Programme has been applied since 1995 to protect consumers against *Salmonella* infections spread through foods of animal origin (Anonymous, 1994). The implementation of the programme has generally kept the annual occurrence of *Salmonella* in production animals and foodstuffs originating from them at levels of less than 1% (EVIRA, 2006b). However, the recent *Salmonella* crisis in Finland, which was due to contaminated feed, further highlighted the importance of monitoring of the entire food chain starting from primary production and feedstuffs.

Moreover, collaboration projects at international level have been established to advance microbiological food safety worldwide. For example, the Global Salm-Surv programme coordinated by the WHO promotes the capacity and quality of *Salmonella* surveillance, serotyping, and antimicrobial resistance testing, and the FAO together with the WHO have performed microbiological risk assessments for *Salmonella* in eggs and broilers (FAO/WHO, 2002).

2.2.3 Methods for the detection of *Salmonella* in food

The detection of foodborne pathogens is challenging for several reasons: target bacteria are often present in low numbers in foods, bacteria may be sub-lethally injured due to

food processing, and the high amount of other bacteria and food matrix can further complicate the analysis. Thus, most methods use sample enrichment to increase the number of live target bacteria in the matrix to detectable levels prior to analysis. Moreover, the legislative demand, the absence of *Salmonella* cells in 25 g (or 10 g) of food, sets strict performance requirements for methods of analysis (Anonymous, 2005a).

In general, the methods used in foodborne pathogen detection can be divided into traditional (also referred to as conventional methods) and alternative methods (also referred to as rapid methods). The traditional methods include culture methods, which still represent the golden standard in the testing of microbiological quality of food. Some culture methods have gained an internationally accepted reference method status in foodborne pathogen detection such as the methods published by the International Organization for Standardization (ISO). Although culture-based methods are selective, sensitive, and detect only viable cells, they are also labour-intensive and time-consuming requiring several days or even weeks to obtain a confirmed result. The terms rapid methods or alternative methods are synonyms referring to a vast array of novel testing methods, which can significantly reduce the analysis time compared to traditional methods. However, because of lengthy sample preparation, most alternative methods cannot be considered to be truly rapid in the original sense of the word. Alternative methods should have characteristics such as speed of analysis, ease of use and/or automation, improved analytical performance (e.g. sensitivity, specificity), possibility for miniaturization, and reduction of total cost, in order to be noteworthy rivals to traditional methods (ISO, 2003). In addition, the success of an alternative method depends on other factors such as robustness, reliability, throughput, overall convenience, and the level of validation and standardization, which all determine the true applicability of the method to routine use. Alternative methods for *Salmonella* provide qualitative information on whether the pathogen is present or not in the sample. If the alternative method gives a presumptive positive test result, confirmation, i.e. further identification and typing of the particular strain using the reference method, is required for epidemiological surveillance and national control programmes.

In 2005, the food industry was estimated to perform nearly 110 million tests for foodborne pathogens and Figure 1 presents the categories of tested pathogens and the methods used (Strategic Consulting, 2005). Traditional culture methods still dominate the field of pathogen testing, although alternative methods are steadily increasing their market share as novel and better methods appear.

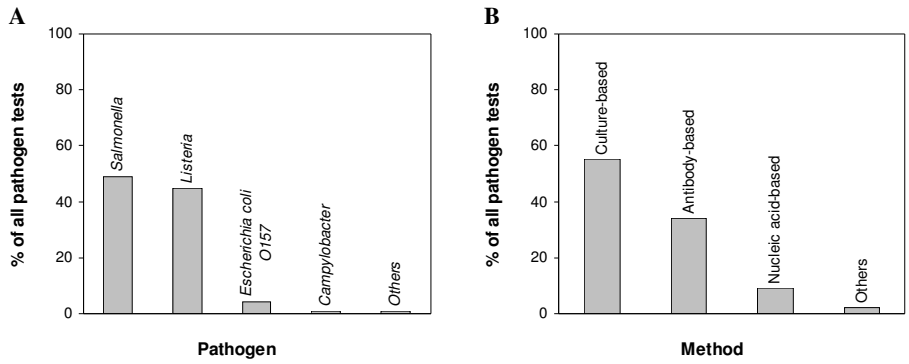

Figure 1. Trends in foodborne pathogen testing worldwide. (**A**) Most commonly tested pathogens. (**B**) Categories of methods used in pathogen testing. Based on Strategic Consulting (2005).

2.2.3.1 Reference method

Culture methods are generally comprised of four steps: pre-enrichment, selective enrichment, isolation (detection), and confirmation. The purpose of non-selective pre-enrichment is to allow the resuscitation and growth of sub-lethally damaged organisms and the selective enrichment step permits the growth of target cells while suppressing other bacteria present in the sample. (Fricker, 1987.) Various media for the detection of *Salmonella* have been introduced over the years (Fagerberg and Avens, 1976; Vassiliadis, 1983; Fricker, 1987; Busse, 1995) and even a universal pre-enrichment broth allowing simultaneous sample processing of *Salmonella* and *Listeria* has been reported (Bailey and Cox, 1992). However, the performance of a particular method can be influenced by many factors, including the food matrix, background flora, original contamination levels, and serovars (Fagerberg and Avens, 1976).

In Europe, the commonly used reference method for the detection of *Salmonella* spp. in food and animal feeding stuffs is based on the standard ISO 6579:2002 (ISO, 2002). The method consists of a two-step enrichment using a non-selective pre-enrichment in buffered peptone water (BPW) followed by a selective enrichment in two broths: Rappaport-Vassiliadis medium with soya and Muller-Kauffmann tetrathionate novobiocin broth. The selective enrichment is carried out in parallel with two media because the performance of the media may be affected by the food matrix and the particular *Salmonella* serovar. Subsequent to plating on two selective media (xylose lysine deoxycholate agar and, e.g. brilliant green agar), suspect colonies are confirmed with appropriate biochemical and serological tests. Negative results are obtained in less than 72 h, but the confirmation of potentially positive samples takes additional days.

2.2.3.2 Alternative methods

The following sections present the main techniques and most prominent advances in the field of alternative methods for the detection of *Salmonella* in food matrices with the emphasis on nucleic acid techniques. All the methods described are used to analyze samples taken directly from the enrichment broth (or sometimes even directly from the food matrix) without the need of isolating and obtaining a pure culture of the pathogen.

Antibody-based methods

The use of antibodies in the detection of *Salmonella* started with agglutination tests forming the basis for serotyping and fluorescent antibody techniques (Thomason *et al.*, 1957; Haglund *et al.*, 1964; Sperber and Deibel, 1969). Krysinski and Heimsch (1977) reported one of the first enzyme immunoassays for *Salmonella*. Since then various types of immunoassays have been routinely applied to the detection of this foodborne pathogen. In addition, anti-*Salmonella* antibodies have been employed in immunomagnetic separation (IMS) where antibody-coated magnetic beads are used to capture target cells from the sample (Skjerve and Olsvik, 1991).

In general, immunoassays for *Salmonella* are based on the heterogeneous non-competitive ELISA format (Mattingly, 1984; Cudjoe *et al.*, 1995; Holt *et al.*, 1995; Valdivieso-Garcia *et al.*, 2003; Fukuda *et al.*, 2005; Magliulo *et al.*, 2007) where the target cells are "sandwiched" between antibodies typically directed against somatic and flagellar antigens. Although the use of enzyme labels is common practice, other types of labels have also been reported for *Salmonella* immunoassays (Tu *et al.*, 2002; Gehring *et al.*, 2008). The detection limits of *Salmonella* immunoassays with food matrix are usually in the range of 10^4-10^6 cells/mL, but with careful selection of detection method and antibody the sensitivity can be further improved. Although the samples have to be enriched prior to immunoassay to allow the target organism to reach a detectable level, the following sample preparation steps are minimal because of the inherent robustness of antibody-based methods. Generally, the enriched samples are used as such or boiled (to inactivate pathogens and release antigens) before addition to the assay. The cross-reactivity of antibodies with antigens in closely related bacteria is considered to be the main disadvantage of antibody-based methods.

There is a vast array of commercial antibody-based assay kits available for the detection of *Salmonella*. Several extensive evaluations have been conducted to show that the performance of the kits is comparable to the reference method to promote the certification of the kits (Curiale *et al.*, 1997; Bird *et al.*, 1999; Hughes *et al.*, 2001; Feldsine *et al.*, 2008). However, as the sample preparation always includes pre-enrichment, which is usually followed by selective enrichment and even post-enrichment depending on the sample matrix and kit, these methods still take at least 24 h to perform. The level of sophistication of these assays ranges from a simple, manual lateral flow device (dipstick) with visual detection to automated systems. The main benefits of the dipstick are the ease of use and speed of analysis because after enrichment the user only adds a drop of broth to the test and the result is ready in a few

minutes without washing or further manipulations. Lateral flow tests for *Salmonella* are available, e.g. from BioControl Systems (Bellevue, WA, USA), DuPont Qualicon (Wilmington, DE, USA), Strategic Diagnostics (Newark, DE, USA), and Neogen (Lansing, MI, USA). Examples of immunoassays amenable to automation include, e.g. microtiter plate based Assurance EIA Salmonella and TRANSIA PLATE Salmonella Gold from BioControl Systems, and bioMérieux (Marcy l'Etoile, France) offers a fully automated immunoanalyzer, the VIDAS system, for the detection of *Salmonella*.

Immunosensors are a commonly used biosensor type in the detection of foodborne pathogens (Patel, 2002; Ricci *et al.*, 2007; Palchetti and Mascini, 2008). In particular, optical immunosensors such as the label-free SPR-based systems have raised interest in this field (Koubová *et al.*, 2001; Bokken *et al.*, 2003; Bergwerff and van Knapen, 2006; Homola, 2008), partly because of the commercially available systems, e.g. Biacore (GE Healthcare, Uppsala, Sweden). In the scientific literature, immunosensors for the detection of *Salmonella* in food matrix have utilized various transducer elements with detection limits typically in the range of 10^3-10^5 cells/mL and assay times of up to 90 min (Seo *et al.*, 1999; Varshney *et al*., 2003; Taitt *et al.*, 2004; Ko and Grant, 2006; Mazumdar *et al.*, 2007). In general, sample matrix effects tend to impair sensor performance in terms of sensitivity, and therefore, the use of proper sample matrices is essential during method development to be able to evaluate the applicability of the method for routine use. Unfortunately, many biosensor methods described for the detection of *Salmonella* in food have not been validated properly, which makes it difficult to estimate the true potential of these methods. The principles and future challenges of biosensors, which were briefly discussed in 2.1.3.2, are also valid for applications for foodborne pathogens. Consequently, although the potential of biosensors in food safety applications has long been recognised (Oh, 1993), the biosensors are only now beginning to fulfil expectations and require further improvements in performance to be truly applicable to food safety testing at the levels required by the authorities.

In conclusion, there are validated and robust antibody-based methods with good performance characteristics available for the detection of *Salmonella* in food. However, in order to achieve the detection limit of 1 CFU/25 g of food, sample enrichment has to be employed prior to assay to increase the number of target cells. Thus, even in the best-case scenario, the time to result approaches 24 h. Although this cannot be considered to be particularly rapid, the assay time is still considerably improved compared to the time required by the reference method.

Nucleic acid-based methods

Following the early hybridization assays for *Salmonella* (Fitts *et al*., 1983; Olsen *et al.*, 1995), in vitro amplification techniques emerged enabling more sensitive detection. Currently, PCR represents the most well-known and established technique (Saiki *et al.*, 1985), although some novel approaches such as the isothermal nucleic acid sequence-based amplification (NASBA) using ribonucleic acid (RNA) targets have

also been applied to foodborne pathogen detection (Cook, 2003). NASBA has the advantage over PCR of having the potential to detect only viable cells. Nevertheless, this novel technique requires further development to be able to follow in the footsteps of PCR.

PCR: advantages and disadvantages

PCR-based methods rely on genes, and thus, are not influenced by the growth state or the environment of the bacteria. Therefore, PCR is considered to be more reliable than the traditional culture-based methods, which use physiological and morphological criteria for identification. Furthermore, with nucleic acid amplification non-cultivable and slow-growing organisms can be detected, which can give a negative result with a culture method. In addition to improved sensitivity, specificity, and rapidity, state-of-the-art PCR techniques also enable automation and high throughput. Despite these advantages, nucleic acid-based methods have not yet been able to shake the reference status of culture methods.

The implementation of PCR into routine use has not been easy because of several disadvantages associated with PCR. These include the high expertise in molecular biology required for performing PCR, the laborious procedures for both sample preparation and PCR, the false positive results due to lack of discrimination power between viable and non-viable cells if DNA is used as a target, the lack of robustness, proper validation and standardization of the methods, and contamination problems. However, there are solutions to all of the above mentioned issues. For example, commercial ready-to-use PCR kits and sample preparation systems for DNA purification easing up the manual work in PCR have been introduced. The problem of false positive results can be circumvented by sample enrichment, by using, e.g. ethidium monoazide, which has been suggested to irreversibly bind to the DNA of damaged cells and prevent amplification (Nogva *et al.*, 2003; Guy *et al.*, 2006), or by reverse transcription (RT)-PCR. In RT-PCR RNA, either messenger RNA (mRNA) or ribosomal RNA (rRNA), is used as a template for amplification because it is a better measure of cell viability than DNA (McKillip *et al.*, 1998; Sheridan *et al.*, 1998; Rijpens *et al.*, 2002). Nevertheless, because RNA, and especially mRNA, is labile compared to DNA, the handling and processing of samples for RT-PCR is challenging and may lead to less sensitive detection. In addition, the physiological state of bacteria can affect the level of transcription, and thereby, the sensitivity of RT-PCR (Szabo and Mackey, 1999). To enhance the status of PCR in foodborne pathogen testing, several PCR methods have recently undergone a thorough validation process (Lübeck *et al.*, 2003; Malorny *et al.*, 2003a; Malorny *et al.*, 2003b; Abdulmawjood *et al.*, 2004; D'Agostino *et al.*, 2004; Malorny *et al.*, 2007) with the ultimate aim of having standardized PCR methods available as is the case with traditional culture methods (Malorny *et al.*, 2003c). The contamination problem was especially highlighted when the detection of amplification products was based on post-PCR processing of samples, using techniques such as gel electrophoresis or heterogeneous hybridization. However, the emergence of homogeneous assay concepts where the detection of the amplicon is

performed with intercalating dyes (Higuchi *et al.*, 1992) or sequence-specific labelled probes or primers (Marras *et al.*, 2006), has provided safer and simpler means for detection. In addition, the use of labelled probes has further increased the specificity of PCR.

General principles of food sample preparation for PCR

Laborious sample preparation is considered to be one of the main reasons why nucleic acid-based methods have not yet gained wider acceptance. Given the variety and complex nature of foods and the range of micro-organisms to be detected, a universal sample preparation procedure would be difficult to design. Thus, various methods for the separation and concentration of foodborne pathogens from complex sample matrices have been developed (for reviews, see Lantz *et al.*, 1994, Benoit and Donahue, 2003, and Stevens and Jaykus, 2004a). Moreover, the inherent sensitivity of PCR to various inhibitors (from sample matrix and/or sample preparation), which interfere with polymerase activity or cell lysis or cause degradation or capture of nucleic acids, makes sample preparation for PCR even more challenging (Rossen *et al.*, 1992; Wilson, 1997).

The use of enrichment can be considered as standard procedure in food sample preparation prior to PCR. The purpose of enrichment is to increase the number of target pathogens, which are often present in low numbers in the sample, and therefore, easily lost among the indigenous microflora. The increase in cell number is particularly important because of the large initial sample volume (typically 250 mL) compared to the small volume (< 50 µL) used in PCR. Consequently, the required cell density for reliable and repeatable amplification in PCR is typically in the range of 10^3 cells/mL. In addition, during enrichment the number of dead cells, which otherwise can generate false positive results in PCR, is diluted. To further improve detection limits, the sample can be subjected to various types of separation and concentration procedures after enrichment with the goal of providing a homogeneous sample with a small volume and high recovery of viable target cells for PCR. If required, the effect of PCR inhibitors can be reduced by dilution or by DNA extraction, using, e.g. commercially available kits producing highly purified DNA. Nevertheless, the performance of the kits can vary in terms of recovery and DNA purity depending on the sample matrix and the manufacturer (Amagliani *et al.*, 2007). PCR has the potential, at least in principle, to replace cultural enrichment through specific amplification. Few approaches have been reported where the detection of foodborne pathogens is performed directly (without enrichment) from a representative food sample using various concentration techniques (Stevens and Jaykus, 2004b; Wolffs *et al.*, 2006). However, the selection of a suitable technique is dependent on the matrix (and organism) in question (Stevens and Jaykus, 2004b). Despite the significant time-savings, the sensitivity of these direct methods requires improvement to be comparable with PCR methods using enrichment.

To sum up, sample preparation continues to be the most labour-intensive step in the detection of foodborne pathogens with PCR. Therefore, efficient sample preparation

methods are required to fully exploit the potential of PCR. It remains to be seen, whether a method will be developed that would either eliminate the need for food samples to be enriched, or at least decrease the enrichment time considerably prior to PCR detection without loss of assay sensitivity. The shortening of the total assay time and simplified sample preparation procedures would certainly enhance the acceptance of PCR in food diagnostics.

PCR assays for Salmonella

Target genes for PCR amplification in the detection of foodborne pathogens include, e.g. rRNA genes, protein genes coding for toxins and enzymes, and repetitive elements. The rRNA genes are prominent targets because the widely available information on rRNA sequences facilitates the design of probes and primers. Furthermore, rRNA sequences contain a mix of conserved and variable segments allowing differentiation at various levels. However, PCR performance in terms of selectivity and sensitivity can vary considerably based on the selected target and primers, even within the same gene (He *et al.*, 1994). For example, some years ago Malorny *et al.* (2003a) evaluated the performance of four previously published primer pairs for *Salmonella*, targeting genes *oriC* (Widjojoatmodjo *et al.*, 1991), *ompC* (Kwang *et al.*, 1996), *invA* (Rahn *et al.*, 1992), and random fragment (Aabo *et al.*, 1993). The primer pair for the *invA* gene located on the pathogenicity island 1 of *Salmonella* encoding proteins of the type III secretion system (Collazo and Galán, 1997) was found to have the best selectivity. This primer set was extensively tested with various *Salmonella* and non-*Salmonella* strains in a collaborative study (Malorny *et al.*, 2003a), followed by a thorough validation with food matrices (Malorny *et al.*, 2003b).

In addition to selecting proper targets, as well as primers (and probes), and optimizing the PCR assay, other challenges include the choice and optimization of sample preparation to obtain the required performance level. Shortened pre-enrichment times for *Salmonella* have been used with varying degrees of success depending on the sample matrix, type of contamination, following sample preparation, and detection method (Soumet *et al.*, 1994; Gouws *et al.*, 1998; Ferretti *et al.*, 2001; Ellingson *et al.*, 2004; Myint *et al.*, 2006; Notzon *et al.*, 2006; Josefsen *et al.*, 2007; Warren *et al.*, 2007). For example, Ellingson *et al.* (2004) described a 12-h method for the detection of *Salmonella* in raw and ready-to-eat beef products using real-time PCR. The sample preparation consisted of a 6-h pre-enrichment step followed by centrifugation of a 15 mL sub-sample and subsequent DNA extraction. Although the results were in 100% agreement with the reference method, the spiking levels were considerably higher than the legislative demand. Warren *et al.* (2007) on the other hand used appropriate spiking levels and obtained a detection limit of approximately 10 CFU/25 g with only a 5-h pre-enrichment. This exceptionally short pre-enrichment time was possible because of a flow-through immunocapture system (Pathatrix, Matrix MicroScience, Newmarket, UK) that allowed the analysis of the entire sample volume of 250 mL. However, during food processing bacteria are exposed to various stresses, which may slow down the

recovery of the cells during pre-enrichment. Accordingly, naturally contaminated samples may require a longer incubation time than their artificially contaminated counterparts. Therefore, naturally contaminated samples or, in the case of artificial inoculation, sub-lethally injured cells should be used in assay validation in order not to overestimate the sensitivity of the assay (Rijpens *et al.*, 1999). Unfortunately, this type of approach is rarely reported.

In scientific literature, numerous PCR methods based on either homogeneous or heterogeneous assay concepts have been described for the detection of *Salmonella* in foods. Nevertheless, in many cases the methods have not been validated properly against the reference method. Moreover, the assays can have significant variations in performance, in terms of specificity and detection limit depending on the overall PCR design, reagent selection, sample matrix, and sample preparation. However, there have also been many excellent reports published over the years and a brief overview of recently published methods with particular points of interest is presented in Table 4. The trend in PCR is clearly towards real-time methods, which are expected to gradually replace traditional PCR methods. Recently, real-time PCR methods enabling multiplexing have emerged in the field of foodborne pathogen detection (Jothikumar *et al.*, 2003; Wang *et al.*, 2004; Wolffs *et al.*, 2007; Elizaquível and Aznar, 2008). Unfortunately, the advantages of multiplexing do not always extend to the sample preparation phase because different pathogens usually require separate enrichment procedures.

Examples of commercial PCR kits for *Salmonella* include the BAX System (DuPont Qualicon), iQ-Check (Bio-Rad, Marnes-la-Coquette, France), Foodproof (Biotecon Diagnostics, Potsdam, Germany), R.A.P.I.D. LT Food Security System (Idaho Technology, Salt Lake City, UT, USA), TaqMan (Applied Biosystems, Foster City, CA, USA), and Assurance GDS (Biocontrol Systems). These state-of-the-art PCR kits provide results in approximately 24 h, which includes the time required for sample preparation consisting of standard pre-enrichment followed by at least a cell lysis step. The kits are generally based on homogeneous PCR with real-time detection and the sensitivities are in the range of 10^3-10^4 cells/mL enabling detection down to 1-10 CFU/25 g of food. The commercial kits have been thoroughly validated against the reference method by a third party to provide objective evidence on the performance characteristics of the kits in various food matrices.

In summary, properly validated PCR assays are fit for purpose and enable specific and sensitive detection of *Salmonella* in food at levels required by the legislation. In general, the homogeneous assay concept (with labelled probes) improves the performance of the assays, and therefore, the application of real-time PCR methods with the capability for quantification is expected to increase in the field of foodborne pathogen detection. Although food samples still have to be enriched, future advances in sample preparation combined with the amplification power of PCR will probably shorten the total assay time considerably, which further strengthens the competitive advantage of PCR compared to antibody-based methods.

Table 4. Recently published PCR methods for the detection of *Salmonella* in food.

Sample matrix	Sample preparation	Assay description	Detection limit	Merits	Reference
Pork and poultry neck skin	Pre-enrichment (8 h, 37 °C in BPW without shaking) and DNA extraction	Real-time PCR with TaqMan probes	1-10 CFU/25 g	Results in 12 h and homogeneous assay concept	Josefsen *et al.* (2007)
Tomatoes, potato salad, and beef	Pre-enrichment (5 h, 37 °C in TSYE with shaking), IMS, and DNA extraction	Real-time PCR with TaqMan probes	Approaching level of < 10 CFU/25 g	Pathatrix IMS system, results in 8 h, and homogeneous assay concept	Warren *et al.* (2007)
Milk, beef, and fish	Pre-enrichment (18 h, 37 °C in BPW without shaking) and DNA extraction	Real-time PCR with hybridization probes	< 5 CFU/25 g	Large-scale validation and homogeneous assay concept	Perelle *et al.* (2004)
Yogurt and cheese	Centrifugation and DNA extraction	End-point PCR with electrophoresis	< 25 CFU/25 g	No enrichment	Stevens and Jaykus (2004b)
Beef and chicken carcass rinse	Pre-enrichment (18 h, 37 °C in BPW without shaking) and DNA extraction	End-point PCR with electrophoresis	< 5 CFU/25 g (or 100 mL)	Large-scale collaborative study using naturally contaminated samples	Malorny *et al.* (2003b)

Abbreviations: BPW, buffered peptone water; CFU, colony forming unit; DNA, deoxyribonucleic acid; IMS, immunomagnetic separation; PCR, polymerase chain reaction; TSYE, tryptic soy broth supplemented with 0.5% yeast extract.

Other methods

Some technologies, being either at the early stages of development or less employed than the previously mentioned mainstream methods because of cost, complexity, or shortcomings in performance, are briefly introduced here.

Impedance microbiology relies on the direct or indirect measurement of electrical changes in culture media or reaction solution, respectively, caused by bacterial growth. The technique has the advantage of detecting only viable cells and it has been applied to the detection of microbial contamination and foodborne pathogens. (Silley and Forsythe, 1996; Wawerla *et al.*, 1999.) However, the classical technique is neither particularly rapid nor sensitive because the changes in impedance can be registered only when the number of bacteria is in the range of 10^6-10^7 cells/mL (Yang and Bashir,

2008). Further, the enrichment medium has to be designed carefully to provide optimal signals for the direct measurement and to support the selective growth of the target bacteria, unless other means of selectivity such as IMS are employed (Yang and Li, 2006). Recently, impedance methods using improved technical solutions and novel assay formats have appeared for the detection of *Salmonella* (Yang *et al.*, 2004; Mantzila *et al.*, 2008; Yang and Bashir, 2008). Nevertheless, more work is required to enhance the performance of impedance-based techniques and to provide assays suitable for routine testing.

Although the number of reported applications is limited, bacteriophages have been used as tools in the detection of pathogens in food. For instance, Lakshmanan *et al.* (2007) utilized phages instead of antibodies as the recognition element in a biosensor construct to detect *Salmonella* directly in spiked milk samples. Favrin *et al.* (2003) described an assay where *Salmonella* cells were captured from enrichment broth using IMS and infected with bacteriophages. The resulting progeny phages were detected indirectly, after they had infected another population of *Salmonella* cells. In general, the sensitivities of phage-based methods are catching up with PCR and the methods based on, e.g. reporter bacteriophages have the additional benefit of enabling the differentiation between live and dead cells (Goodridge and Griffiths, 2002; Hagens and Loessner, 2007).

Flow cytometry, a technique for sorting cells based on their optical properties, has been applied to some extent to the detection of foodborne bacteria. However, the cost and complexity of the instrumentation have limited its use in routine applications in the past (Veal *et al.*, 2000). Because most micro-organisms are too similar to be distinguished optically, the discrimination of specific targets can be done, e.g. by using fluorescently labelled antibodies. The technique has been applied to the detection of *Salmonella* in liquid food samples such as eggs, milk, and chicken carcass rinse (McClelland and Pinder, 1994; Wang and Slavik, 1999).

In the future, DNA microarray technology, which can provide detailed genetic information on pathogens and, e.g. their virulence factors and antibiotic resistance profiles, may extend outside research applications and find use in food safety testing. Nevertheless, there are several problems relating to sample matrix interference, target cell concentration, assay functionality, and cost of data analysis that need to be addressed before this technique can be of value in food diagnostics. (Olsen, 2000; Kostrzynska and Bachand, 2006.) Altogether, DNA microarrays may ultimately be more suited for identification and subtyping purposes than for routine foodborne pathogen detection.

2.2.4 Future trends in foodborne pathogen testing

A common theme for all assay development done in the field of foodborne pathogens is the pursuit for robust, simple, and rapid methods with good detection limits. The methods should be properly validated and provide possibilities for high throughput,

multiplexing, and on-site testing. The next-generation methods in the field of alternative methods include biosensors and nucleic acid assays. Biosensor technology has raised a lot of interest and when the problems associated with the technology and cost issues are solved biosensor applications may overtake traditional immunoassays (Lazcka *et al.*, 2007). The significant growth already seen in the use of nucleic acid-based assays is expected to continue in the future (Strategic Consulting, 2005). Although culture methods have retained the status of reference methods so far, the situation may change especially as the standardization of PCR methods progresses.

In general, alternative methods are more expensive than traditional culture methods and their performance should therefore be significantly better to be a favourable cost/performance trade-off. The reduction in time-to-results is one of the most valued properties for the food industry. However, because of the lengthy sample preparation involved, alternative methods rarely provide results within the same day. Thus, future efforts should also concentrate on solving sample preparation issues. Although alternative methods still need to prove their value to the market, the future for these methods looks bright, especially when the time issues relating to sample preparation are solved.

3 AIMS OF THE STUDY

The general aim of the present study was to provide rapid and user-friendly molecular methods for food safety testing with regard to chemical and biological hazards in foods. The study relied on two separate techniques: immunoassays and PCR, which were used for the detection of selected veterinary residues (**I-III**) and a commonly occurring foodborne pathogen (**IV, V**), respectively.

More specifically, the aims were:

1. To develop and validate rapid, competitive time-resolved fluoroimmunoassays for selected anticoccidial residues in food.

2. To demonstrate the applicability of the dry chemistry assay concept for competitive immunoassays and PCR.

3. To explore the potential of a novel, automated PCR instrument platform with a built-in fluorometer for rapid nucleic acid testing, and to evaluate the performance characteristics of the platform.

4. To develop and validate a homogeneous time-resolved fluorescence PCR assay for the detection of *Salmonella* spp. in food using the automated PCR platform.

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods are described in the original publications **I-V.** Only a brief summary and some additional information, in particular regarding publications **IV** and **V**, are presented here.

4.1 IMMUNOASSAYS

4.1.1 Antibodies (I-III)

The polyclonal rabbit antibodies against nicarbazin (**I**) (Connolly *et al.*, 2002) and halofuginone (**II**) were produced and supplied by the partners of the "Poultry-check" project (QLK1-CT-1999-00313) funded by the European Commission. The polyclonal rabbit antibody against monensin (**III**) was kindly provided by Prof. Elliott (Queen's University Belfast, Belfast, UK).

4.1.2 Preparation of labels (I-III)

The labels consisted of protein carriers coupled with europium chelates and analytes (or their derivatives). The intrinsically fluorescent 7-dentate europium chelate (Takalo *et al.*, 1994), kindly provided by Jaana Rosenberg (Department of Biotechnology, University of Turku, Turku, Finland) and Innotrac Diagnostics (Turku, Finland), was used for the labelling of the carrier proteins, ovalbumin (**I, II**) and apo-transferrin (**III**) (both from Sigma, St. Louis, MO, USA). The structure of the chelate is presented in Figure 2.

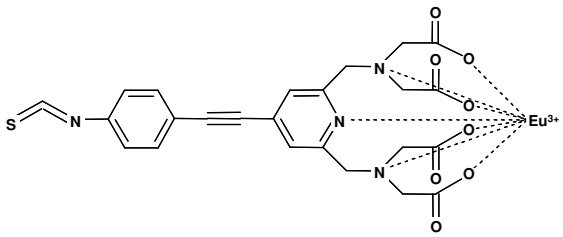

Figure 2. Structure of the 7-dentate europium chelate, {2,2',2",2'"-{[4-[(4-isothiocyanatophenyl)ethynyl]-pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetato)}europium(III). The broken lines indicate the non-covalent bonds between the ligand and the lanthanide ion.

The selected analytes were DNC (**I**), the marker residue for nicarbazin, halofuginone (**II**), and monensin (**III**) (Figure 3). For the first two analytes, derivatives, a DNC mimic (4'-nitrosuccinanilic acid, Sigma) and a succinyl derivative of halofuginone (a gift from Prof. Elliott, Queen's University Belfast) were used in the labelling instead of the less reactive parent compounds. Monensin (Sigma) could be used as such because it has an intrinsic carboxyl group.

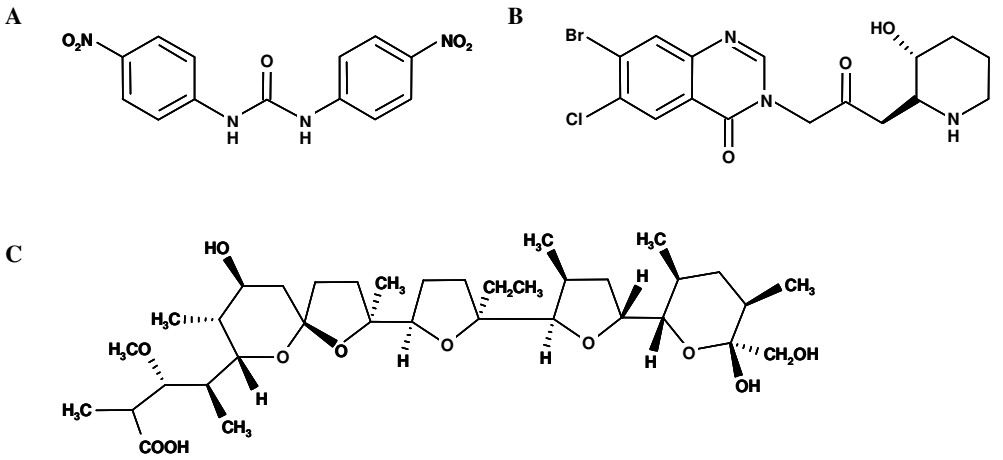

Figure 3. Chemical structures of the anticoccidials (**A**) DNC, (**B**) halofuginone, and (**C**) monensin.

Two strategies were employed in the preparation of the labels: either proteins were first labelled with lanthanide chelates, after which activated analytes were coupled to the labelled proteins (**I, II**) or the analyte coupling was performed prior to labelling (**III**). The carboxyl group present in the analytes (or their derivatives) was activated in organic solution using a 1.1-fold molar excess of N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) (both from Acros Organics, Geel, Belgium) in order to facilitate the formation of an amide bond between the analyte and the protein (Langone and Van Vunakis, 1982). The coupling reaction was performed in 50 mM phosphate buffer, pH 7.0-7.2, with optimized amounts of activated analyte (a 200-fold molar excess (**I**) or a 100-fold molar excess (**II, III**)). The protein carrier was labelled with a 60-fold molar excess of europium chelate in 50 mM carbonate buffer, pH 9.2-9.8. All labels were purified by gel filtration and stored at 4 °C.

4.1.3 Dry chemistry wells (I, II)

The dry chemistry assay concept was used as the basis in the preparation of ready-to-use assay wells for nicarbazin (**I**) and halofuginone (**II**) (Lövgren *et al.*, 1996). First, analyte-specific antibodies (50 µL/well) were attached to single microtiter wells, which were pre-coated with goat anti-rabbit immunoglobulin G (IgG) directed against heavy chains of rabbit IgG (BiosPacific, Emeryville, CA, USA). After a washing step, a 50 µL aliquot of optimized buffer solution containing, e.g. sugar, detergent, and protein was dispensed and dried in the wells to form an insulation layer. The label was carefully added on top of the layer in a volume of 1 µL and dried. The dry wells were packed into barcode-labelled pens designed to be used in the Aio! immunoanalyzer (Innotrac Diagnostics) and stored at 4 °C in sealed packages with desiccant for routine use.

4.1.4 Sample preparation (I-III)

The sample matrices, eggs and chicken liver, were obtained from local supermarkets and Broilertalo (Eura, Finland), respectively. In brief, homogenized and weighed samples were spiked when required with appropriate concentrations of standard solutions, DNC (**I**) (Sigma), halofuginone hydrobromide (**II**) (a gift from Prof. Elliott, Queen's University Belfast) or monensin (**III**). The extraction was performed with acetonitrile combined with sonication (**I, II**) or shaking (**III**). After centrifugation, the supernatants were collected and evaporated to dryness in a heating block under a stream of nitrogen. Liver samples were further purified using a hexane and methanol-water mixture (**I, II**), and subsequent to incubation, the liver samples were centrifuged and the aqueous layer was collected for evaporation. All dried samples were reconstituted in water (**II, III**) or methanol-water mixture (**I**).

4.1.5 Assay procedures (I-III)

The immunoassays were performed as heterogeneous competitive assays in a one-step format, i.e. all immunoreagents were incubated simultaneously. The details of assay procedures are summarized in Table 5. The assays for nicarbazin (**I**) and halofuginone (**II**) were carried out in the fully automated Aio! immunoanalyzer with a custom-made assay protocol employing the ready-to-use dry chemistry wells and surface measurement. The monensin assay (**III**) was performed manually in a microtiter plate format starting with the attachment of the analyte-specific antibody to the anti-rabbit IgG-coated wells and ending with signal measurement using dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) technology and a Victor 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Turku, Finland).

Table 5. Assay procedures for the developed immunoassays.

Original publication	**I**	**II**	**III**
Analyte	Nicarbazin	Halofuginone	Monensin
Antibody coating	Ready-to-use wells	Ready-to-use wells	50 µL of antibody, 1 h, RT, SS, wash 4x
Assay volume	50 µL of sample	50 µL of sample	25 µL of sample + 25 µL of label
Incubation	15 min, 36 °C, SS	15 min, 36 °C, SS	30 min, RT, SS
Wash	6x	6x	6x
Procedure prior to detection	Drying: 40 s, 95 °C	Drying: 40 s, 95 °C	Signal generation: 200 µL of enhancement solution, 15 min, RT, SS
Detection	TRF, surface readout	TRF, surface readout	TRF, DELFIA technology
Assay time [a]	< 20 min	< 20 min	< 60 min
Sample matrix	Egg and liver	Egg and liver	Egg
Sample dilution	100x egg, 200x liver	10x egg and liver	No dilution

[a] Starting from the addition of the sample and ending with obtaining of the result. *Abbreviations:* DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; RT, room temperature; SS, slow shaking; TRF, time-resolved fluorometry.

4.2 PCR ASSAY

4.2.1 Oligonucleotides (IV, V)

The multiplex assay targeted the *invA* gene of *Salmonella* spp. (Galán *et al.*, 1992): the forward primer was previously published by Rahn *et al.* (1992) and the reverse primer, probe, and quencher were designed based on an article by Perelle *et al.* (2004). The primers for internal amplification control (IAC) and IAC target plasmid (pGEM3-mmPSA) were as described by Nurmi *et al.* (2000a), but the original sequences of the IAC probe (Nurmi *et al.*, 2000a) and the quencher probe (Nurmi *et al.*, 2002) were slightly modified for this work. The sequences and modifications of primers, probes, and quencher probes (all from Thermo Fisher Scientific, Ulm, Germany) are presented in Table 6. (**Unpublished.**)

Table 6. Oligonucleotide primers, probes, and quencher probes used in the PCR assay.

Oligonucleotide	Sequence from 5' to 3' end	Modifications	Nucleotide position
Salmonella forward	GTG AAA TTA TCG CCA CGT TCG GGC AA	--	371-396
Salmonella reverse	CVG CRA CRC GYT CTG AAC CTT T	--	475-496
Salmonella probe	ACY GTS GTY CAG TTT ATC GTT ATT AC	5' Aminolink C6 3' Phosphate	448-473
Salmonella quencher	ACG ATA AAC TGR ACS ACR GT	3' Dabcyl	448-467
IAC forward	TGA ACC AGA GGA GTT CTT GCA	--	523-543
IAC reverse	CCC AGA ATC ACC CGA GCG A	--	667-685
IAC probe	CCT TCT GAG GGT GAT TGC G	5' Aminolink C6 3' Phosphate	597-601, 604-617
IAC quencher	AAT CAC CCT CAG AAG G	3' Dabcyl	600-601, 604-617

Nucleotide positions refer to GenBank accession numbers M90846 (for *Salmonella*) and X05332 (for IAC). Codes for wobble bases are as follows: R = A and G; S = G and C; V = A and G and C; Y = C and T. Underlined letters denote bases that are inverted in the IAC sequence compared to X05332. Missing nucleotide positions in the IAC probe and quencher denote a deletion of two base pairs in the original sequence (Ylikoski *et al.*, 1999). Dabcyl, 4-((4-(dimethylamino)phenyl)azo)benzoic acid.

4.2.2 Labelling of probes (IV, V)

The amino groups of the probes were labelled with intrinsically fluorescent 9-dentate GenomEra chelates (Abacus Diagnostica, Turku, Finland): terbium chelate (Takalo *et al.*, 2008) was employed in the detection of the *Salmonella* target sequence and europium chelate (von Lode *et al.*, 2003) was used for the IAC. The structures of the chelates are shown in Figure 4.

Probes were labelled with an excess of chelate at a DNA concentration of 2 µg/µL in 50 mM carbonate buffer, pH 9.8. After an overnight incubation at 37 °C probes were purified with reversed-phase high performance liquid chromatography (HPLC) using a µRPC C2/C18 ST 4.6/100 column (GE Healthcare) and a 16-minute linear gradient of 14-30% acetonitrile in 50 mM triethylammonium acetate buffer (Fluka, Buchs, Switzerland) at a flow rate of 1 mL/min. The probes were dried, dissolved in 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, quantified spectrophotometrically, and stored in small aliquots at −20 °C. (**Unpublished.**)

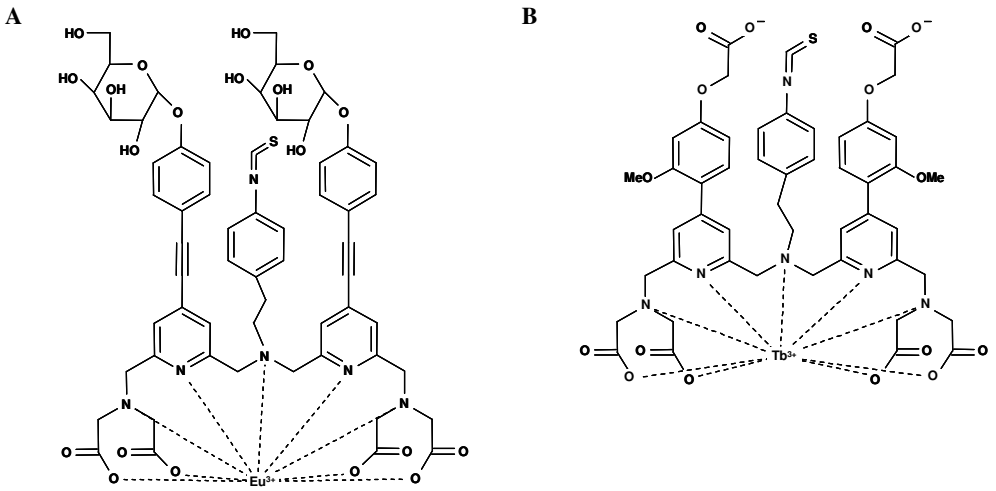

Figure 4. Structures of the GenomEra chelates. The broken lines indicate the non-covalent bonds between the ligand and the lanthanide ion.

(**A**) {2,2',2",2"'-{[2-(4-isothiocyanatophenyl)ethylimino]bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]-ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III)

(**B**) {2,2',2",2"'-{[2-(4-isothiocyanatophenyl)ethylimino]bis(methylene)bis{4-[4-(carboxymethoxy)-2-methoxy-phenyl]-pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}terbium(III)

4.2.3 Dry chemistry vessels (IV, V)

The ready-to-use PCR kits (Abacus Diagnostica) for the detection of *Salmonella* were a part of the Magda™ CA Salmonella system (Raisio Diagnostics, Turku, Finland). The PCR kits consisted of GenomEra reaction vessels containing all the reagents for the *Salmonella* assay in dry form (Korpimäki *et al.*, 2005; Nurmi *et al.*, 2005). The preparation of the dry chemistry vessels started with the application of the master mix and polymerase as separate drops in small volumes to the reaction chamber of the vessel. After an overnight drying in a vacuum desiccator at room temperature (RT), the lid and the back of the vessel were heat-sealed with foil and the vessel was barcoded. The vessels were packed in aluminium foil packages with desiccant and stored at 4 °C for routine use. (**Unpublished.**)

4.2.4 Sample preparation (V)

The selected sample matrices were raw meat (sliced or minced beef, pork, and poultry) and ready-to-eat meals (sandwiches, meatballs, and chicken pasta). The sample preparation was performed with the Magda CA Salmonella system, which contained reagents and consumables for sample pre-enrichment and IMS. In brief, food samples were mixed, weighed (25 g) and when required, artificially contaminated with various strains of *Salmonella* spp. (obtained from Raisio Diagnostics) at a level of approximately 1-10 CFU/25 g. The samples were diluted 1:10 in BPW containing the

Magda CA enrichment supplement and homogenized. After a 6-h incubation at 37 °C with shaking (300 rpm), a 10 mL sub-sample of the broth was collected for IMS. During the IMS step (1 h, 37 °C, 300 rpm), the target cells were captured using superparamagnetic particles coated with *Salmonella*-specific antibodies followed by the collection of the particles with a PickPen magnetic tool (Bio-Nobile, Turku, Finland). Subsequently, the particles were washed twice with Magda CA wash buffer and once with Magda CA elution buffer prior to suspension in a 60 µL volume of the same elution buffer for PCR analysis.

4.2.5 Assay protocol (IV, V)

The homogeneous Magda CA Salmonella PCR assay was performed with a novel PCR instrument platform, GenomEra (Abacus Diagnostica). The assay was started by manually adding a 30 µL aliquot of the sample to the ready-to-use dry chemistry vessel. Thus, the amplification reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5.5 mM $MgCl_2$, 0.32 mM dNTPs, 0.50 µM *Salmonella* primers, 0.015 µM *Salmonella* probe, 0.10 µM *Salmonella* quencher, 0.15 µM IAC primers, 0.030 µM IAC probe, 0.11 µM IAC quencher, 10 000 copies of IAC template, 2 U of polymerase, 2 g/L bovine serum albumin (BSA), 25 g/L trehalose, and 0.01% NaN_3 (**unpublished**). The vessel was immediately inserted into the instrument where the correct assay protocol was identified and initiated based on the barcode on the vessel. The vessel was automatically and irreversibly sealed with heat inside the instrument before thermal cycling. The PCR consisted of 45 cycles, during which the vessel was transferred between blocks maintained at constant temperatures. For the end-point time-resolved fluorescence measurement, all oligonucleotides in the reaction vessel were denaturated and the vessel was then transferred to the measurement block. The fluorescence signal was measured twice at selected time intervals in order to record the signals after denaturation (D) and after hybridization (H) and the results (R) for each label were calculated with the following formula R = H/D*100 (Nurmi *et al.*, 2008). The qualitative results were automatically reported by the instrument software based on the comparison of the result to the pre-determined cut-off value embedded in the lot-specific barcode. The total assay time was 45 min.

4.2.6 Reference method (V)

All food samples analyzed with PCR were also tested with the slightly modified culture-based reference method ISO 6579:2002 (ISO, 2002).

5 SUMMARY OF RESULTS AND DISCUSSION

5.1 IMMUNOASSAY CHARACTERISTICS

5.1.1 Assay format (I-III)

Immunoassay is an analytical technique employed to detect and quantify biomolecules using specific antigen-antibody interactions (for a comprehensive review, see Price and Newman, 1997). The assays can be classified as heterogeneous or homogeneous based on the requirement for separation of bound and unbound reagents prior to detection; the homogeneous assay format relies on a simple "mix and measure" principle without any separation steps, whereas the heterogeneous assay format relies on separation, which is typically achieved by immobilizing one of the reagents (either antibody or antigen) to a solid phase and by removing the unbound reagents with washing. Consequently, the separation and washing steps slow down the heterogeneous assays, but with the benefit of improved detection limits. The homogeneous assays on the other hand are rapid because of fast reaction kinetics and lack of separation. Furthermore, the simplicity of the assay concept makes homogeneous assays particularly amenable to automation. However, the dynamic range and sensitivity of many homogeneous assays is restricted by matrix interferences. (Hemmilä, 1985; Hemmilä and Webb, 1997.)

Immunoassays can be further divided into two major categories: competitive (limited reagent) and non-competitive (excess reagent or sandwich) assays based on the measurement of antibody binding site occupancy by the analyte (Ekins, 1997). In the non-competitive assay format two antibodies are used to bind the analyte and the signal detected is directly proportional to the concentration of the analyte, leading to improved sensitivity, precision, and specificity, faster kinetics, and a wider working range (Ekins and Dakubu, 1985). The competitive assay format is usually applied to the analysis of haptens where the simultaneous binding of two antibodies is not possible because of the small size of the molecule. Although some innovative non-competitive assays for small molecules have been described (Barnard and Kohen, 1990; Self *et al.*, 1994), these approaches have not been widely employed. In the competitive assay system the signal is inversely proportional to the analyte concentration, and consequently, the maximum signal level is obtained in the absence of the analyte. The main factors influencing sensitivity are the affinity and amount of the antibody, and to some extent the specific activity of the label. In general, the performance of competitive assays is limited in many ways compared to their non-competitive counterparts (Ekins and Dakubu, 1985). For example, competitive assays have a narrower working range, are usually less sensitive, and require longer incubation times than non-competitive assays.

All the developed heterogeneous immunoassays were used for the detection of haptens, and therefore, the assays were based on the competitive assay format where a fixed

amount of labelled analyte (or labelled derivative) competed with the unlabelled analyte present in the sample for the limited number of antibody binding sites.

5.1.2 Labels and time-resolved fluorescence detection (I-III)

A wide range of lanthanide chelates has been developed over the years to suit various heterogeneous and homogeneous assay applications and to improve the time-resolved fluorescence detection (for reviews, see Hemmilä and Mukkala, 2001 and Hemmilä and Laitala, 2005). The unique fluorescence properties of lanthanide chelates, e.g. sharp emission lines, well separated excitation and emission wavelengths (i.e. large Stokes' shift), and long and distinct fluorescence lifetimes enable spectral and temporal resolution in the measurement. As a result, a significant reduction in the background fluorescence mainly caused by autofluorescence from biological sample material and light scattering is achieved, leading to improved signal-to-noise ratios (S/N), and subsequently, increased assay sensitivity. (Siitari *et al.*, 1983; Soini and Lövgren, 1987.) Consequently, TRF in combination with lanthanide chelate labels has been regarded as one of the most sensitive non-isotopic label technologies (Madersbacher *et al.*, 1993).

The success of TRF began with DELFIA technology, which was introduced in the 1980s (Hemmilä *et al.*, 1984; Soini and Lövgren, 1987). The defining characteristic of DELFIA is the use of practically non-fluorescent chelates for labelling and completing the bioaffinity reaction, after which lanthanide ions are dissociated into an acidic aqueous solution (enhancement solution) to form a strongly fluorescent secondary complex prior to signal measurement. DELFIA is a strictly heterogeneous assay technology enabling highly sensitive assays with detection limits almost down to the femtomolar range (Hemmilä *et al.*, 1984). Nevertheless, it has some disadvantages such as the final liquid handling step, which slows down the assay and makes the assay susceptible to contamination from external lanthanide ions, and the loss of spatial localization of the signal. Assays based on DELFIA principle have been utilized in various fields, including veterinary residue analysis (Elliott *et al.*, 1994; Crooks *et al.*, 1998; Tuomola *et al.*, 2002; Korpimäki *et al.*, 2004) and clinical diagnostics (Lövgren *et al.*, 1984; Qin *et al.*, 1997; Eriksson *et al.*, 2000; Nurmikko *et al.*, 2001).

The emergence of intrinsically fluorescent chelates such as the 7- and 9-dentate europium chelates by Takalo *et al.* (1994) and von Lode *et al.* (2003), respectively, provided the possibility to direct surface measurement (Lövgren *et al.*, 1996; Meriö *et al.*, 1996; Pettersson *et al.*, 2000). However, the 7-dentate lanthanide chelate was sensitive to aqueous quenching, i.e. water molecules deteriorated the signal intensity, and therefore, a drying step was required prior to surface readout (von Lode *et al.*, 2003). Nevertheless, the simplified surface readout requiring no separate signal enhancement facilitated the automation of heterogeneous assays and enabled the development of more rapid assay protocols (Pettersson *et al.*, 2000). The combination of surface measurement and intrinsically fluorescent chelates has been utilized

particularly in research and clinical diagnostics (Pettersson *et al*., 2000; Eriksson *et al*., 2003; von Lode *et al*., 2004; Ylikotila *et al*., 2005; Wittfooth *et al.*, 2006).

In all the immunoassays developed in the current work, one of the underlying themes was the use of TRF and intrinsically fluorescent 7-dentate europium chelates. All the selected analytes had only one reactive group available and the direct labelling of this group did not provide adequate signal levels for the surface measurement (**unpublished**). However, the labelling degree could be increased by using carrier molecules with several reactive groups. This type of multiple labelling has been shown to enhance the sensitivity of the assays despite some unwanted side effects such as increased non-specific binding (Diamandis, 1991; Qin *et al.*, 2001). Therefore, to obtain a better labelling degree, protein carriers were used in the preparation of the labels. The analytes (or their derivatives) and intrinsically fluorescent lanthanide chelates were sequentially conjugated to these carriers. Because both the isothiocyanate group of the chelate and the DCC/NHS activated carboxyl group of the analyte reacted with free amino groups on the protein carrier, the amounts of the analyte and the chelate were roughly optimized for the reaction. In particular, the amount of lanthanide chelates had to be high enough to obtain a sufficient signal level for the surface measurement. Differences in assay performance characteristics, i.e. in signal level and inhibition profile, were observed between label batches due to inherent variation in the sequential coupling reaction. Thus, the particular label concentration used in the assay was re-optimized every time a new label batch was taken into use.

If surface measurement is used together with conventional antibody coating procedures, only a fraction of the bound label is actually situated within the area of the excitation beam as opposed to dissociation-enhanced measurement where the signal is measured from solution covering the entire area of the bound label. Thus, surface readout can be more susceptible to local variations in antibody coating. (Soukka *et al.*, 2001.) The shortcomings of surface measurement can in part be compensated by optimizing washing and shaking procedures, by producing a dense and correctly orientated binding surface preferably with (site-specifically oriented) antibody fragments instead of monoclonal or polyclonal antibodies, and/or by confining the capture antibody to the area of the excitation beam, i.e. spot coated wells, to further concentrate the signal and improve assay sensitivity (Soukka *et al.*, 2001; Peluso *et al.*, 2003; Ylikotila *et al.*, 2006; Välimaa *et al.*, 2008). All the assays developed relied on the use of polyclonal antibodies mainly because of the simplified, economical, and rapid production scheme. The assays were performed in microtiter wells pre-coated with anti-rabbit IgG. This secondary antibody was treated with acidic solution to increase the hydrophobicity of the Fc portion of the molecule, and thus, to direct its attachment to the solid surface (Conradie *et al.*, 1983; Kakabakos *et al.*, 1990; van Erp *et al.*, 1992). Accordingly, the analyte-specific rabbit antibody was correctly orientated with reduced loss of antibody conformation or activity. The intra-assay variation of the assays was typically below 10%, whether the surface readout (**I, II**) or dissociation-enhanced measurement (**III**) was used, which indicated that the applied

coating procedure with polyclonal antibodies was feasible in terms of variation despite the measurement mode.

Although the monensin assay (**III**) was performed manually using DELFIA technology, it could have been performed in the same format as the other assays because of the intrinsically fluorescent label. The effect of the measurement mode, dissociation-enhanced measurement and surface readout on assay performance was tested with various incubation times in a buffer system. The signal level and S/N were approximately two times higher with DELFIA, which resulted in an improved limit of detection (LOD). However, the IC_{50} (concentration that inhibits 50% of the maximum signal) of the assay was not significantly affected by the measurement mode (**unpublished**). Because the matrix effects might further lower the signal levels, the measurement based on DELFIA principle was selected for the monensin assay. Nevertheless, the benefits of surface readout such as shorter and simpler assay protocols would generally favour its use.

5.1.3 Dry chemistry (I, II)

The aim of the all-in-one dry chemistry immunoassays was to facilitate and speed up straightforward one-step assays by drying all assay-specific reagents into microtiter wells in advance (Lövgren *et al.*, 1996). In the dry chemistry assays developed, an insulation layer consisting mainly of buffer components and optimized amounts of sugar and protein was required to prevent the premature binding of the label to the antibody. The assay performance was significantly deteriorated if the labelled analyte leaked through the layer during storage. This effect might partly be derived from the differences in the affinity of the antibody for the analyte and the labelled analyte-protein conjugate and the incubation time used in the assay. Consequently, the insulation layer was designed to be dense: 50 µL of buffer containing trehalose (6-8%) and casein (0.2%) was dried in each well to cover the entire bottom of the well. Furthermore, the label was applied on the edge of the well where the layer was thicker than in the middle. The careful optimization of the components of the insulation layer ensured that on the one hand, the performance characteristics of the assay were not compromised due to the reagent composition, and on the other hand, the wells remained stable even during long-term storage (Lövgren *et al.*, 1996).

The stability of the all-in-one dry chemistry wells at RT was monitored during a period of one year at selected time points by analyzing standard solutions at three different concentration levels. The data from the stability study for halofuginone is presented in Figure 5. During the study no significant changes in the performance of the assays, with regard to signal level and inhibition profile were observed. Thus, the wells required no special storage conditions and were stable for at least 12 months at RT. This compares well with the stability reported for other immunoassays using the same dry chemistry concept (Pettersson *et al.*, 2000; von Lode *et al.*, 2004).

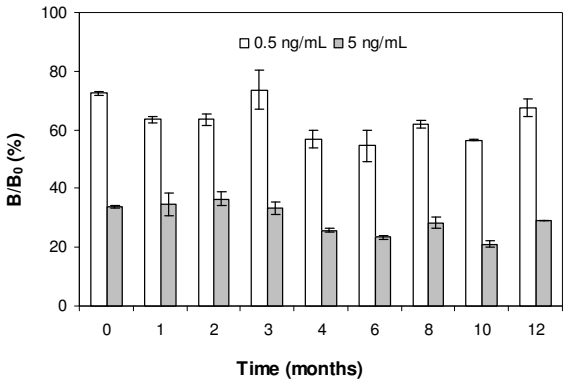

Figure 5. Real-time stability study for the halofuginone assay. The wells containing the dried reagents were stored at RT. The columns represent the B/B_0 values (calculated by dividing the fluorescence signal of the standard by the signal of the zero standard) for each time point analyzed with two or four replicates. (**Unpublished.**)

5.1.4 Sample preparation (I-III)

The purpose of sample preparation was to remove interfering substances, to concentrate the amount of target analyte, and to produce a homogeneous, representative sample for analysis. The selected sample matrices were poultry liver (**I, II**) and eggs (**I-III**). Liver was preferred as a test material over muscle because muscle generally contains lower concentrations of residues than liver. For example, nicarbazin residue content in liver has been found to be approximately 10 to 30 times higher than the concentration in the corresponding muscle samples (Veterinary Residues Committee, 2007).

A relatively straightforward sample preparation procedure was developed for all assays using standard sample preparation techniques for residue analysis (Stolker and Brinkman, 2005). The sample preparation for both matrices started with sample homogenization, after which residues were extracted from the sample matrix with acetonitrile. Shaking (**III**) or sonication (**I, II**) was used to improve the efficiency of the extraction. In the case of liver samples, additional purification, i.e. hexane wash, was required to reduce the matrix effects (**I, II**). The dilution of the samples prior to assay adjusted the assay sensitivity to the appropriate concentration range and further reduced the amount of interfering substances (**I, II**). The sample preparation also included evaporation steps, which considerably slowed down the procedure. Nevertheless, the evaporation step prior to reconstitution could be omitted if the assay sensitivity was adequate to analyze more dilute samples. Accordingly, the sample preparation procedure could be shortened if the extract was simply diluted to eliminate the harmful effects of organic solvents on antibodies and applied directly to the assay. This approach might prove to be useful at least for the nicarbazin assay (**I**) where significant sample dilution is used.

5.1.5 Assay performance (I-III)

At the time of the assay development in the current work, there were no official MRLs in the EU for the residues of monensin, halofuginone, and nicarbazin used as feed additives in poultry production. Consequently, the sensitivity requirements for the assays, that is the concentration levels the assays should be able to detect, were selected based on existing guidelines. In the monensin assay (**III**), the target was based on the 10 µg/kg action level used in the Finnish national residue control programme (National Food Agency *et al.*, 2005). An MRL of 30 µg/kg for halofuginone residues in bovine liver was used as a guideline in the development of the halofuginone assay (**II**) for both sample matrices (European Medicines Agency, 2001). For the nicarbazin assay (**I**), the targets for DNC residues were set to 200 µg/kg (Joint FAO/WHO Expert Committee on Food Additives, 1999) and 100 µg/kg (Veterinary Medicines Directorate, 2000) in liver and eggs, respectively.

In addition to the sample preparation, the assay parameters, including buffer composition, concentration of the antibody and label, and assay incubation time and temperature were optimized in order to achieve the required detection limits. All assays were performed in a simple one-step format where the label and sample were incubated simultaneously in the antibody-coated wells. The dry chemistry assays (**I, II**) were performed with the fully automated immunoanalyzer, which enabled a user-friendly assay with a total turn-around-time of less than 20 min. The incubation time of the monensin assay (**III**) was set to 30 min, although the washing, enhancement, and measurement steps somewhat lengthened the procedure. As discussed earlier, the monensin assay could also have been transferred to the automated assay format. The shortening of the incubation time from 30 min to 15 min as with the other analytes should also be feasible because of the elevated incubation temperature in the immunoanalyzer speeding up the reaction rate. Moreover, additional assay optimization could further compensate the different measurement mode. The monensin assay was carried out in a conventional microtiter plate format, whereas the automated dry chemistry assays relied on the use of single microtiter wells. Therefore, although monensin assay was slower to perform, it actually had a better assay throughput than the automated assays.

Although the assays were designed to function primarily as screening assays providing qualitative results, quantitative data could also be obtained. The standards used in both approaches were prepared in negative sample matrix and treated and diluted similarly to unknowns (Tuomola, 2000). In the qualitative assay approach, a positive control spiked at a selected concentration level provided the threshold signal, which was used to classify the unknown samples as negative or potentially positive (Figure 6). In the quantitative approach, a standard curve was established.

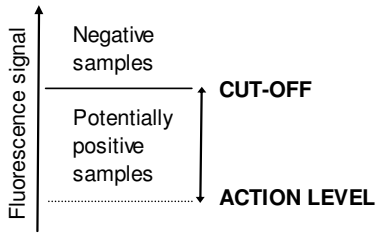

Figure 6. Principle of sample classification in the qualitative assay approach. The positive control provides the signal level (cut-off), to which unknown samples are compared. If the signal is higher, the sample is negative (compliant). If the signal is lower, then the sample is potentially positive (non-compliant) and should be subjected to confirmatory analysis. The safety marginal (arrow) between the cut-off and actual action level (such as MRL) can be adjusted accordingly.

The LOD (**I, II**) and CCα (**III**) of the assays were based on the mean concentration of blank samples + 3 SD. The limit of quantification (LOQ) corresponded to a particular B/B_0 ratio, which was selected to be between 0.80 and 0.85 (**I, II**). The CCβ (**I-III**) was determined according to Commission Decision 2002/657/EC based on the rate of false compliant results (Anonymous, 2002a). A summary of assay performance characteristics is presented in Table 7. In general, the sensitivities obtained compared well to the current requirements. Recently, EFSA was given the responsibility for the MRL evaluation of feed additives (Anonymous, 1990, 2003b). Thus, if the sensitivity requirements were to change in the future, the LOQ/CCβ of the assays could be re-adjusted, e.g. by modifying the dilution factor of the samples. The intra-assay variation of all assays was typically below 10% and the interassay variation was less than 15%. Furthermore, all assays had a good recovery of added standards; the recoveries varied between 87% and 116% depending on the analyte and matrix. To properly assess whether true unknown samples behave similarly to their spiked counterparts, incurred samples should be included in the validation process. During the current study, however, the acquirement of incurred samples was difficult, and therefore, only a small number of samples containing DNC residues could be tested (**I**). The preliminary data indicated that the spiked and incurred samples performed alike in the assay, which was also in line with the recovery results. Moreover, the results from the immunoassay were in agreement with an LC-MS-MS method used to analyze the same samples (Yakkundi *et al.*, 2001). Nevertheless, a larger panel of incurred samples should be analyzed prior to any further conclusions.

Table 7. Summary of selected assay parameters and performance characteristics (**I-III, unpublished**).

Original publication	**I**		**II**		**III**
Analyte	Nicarbazin		Halofuginone		Monensin
Sample matrix	Egg	Liver	Egg	Liver	Egg
Dilution	100x	200x	10x	10x	No dilution
LOD/CCα (µg/kg)	3.2	11.3	1.7	1.0	1.2
LOQ (µg/kg)	10.0	35.0	2.5	2.0	n.d.
CCβ (µg/kg)	< 100	< 200	< 30	< 30	< 2
Upper assay range (µg/kg)	150	400	100	100	100

Abbreviations: CCα, decision limit; CCβ, detection capability; LOD, limit of detection; LOQ, limit of quantification; n.d., not determined.

5.2 PCR PLATFORM AND ASSAY CHARACTERISTICS

5.2.1 Principle of the GenomEra platform (IV)

The general obstacles in PCR, including the lack of assay robustness, the high level of expertise required to perform the assays, labour-intensive procedures, and the risk of contamination, have limited the application of PCR for routine use. The GenomEra platform, including the PCR instrument with integrated fluorometer and ready-to-use assay kits, addressed these issues by providing an automated and simple assay system specifically designed to accommodate qualitative small-scale analysis of nucleic acids.

The GenomEra assay kits such as the one utilized in the Magda CA Salmonella system consisted of individual polypropylene reaction vessels containing all assay reagents in dry form. The assays were easy to perform: only the addition of the sample was required to start the assay. Furthermore, the use of the PCR instrument was simple as the dedicated software guided the end user through assay initiation, selected the correct assay based on the barcodes on the vessels, and finally reported the results automatically in an unambiguous manner. The thermal cycling was performed using five blocks positioned in a linear fashion and set at constant temperatures; two of the blocks were used for the actual cycling, one for the measurement, and the remaining two for speeding up the temperature changes. The homogeneous assay concept relying on a simple "mix and measure" principle enabled detection from the liquid phase without any separation steps. In addition, the homogeneous assay concept significantly reduced the risk of contamination because there was no need for post-PCR processing of amplicons, and in particular, the irreversibly sealed vessels used in the platform enabled the safe disposal of waste. The detection limit (background + 3 SD) of the instrument, determined with europium and terbium chelate solutions, was 19 and

22 pM for europium and terbium, respectively, and the dynamic range of the instrument covered nearly four orders of magnitude (Figure 7).

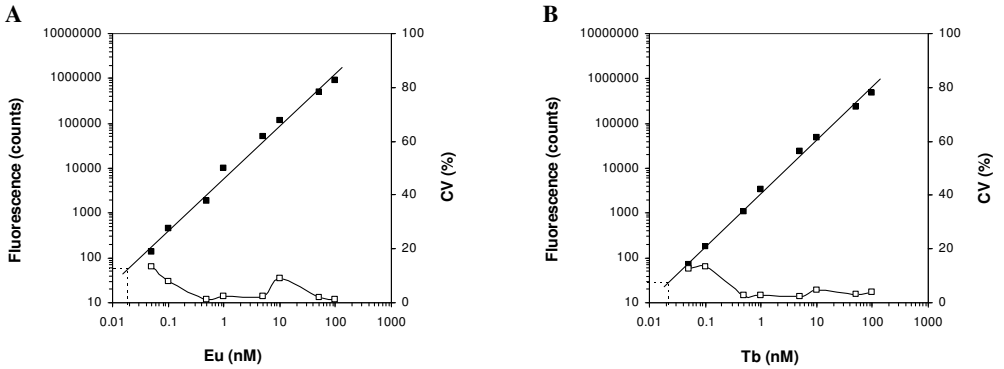

Figure 7. Calibration curves (black symbols) and precision profiles (white symbols) for (**A**) europium and (**B**) terbium chelates using the GenomEra instrument. The dotted lines indicate the detection limit (background + 3 SD). The values shown are the mean of three replicates. (**IV, unpublished.**)

5.2.2 Internal amplification control (IV, V)

An IAC, which is a non-target DNA sequence co-amplified in the same reaction vessel as the target, is recommended to be used in PCR in order to screen for PCR failure, which can be a result of instrument malfunction, reagent failure or PCR inhibition (ISO, 2005). There are two main strategies in designing IACs for PCR: the IAC can be either competitive or non-competitive. The competitive design employs the same primers for the target and IAC with the advantage of prevention of non-specific interactions between multiple primers and is used especially in quantitative assays (Zimmermann and Mannhalter, 1996). Nevertheless, this approach may deteriorate detection sensitivity and the amount of competitive IAC must therefore be carefully controlled in order to avoid false negative results due to weak inhibition or competition (Rosenstraus *et al.*, 1998; Malorny *et al.*, 2003a). The advantage of the non-competitive approach where the target and IAC are amplified using different primers is that the same IAC can be applied to various assays. However, the use of several primers (and probes) in the same reaction complicates the assay design and the PCR conditions may be suboptimal for one or both reactions. As a result, accurate quantitative information cannot usually be obtained with the non-competitive approach. (Hoorfar *et al.*, 2004.)

In the GenomEra platform, the non-competitive IAC was pre-dried into the reaction vessels to control the quality of the testing process. To balance the competition for other assay reagents, the amplification of the target was facilitated by increasing the concentration of target primers (0.50 µM) compared to IAC primers (0.15 µM) (Hoorfar *et al.*, 2004). Moreover, the IAC was designed to be slightly longer than the target in order to favour the amplification of the latter (Ballagi-Pordány and Belák,

1996; Hoorfar *et al.*, 2004). However, in retrospect the reduction of the IAC copy number, which was designed to be high to compensate for the losses during storage, might be an even more efficient way to limit the competition for general reagents with the additional benefits of enhanced assay sensitivity and easier identification of false negative results. This type of approach could be useful particularly for assay kits stored in cold conditions where reagent losses are reduced compared to storage at RT (Trapmann *et al.*, 2004). (**Unpublished.**)

5.2.3 Labels and time-resolved fluorescence detection (IV, V)

The emergence of stable fluorescent lanthanide chelates requiring no ion dissociation provided potential for homogeneous assays based on TRF. Early reports of homogeneous immunoassays using environmentally sensitive fluorescent lanthanide chelates exhibiting different signal intensity depending on the immediate chemical environment were published in the late 1980s (Hemmilä *et al.*, 1988; Barnard *et al.*, 1989) and later these types of chelates were applied to nucleic acid assays (Nurmi *et al.*, 2000b). Subsequently, chelate structures such as 9-dentate chelates forming a nine-coordinate complex with the lanthanide ion to protect the lanthanide ion from aqueous quenching (von Lode *et al.*, 2003; Hakala *et al.*, 2005; Takalo *et al.*, 2008), and even 10-dentate chelates (Takalo and Rosenberg, 2006), have been developed that are particularly suitable for homogeneous assay concept.

In the developed nucleic acid assay, intrinsically fluorescent 9-dentate chelates were used for labelling of the oligonucleotide probes. The designing of the probes was relatively simple because only one label moiety without any special secondary structures such as in Molecular Beacon probes and Scorpion primers was required. After labelling, the probes were purified with HPLC in order to separate the non-reacted label and non-labelled oligonucleotides from the labelled product. Thorough HPLC purification decreased the non-specific background, which is particularly important in homogeneous assays with no separation. The *Salmonella* assay utilized two different sets of lanthanide chelate labelled probes and their complementary quencher probes; the target-specific probe was labelled with terbium, the IAC-specific probe with europium, and both the quenchers with a non-fluorescent quencher, dabcyl. The probes were designed not to hybridize to the template during thermal cycling. The time-resolved fluorescence detection in the homogeneous *Salmonella* assay was performed as end-point measurement based on the competitive hybridization principle (Morrison *et al.*, 1989), which has also been used successfully in combination with TRF previously (Kiviniemi *et al.*, 2003; Ylikoski *et al.*, 2004; Kiviniemi *et al.*, 2005; von Lode *et al.*, 2007). The principle was simple: if no PCR products were present during the measurement, the fluorescent probes were hybridized to their complementary quenchers giving only a low level of signal. However, if a target was present and amplified, the proportion of labelled probes bound to the target were not quenched and caused an increase in the fluorescence signal. Thus, the generated signal was dependent on the amount of the amplified product at the end of the PCR. The results for *Salmonella* and IAC amplification were calculated based on

two sequential end-point time-resolved fluorescence measurements where the signal was recorded first after denaturation and then again after hybridization at a constant temperature (Nurmi *et al.*, 2008). The first measurement provided the maximum obtainable signal with all probes free in solution and during the second measurement the signal level depended on the amount of amplified product present. The results calculated for both *Salmonella* and IAC were compared to lot-specific cut-off values in order to classify the sample as positive, negative or inconclusive. For the sample to be positive for *Salmonella*, only a successful amplification of the *Salmonella* target providing a result above the cut-off was required.

5.2.4 Dry chemistry (IV, V)

The labour-intensive phase in PCR reagent setup can be avoided by using ready-to-use mixtures, which facilitate automation and offer speed and reproducibility to the assay. Furthermore, the risk of pipetting errors and contamination is minimized and the amount of waste due to unused materials is reduced compared to bulk reagent analyses. In particular, the possibility to store reagents at RT, which can be accomplished with pre-dried reagents, can be advantageous for the end user. In general, the commercial PCR kits for *Salmonella*, as discussed in the literature review, still require several pipetting steps before the sample can be added to the assay. Thus, most kits cannot be characterized as truly ready-to-use systems, although exceptions exist, such as the kits by DuPont Qualicon and Idaho Technology. The dry chemistry assay developed in the current work provided a genuinely ready-to-use system because only the addition of the sample was required to start the assay.

Freeze-drying (lyophilization) is a commonly used method for drying PCR components. During freeze-drying, reagents are significantly concentrated and protective reagents are, therefore, recommended to be included in the mixture (Franks, 1990). Trehalose has proven to be especially efficient in protecting and stabilizing proteins (Roser, 1991; Colaço *et al.*, 1992; Sola-Penna and Meyer-Fernandes, 1998; Kaushik and Bhat, 2003). Accordingly, trehalose has been included in freeze-dried PCR mixtures (Klatser *et al.*, 1998; Tomlinson *et al.*, 2005; Aziah *et al.*, 2007), which have been stable even for 12 months at RT (Klatser *et al.*, 1998). However, because freeze-drying is a rather cumbersome and expensive technique, other approaches to produce dried components for PCR have been reported; reagents have been embedded in wax and dried using conventional methods with or without protective reagents (Kaijalainen *et al.*, 1993; Blair *et al.*, 1994; Wolff *et al.*, 1995; Nurmi *et al.*, 2001).

The dry chemistry concept in combination with TRF, first described in the immunoassay field (Lövgren *et al.*, 1996) and later in PCR (Nurmi *et al.*, 2001), has been further modified to suit the GenomEra system (Korpimäki *et al.*, 2005). In the current work the master mixture and enzyme were also dried as separate spots in order to avoid unwanted side effects during drying and immediately after adding the sample. This separation of key PCR components mimicked a hot-start like property even if a standard DNA polymerase was used. In addition to its stabilizing effect, trehalose is

recommended, e.g. by Cepheid (Sunnyvale, CA, USA) to be included in PCR to improve the productivity of amplification. Furthermore, proteins such as BSA have been shown to decrease non-specific binding of reagents and relieve PCR inhibition (Kreader, 1996; Khandurina *et al.*, 2000; Erill *et al.*, 2003; Kricka and Wilding, 2003). Therefore, protein and sugar were added to the reaction mixture with the aim of protecting the reagents, enhance the stability and assay performance, and also aid in the dissolution of the reagents upon addition of the sample (**unpublished**).

Stability testing was performed with dried reagents stored at three temperatures (−20 °C, 4 °C, and RT). The results for the kits stored at 4 °C and RT are shown in Figure 8. In general, the results were similar for assay kits whether they were frozen or cold stored. The performance of the assay kits stored at 4 °C did not significantly change during the one year stability study. However, the performance of the kits stored at RT started to weaken as early as at four months. Thus, the stability of the reagents was highly dependent on the storage conditions.

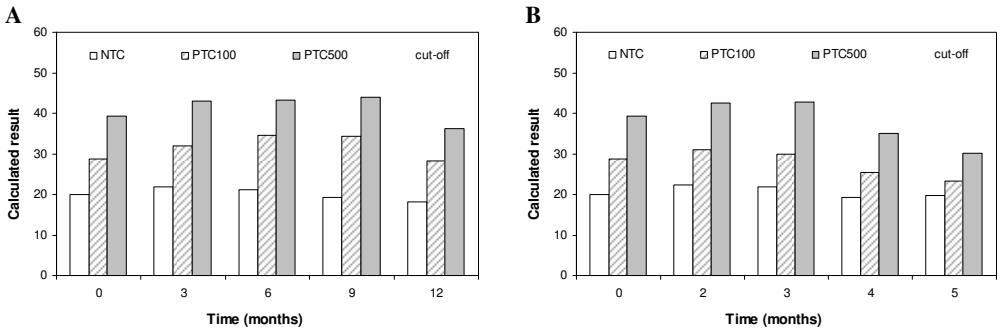

Figure 8. Real-time stability study for the *Salmonella* dry chemistry assay kits stored at (**A**) 4 °C and (**B**) RT. The data shown is based on the measurement of terbium fluorescence from the analyte-specific probe. The performance of the assays was tested at various time points with three types of controls: no template control (NTC) and positive template controls (PTC) containing 100 and 500 copies of *Salmonella* DNA, using at least four replicates. Three different lots with individual lot-specific cut-off values, indicated by lines, were used throughout the study. The cut-offs were used to classify the samples as *Salmonella* positive or negative based on the time-resolved fluorescence measurements. (**IV, unpublished.**)

5.2.5 Sample preparation (V)

As discussed in the literature review, most PCR-based methods used in food diagnostics still rely on traditional sample enrichment prior to detection. PCR-based methods are particularly sensitive to inhibitory effects resulting from substances in the sample material, from sample preparation or both (Rossen *et al.*, 1992; Wilson, 1997). Therefore, proper sample preparation provides the key to the robustness and overall performance of PCR. Consequently, the aim of sample preparation is to produce a homogeneous, representative sample for PCR analysis containing a high amount of target and a minimal amount of interfering substances. In the Magda CA Salmonella

system, sample preparation was based on a short non-selective pre-enrichment in supplemented BPW followed by IMS of target cells.

Because bacterial cells in naturally contaminated food samples are frequently damaged due to adverse conditions during food processing, sub-lethally injured cells were used in the current work for spiking to mimic the real situation. Different types of stress treatments causing sub-lethal injury and their effect on the recovery and analysis of *Salmonella* and other foodborne pathogens have been reported (Mansfield and Forsythe, 1996; Rijpens *et al.*, 1999; Brashears *et al.*, 2001; Wuytack *et al.*, 2003; Jasson *et al.*, 2007). The selected stress treatments in this work were heat (30 min, 55 °C) and freezing (24 h, −20 °C). Despite the use of sub-lethally injured cells, the pre-enrichment step used in the Magda CA system could be considerably shortened compared to the time (18±2 h) utilized in the reference method (ISO, 2002). This was achieved by optimizing the protocol with regard to broth composition and aeration. In contrast to our studies, shaking during pre-enrichment has been reported not to have a beneficial effect on the growth of *Salmonella* during short-term incubation (Josefsen *et al.*, 2007). However, the shaking speed in the Magda CA Salmonella system was faster than reported by Josefsen *et al.* (2007), which might have improved the accessibility of nutrients, and thus, increased the growth rate of *Salmonella* along with other bacteria.

IMS is based on the use of antibody-coated paramagnetic particles or beads, which enable the separation and specific collection of the target bacteria from the sample, the reduction in the amount of sample components, background micro-organisms and PCR inhibitors, and the concentration of the sample by reducing the sample volume (Olsvik *et al.*, 1994; Šafařík and Šafaříková, 1999). Anti-*Salmonella* antibody-coated beads can be tailor-made or obtained in ready-to-use format, e.g. from Invitrogen (Carlsbad, CA, USA). In general, the choice of antibody depends on the application and whether the aim is to capture one or multiple species from a particular genus. In the case of genus *Salmonella*, the situation is particularly challenging because the number of identified serovars is already over 2 500 (Grimont and Weill, 2007). Early publications describing the use of antibody-coated magnetic particles in the detection of *Salmonella* utilized both monoclonal (Mattingly, 1984; Mattingly and Gehle, 1984) and polyclonal antibodies (Skjerve and Olsvik, 1991). The magnetic particles employed in the Magda CA Salmonella system were coated with polyclonal antibodies against common structural antigens of *Salmonella* (**unpublished**). Due to batch-to-batch variation of the antibodies, every new batch was tested before use to confirm the performance of the antibody.

Generally, IMS is carried out in a volume of 1 mL or less in order to allow easy handling of samples in microtiter wells or microtubes. Nevertheless, the use of larger volumes for IMS can be beneficial, i.e. improve the recovery of target cells, and thereby enhance the sensitivity of the assay (Skjerve and Olsvik, 1991; Fluit *et al.*, 1993; Ogden *et al.*, 2000; Rotariu *et al.*, 2005; Notzon *et al.*, 2006), although the amount of background flora and PCR inhibitors also tend to increase with the sample volume. An interesting commercial example of automated IMS is the Pathatrix system

(Matrix MicroScience) where the entire sample volume of 250 mL is re-circulated through a magnetic capture phase using a high flow rate, which in combination with high volume washing is claimed to efficiently reduce non-specific binding (Yuk *et al.*, 2006; Warren *et al.*, 2007). Compared to the normal type of IMS, where only a small fraction of the total sample volume is used for IMS, Pathatrix enables more sensitive assays with shorter enrichment times. Ogden *et al.* (2000) used a traditional IMS system and reported that the use of a 10 mL sample in IMS provided a 9-fold improvement in the recovery of target cells compared to a 1 mL sample. The recovery was not further improved, however, with larger sample volumes (50 mL) due to less effective collection of beads and less probable collision of the beads with the target cells. These drawbacks could partly be compensated for by using a more powerful magnet and by increasing the mixing time or the amount of magnetic particles. Nevertheless, prolonging the time of immunoreaction tends to concurrently increase the amount of non-specific binding due to sample matrix and background flora (Skjerve *et al.*, 1990; Fratamico *et al.*, 1992; Vermunt *et al.*, 1992). This non-specific binding in IMS can be reduced, for example, with detergents and optimized washing procedures (Morgan *et al.*, 1991; Vermunt *et al.*, 1992). Because of the short pre-enrichment step in the Magda CA system, it was particularly important to have good recovery at the IMS phase, which replaced the selective enrichment. Therefore, a sub-sample volume of 10 mL was used for IMS to increase the recovery of target cells without substantial negative effects from the accumulation of inhibitory material compared to a 1 mL sample. The selected incubation time for the beads was based on the optimized combination of assay time, sample volume, amount of beads, recovery, and amount of non-specific binding. Several authors have reported IMS-PCR assays for various matrices where a separate lysis step (Widjojoatmodjo *et al.*, 1991; Widjojoatmodjo *et al.*, 1992; Fluit *et al.*, 1993) or even DNA extraction (Notzon *et al.*, 2006; Yuk *et al.*, 2006) has been performed prior to PCR. However, in the Magda CA system with robust time-resolved fluorescence detection the immunomagnetic beads could be added to PCR as such without any additional steps and the DNA from the cells was released into the solution during the initial denaturation.

As discussed, there are several factors affecting IMS, e.g. duration, composition, and temperature of the enrichment phase, number of background micro-organisms, sample matrix, amount, size, and surface area of the antibody-coated magnetic particles, specificity and affinity of the antibody, and the volume, duration, temperature, and mixing rate of the immunoreaction. IMS has been considered to be laborious, not suitable for high throughput applications, and expensive because of the antibodies and disposable beads. However, the use of the PickPen magnetic tool has been shown to increase the sample throughput and improve the efficiency of the IMS procedure (Nou *et al.*, 2006). In addition, the cost-effectiveness of the assay can in part be targeted by optimizing the amount of beads required per reaction.

5.2.6 Assay performance (V)

According to legislation, there should be no *Salmonella* cells present in 25 g of food (or 10 g depending on the food category) (Anonymous, 2005a). Consequently, this places considerable demands on assay performance. The PCR assay developed was optimized by selecting proper reagents, reaction conditions, and sample preparation system. In addition to the primers targeting the *invA* gene, the specificity of PCR was further increased with probes recognizing correctly amplified target DNA. Based on the results of the inclusivity and exclusivity tests, the selectivity of the Magda CA assay system was 100%. The inherent sensitivity of PCR to inhibition was taken into account in the sample preparation where a final sample volume of 60 µL was obtained. Therefore, in the case of an inconclusive result (neither target nor IAC is amplified), the test could easily be repeated with different dilutions of the sample, although at the cost of assay sensitivity. The validation of the Magda CA Salmonella system was performed with various food samples (n=107), which were artificially contaminated with *Salmonella* cells approximately at a level of 1-10 CFU/25 g of food. Unfortunately, the use of naturally contaminated samples was not possible because *Salmonella* contamination is very rare in Finland (EVIRA, 2006b). The overall relative accuracy, sensitivity, and specificity of the system were 99.1%, 98.4%, and 100%, respectively. Further studies should, however, be conducted to assess the assay performance more thoroughly with a larger sample panel using various sample matrices and naturally contaminated samples before the interlaboratory study, which is required to complete the validation process, can be started (ISO, 2003). Nevertheless, the validation data obtained from the method comparison study holds a promise that performance comparable to commercial assays and other reported assays for foodborne pathogens can be achieved (Malorny *et al.*, 2003b; Abdulmawjood *et al.*, 2004; Malorny *et al.*, 2004; Malorny *et al.*, 2007). The main advantage of the system was the exceptional rapidity enabling the entire process to be completed within one workshift of 8 hours. This compares favourably with the best commercial PCR assays, which still require approximately 24 h for obtaining the result, not to mention the time required by the reference method. Consequently, the system developed could be of particular interest to the food industry where prompt reactions to contamination and decreased storage times are valued.

6 CONCLUSIONS

Food safety policy in the EU relies on the integrated "from farm to fork" approach aiming to provide a high level of food safety throughout the food chain. As part of this effort, foodstuffs are routinely monitored for various chemical and biological hazards, and therefore, reliable and rapid methods of analysis are required.

In this study, rapid and user-friendly molecular methods using TRF as the detection technology were developed for two groups of food-related hazards; immunoassays were employed in the screening of selected veterinary residues and a novel PCR platform suitable for rapid nucleic acid testing was developed for the detection of *Salmonella*. In addition, the applicability of the dry chemistry assay concept to both types of methods was evaluated.

The main conclusions based on the original publications are as follows:

- The combination of lanthanide chelates and time-resolved fluorescence detection provided a robust assay concept applicable to immunoassays and PCR with the possibility for multiplexing by using the europium and terbium chelates. The 7-dentate intrinsically fluorescent europium chelates utilized in the heterogeneous immunoassays allowed signal measurement either using DELFIA technology or simple surface readout after a drying step. The detection could be further simplified with the 9-dentate chelates enabling the homogeneous assay concept with signal measurement directly from the liquid phase.

- The dry chemistry assay concept based on ready-to-use, stable, dried reagents provided a simple and user-friendly assay system for immunoassays and PCR alike. The concept facilitated automation, which further simplified and accelerated the assays.

- Sample preparation was the most laborious step in all assays, although the procedures were designed to be simpler than those traditionally used. Sample preparation for the immunoassays was based on the extraction of coccidiostat residues from eggs and chicken liver with organic solvents. Further time-savings could be achieved by replacing the final evaporation step in the procedure with simple dilution, although at the expense of assay sensitivity. The sample preparation for the *Salmonella* assay was comprised of the non-selective pre-enrichment of food samples followed by the IMS step. Compared to the reference method, significant reductions in the sample preparation time were achieved as the pre-enrichment time was shortened to 6 h and IMS replaced selective enrichment. However, the basic need for sample enrichment still represents a major bottleneck in sample preparation.

- The competitive immunoassays for the selected anticoccidials were based on the heterogeneous assay format. The automated assays for nicarbazin and halofuginone with a turn-around-time of less than 20 min consisted of five steps: sample addition, incubation, washing, drying, and signal measurement from the dried surface. The assays represented the first competitive time-resolved fluoroimmunoassays for residue analysis utilizing the full potential of the dry chemistry concept and the automated immunoassay platform with surface measurement. The monensin assay was performed manually using DELFIA technology. Although the actual assay procedure was slower because of the additional signal generation step, the microtiter plate format provided a higher throughput than the single wells used in the automated assays. All assays could be used either in qualitative or quantitative mode with acceptable performance characteristics.

- The multiplex PCR assay for *Salmonella* was performed with a novel PCR instrument platform using homogeneous end-point detection based on the competitive hybridization principle and the use of TRF. The assay developed provided qualitative assay results for *Salmonella* spp. with a detection limit of 1-10 CFU/25 g of food. The entire process could be performed within 8 h. Thus, the system which included rapid sample preparation together with a sensitive PCR assay offered significant time-savings compared to other methods available for the detection of *Salmonella*.

Altogether, the main issues to be solved in the field of food safety testing relate to sample preparation, assay concepts, and performance requirements, as well as instrumentation and automation where small-scale systems for field testing and high throughput systems with the possibility for multi-analyte analysis are required. In particular, simplicity, sensitivity, and speed of sample preparation and assays are of key importance. In this study, several steps were taken towards these goals. The sample preparation procedures were simplified in order to reduce the total assay times and the combination of dried reagents and TRF provided a sensitive, rapid, and easy-to-use assay concept amenable to automation. The general performance characteristics of the assays were good and the target levels set for detection were achieved. All in all, current and future advances in food diagnostics could be expected to lead to an increased testing rate, which would in turn give us a more comprehensive picture of the state of food safety and the opportunity to improve it further.

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