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INNOVATIVE IMMUNOASSAY FORMATS FOR THE DETECTION OF FOOD CONTAMINANTS AND PATHOGENIC BACTERIA

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CONTENTS

CHAPTER 1 – Food contaminants: an overview

1.1 Introduction

The contamination of food is a worldwide public health concern and is a leading cause of trade problems internationally. Contaminants are substances that, usually, have not been intentionally added to food. These substances may be present in food as a result of the various stages of its production, packaging, storage, transport or holding. In addition, contamination may occur through environmental pollution of the air, water and soil, such as the case with toxic metals, PCBs and dioxins, or through the intentional use of various chemicals, such as pesticides, veterinary drugs and other agrochemicals.

The range of toxicants found in food can be therefore broadly subdivided into:

- Contaminants or those substances that inadvertently get into our food as by-products of industrial manufacturing or waste or as residues from intentional food chemicals, such as pesticides.
- Naturally-occurring toxicants or those substances that are found naturally in our food because they are products of plants, animals, and other organisms.
- Food processing toxicants or those substances that incidentally become part of our food as by-products of various processing situations. Also, other substances added intentionally to the food for some benefit could potentially, if added in excess, adversely affect the consumer.

Since food additives and contaminants resulting from food manufacturing and processing can adversely affect health, WHO has implemented the Global Environment Monitoring System - Food Contamination Monitoring and Assessment Programme (GEMS/Food), which has informed governments, the Codex Alimentarius Commission and other relevant institutions, as well as the public, on levels and trends of contaminants in food, their contribution to total human exposure, and significance with regard to public health and trade.

1.2 Classes of Contaminants

Contaminating substances may enter the food chain at different stages: Through various constituents like fertilizer ingredients pesticides can enter food crops through plant roots, and the irrigation water derived from waste water treatment plants. Contaminants in

forages and other feeds can be transmitted to animal products. Veterinary drugs can leave residues in animal products. Environmental chemicals such as heavy metals from many sources have sometimes been found as food contaminants.

1.2.1 Pesticides

Pesticides play an important role in food production by controlling insects, weeds, organisms responsible for plant diseases, and other pests. In protecting food plants, pesticides assist in ensuring substantial yield and providing consumers with a wide variety of foods at inexpensive prices. Pests are living organisms that occur where they are not wanted or that cause damage to crops, humans, or other animals. Most pesticides create some degree of risk of harm to humans, animals, or the environment because they are inherently designed to kill or otherwise adversely affect living organisms but be selective with regard to target species (Albert, 1979). At the same time, pesticides are useful to society, destroying potential disease-causing organisms and control insects, weeds, and other pests. Understanding the modes of action of pesticides is important to the food toxicologist both for the design of compounds to be specific for the targeted species and for understanding potential adverse human health effects.

Pesticides have several modes of action. They may physically alter the organism in a mechanical way, which blocks their cellular processes. Examples include oil (petroleum products) that are used as sprays, which clog the respiratory mechanism of insects, or compounds like bypyridylium herbicides, which act by destroying the cell membranes of plants causing desiccation. Petroleum oils can also deter feeding or egg lying in some insects. Some pesticides are metabolic system inhibitors and restrict the transfer of energy within the target organisms. Inhibitory pesticides in this category are many, such as rotenone and cyanide, which disrupt respiratory functions in animals, or herbicides that inhibit seed germination or plant growth, and various fungicides that inhibit germination of spores. Some pesticides require metabolic activation into the toxic form before they become toxic to the target pest. For example, sodium monofluoroacetate is converted to fluorocitrate before it becomes toxic to vertebrates. Other pesticides owe their effectiveness to the alteration of protein synthesis or enzyme disruption. Proteins, as the basic building blocks of all cell components, are crucial and substances made up of proteins, such as enzymes, control many important cell functions. Pesticides can disrupt enzyme processes or denature proteins. Examples include inorganic copper compounds, dithiocarbamate fungicides, and phosphor amino acid herbicides, such as glyphosate and organophosphate insecticides. Other pesticides alter hormone actions that control many of

the biological functions of organisms, including growth and reproductive cycles. Several pesticides simulate or otherwise interfere with hormones to disrupt these cycles, for example phenoxy herbicides that interfere with plant growth hormones, and insect growth regulators that interfere with cuticle formation in insects during molting. Nervous system disruptor pesticides affect mainly animal groups such as insects, nematodes, and rodents. Some pesticides, such as some fumigant pesticides, are narcotics. Others disrupt the movement of nerve impulses, such as the organophosphate, carbamate, and pyrethroid pesticides. Some pesticides affect photosynthesis, the process of using sunlight energy to create carbohydrates from carbon dioxide and water. Pesticides that disrupt photosynthesis prevent the plant from producing or storing energy and ultimately kill the plant, for example triazine, substituted urea and uracil herbicides. Finally, some pesticides work in more than one way and fall into more than one of these categories. The modes of action of many pesticides are not fully understood. Some pesticides are residual in action and continue to be effective for days, weeks, or months after their application. Examples are the triazine herbicides that persist in the soil and kill emerging weeds over the lifetime of a crop, or insecticides that remain active for several years as a barrier to termites entering buildings.

Modern pesticides are designed not to persist for long in the environment, which was a problem associated with organochlorines like dichlorodiphenyltrichloroethane (DDT). Modern pesticides act quickly and are then degraded to innoxious substances by chemical or microbial processes. This helps prevent their build-up in crops or other organisms. Pesticide breakdown depends on the compound's chemical properties, how much is applied, and how it is distributed, as well as environmental factors such as temperature, moisture, soil pH, and the availability of microorganisms.

1.2.2 Metals

Many metals, including heavy metals which have particular concerns for health problems, end up in our food supply and can be traced back to industry via effluent, sewage, dumps, and dust into the environment, and eventually into the food chain. Not all the traces of heavy metals in plants and animals are subsequent to human activity. Metals arise through absorption processes of naturally occurring soil components. Arsenic, cadmium, lead, mercury, and nickel are the most prevalent heavy metals with health concerns. The definition of a heavy metal is one that has a specific gravity of more than 5 g/cm^3. By definition this would account for 60 metals, several of which are biologically essential, and

many others lack sufficient information regarding toxicity, including platinum, silver, and gold.

Arsenic

Arsenic is found in the environment and originates from both natural and industrial sources. Sources of arsenic exposure include air pollution, antibiotics given to commercial livestock, certain marine plants, chemical processing, coal-fired power plants, defoliants, drinking water, drying agents for cotton, fish, herbicides, insecticides, meats (from commercially raised poultry and cattle), metal ore smelting, pesticides, seafood (fish, mussels, oysters), specialty glass, and wood preservatives.

Chronic arsenic poisoning occurs throughout the world because of high arsenic content in drinking water (greater than 1000 μg/L), in such places as Taiwan, Hungary, Chile, Inner Mongolia, Mexico and Bangladesh. In some Western United States, such as New Mexico, Arizona, Nevada, Utah, and Southern California, arsenic levels are in the 50±100 μg/L range and the health effects may be less pronounced. Epidemiological evidence implicates arsenic as an inducer of skin and bladder cancer and affecting heart disease, neurological, vascular, and cognitive function.

Cadmium

Cadmium is ubiquitous and is actively extracted from ores for commercial purposes. Contributions to residues include industrial processes such as metal refining, coal and oil industry, and electroplating plants. Other sources of cadmium exposure include air pollution, art supplies, bone meal, cigarette smoke, food (coffee, fruits, grains, and vegetables grown in cadmium-laden soil, meats, or refined foods), freshwater fish, fungicides, highway dusts, incinerators, mining, nickel-cadmium batteries, oxide dusts, paints, phosphate fertilizers, power plants, seafood (crab, flounder, mussels, oysters, scallops), sewage sludge, softened water, smelting plants, tobacco and tobacco smoke, and welding fumes.

Cadmium is concentrated in the kidneys, liver, lungs, and in blood-forming organs. Ingestion of cadmium can lead to kidney damage and metabolic anomalies usually through enzyme inhibition. Itai-itai disease in Japan is an example of an illness caused by rice highly contaminated with cadmium. Cadmium is a cumulative poison but unlike lead, the definition of an exact toxicity limit has been difficult to determine because of bioavailabiliity questions.

Lead

The main sources of lead pollution in the environment are industrial production processes and their emissions, road traffic with leaded petroleum, the smoke and dust emission of coal and gas. Other sources of lead exposure include air pollution, ammunition (shot and bullets), bathtubs (cast iron, porcelain, steel), batteries, canned foods, ceramics, chemical fertilizers, cosmetics, dolomite, dust, foods grown around industrial areas, gasoline, hair dyes and rinses, leaded glass, newsprint and colored advertisements, paints, pesticides, pewter, pottery, rubber toys, soft coal, soil, solder, tap water, tobacco smoke, and vinyl mini-blinds.

Both the acute and chronic forms of lead poisoning occur. Acute toxicity occurs by the consumption of large single doses of soluble lead salts. Chronic toxicity can occur through the regular consumption of foodstuffs which may be only slightly contaminated with lead and represent the greater problem of cumulative poisoning. The mode of action of lead is related to the metal's high affinity for proteins, such as hemoglobin, plasma proteins, and the subsequent inhibition of protein-mediated processes. Lead ingestion leads to inhibition of the synthesis of red blood cells, which in turn compromises oxygen transport. When the binding capacity of blood proteins are exceeded, lead affects other organs, such as bone-marrow, liver, nervous system, reproductive tissues, and kidney. Organic lead compounds are particularly troublesome and likely lead to injuries to mental development with reduction in intelligence, growth, and cognitive function. Although lead has been largely eliminated from gasoline and the lead content of populations has fallen sharply, lead in water continues to be a problem in many countries, such as some parts of the United Kingdom. Thus, foodstuffs contribute to the lead burden, particularly, lead found in vegetables, fruits, drinking water, beverages (wine), and cereal products. Fruits and vegetables mostly acquire their lead contamination through impurities in the air, which can be greatly reduced by washing.

Mercury

Mercury has been extracted since ancient times and is used in electrical devices, catalysts, thermometers, and pigments. Other sources of mercury exposure include air pollution, batteries, cosmetics, dental amalgams, diuretics (mercurial), electrical devices and relays, explosives, foods (grains), fungicides, fluorescent lights, freshwater fish (especially large bass, pike, and trout), insecticides, mining, paints, pesticides, petroleum products, saltwater fish (especially large halibut, shrimp, snapper, and swordfish), shellfish, and tap water.

Mercury passes into the environment through emissions from chemical plants, power stations, often as effluents and sludge. Mercury becomes concentrated in shellfish, crustaceans, and fish, and passes on in the food chain in its highly toxic form, methylated mercury. The Minamata sickness in Japan in 1965 was the result of consumption of fish taken from regions of the sea contaminated by industrial effluent.

Methyl mercury compounds are the most toxic of heavy metals. Ingestion of organic mercury results in distribution to the liver, kidneys, and brain. Salts of mercury cause tiredness, loss of appetite, weight loss, muscular weakness, perhaps paralysis, and eventually kidney failure. Methyl mercury damages the central nervous system and the immune system, and has been shown to produce teratogenic effects.

Nickel

Sources of nickel exposure include appliances, buttons, ceramics, cocoa, cold-wave hair permanent, cooking utensils, cosmetics, coins, dental materials, food (chocolate, hydrogenated oils, nuts, food grown near industrial areas), hair spray, industrial waste, medical implants, metal refineries, metal tools, nickel-cadmium batteries, orthodontic appliances, shampoo, solid-waste incinerators, stainless steel kitchen utensils, tap water, tobacco and tobacco smoke, water faucets and pipes, and zippers.

High nickel concentrations in tissues has been associated with depression of prolactin secretion, and inhibition of insulin secretion. Nickel has carcinogenic effects when inhaled, producing lung and nasal cancers.

1.2.3 Industrial by-products and waste

As noted above, many of the metals may find their way into our food as industrial by-products or waste. Since the Industrial Revolution, industrial and mining operations have been accompanied by industrial waste, which may be toxic, ignitable, corrosive, or reactive. Obviously, proper management is the key and improperly managed waste can pose dangerous health and environmental risks. It is estimated that, in the United States, the amount of hazardous waste generated by manufacturing industries has increased from 4.5 million tons annually after World War II to some 265 million tons by 1990 (Council on Environmental Quality, 1981). Waste is generated at every stage in the production process, use, and disposal of manufactured products. The introduction of many new technologies for the home and office (computers, drugs, textiles, paints and dyes, plastics) in recent times has introduced more hazardous waste, including toxic chemicals, into the

environment. The EPA estimated in 1980 that more than 70,000 different chemicals were being manufactured in the United States, with some 1,000 new chemicals being added each year (Petulla, 1988). The human health and environmental impacts of many of these chemicals are largely unknown. Industrial waste may be solid, liquid, or gaseous and is divided into hazardous and non-hazardous waste. Hazardous waste may result from manufacturing or other industrial processes. Certain commercial products such as cleaning fluids, paints, or pesticides discarded by commercial establishments or individuals can also be defined as hazardous waste. The definition of hazardous waste has been further refined through regulations. Non-hazardous industrial waste is that which does not meet the EPA's definition of hazardous waste and is not municipal waste.

1.3 Naturally-occurring toxicants

Natural or raw foods contain an array of chemicals and only a few of these chemicals actually possess nutritional importance. Most of the chemicals found in food neither enhance nor detract from the wholesomeness and nutrient quality of the food. They move through the gastrointestinal tract unchanged and exert little to no effect on surrounding tissues or other nutrients they pass. However, there is a group of chemicals found in foods that can affect health by impairing nutrient absorption, inhibiting or destroying nutrients (antinutrients), or acting as mutagens, teratogens or carcinogens. Such chemicals occur in food in small quantities and if the diet is nutritionally adequate and widely varied, these minute amounts of otherwise toxic compounds can be tolerated or rendered harmless.

1.3.1 Plant and animal toxicants

Many plant and animal foods contain natural or processing-induced constituents with biological activity in mammals. Among the plant family these are glycoalkaloids, amines, glucosinolates, cyanogenic glycosides, protease inhibitors, oxalates, coumarins, polyphenols, cyclopropenoid fatty acids, phytates, xanthines, and essential oils. Recently, various lines of evidence have suggested that plant-derived constituents may play an important role in determining spontaneous rates of genetic damage and tumor incidence. On the other hand, genotoxic constituents such as certain flavones, anthraquinones, browning products, benzoxazinones, and acetals have been identified in plant-derived foods, and unsaturated oilseed lipids have been found to increase cancers of certain sites, particularly intestine and breast, in laboratory animals.

Whether these constituents play a significant quantitative role in human health is a key scientific issue which is at present not adequately resolved. With the increase in fish consumption for health or other individual preferences, concerns for higher exposures to animal toxins are increased. As will become apparent, whether to classify these as true animal toxins is debatable since they seem to be more microbial in their origin. Ciguatera is a form of poisoning caused by the consumption of finfish (barracudas, snappers, jacks, mackerel, and triggerfish) which have accumulated naturally-occurring toxins that originate from several dinoflagellate (algae) species that are common to ciguatera. Manifestations of ciguatera usually involves a combination of gastrointestinal, neurological, and cardiovascular disorders. Shellfish poisoning is caused by a group of toxins elaborated by planktonic algae, dinoflagellates, upon which the shellfish feed. The toxins are accumulated and metabolized by the shellfish. The 20 toxins responsible for paralytic shellfish poisonings (PSP) are all derivatives of saxitoxin. The effects are predominantly neurological and include tingling, burning, numbness, drowsiness, incoherent speech, and respiratory paralysis. Scombroid poisoning is caused by the ingestion of fish that contain high levels of histamine and other vasoactive amines and compounds. Histamine and other amines are formed by the growth of certain bacteria in the fish or any food that contains the appropriate amino acids. Fish poisoning can also occur by consumption of members of the pufferfish family.

Tetrodotoxin has also been isolated from widely differing animal species, including the California newt, parrotfish, frogs of the genus Atelopus, the blue-ringed octopus, starfish, angelfish, and xanthid crabs. Recent reports of the production of tetrodotoxin/anhydrotetrodotoxin by several bacterial species, including strains of the family *Vibrionaceae*, *Pseudomonas sp*., and *Photobacterium phosphoreum*, point toward a bacterial origin of this family of toxins.

1.3.2 Microbial toxicants

Microbiological contamination of food should cause more concern than any other food safety hazard. Bacteria are by far the most common and are responsible for many diverse foodborne diseases (Altekruse, 1997). According to the national FoodNet database, 76 million persons were estimated to have contracted foodborne diseases in the United State in 2001. Because only a small minority of the cases seek medical attention, the number is likely higher. Many people who experience foodborne disease are unaware since the characteristics are similar to those of the `flu'.

In 2001, the Centers for Disease Control and Prevention (CDC) released a report showing a decline in the major bacterial foodbone illnesses. According to the data, the four major bacterial foodborne illnesses *Campylobacter*, *Salmonella*, *Listeria*, and *E. coli O157*, posted a 21% decline in the past six years. *Campylobacter* infections dropped 27%, infections from *Listeria* fell 35%, and *Salmonella* infections decreased by 15%. *E. coli O157* infections dropped 21%, but all of that decline occurred since 2000. These declines signify important progress toward meeting the Healthy People 2010 objectives for reducing the incidence of disease caused by these bacterial infections. Other less common bacterial foodborne illnesses also showed significant declines since 1996. *Yersinia* infections decreased 49% and *Shigella* infections dropped by 35%. CDC notes the data do not show a sustained decline in some infections indicating that increased efforts are needed to further reduce the incidence of foodborne illness. CDC notes several factors have contributed to the decline in foodborne illnesses. These include enhanced surveillance, the implementation of Pathogen Reduction/Hazard Analysis Critical Control Point (HACCP) regulations in meat and poultry plants, HACCP regulation of fruit and vegetable juices, seafood HACCP, publication and outreach of good agricultural practices for fresh produce, increased regulation of imported food, and last, but certainly not least, extensive food safety education. Food safety education and research have played important roles with consumer educational efforts in this area (IFT expert report, 2000).

1.3.3 Mycotoxins

Animal and human health problems related to food products contaminated with toxic metabolites produced by fungal growth have long been recognized. The Food and Agriculture Organization has estimated that at least 25% of the world's food crops are affected by mycotoxins annually (Food and Agriculture Organization of the United Nations, 1996). Thus, many scientific reports have been published concerning the occurrence of mycotoxins in foods and feeds, and their impact on human and animal health.

Recently, there have been reports on cocontamination of various toxins, i.e., aflatoxin B_1/fumonisin B_1 and ochratoxin A/aflatoxin B_1, among others (Park, 1996; Harvey, 1989; Brownie, 1988). Although more documentation is needed on the levels and effects of mycotoxin cocontamination, it is important to consider that food commodities are a complex environment and that the individual effect of each toxin might be affected by the presence of other toxins or food constituents (Rice, 1994). Examples of mycotoxins affecting health include:

Aflatoxins: Historically, aflatoxins have undoubtedly been the group of mycotoxins of most concern because they have been shown to be both potent hepatotoxins and carcinogens in many species. Aflatoxin contamination of foods and feeds occurs when aflatoxigenic species of *Aspergillus* sp. successfully colonize a commodity, grow and find conditions appropriate for toxin production. The three species of *Aspergillus* that produce aflatoxins are *A. flavus*, *A. parasiticus*, and *A. nomius* (Wilson, 1994). *A. flavus* is a common constituent of the microflora in air and soil throughout the world. It is prevalent in stored wheat, corn, cottonseed, rice, barley, bran, flour, peanuts, soybeans, sorghum, chili peppers, copra, millet, tree nuts, and green coffee beans, among other commodities. Growth can occur even when products are stored under relatively low moisture, which eliminates the growth of competing species such as *Penicillium* and *Fusarium*. However, storage in hot or humid conditions can aggravate toxin formation. Aflatoxin contamination also may be severe when developing crops are exposed to drought conditions (Shibamoto, 1993).

Chemically, aflatoxins are defined as a series of 18 known bisulfuran polycyclic compounds that fluoresce strongly in ultraviolet light (ca. 365 nm). Aflatoxins B_1 and B_2 produce blue fluorescence, whereas G_1 and G_2 produce green fluorescence. Four other aflatoxins, M_1, M_2, B_2A, and G_2A are produced in small amounts. In some animal species, such as dairy cattle, aflatoxins B_1 and B_2 are partially metabolized to give the hydroxylated derivatives: aflatoxins M_1 and M_2, respectively. Other metabolic derivatives are aflatoxin P_1 and Q_1 (Eaton, 1994).

The effect of aflatoxins on animals is quite different depending on age, sex, species, nutritional condition of the animal, dosage level, frequency, and composition of the diet. Sensitivity to the toxins varies greatly from species to species (i.e., the LD_{50} ranges from 0.5 mg/kg for the duckling to 60 mg/kg for the mouse). Rats, poultry, and trout are highly susceptible to the effects of aflatoxin, whereas sheep, hamsters, mice, and pigs are fairly resistant. The organ primarily affected is the liver, but changes can be seen in most organs.

The carcinogenicity, mutagenicity, teratogenicity, and acute toxicity of aflatoxins have been well documented (Ellis, 1991). Aflatoxin B_1 is the most important in terms of occurrence and toxicity, and the most potent of the naturally occurring carcinogens. In susceptible experimental animals, cancer has been induced in low doses that are comparable to levels present in contaminated human diets (Buss, 1990).

Preharvest prevention of aflatoxin formation is difficult; therefore, aflatoxins in foods and feeds are considered a continuous risk. Discontinuing the use of aflatoxin-contaminated

grains and oilseeds is not always practical.

There is a need to manage the risks associated with aflatoxin contamination before using these products as animal feed or human food. Thus, several methods for decontamination and postharvest control have been reported (Lopez-Garcia, 1998).

The use of ammonia-heat treatments has shown effective reduction of aflatoxin (Jorgensen, 1981). Other chemicals such as monomethylamine, sodium hydroxide, sodium hypochlorite, and hydrogen peroxide also have resulted in acceptable detoxification in several commodities. During fermentative production of ethanol, little degradation of the toxin was achieved. Other decontamination approaches include food and feed processing such as thermal inactivation, irradiation, solvent extraction, mechanical separation, density segregation, and reduction in bioavailable aflatoxin by selective chemisorption. Biocontrol methods and microbial inactivation have been suggested as well as decontamination procedures (Phillips, 1994).

Aflatoxins have become generally accepted to be poisonous and deleterious, and are now widely regulated in foods. In the U.S., the Food and Drug Administration (FDA) regulates feed and food containing aflatoxins at regulatory levels of 20 ppb of aflatoxin B_1 for human foods and selected animal feed.

Levels up to 300 ppb are permitted for specific commodities and under selected animal feeding operations, and 0.5 ppb of aflatoxin M_1 in milk (Pitt, 1993).

Fumonisins (Fumonisin B_1 and Fumonisin B_2): are natural toxins produced by *Fusarium moniliforme* and other *Fusarium* species; these molds are common natural contaminants of corn. Fumonisins have been linked to fatalities in horses and swine. Recent studies have demonstrated the presence of fumonisins in human foods, including corn meal and breakfast cereals. Epidemiological investigations demonstrating a possible association of *F. moniliforme* with esophageal cancer and recent animal studies indicating the carcinogenicity of fumonisin B_1 have highlighted the need to ensure that foods do not contain excessive amounts of fumonisins. Deoxynivalenol (DON), commonly called vomitoxin, is a natural toxin produced by several molds of the genus *Fusarium*, especially *F. graminearum*, which is a common contaminant of several grains, including wheat, corn, barley, and rye. DON has been associated with a number of adverse health effects in humans and animals. Several adverse weather related DON contamination episodes in the United States motivated the FDA to issue advisory levels for food (wheat) and feed in 1982 and updated levels in 1993. FDA is continuing to study the scope and toxicological significance of the DON problem in order to make a determination as to whether further

regulatory measures are needed to control DON in food and feed products.

Ochratoxin A: is a naturally-occurring nephrotoxic fungal metabolite produced by certain species of the genera Aspergillus and Penicillium. It is mainly a contaminant of cereals (corn, barley, wheat, and oats) and has been found in edible animal tissues as well as in human blood sera and milk. Studies indicate that this toxin is carcinogenic in mice and rats. It is not completely destroyed during the processing and cooking of food, therefore the implication for risk to human health and safety must be considered.

Patulin: is a mycotoxin produced by several species of mold fungi including *Penicillium expansum*, the causal organism of apple rot. Apple juice prepared from apples contaminated with *Penicillium expansum* could be a possible source of patulin in the human diet. The World Health Organization (WHO) has recommended a maximum patulin level of 50 ppb based on a toxicological assessment. At least 12 countries regulate patulin at 30-50 ppb. The United Kingdom's Food Advisory Committee announced recently its intention to establish control measures on patulin. FDA identified patulin as a potential hazard in fruit juice in its juice HACCP proposal. In the Codex Alimentarius, FDA participated in negotiations as to what limitation on patulin in apple juice is appropriate for apple juice and concentrate in international commerce.

1.4 Food processing toxicants

During the processing of foods, products may be produced that, if present in large amounts, could potentially adversely affect health. For example, cooking certain meats at high temperatures creates chemicals that are not present in uncooked meats. A few of these chemicals may increase cancer risk, such as polycyclic aromatic hydrocarbons and heterocyclic amines. Another example is when nitrates and nitrites react with secondary amines to form nitrosamine. Nitrosamines are mutagens which have been linked to cancers. Nitrates and nitrites are used to preserve meats and contribute to prevention of growth of *Clostridium botulinum*, the bacterium responsible for producing the highly potent botulinum toxin.

1.4.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are produced when any incomplete combustion occurs. Thus, they are found in polluted air, cooking oil fumes, tobacco smoke, smoked foods and foods cooked at high temperature.

Most PAHs are not carcinogenic, although a few are, for example benzo(a)pyrene. They appear mainly in meats cooked during high temperature grilling. Microwaving does not produce PAHs, and foods other than meats contain negligible amounts of PAHs. Foods low in fat, or cooked beneath the source of heat, contain many fewer PAHs, so the type of food cooked and the method of cooking are important determinants of PAHs.

Breathing air containing PAHs can occur in the workplace of coking, coal-tar, and asphalt production plants, smokehouses, and municipal trash incineration facilities. Breathing air containing PAHs can also occur from cigarette smoke, wood smoke, vehicle exhausts, asphalt roads, or agricultural burn smoke. Coming in contact with air, water, or soil near hazardous waste sites can also increase exposure to PAHs. Eating grilled or charred meats, contaminated cereals, flour, bread, vegetables, fruits, meats, and processed or pickled foods increases an individual's exposure to PAHs. Drinking PAH-contaminated water or cow's milk can increase an individual's exposure to PAHs. Nursing infants of mothers living near hazardous waste sites may be exposed to PAHs through their mother's milk.

1.4.2 N-nitrosamines

Nitrosamines are a class of chemical compounds that were first described over 100 years ago. In 1956 it was reported that dimethylnitrosamine produced liver tumors in rats. Approximately 300 of these compounds have been tested and 90% of them have been found to be carcinogenic in a wide variety of experimental animals. Dimethylnitrosamine (DMNA) (also called N-nitrosodimethylamine or NDMA) is a member of a group of chemicals known as nitrosamines which are recognized as cancer-causing substances. DMNA is a volatile liquid, which dissolves easily in water and in oil. It can be broken down by light or microorganisms but, in their absence, DMNA can persist in water for a very long time. Even in a sewage treatment plant, DMNA degrades only very slowly. Nitrosamines (including DMNA) are also formed in tobacco smoke. An interesting observation is that the concentration of nitrosamines in side-stream smoke from a cigarette has 20-200 times more nitrosamines than the mainstream smoke. Exposure to very smoky rooms can result

in as much DMNA exposure as smoking. Most nitrosamines are mutagens and a number are transplacental carcinogens. Most are organ-specific. For instance, DMNA causes liver cancer in experimental animals, whereas some of the tobacco-specific nitrosamines cause lung cancer. Since nitrosamines are metabolized the same in human and animal tissues, it seems highly likely that humans are susceptible to the carcinogenic properties of nitrosamines.

Foods which contain nitrogen sources and amines have particularly high levels of DMNA and other nitrosamines. Amines occur commonly and sodium nitrite is added to cured meats to prevent toxin production by *Clostridium botulinum*. Such foods are bacon, prepared meats, fish, tomatoes, spinach, beer, and many others. In the body, nitrosamines are formed as a result of these nitrates and nitrites (present in meat and other food and vegetables as preservatives) reacting with saliva in the mouth or with gastric juices in the stomach. DMNA can also be formed naturally as a result of certain biological processes associated with bacteria.

Cured meats can contain nitrosamines because meats contain amines and sodium nitrite, a source of nitrosating agents added to cured meats as a preservative. Of all the cured meats, bacon has received the most attention. It almost always contains detectable levels of nitrosamines, principally nitrosopyrrolidine and, to a lesser extent, DMNA. The very high cooking temperatures used to fry bacon are conducive to nitrosamine formation. In the late 1970s, extensive attention was focused on the issue of nitrosamines in cured meats and the removal of sodium nitrite as a food additive was considered.

However, the prospect of sodium nitrite removal presented a formidable dilemma for the regulatory agencies. Removal of sodium nitrite would prevent nitrosamine formation, but it might also increase the risk of botulism poisoning.

Sodium nitrite and sodium chloride together are particularly effective against *Clostridium botulinum*. The solution to the dilemma was to limit the addition of sodium nitrite to 120 ppm, the lowest level found to be effective in controlling growth and toxin production by *Clostridium botulinum*.

Ascorbic acid also inhibits nitrosamine formation. The addition of 550 ppm of ascorbic acid is now required in the manufacture of cured meat in the United States. Actually, most cured meat manufacturers add erythorbic acid (a less expensive isomer of ascorbic acid) rather than ascorbic acid. Another antioxidant, alpha-tocopherol (vitamin E), is added to some cured meats to inhibit nitrosamine formation. As a result of these strategies, there are now significantly lower levels of nitrosamines in fried bacon and other cured meats than there were some years ago. Ascorbic acid, erythorbic acid, and alpha-tocopherol

inhibit nitrosamine formation due to their oxidation and N reduction properties.

Dimethylnitrosamine has been found in beer, formed by direct-fire drying of barley malt, an ingredient used in making beer. By converting the process from direct-fire drying to indirect-fire drying, the nitrosating agents and the formation of DMNA were markedly reduced. Thus beer now contains only 2% of the DMNA that was present 20 years ago.

Nitrosamines can form in the gastric juice of the human stomach, referred to as endogenous nitrosation. Bacteria in the mouth chemically reduce nitrate, which is prevalent in many vegetables, to nitrite, which in turn can form nitrosating agents. Many foods contain amines that can react with nitrosating agents in the acidic stomach to form nitrosamines.

Nitrosamines are carcinogenic in animals. Current exposure is probably closer to 0.1 μg per day due to successful efforts over the past 20 years to reduce nitrosamine formation in foods and beverages. In contrast, there is an estimated exposure of 17 μg per day from cigarette smoking. An enormous amount of indirect evidence indicates that nitrosamines are human carcinogens. For instance, tobacco-specific nitrosamines are one of the major groups of chemical carcinogens in tobacco products and no doubt remains about the causal link between tobacco use and cancer. But, it is difficult to evaluate the risk of cancer from daily exposure of 1 μg from foods and beverages. The same difficulty applies to the risk assessment of the exposure to minute amounts of aflatoxin, PAHs, and heterocyclic amines in a variety of foods and beverages.

1.4.3 Heterocyclic amines

Heterocyclic amines (HCAs) are the carcinogenic chemicals formed from the cooking of muscle meats such as beef, pork, fowl, and fish (Bjeldanes, 1982, 1983; Felton, 1994; Sugimura, 2002). HCAs form when amino acids (the building blocks of proteins) and creatine (a chemical found in muscles) react at high cooking temperatures. Some 17 different HCAs have been found, resulting from the cooking of muscle meats, that may pose human cancer risk. Research conducted by the National Cancer Institute (NCI) as well as by Japanese and European scientists indicates that HCAs are created within muscle meats during most types of high temperature cooking. Further evaluation is needed of the relationship between methods of cooking meat and the development of specific types of cancer. One study conducted by researchers from NCI's Division of Cancer Epidemiology and Genetics found a link between individuals with stomach cancer and the consumption of cooked meats. Those who ate their beef medium-well or well-done

had more than three times the risk of stomach cancer than those who ate their beef rare or medium-rare. They also found that people who ate beef our or more times a week had more than twice the risk of stomach cancer than hose consuming beef less frequently. Additional studies have shown that an ncreased risk of developing colorectal, pancreatic, and breast cancer is associated with intakes of well-done, fried, or barbequed meats.

1.5 Novel foods and natural toxicants

Functional food has been defined as food either natural or formulated, which will enhance physiological performance or prevent or treat diseases and disorders (Wildman, 2001) or as defined by the Food and Nutrition Board of the National Academy of Sciences, any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. The functional food market has likely doubled in the past ten years to over $10 billion and many food companies are taking full advantage of the public's growing awareness of food in health. Thus, a natural extension of the functional food area is modifying or cultivating food plants and animals to maximize their ability to produce the desirable compounds that are important to health, for example more vitamin E, vitamin A. Genetic modification (GM) of plants or animals for the purpose of producing more functional foods, is at the cutting edge of food science (Reilly, 2000; Harlander, 2002). Some have claimed that this is a new “second generation” of GM crops, being developed to be genetically modified to express traits for delivering apparent benefits to the consumer and unlike the “first generation” developed for delivering only producer benefits. Others will contend that because the density of nutritive and health-beneficial functional chemicals can vary dramatically among plants and animals, with levels often being quite low or non-existent, there is a need to develop strategies that would improve the composition of such chemicals in certain foods. Conventional breeding techniques can be used to achieve this goal, but GM is an attractive alternative that would enable investigators to overcome the genetic diversity within existing germplasm that limit the extent of improvement that can be achieved through conventional techniques. Examples include the engineering of beta-carotene in rice grains, elevating iron content in rice, enhancing vitamin C content in lettuce, increasing flavonols in tomato.

The research is complex because often there is a need not only to understand the biochemical pathway for producing such metabolites, but also a need to appreciate the complexity of the metabolites trafficking through the organism, which likely require the identification of several genes for use in a transgenic improvement strategy.

Safety considerations for a functional food derived by GM should not be treated any differently from the functional food derived by conventional plants or animals. Alterations introduced into the genome are very specific and minor, altering one or a few genes and their gene products. The overall safety evaluation of a GM functional food will be conducted under the concept known as “substantial equivalence” where GM products with one or a few new introduced traits remain substantially equivalent to their traditional counterparts (Cockburn, 2002; Kuiper, 2002).

Over 50 GM crops have undergone such evaluation and been judged safe, via World Health Organization and the Food and Agriculture Organization of the United Nations (FAO/WHO, 2000). Overall, the safety assessment is focused on those genes and their products that are introduced into the new variety. Typically, the emphasis is placed on proteins expressed by the introduced atypical gene.

Consumption of DNA, regardless of the source, is considered highly digestible and safe (Beever, 2000; Jonas, 2001). For novel organisms not judged substantially equivalent to the traditional counterpart, which might be the situation when a large number of genes are introduced or the nutritional content has been altered substantially, a more thorough safety evaluation will be needed.

If the parent plant is known to have antinutrient properties, natural toxicants, or allergenic substances, the novel organism will be tested to determine the concentration of such compounds. Currently, the safety assessment approach for such products is voluntary in the United States; however, a mandatory system is likely to be imposed by the FDA in the near future.

1.5.1 Natural toxicants

Plants have in their arsenal an array of thousands of chemicals noxious or toxic to bacteria, fungi, insects, herbivores, and or other species feeding upon them.

Fortunately for humans, this chemical diversity also includes many compounds that are beneficial to health: nutrients, antioxidants, anticarcinogens, and many compounds with medicinal value. Most plant species in the world are not edible, many because of the toxins they produce. Plant domestication has gradually reduced the levels of these compounds so that the plant foods we eat today are far less toxic than their wild parents. However, such domestication has resulted in modern food plants being much more susceptible to disease. Toxins range from known carcinogens to skin irritants. A few lipid-soluble plant toxins are capable of bioaccumulation, for example, solanine from potatoes.

Toxin concentrations in a plant can vary tremendously and the concentration can be dictated dramatically by environmental stress on the plant (drought, heat/cold, mineral deficiencies, etc.), and disease. Also, different varieties of plant species can differ in the levels of toxins and nutritional value.

1.5.2 Antinutrients

Antinutrients, although not necessarily toxic per se, are plant compounds which decrease the nutritional value of a plant food, usually by making an essential nutrient unavailable or indigestible when consumed by humans/animals, i.e. cause nutritional deficiencies. For example, phytate, a common component of most seeds and cereals, forms a complex with many important minerals, making less of the minerals available or making an antimineral substance. Antinutritives can have important health implications particularly with populations facing malnutrition or in marginal nutritional states (Janssen, 1997).

Several substances are antinutrients because they interfere with protein digestion, or the absorption or utilization of amino acid Phytohaemagglutinin are compounds referred to as lectin or hemagglutinin and have been used by immunologists for years to trigger DNA synthesis in T lymphocytes, and more recently, to activate latent human immunodeficiency virus type 1 (HIV-1, AIDS virus) from human peripheral lymphocytes. In addition to inducing mitosis, lectins are known for their ability to agglutinate many mammalian red blood cell types, alter cell membrane transport systems, alter cell permeability to proteins, and generally interfere with cellular metabolism. Lectins originate from plants, particularly legumes. Bean lectins, extracted from raw beans, bind to intestinal mucosal cells and interfere with absorption of amino acids, thyroxine, and various lipophilic compounds. Ricin, found in the castor bean, is toxic and causes intestinal cell necrosis. Protease inhibitors are proteins that inhibit proteolytic enzymes usually by binding to the active sites. Ovomucoid and ovoinhibitor are protease inhibitors found in raw eggs, which inactivate trypsins. Elastase is inhibited by protease inhibitors found in soybeans, kidney beans and sand potatoes. Many are heat-labile but some are heat-resistant.

1.5.3 Antiminerals

Besides phytic acid, which can interfere with bivalent and tervalent metal ions, oxalic acid, gossypol, glucosinolates, and dietary fiber are other known substances that can compromise mineral status. Oxalic acid reduces the availability of essential bivalent ions and is rich in rhubarb, spinach, and celery.

Foods rich in oxalic acid exhibiting an oxalic acid to calcium ratio higher than 1 may decrease calcium availability. Foods rich in glucosinolates such as cabbage, legumes, rutabaga, and turnips may compromise iodine absorption promoting goiter. Gossypol is found in the cotton plant and forms insoluble chelates with many essential metals, such as iron. However, processing of cotton seed removes up to 99% of the gossypol. Dietary fiber has the capacity to complex with various metals, such as calcium, magnesium, zinc and phosphorus.

1.5.4 Allergenic compounds

Genetic modification of foods ultimately results in alteration of protein products either in quantity or the introduction of new proteins. The synthesis of new proteins into foods may present concerns about safety, particularly allergenicity (Hefle, 1996; Taylor, 1997; Wal, 1999). Although all allergens are not proteins, most are and, fortunately, only a few of the many diverse proteins found in foods are allergenic when ingested. It is important that the potential allergenicity of an introduced protein be evaluated because the incidence of food allergies can be traumatic and life-threatening. A task force of the International Food Biotechnology Council (IFBC) and the Allergy and Immunology Institute of the International Life Sciences Institute (ILSI) developed a decision tree approach for the assessment of potential allergenicity of plants produced by GM in 1996. The decision tree approach was modified by the FAO/WHO and focuses on evaluating the source of the gene, the sequence homology of the newly introduced protein to known allergens, the immunoreactivity of the novel protein with serum IgE from individuals with known allergies to the source of the transferred genetic material (Taylor, 2001). It is useful to have information regarding various physical and chemical properties of the novel protein, for example heat and digestive stability. It is likely that application of such criteria to GM foods will provide assurance that the novel protein in question will be assessed adequately for safety. However, some consumers and interest groups who are opposed to agricultural biotechnology have repeatedly called for mandatory labeling of such foods. Labeling would

be generic and of limited value to consumers with respect to information regarding specific food allergies.

1.6 Pharmaceuticals and other emerging contaminants

A large amount of drugs and their metabolites are released in the environment and potentially enter in the food chain. These class of compounds are classified as “emerging contaminants” and are under observation all around the western countries. Antidepressants, anti-inflammatory drugs, antibiotics, sexual steroids**,** birth control pills, seizure medication, cancer treatments, pain killers, tranquilizers and cholesterol-lowering compounds have been detected in varied water sources.

Pharmaceutical industries, hospitals and other medical facilities are obvious sources, but households also contribute a significant share. People often dispose of unused medicines by flushing them down toilets, and human excreta can contain varied incompletely metabolized medicines. These drugs can pass intact through conventional sewage treatment facilities, into waterways, lakes and even aquifers.

Personal care products also are showing up in water. Generally these chemicals are the active ingredients or preservatives in cosmetics, toiletries or fragrances. For example, nitro musks, used as a fragrance in many cosmetics, detergents, toiletries and other personal care products, have attracted concern because of their persistence and possible adverse environmental impacts. Some countries have taken action to ban nitro musks. Also, sun screen agents have been detected in lakes and fish.

Some scientists believe pharmaceuticals do not pose problems to humans since they occur at low concentrations in water. Other scientists say long-term and synergistic effects of pharmaceuticals and similar chemicals on humans are not known and advise caution. They are concerned that many of these drugs have the potential of interfering with hormone production. Chemicals with this effect are called endocrine disrupters and are attracting the attention of water quality experts.

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CHAPTER 2 – Analytical techniques for food contaminants

2.1 Introduction

Numerous analytical systems for screening, identifying, and quantifying contaminants in foods have been developed over the years. The more appropriate term is “microcontaminants” since in many cases they are present at a relatively low concentration. They represent almost all kinds of known analytical approaches including microbiological, immunochemical, and physicochemical techniques. Each of the existing techniques has its own advantages and drawbacks that must be carefully considered in the selection of the most convenient detection/confirmation system for a particular analyte in a given matrix.

Contaminants show large variation in molecular structure and, consequently, in physicochemical properties and biological activity. In addition, they have in common their presence in foods at relatively low, often sub-ppm levels, and their tendency to coexist with derived breakdown products which in many cases also must be included in the analysis.

Application of microbiological or immunochemical techniques offers the advantage of screening contaminants in foods with little or no previous sample preparation. Application, on the other hand, of physicochemical techniques, allows quantification and more tentative identification of residues in those samples found positive. The analytical problem for food contaminants is complicated by the fact that it is not known whether contaminants exist, and if they exist, the type and quantity are not known.

The analytical methods are standardized by such organizations as the U.S. Food and Drug Administration (FDA), U.S. Department of Agriculture (USDA), U.S. Environmental Protection Agency (EPA), Association of Official Analytical Chemists (AOAC), Institute of Food Technologists (IFT) and National Food Processors Association. Similar Institution are present at EU level.

In the White Paper on Food Safety of January 12th 2000 the European Commission announced a series of initiatives to improve and complete the legislation on food and feed controls. In this regard, Regulation (CE) N° 882/20 04 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules was adopted by the European Parliament and the Council on 29 April 2004.

2.2 Sample selection

The first step in food analysis is sample selection. Ideally, a sample will be identical to the material from which it has been removed. Samples can be chosen at random, by judgment of the analyst, or according to a system based on timing or location (such as daily at noon, or within a specific portion of the product or its container).

Samples must be representative, collected without contamination, and properly handled for the analytical results to be meaningful. If the analysis is not to be performed immediately, the sample will probably have to be preserved refrigerate or freezed to prevent deterioration.

Preservation involves the control of temperature, moisture, oxygen, and light, and may be as simple as sealing the sample in a container and placing it in a refrigerator. Containers must be dry, sterile, and unbreakable.

2.3 Extraction

Food samples frequently have to undergo extraction, separation, or concentration procedures prior to analysis. Foods contain a myriad of compounds and are not homogeneous, often forcing the removal of interfering components and the isolation of the analyte before an analysis is attempted. Common procedures include distillation, filtration, and precipitation. The sample may also have to be homogenized, ground, or treated in some other way. Buldini and Smith reviewed a number of modern extraction techniques (Buldini, 2002; Smith, 2003), which include the following:

Digestion

- Microwave oven digestion, with acids such as nitric or sulfuric, for solubilizing and oxidizing organic compounds to obtain free ions. Digestion by microwave is faster than the classical wet digestion.
- UV photolysis digestion, with hydrogen peroxide, for degrading organic compounds with hydroxyl radicals to obtain free ions. Small amounts of reagents are required, but digestion time is longer.

Membrane

- Microfiltration or ultrafiltration, based on size exclusion. Polymer membranes are often used for separation.

- Dialysis, based on ionic charge and size exclusion. Cellulose membranes are typically used.

Solvent

- Solvent extraction, for dissolving compounds of interest.
- Pressurized fluid extraction, at the near-supercritical region, where extraction is faster and more efficient.
- Supercritical fluid extraction, above the critical pressure and temperature of carbon dioxide, which is nontoxic and nonpolluting. Extraction is completed in minutes instead of hours, and thermal degradation is reduced.
- Microwave-assisted extraction, usually requiring < 15 mL solvent, < 10 min extraction time, and no elevated pressure.

Sorbent

- Solid-phase extraction and microextraction, where analytes are held by sorbents such as silica or polymers, and then solubilized and eluted.

Headspace

- Purge and trap (or dynamic headspace), in which the analyte is flushed from a liquid or gaseous sample and concentrated in a cryogenic trap.
- Adsorbent trap, where a synthetic porous polymer adsorbs a gas which is then desorbed.

The digestion methods are employed for extracting ions, and the other methods are used to extract compounds. Dialysis is used for both. Some of the extraction methods, such as supercritical fluid and sorbent, are used in conjunction with analytical techniques.

2.4 Instrumental technique selection

The analyst decides the problem to be solved and plans the analyses required, choosing techniques for their appropriateness using criteria in a number of categories:

Ability to conduct analysis

Sample size, reagents, instruments, cost, final state of sample (destroyed or intact)

Fundamental characteristics

Precision, accuracy, sensitivity, specificity, detection limit, reproducibility.

Personnel concerns

Safety, simplicity, speed

Technique status

Official method, in-house method

Official methods are developed by being comprehensively studied and compared between laboratories. Standardized official methods include those published by AOAC International, American Association of Cereal Chemists and American Oil Chemists' Society (Official Methods of Analysis of AOAC International, 2003; Approved Methods of the AACC 2001). More specialized method collections, such as Food Chemical Codex for determination of additives, have also been compiled (Food Chemical Codex, 2004). Many techniques are not listed as official because they are relatively new or have not yet been applied to certain types of samples. In these cases, in-house methods may be used if they have been validated (Wood, 1999).

2.5 Analytical techniques

A multitude of analytical techniques are available for food. The number of instrumental methods has been steadily growing, and can be broadly categorized as chromatographic, spectroscopic, physical, and biological.

2.5.1 Chromatographic techniques

Chromatography is based on distribution or partition of a sample solute between stationary and mobile phases. Chromatographic techniques in common use today in food analysis include gas chromatography (GC), high-performance liquid chromatography (HPLC), and supercritical fluid chromatography (SFC). These often serve as a separation method when connected to another instrument such as a mass spectrometer, which serves as the detector.

Gas Chromatography (GC)

GC was introduced in the 1950s and has been applied to a wide range of foods. It is applicable to volatile substances that are thermally stabile; LC and SFC are more appropriate chromatographic methods for analysis of amino acids, peptides, sugars, and vitamins. GC is useful for analysis of nonpolar compounds, although polar compounds may be analyzed if derivatized first.

Isolation of the analyte from the sample matrix is particularly important in GC to avoid false responses from matrix degradation products. Headspace methods (including direct sampling of the headspace), distillation, and solvent extraction are often employed. Detectors include thermal conductivity (which is nonspecific), flame ionization (for most organic compounds), electron capture (mainly for pesticide residues), and flame photometric (for pesticides and sulfur compounds). The most common food analysis applications for GC involve carbohydrates, drugs, lipids, and pesticides (Lehotay, 2002).

Improvements in chromatography are constantly occurring. For instance, a new approach is comprehensive chromatography, which allows a sample to be separated along two independent axes. Comprehensive two-dimensional gas chromatography, GC X GC, consists of a high-resolution column with a nonpolar stationary phase, a modulator for separating the eluate into many small fractions, and a second column which is short, narrow, and polar. This technique has been applied to fatty acids, flavors, and pesticides (Dallüge, 2003).

High-Performance Liquid Chromatography (HPLC)

HPLC was developed in the 1960s as an improvement over column liquid chromatography and has been used to measure non-volatile food components. Spectroscopic detectors are often employed and nowadays mass spectrometry with electrospray interface is the more popular hyphenated configuration for an almost universal applicability. Normal-phase HPLC, in which the stationary phase is a polar adsorbent and the mobile phase is a non-polar solvent, is often used for fat-soluble vitamins and carbohydrates. Reversed-phase HPLC, with a non-polar stationary phase and polar mobile phase, is more popular because of its wider application. Ion-exchange HPLC, with a functionalized organic resin as packing material, is used for detection of inorganic ions and analysis of carbohydrates and amino acids. HPLC is currently the most popular food analysis technique (GC is second) and is most used for amino acids, carbohydrates, drugs, lipids, and proteins.

A new application of this technique is comprehensive two-dimensional liquid chromatography gas chromatography, LC X GC. Triglycerides can first be separated

according to double bond content and then by carbon number. Janssen et al. demonstrated fingerprinting of olive oil, which can be applied to place of origin analysis, by separation into mono-, di-, and triglycerides as well as sterols, esters, and other compound classes (Janssen, 2003).

Supercritical Fluid Chromatography (SFC)

Supercritical carbon dioxide serves as the mobile phase in SFC; an open tubular column or a packed column is employed as the stationary phase, and any GC or LC detector is used. Instrumentation first became available in the 1980s. Smith reviewed the history and applications of supercritical fluids, citing its use in separating lipids from food matrices as a chief advantage over other methods (Smith, 1999). However, SFC is prone to operational difficulties and is a normal-phase method; reversed-phase HPLC is often viewed as preferable.

2.5.2 Spectroscopic techniques

Spectroscopy is based on interactions of matter with electromagnetic radiation. Interactions can take the form of absorption and emission, and can be detected by using emission, transmission, and reflection designs. Food scientists most often deal with the ultraviolet (UV), visible (Vis), infrared (IR), radio (nuclear magnetic resonance, NMR), and microwave (electron spin resonance, ESR) regions of the spectrum, and use spectroscopic techniques for quantitative and qualitative analyses.

UV, Vis, and Fluorescence

UV and Vis spectroscopy measure absorbed radiation and have been used in food laboratories for many years. A food component that absorbs in the ultraviolet or visible range may be analyzed at its characteristic wavelength in a UV-Vis spectrophotometer, as long as there are no interfering compounds.

Fluorescence spectroscopy deals with emitted radiation, and can be three orders of magnitude more sensitive than UV or Vis spectroscopy. Many organic molecules fluoresce, including bacteria and some pesticide residues, making fluorescence spectroscopy an option for detecting food contamination.

Infrared (IR)absorption

Many molecular groups absorb IR light at specific wavelengths in an infrared spectrum, with the fingerprint region of the spectrum leading to positive identification of compounds. This long-standing technique has been expanded upon in recent years.

Fourier transform infrared (FTIR) spectrometers are now used on production lines for determining concentrations of fat, protein, and moisture. This on-line method of analysis has a large advantage over other techniques because the sample does not have to be extracted or treated in any way. Attenuated total reflectance (ATR) deals with internal reflection of IR light, and it has been used to examine sugars and trans fatty acids. High-pressure and high-temperature ATR cells have been developed.

This technique can be enhanced by using multiple internal reflection (MIR), in which light is bounced off the surface several times. Further developments in the optical components are needed before this method can be used more extensively on foods.

The newest IR technique is diffuse reflectance infrared Fourier transform (DRIFT), which measures the sum of surface-reflected light and light that has been absorbed and reemitted (Wilson, 1999). DRIFT has been employed recently to monitor production and detect compounds in coffee.

Raman

Raman spectroscopy is a complementary technique to IR spectroscopy. IR absorption depends on changes in dipole moment, meaning that polar groups have strong IR responses. Raman scattering deals with changes in polarizability of functional groups, so non-polar groups produce intense responses. Proteins and amino acids lend themselves to Raman spectroscopy, and carbohydrates, lipids, and minor food components are also examined by this technique. In addition to basic research on molecular structure, Raman spectroscopy is now being used for industrial process control (Li-Chan, 1996).

Atomic Absorption and Atomic Emission

Atomic absorption spectroscopy (AAS) is based on absorption of UV-Vis radiation by atomized minerals, whereas atomic emission spectroscopy (AES) uses the emission of radiation by a sample. Samples must usually be ashed, dissolved in water or dilute acid, and vaporized. In AAS, samples are atomized by nebulizer and burner (flame AAS), or by a graphite furnace (electrothermal AAS). Electrothermal AAS uses smaller samples and has much lower detection limits than flame AAS, but it is more costly and less precise. In AES, atomization and excitation can be performed by flame or by inductively coupled

plasma (ICP), where samples are heated to over 6000 K in the presence of argon. Both AAS and AES measure trace metal concentrations in complex matrices with excellent precision and accuracy. AAS is the more established technique, with a wider variety of instruments available, but ICP-AES can be used to measure more than one element in a sample and can measure compounds that are stable at high temperatures. Both AAS and AES have supplanted classical methods for detecting minerals in food.

Mass Spectrometry (MS)

A mass spectrometer ionizes molecules to produce charged fragments that are separated by size and charge. MS has been used for identification and analysis of complex compounds since the early 1960s. The coupling of separation techniques with MS, which began in the 1970s, has overcome the main analytical problem with chromatographic techniques — namely, ambiguity about the identity of the analyte. MS is frequently used in combination with GC, HPLC, ICP, and capillary electrophoresis, and there are tandem MS-MS instruments. Three new ionization techniques used in food analysis are electrospray ionization (ESI, where multiply charged ions are produced by repeated formation and explosion of charged droplets), heated nebulizer-atmospheric pressure chemical ionization (HN-APCI, where a gas-phase ion-molecule reaction process allows the analyte molecules to be ionized under atmospheric pressure), and matrix-assisted laser desorption/ionization (MALDI, where a sample is crystallized in a matrix of small aromatic compounds, and the crystal is subjected to a pulsed ultraviolet laser that fragments the molecules). MS techniques have been used to analyze the gamut of food components, including antioxidants, aroma compounds, carbohydrates, drug residues, lipids, peptides and proteins, toxins, and vitamins (Careri, 2002).

Nuclear Magnetic Resonance (NMR) and Electron Spin Resonance (ESR)

NMR is a spectroscopic method in which atomic nuclei that are oriented by a magnetic field absorb characteristic frequencies in the radio range. ESR deals with electrons and microwave frequencies. These techniques have several advantages: they are non-destructive, do not usually require sample separation or extraction, and can analyze the interior of a sample. Drawbacks include lower sensitivity and selectivity than some other techniques. NMR experiments are performed using continuous wave (magnetic field held constant and oscillating frequency varied, or vice versa) or pulse (short time, large amplitude) methods; ESR uses continuous wave. Available NMR instruments include low-resolution (for moisture or oil content), high-resolution liquid (analysis of liquid phase),

high-resolution solid (analysis of solid phase), and magnetic resonance imaging (three-dimensional views of cross sections of foods).

Virtually any food can be analyzed by NMR and ESR. NMR is often used to examine physical properties such as melting, crystallization, polymorphism, and oil content, and ESR is used for detecting free radicals produced in physical and chemical processes. Mannina et al. summarized the principles of NMR and applied the technique to analyzing free acidity, fatty acid profile, and sterol, squalene, and chlorophyll content as methods of authenticating olive oil (Manina, 2003).

Other Spectroscopic Techniques

Consumers rely on color, flavor, odor, and texture to determine the quality of food. Colorimeters are used to qualitate and quantitate food color, with measurements based on hue, lightness, and saturation, and are often used in conjunction with sensory and shelf-life studies. A digital camera and computer graphics software have recently been applied to the analysis of surface color of food (Yam, 2003).

Refractometry is based on the change in velocity of light by the analyte. Refractive index measurements are useful in determining concentrations of beverages, sauces, and other liquid foods. HPLC instruments sometimes have refractometer detectors.

Polarimetry is the study of the rotation of polarized light by optically active substances. Polarimetry is used to distinguish optical isomers, identify and characterize optically active substances, and measure their change in concentration during reactions. It is commonly applied to the measurement of oils in the flavor industry, sugars, and starches. Circular dichroism and optical rotatory dispersion are based on the interaction of circularly polarized light with optically active species; the former depends on wavelength and the latter on molar absorptivity. These techniques are often applied to amino acids, peptides, proteins, and complex natural products.

Ultrasonic sensors have been applied to the determination of compositional and textural properties by measurement of velocity of ultrasound waves through a sample. Ultrasonic imaging is used to examine structure in foods, but is too time-consuming for routine inspections (Coupland, 2003).

2.5.3 Physico-chemical techniques

Electrochemical

The most common electrochemical technique is the familiar pH electrode. An alternative to AAS and AES is the ion-selective electrode, which is sensitive to a particular ion. This technique is simple, rapid, and relatively inexpensive. However, these electrodes are not ion-specific, as there may be interference from ions other than those being examined.

Electrophoresis

The basis for gel electrophoresis, developed in the 1950s, is the separation of charged molecules when an electric field is applied. The main types of electrophoresis are non-denaturing, where separation is according to charge, shape, and size; denaturing or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), where the separation is primarily by molecular weight; isoelectric focusing, which separates by charge; and two-dimensional, with separations in perpendicular directions by isoelectric focusing and SDS-PAGE. The analysis is carried out on a porous gel and is mainly used for studying nucleic acids and proteins.

Capillary electrophoresis (CE), developed in the 1980s, uses a capillary tube and photometric detection. CE techniques include capillary zone electrophoresis, for charged analytes, and micellar electrokinetic chromatography, for neutral analytes. CE has been applied to analysis of amino acids, carbohydrates, proteins, and vitamins, as well as additives, natural toxins, and antibiotic and pesticide residues. Detection limits are relatively high, however, because of the low sample volume (Dong, 1999).

2.5.4 Biological techniques

Catalytic biosensors: Enzyme and Microbial Sensors

Biosensors consist of a biological recognition element that produces a quantifiable response in a signal transduction element when in contact with the analyte. Enzyme biosensors use enzymes to generate products that are detected by acoustic, electrochemical, optical, and photothermal transduction elements. Microbial biosensors use genetically modified microorganisms that are immobilized on a membrane or trapped in a matrix, with the transduction mechanism consisting of an oxygen or pH electrode, or a luminometer if luciferin is added. A popular type of optical element developed in the 1990s is surface plasmon resonance. These types of biosensors are often applied to detection of

contaminants such as herbicides, pesticides, pathogens, and toxins, as well as food components such as carbohydrates and amino acids. Improvements in response time, sensitivity, and specificity are needed for wider acceptance of this technique (Patel, 2002; Mello, 2002).

Affinity biosensors:Immunosensors

Immunosensors are biosensors in which the biological recognition elements are antibodies that are attached to a solid support and bind to a particular antigen or antibody in the sample. The most common immunoassay is enzyme-linked immunosorbent assay (ELISA), in which an enzyme-linked antibody is applied after the antigen or antibody is bound. The antibody/antigen interaction is specific enough to allow detection of species of origin, and it is also used to detect allergens, enzymatic inactivation, genetically modified organisms, microbial contamination, and toxins.

The principles of the immunological methods are presented in the next chapter.

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CHAPTER 3 – Immunoassays in food analysis

3.1 Introduction

For the first screening of contaminants in foods several technologies were used as an alternative to conventional reference analytical methods. Of these, immunological methods are probably the best known and a high number of immunoassays are now available.
The concept of immunoassay was first described in 1945 when Landsteiner suggested that antibodies could bind selectively to small molecules (haptens) when they were conjugated to a larger carrier molecule. This hapten-specific concept was explored by Yalow and Berson in the late 1950s, and resulted in an immunoassay that was applied to insulin monitoring in humans. This pioneering work set the stage for the rapid advancement of immunochemical methods for clinical use.
The first application of immunologically based technology to pesticides was not reported until 1970, when Centeno and Johnson developed antibodies that selectively bound malathion. A few years later, radioimmunoassays were developed for aldrin and dieldrin and for parathion. In 1972, Engvall and Perlman introduced the use of enzymes as labels for immunoassay and launched the term enzyme-linked immunosorbent assay (ELISA). In 1980, Hammock and Mumma described the potential for ELISA for agrochemicals and environmental pollutants. Since then, the use of immunoassay for food analysis has increased dramatically. For example, immunoassay technology has become a primary analytical method for the detection of products containing genetically modified organisms (GMOs).

The advantages of immunoassay technology relative to other analytical techniques include the following:

- low detection limits
- high analyte selectivity
- high throughput of samples
- reduced sample preparation
- versatility for target analytes
- cost effectiveness for large numbers of samples
- adaptability to field use.

As is the case with every analytical method, immunoassay technology has limitations, including:

- interferences from sample matrices
- cross reactivity to structural analogs of the target analyte
- low availability of reagents
- longer assay development time than some classical analytical methods.

The immunoassay is clearly not the best analytical method for all analytes in all situations. However, immunoassay technology is important for the analyst because it complements the classical methods, thus providing a screening method for many compounds and the only reasonable analytical choice for others. Most immunoassays can be used to obtain quantitative results with similar or greater sensitivity, accuracy and precision than other analytical methods. They are generally applicable to the analysis of small molecules, including pharmaceuticals and pesticides, identification of pest and beneficial species, characterization of crop quality, detection of GMOs, product stewardship, detection of disease and even monitoring for bacteria and bioterrorism.

3.2 Principles of immunoassays

3.2.1 Binding reagent

The basis of all immunological methods is the recognition of an analyte, the antigen (Ag), by a reagent, the antibody (Ab), directed against it.
Antibodies are produced by lymphocytes of vertebrates as part of the defence reaction to the invasion of bacteria, viruses, foreign proteins and other macromolecules, the so-called antigens. The sera of these vertebrates contain a number of antibodies (i.e. polyclonal antibodies) which are specific for the same antigen, but as they are produced by different cells, they may belong to different antibody classes and may recognize different parts of the molecule, i.e. different epitopes with different affinities. The selection of a single cell which produces only one type of antibody with the desired specificity and affinity and its fusion to immortalized cells leads to the production of monoclonal antibodies. These can be produced in cell culture in large amounts with a constant quality (Campbell, 1991).
Most of the chemical contaminants found in food are too small (less than approximately 1000 Da) to be antigenic, i.e. to activate the immune system of vertebrates. Therefore,

they are called haptens. Antibodies against haptens can be obtained only if they are covalently coupled to a carrier protein (Hock, 2000; Vanderlaan, 1988). After injection of this immunoconjugate a number of antibodies with different specificities are produced by the animal. Among these antibodies are some recognizing the hapten and some binding only epitopes on the carrier protein or the linker between protein and hapten. Careful selection leads to the desired cell lines.

Antibodies are glycoproteins with a Y-structure and two binding sites for the antigen. The binding of the antigen is non-covalent using all forms of bonding which mediate protein-protein interaction including hydrophobic interaction (Rabbany, 1994). Structural loops which are responsible for the high affinity of these interactions can be formed in both molecules. It was also found that in the majority of binding reactions both the antigen and the antibody change their structure to optimize the interaction, thus resembling more of a handshake than a 'key-lock' mechanism (Campbell, 1991).

The reaction between free antigen and antibody leading to the antibody-antigen complex is treated as an equilibrium reaction. The affinity constant (K_A) is then defined as the ratio of the kinetic constants of the association reaction and of the dissociation reaction.

Affinity describes the amount of antibody-antigen complex that will be found at equilibrium. High-affinity antibodies (i.e. K_A 10^{12} $L.mol^{-1}$) will bound larger amounts of antigen in a shorter period of time then low-affinity antibodies (i.e. K_A 10^4 $L.mol^{-1}$).

In immunoassays for food analysis polyclonal (PAb) and monoclonal (MAb) antibodies are used. Thanks to their specificity of binding, their homogeneity and their ability to be produced in unlimited quantities, MAbs are preferred to polyclonal ones, but they have several disadvantages:

- Difficulty selecting a suitable clone
- Difficulty scaling up production to commercial levels
- The need to separate the single Ab produced from other cell proteins
- Practical limitation to rodent species

Many of these problems are addressed by new molecular biologic techniques that have revolutionized the production of Ab-like molecules (Jones, 1986; George, 1997; Dall'Acqua, 1998).

By using recombinant DNA and amplification technologies, binders can be produced from "naïve" DNA libraries, eliminating the need for immunization. Variable regions of the Ab, making up the complementarity determining regions, can be linked with constant regions to mimic a native Ab or can be linked in a much simpler fashion to produce a single chain fragment variable region Ab, which can be joined to produce multivalent binders (one type,

the chelating recombinant Ab, achieves higher binding affinity by recognizing adjacent epitopes on a large Ag) (Neri, 1995) or modified in other ways desired for a particular assay. Selection of suitable clones is made easier by phage display and other display technologies, and production can be performed in *Escherichia coli* or in completely acellular systems.

Investigators also have looked toward entirely different classes of molecules as possible binding reagents. Considerable promise has been shown by aptamers, which are polynucleotides with an affinity for a particular ligand (Osborne, 1997; Ellington, 1990; Famulok, 1999).

From a mixture of random polynucleotides, the ligand is used to pull out sequences for which it has affinity, then these are amplified using the polymerase chain reaction (PCR). After inducing further variations in the selected sequences, the cycle can be repeated. This process, termed SELEX (systemic evolution of ligands by exponential enrichment), eventually can yield molecules with affinities and specificities comparable to those of MAbs.

The affinity of aptamers can be enhanced further by chemical modification; for example, an aptamer directed against an enzyme can be tethered to an inhibitor that binds tightly to the active site.

Molecular imprinting is another approach to producing polymers with desired binding properties (Ansell, 1996; Andersson, 1997; Haupt, 1998). The polymerization of some type of versatile, monomeric unit is guided by a molecular template made up of the analyte of interest. Polymerization typically is allowed to proceed to formation of a solid mass, from which a particulate binding reagent is produced by grinding. Hence, this technique does not offer the advantage of a pure, defined binder, although it seems that binding properties can be reproduced fairly well. Molecular imprinting may be especially well suited to small molecules with limited aqueous solubility.

Synthetic soluble ligand binders ultimately could have the advantages of a completely defined structure, completely defined binding properties, and lower production costs. They also could be tailored to the overall assay design, for example with built-in fluorescence or enzymatic labeling. However, the cost of developing such ideal binders will be high, and the incentive has been diminished by the generally satisfactory performance of Abs in existing assays.

3.2.2 Imunoassay formats

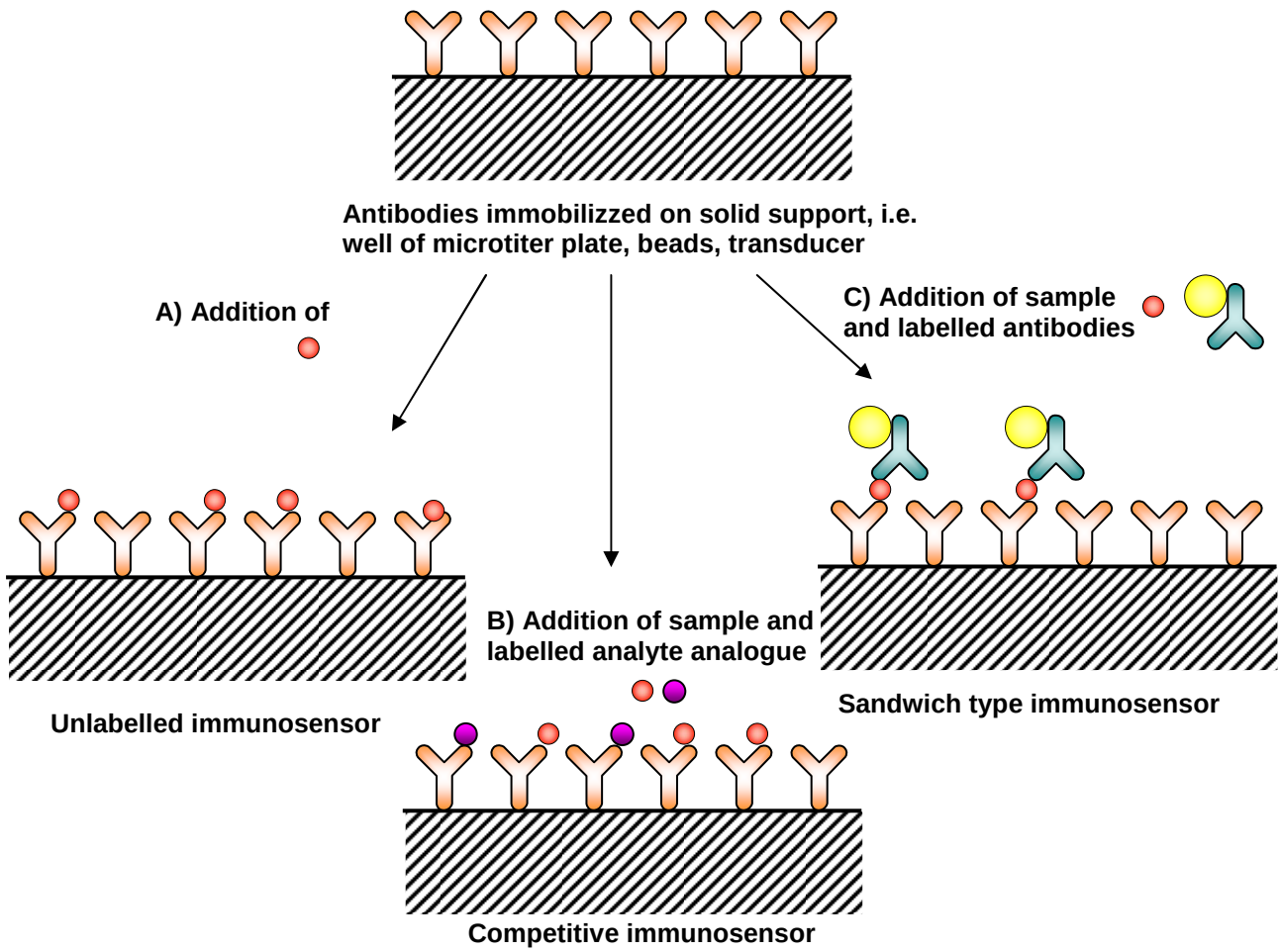
The use of antibodies as analytical reagents leads to particular assay formats and detection principles, which can generally be divided in those relying on the determination of labels and those managing without labels. Label-free assays are based on either secondary immune reactions such as precipitation and agglutination or physical transducers such as piezoelectric crystals or integrated optical elements (Rogers, 1998). Antibodies (Figure 1A) or antigens or corresponding conjugates are immobilized onto the transducer, which allow real-time monitoring of the binding reaction. This principle is directly related to the development of immuno- or, more in general, of affinity sensors. It gained attention because it requires only the addition of the sample to the immobilized antibody followed by the regeneration of the transducer surface. However, at present it is applicable only if the binding molecule has a sufficient size or molecular weight.

Therefore, in particular for the determination of rather small molecules such as pesticides, mycotoxins, antibiotics, or hormones indirect formats based on labelled compounds are most commonly used, with the enzyme linked immunoassay (ELISA) being the best established one (Tijssen, 1985; Martlbauer, 1998). Due to the small size of these compounds, binding of only one antibody at a time is possible. Thus, the competitive assay format is used with the analyte from the sample competing either with an analyte enzyme conjugate for the limited number of immobilized antibody-binding sites (Figure 1B) or with immobilized analyte (derivatives) for binding sites of added labeled antibodies (this format is also called inhibition assay). The signal is obtained from the addition of enzyme substrates which are turned into colored, fluorescent or chemiluminescent active products. It decreases with increasing analyte concentration.

In some cases, e.g. the detection of toxins being proteins originating from pathogenic bacteria or of proteins being indicative for infection with a disease or a pathogen, the analyte possesses more than one epitope and is of sufficient size to allow simultaneous binding of two antibodies. This leads to so-called sandwich-type immunoassays, in which the analyte is first bound to an immobilized capture antibody and then by an enzyme-labeled detection antibody. Again, the signal is obtained after adding the enzyme substrates, yet here the signal increases with analyte concentration since binding of the detection antibody occurs only at those sites where the analyte was previously captured (Figure 1C). This type of assay is approximately one to two orders of magnitude more sensitive than competitive assays. A number of assays based on these principles are commercially available either as 96 well microtiter plate assays allowing the parallel

quantitative analysis of a number of samples or as dipstick formats for semi-quantitative screening of single food samples.

Figure 1: Immunosensor principles based on immobilized antibodies; (**A**) direct sensing of the binding reaction of the analyte; (**B**) competitive assay formats based on analyte tracers, the label can be an enzyme, a fluorophore or even a radioisotope; (**C**) sandwich assay formats based on a second labeled antibody.



Fluorescent dyes can also be used instead of enzymes as labels, the assays are then called fluorescence immunoassays (FIA). Detection can be based on the determination of the intensity of light emitted by the fluorophore used as label or being formed after the addition of enhancer solutions, or by energy transfer between two suitable dyes, on the influence on polarized light, or on time-resolved measurements taking advantage of the longer fluorescence lifetimes of the fluorescent probe compared to the non-specific background (Hage, 1999). Additional assay formats are possible if anti-idiotypic antibodies are available (Hennion, 1998) and by combining homogeneous incubation of reagents with

heterogeneous affinity reactions.
The same basic principles and the advantages of the specificity of antigen antibody recognition are also utilized in immunoaffinity chromatography (IAC) or other principles of immunocapture (IC). The recognizing antibody is immobilized on a suitable support, which may be magnetic beads or chromatographic material filled in a column, and then incubated with the sample. Separation of this support from the sample matrix leads to purification and enrichment of the analyte simultaneously. The analysis is often done by conventional methods such as HPLC after eluting the analyte.

3.2.3 Antibodies immobilization

Antibodies are proteins and as such they can be immobilized by all methods described for protein immobilization. However, in practice only some methods are often used.
Conventional ELISAs are performed in microtiter plates, which are made of polystyrene activated for protein adsorption. Thus, the wells of these plates are coated with antibodies by incubating a defined volume of an antibody solution for several hours. Antibodies not adsorbed after the incubation are removed by washing the wells with a suitable buffer.
The microtiter plates are used as disposables requiring no regeneration.
Most of the immunosensor systems are designed for repeated use. Therefore, the proteins are often covalently immobilized using carriers with suitable functional groups.
The latter can be introduced by modification with silanising reagents. Chemical binding of proteins may cause severe conformational change on the protein structure due to, for example, multiple binding sites and thus may cause a significant decrease of binding capacity. Modification of proteins with low molecular weight compounds such as biotin has proven to be a more gentle procedure, and since tight immobilization of proteins can be accomplished by taking advantage of the strong affinity between biotin and biotin-binding proteins (avidin, streptavidin, neutravidin), this method has become established as an alternative to covalent binding. Moreover, the amount of immobilized protein can easily be defined by the amount of biotinylated protein, as quantitative binding can be expected.
Regeneration of the antibody analyte binding sites can be achieved by a number of solutions, such as those containing ethylene glycol, urea, or thiocyanate or buffers of different pH. However, these regeneration protocols usually affect the binding capacity of the immobilized protein (Dietrich, 1995; Kramer, 1997), leading to a limited reusability of the system (Ivnitski et al., 1999) and requiring reversible immobilization of the antibodies. Thus, alternatively, the binding of antibodies to materials used for affinity purification of

antibodies, such as carriers with covalently immobilized binding proteins, e.g. protein G or protein A, is used as a quasi-reversible antibody immobilization method, as the antibodies can be eluted without damage of the binding material and freshly reloaded prior to the next assay (Rogers, 1998).

Immobilization of antibodies, antigens or binding proteins leads to affinity reactors, through which all solutions are pumped or in which all solutions are incubated and where the affinity reactions and reactions of the tracers take place leading to detectable signals.

These reactors are flow-through columns filled with beads (Dietrich, 1995; Kramer, 1997), glass capillaries, silicon chip reactors, or simple conical propylene cells in which antibodycontaining electrodes were inserted (Pemberton, 1998). They can be microtiter wells to which optical fibers are attached for monitoring the optical densitiy of the solution or in which an electrode is integrated to determine the amount of electrochemically active product of the enzyme reaction (Skladal, 1995; Dequaire, 1999). Even a membrane or the surface of a transducer can serve as an affinity reactor.

3.3 Immunosensor systems requiring no labels

Acoustic sensors

Piezoelectric crystals couple mechanical deformation and electric voltage. They oscillate at a particular vibration frequency, generally in the range of acoustic waves between 9 and 14 MHz, with the resonant frequency being directly related to the mass deposited on the exciting electrodes.

Different types of waves can be generated, allowing distinction between bulk acoustic wave (BAW) sensors and surface acoustic wave (SAW) sensors (O'Sullivan, 1999). Since SAW devices are operated at higher frequencies, they are intrinsically more sensitive than BAW devices. Yet the latter ones are usually applied to food-relevant analyses probably because of the good availability of transducers and well-established measuring principles. The sensors are quartz wafers in the form of 10–16 mm disks, squares or rectangles, which are approximately 0.15 mm thick and sandwiched between two electrodes made of gold (Guilbault, 1994).

These electrodes are used to induce an oscillating electric field perpendicular to the surface of the wafer. The electric field then produces a mechanical oscillation, a standing wave, in the bulk of the quartz wafer. When placed in an electronic oscillator, the portion of the quartz wafer located between the electrodes vibrates with its precise fundamental frequency. The frequency output from the oscillator is identical to the resonant frequency

of the crystal and can be measured with a frequency counter. Binding of compounds to the electrodes due to, e.g., a biochemical binding reaction leads to an increase in mass and consequently, to a decrease of the resonant frequency. First reports were based on measurements in the gas phase only, requiring careful drying of the sensor surface after its incubation in a liquid sample. Later flow-through cells were developed, where only one side of the crystal is in contact with the flowing solution (Horacek, 1997). This allows real-time monitoring of the affinity reaction (O'Sullivan, 1999).

Optical immunosensors

Optical immunosensors described here are based on optical waveguide structures which are used as substrates for biospecific layers, e.g. for immobilized antibodies.

Electromagnetic waves propagate in a dielectric material which is embedded in a material of lower refractive index by multiple internal reflection at the boundary of the two materials. An external light beam is only then able to establish a guided wave in a dielectric if energy transfer occurs between the light beam and the guided mode. Thus, a laser beam is focussed either on the end face of the waveguide or, more commonly, prism, lens or grating coupling is used. Changing the angle of the external light beam with respect to the boundary of the waveguide modifies the exciting electromagnetic field until it matches the guided mode (Kooyman, 1997).

An optimal angle can be defined for each waveguide structure. From an optical viewpoint surface binding processes change the waveguide configuration, in particular the refractive index profile, and by this the propagation velocity of the guided light wave. This can be measured, for example, through changes in the coupling conditions, i.e. through changes of the angle of the light beam leading to an excitation of guided modes.

There is a strong similarity between the excitation of guided modes in a dielectric material, i.e. the coupling of light into a waveguide, and the excitation of a surface plasmon in a metal layer. Both interact by the evanescent part of the electromagnetic field with the sample contacting the metal or the dielectric layer and hence both are used in label-free immunosensor systems (Tiefenthaler, 1992) as they can measure binding of molecules to the waveguide surface through resulting changes of the refractive index. The lower detection limit for a given compound depends on the sensitivity of the device to monitor small changes of refractive index and also on the molecular weight of the compound of interest, because changes of the refractive index are directly proportional to the increase in the mass loading on the waveguide surface due to binding of the analyte. Thus, up to now this approach is applicable only for the detection of high molecular weight analytes.

However, these sensing principles offer additional features, as they show an inherent surface sensitivity, and hence both the immobilization of the biorecognition layer on the surface and the following binding of the corresponding analyte can be monitored in real time. This allows immobilization under more or less standardized conditions as well as the evaluation of kinetic data, i.e. association and dissociation rate constants, and steady-state data suitable for quantitative analysis.

3.4 Immunosensor systems using labels

The integration of labels or labeled compounds in immunoanalysis is well-established in ELISA and FIA (fluorescence immunoassay), and bead-coupled (e.g. latex, gold) immunoassay protocols. Compared to label-free formats detection of specific properties of the labels leads to improvements of the specificity, sensitivity, and lower detection limit of the assay. Hence, labeled compounds, i.e. tracers, are also introduced in immunosensor systems, although they require at least the tracer as additional reagent, often even additional incubation and washing steps and, consequently, more complex devices if they are to be fully automated. In the following, the most important principles of such systems are described considering only enzymes and fluorophores as labels.

Fluorescence sensor systems

Fluoroimmunoassays have increased in interest for almost 20 years as an alternative to radioimmunoassays in using cheap, stable and safe reagents and resulting in rapid and sensitive assays (Hemmila, 1985). Moreover, fluorescence detection is well-established for DNA-analysis using fluorescent intercalators or fluorescence-labeled primers.

Fluorescence sensors are based either on optical fibers or on integrated optical devices. As described above, light propagates in dielectric materials if the electromagnetic waves of the light and the guided modes in the dielectric match. Traveling of the light beam in the waveguide leads to an electric field also in the lower refractive index material; this field decreases exponentially with increasing distance to the boundary between the materials. This electric field is called evanescent field and has a penetration depth of 50–500 nm for visible light. It can be used to excite fluorescent dyes and to collect emitted fluorescent light if the optical properties match. Thus the use of optical fibers or waveguides as transducers in fluorescence affinity sensors allows a separation of bound and unbound tracers through the evanescent field. As antibody labeling with fluorescent probes is an established procedure of immunostaining techniques, most of the fluorescent

immunosensor systems are based on either an inhibition assay format (Bier, 1992; Klotz, 1998) or a sandwich format (Rowe, 1999), depending on the analyte to be determined. However, the use of labeled analyte analogues is also reported (Meusel, 1998; Charles, 1999).

Chemiluminescent systems

Exploitation of chemiluminescent (CL) or bioluminescent (BL) reactions in immunoassay falls into two main areas: components of a chemiluminescent (e.g., luminol) or bioluminescent reaction (e.g., a luciferase, aequorin) can be used as a label, or a chemiluminescent or bioluminescent reaction can be used to monitor an enzyme label or its products.

Chemiluminescent molecules exploited as labels include luminol, isoluminol, acridinium esters, thioesters and sulfonamides, and phenanthridinium esters. Separation and nonseparation assays have been devised, based on isoluminol and acridinium ester labels (Kriska, 1991). Isoluminol derivatives (ID) have been most widely studied in the preparation of tracers for immunoassay because of their high quantum efficiency, low molecular weight, well-known chemical structure, low cost and high stability. Moreover the light efficiency of some ID may be modified by specific binding to the antibody, thus allowing the development of homogenous immunoassays requiring no bound/free separation step. Recently the labelling of streptavidin with amino-buty-ethyl-isoluminol allowed the development of very sensitive immunoassay methods which take advantage of the biotin-avidin system (Messeri, 2005).

To provide a highly sensitive analytical system, CL and BL can be used to analyze the amount of product generated by enzyme labels. CL and BL methods have been developed for many enzyme labels, e.g., alkaline phosphatase, glucose-6-phosphate dehydrogenase, horseradish peroxidase, Renilla luciferase, and xanthine oxidase. Currently, the most successful enzyme assays are the enhanced CL method for a peroxidase label involving a mixture of luminol, hydrogen peroxide, and an enhancer (e.g., p-iodophenol) and the direct CL method for alkaline phosphatase, with an adamantyl 1,2-dioxetane phenyl phosphate as substrate. Both systems are very sensitive and produce long-lived light emission.

Enzymes as labels

Immunosensor systems based on the use of enzymes as labels can follow the same basic principles as enzyme immunoassays (Hage, 1999; Hennion, 1998) performed in microtiter plates. The sensor systems differ from ‘conventional’ immunoassays in the degree of

automation, the detection principle, the solid phase to which the antibody is bound, and the reusability of the bio-recognition layer. Reports deal with either horseradish peroxidase (HRP), alkaline phosphatase (AP), or urease as label, for which chromogenic, fluorogenic, chemiluminescent and electrochemical substrates are described. Thus, a protocol realized in a certain sensor system may be comparable to that of a microtiter plate assay comprising the steps of (1) antibody immobilization, (2) addition of sample, (3) addition of enzyme tracer (both solutions may be premixed and applied simultaneously, (4) washing, (5) addition and incubation of enzyme substrates, and (6) recording of the signal. A final step of (7) regeneration is included in reusable devices.

Due to the complexity of the resulting procedure automation is achieved by the use of flow systems in which the various solutions are supplied and incubated in the ‘immunoreactor‘ through computer-controlled pumps and valves. Systems are described which allow the automated performance of the whole assay and which are thus suitable for direct coupling of the device to a sample line and thus for on-line monitoring of a process (Dietrich, 1995; Kramer, 1997). Most of the systems were described for the determination of pesticides in water. But there are also reports on the application to milk (progesterone) (Pemberton, 1998), poultry (Salmonella) (Dill, 1999) and other food (Brooks, 1992).

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CHAPTER 4 – Chemiluminescence

4.1 Introduction

Chemiluminescence (CL) is the light emission produced by a chemical reaction in which chemically excited molecules decay to the ground state and emit photons. Bioluminescence (BL) is a type of CL which naturally occurs in living organisms and is also used in vitro. Measurement of light from a chemical reaction is very useful from an analytical point of view, because in appropriate experimental conditions the light output intensity is directly related to the analyte concentration, thus allowing precise and accurate quantitative analysis. In addition, the kinetics of the light emission is usually a steady-state glow type, which simplifies sample handling and measurement procedures.

CL as an analytical tool has several advantages over other analytical techniques that involve light (mainly absorption spectroscopy and fluorometry): high detectability, high selectivity, wide dynamic range and relatively inexpensive instrumentation.

The superior detectability of CL measurements is partly due to a low background. In luminescence measurements two components of light reach the detector: the first one (i.e. the net analytical signal) is proportional to the analyte concentration, while the second component (i.e. the background) is an approximately constant light level due to various factors such as the phosphorescence of plastics, impurities in the reagents, emission from other sample components, detector dark current. Warm-up and drift of light source and detector, and interference from light scattering present in absorption and fluorescence methods are absent in CL, making the background light component much lower and thus achieving a significant gain in sensitivity.

Selectivity derives from the fact that the analyte of interest generates its signal in the presence of compounds, which normally interfere in fluorescence measurement, and that do themselves not produce light when the chemiluminescent reagents are mixed together.

Wide dynamic ranges allow samples to be measured across decades of concentrations without dilution or modification of the sample cell. This is due to the way the chemiluminescent signal is generated and measured, i.e. using no excitation source for light production and a phototransducer with an inherent wide range of response for light detection.

The light emitted from chemi- and bioluminescent reactions is typically measured using a luminometer. Luminometers are simple, relatively inexpensive instruments designed to

measure sample light output, generally by integrating light emission for a given period of time. All luminometers basically consist of a sample chamber, a detector and a signal processing apparatus, and are used to measure emission from different sample formats (the most common being single tubes and microtitre plates).

Photodiodes and photomultiplier tubes (PMTs) are the detection devices commonly found in commercial luminometers. Even if improvements in photodiodes have made them suitable for some applications, PMTs are still the detectors of choice for measuring extremely low levels of light.

A relatively recent advancement in light detection technology for analytical purposes is represented by low-light imaging devices based on intensified Vidicon tubes or high sensitivity charge-coupled devices (CCDs). These luminescence imaging instruments, also known as luminographs, allow not only the measurement of light intensity at the single-photon level, but also the spatial distribution of the light emission on a target surface to be evaluated.

CL is utilized in various analytical techniques in which small amounts of analytes are detected and quantitated by measurement of the light emission (DeLuca, 1978; Kricka, 1984; Baeyens, 1990). Chemiluminescent reaction systems often involve enzymes, such as alkaline phosphatase (AP) and horseradish peroxidase (HRP), and suitable CL substrates which allow for the detection of enzymes with very high efficiency (Bronstein, 1989; Beck, 1990; Thorpe, 1986; Thorpe, 1987). These enzymes are widely used as labels in the development of immunoassays (Kricka, 1988; Ashihara, 1994) blotting (Schneppenheim, 1987; Bronstein, 1990) and gene probe assays (Matthews, 1985). Coupled enzymatic bio- and chemiluminescent analytical methods have also been developed: in particular, ATP involving reactions (kinases) have been coupled with the firefly luciferin-luciferase system, NAD(P)H producing or consuming enzymatic reactions (dehydrogenases) with bacterial luciferases, and the luminol/H_2O_2/HRP system has been coupled with oxidase enzymes (Roda, 1989; Carrea, 1986). Advances in molecular biology and the increasing need of ultrasensitive assays have led to the development of novel luminescence systems for a wide variety of applications in genetic research, food technology, environmental monitoring and clinical chemistry, so that CL-based immunoassay and gene detection kits became commercially available and routinely used (Kricka, 1988; Ashihara, 1994; Matthews, 1985; Bronstein, 1990).

CL imaging also represents a very promising detection system which is increasingly used for the ultrasensitive quantitation and localization of analytes in a wide range of applications (Nicolas, 1994; Hooper, 1994). CL imaging is suitable for filter membrane

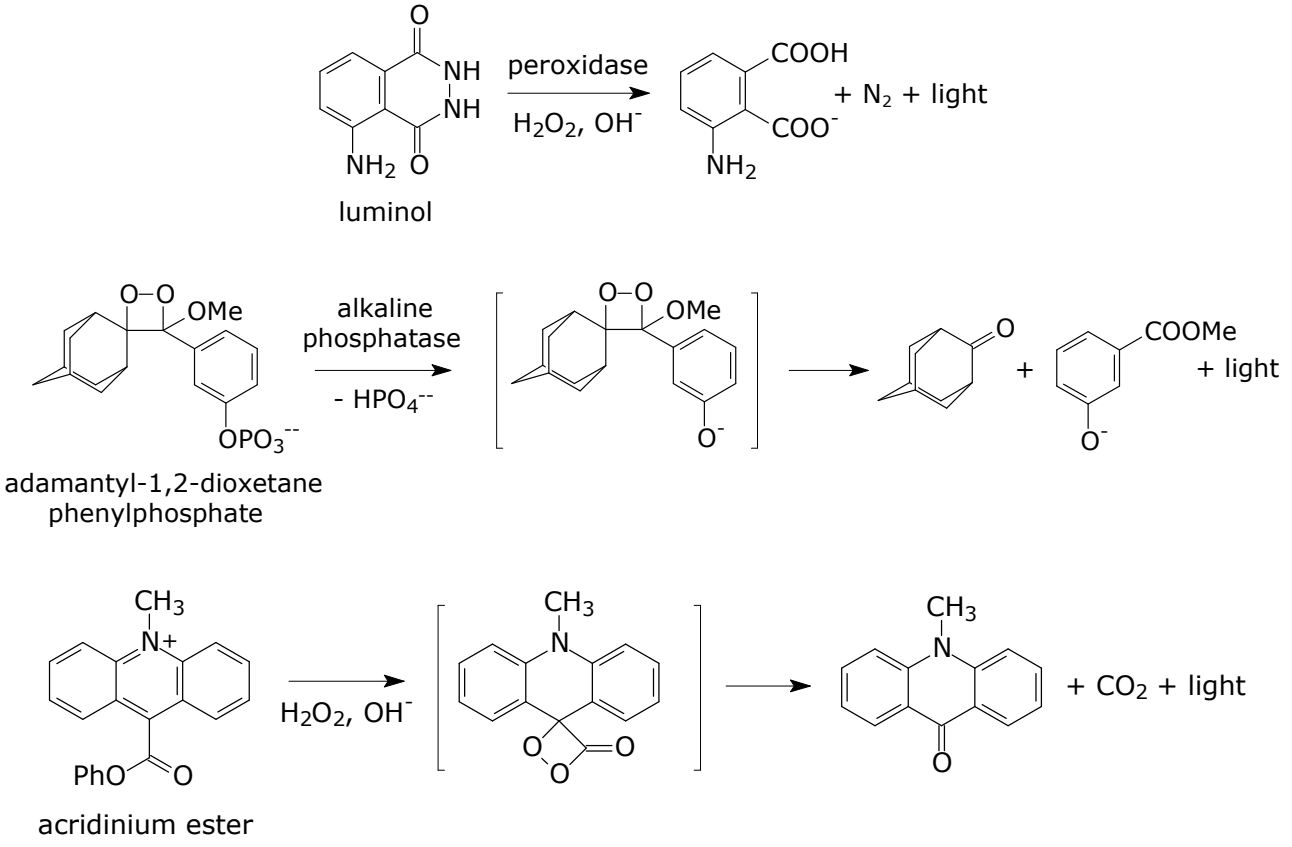
biospecific reactions, such as the Southern, Northern, or Western blot tests, and dot blot hybridization reactions. In these techniques nucleic acids or proteins are either blotted on filter membrane after separation by gel electrophoresis, or directly dotted on the membrane. Nucleic acids are then hybridized with a complementary gene probe labeled with a hapten and detected by anti-hapten antibody conjugated with an enzyme and CL substrate; proteins are detected by specific antibody followed by anti-antibody conjugated with an enzyme and CL substrate. The main advantage with respect to other detection systems (i.e., colorimetric or even chemiluminescent with photographic detection) is the direct and rapid quantitative evaluation of the signal over a wide dynamic range. It can be used for the measurement of CL signals in microtitre plates, with the advantage of a one-step measurement of the emission from the whole plate (differently from conventional luminometers, that usually read the emission well by well or strip by strip). This may be an important advantage in analytical methods relying on the kinetic behaviour of the CL emission. CL imaging is also used to detect BL and CL in whole organs or plants, and on any kind of surfaces. It should be noted that CL imaging devices do not possess the very wide dynamic range characteristic of photomultiplier tube-based instruments. However, this limitation can be partly overcome by taking advantage of the intrinsic wide dynamic range of CL as detection principle.

When coupled with an optical microscope, CL imaging is a very potent analytical tool for the development of ultrasensitive enzymatic immunohistochemistry (IHC) and *in situ* hybridization (ISH) assays, allowing spatial localization and semiquantitative evaluation of the distribution of the labeled probe in tissue sections or single cells to be performed.

4.2 Chemiluminescence reagents

Labeling with enzymes is generally preferred to labeling with CL substances because of the possibility to obtain a higher sensitivity due to the amplification of the CL signal in the presence of an excess of the CL substrate. Moreover, with enzyme labels glow-type light emission kinetics can be usually obtained (e.g. the CL emission reaches a steady-state intensity), which permits both easy handling and standardization of the experimental conditions and quantitation of the labeled probe under investigation, because the steady-state light intensity is directly related to the enzyme activity.

Figure 1: CL reactions commonly used in analytical chemistry



AP and HRP are probably the most widely used CL labels, thanks to the availability of many different CL substrates which originate glow-type emission kinetics and permit the detection of very small amounts (in the order of 10^{-18}-10^{-21} moles) of CL label. The most sensitive and widely investigated CL substrates for AP are based on adamantyl-1,2-dioxetane aryl phosphate derivatives (García-Campaña, 2001; Ito, 2003) while commercial substrates for HRP are mainly luminol-based reagents (**Figure 1**). Adamantyl 1,2-dioxetane aryl phosphates are dephosphorylated by AP to give an unstable intermediate; decomposition of this intermediate produces an excited-state aryl ester that emits light. Luminol and its derivatives are oxidized by peroxides (usually hydrogen peroxide) giving light emission. This CL reaction can be catalyzed by enzymes other than HRP (e.g. microperoxidase and catalase) and by other substances (haemoglobin, cytochrome *c*, Fe(III) and other metal complexes). The presence of suitable molecules such as phenols (*p*-iodophenol), naphthols (1-bromo-2-naphthol) or amines (*p*-anisidine) increases the light production deriving from the HRP-catalyzed oxidation of luminol and produce glow-type kinetics (Van Dyke, 2002; Haddock, 2000). The use of other enzymes, such as glucose-6-

phosphate dehydrogenase (Roda, 2000), β-galactosidase (O'Connell-Rodwell, 2002) and xanthine oxidase (Laxman, 2002; Paulmurugan, 2002; Contag, 2002) as CL labels has been reported.

4.3 Chemiluminescence and immunoassay

The sensitivity and detectability demonstrated by luminescence immunoassays are similar to, or even better than, those of radioisotopes. Indeed, for routine clinical analysis, radioimmunoassays have been almost completely replaced by luminescent immunoassays that are performed by fully automated, random access systems optimized for clinical chemistry. Chemiluminescent immunoassays are also being utilized in other fields as well, including environmental and food analysis. Among the various immunoassay formats, CL offers by far the best performance when applied to sandwich-type assays, the sensitivity of which is determined primarily by the detection limit of the label. Most CL immunoassays are in a heterogeneous format, which requires separation between bound and free tracer that is usually achieved by immobilizing an immunoreagent on a solid-phase such as assay tubes, microtiter plate wells, or microparticles. The separation step also removes the sample constituents which will thus not affect the subsequent CL reaction.

Chemiluminescent immunoassays employ tracers obtained either by direct labeling of antigens or antibodies with CL molecules, or labeling with enzymes detectable with CL substrates. Alternatively, tracers may be obtained labeling antigens or antibodies with ruthenium (II) tris(bipyridyl) which is detected by electrogenerated chemiluminescence. Very efficient CL substrates are available for HRP and AP, which are the label enzymes most commonly used in immunoassay. Utilizing dioxetane phosphate substrates, 10-20 moles of AP, corresponding to about 6000 molecules, was detected, which is the lowest detection limit so far achieved in CL enzyme detection (Bronstein, 1989). Recently, Maeda et al. reported the development of a new bioluminescent enzyme immunoassay (BLEIA) for hormones that utilizes acetate kinase as a label and in which the ATP produced by the label-catalyzed reaction is measured with the conventional luciferin/luciferase system: with this method a detection limit of $1\text{-}2\text{x}10^{-20}$ moles of analyte was achieved (Ito, 2003).

The future of BL/CL immunoassays looks bright, thanks to the availability of new biotechnological tools. BL immunoassays have been developed using photoproteins (e.g. recombinant aequorin) (Mirasoli, 2002) or even a firefly luciferase-coding DNA fragment as labels (Chiu, 1999). Various bifunctional bioluminescent molecules have been prepared and proposed for the development of immunoassays. Examples include: a thermostable

firefly luciferase–biotin complex obtained by fusing the BL protein with a biotin acceptor peptide (Tatsumi, 1996); chimeric antibodies incorporating a specific antibody and a BL label (Casadei, 1990); and BL/CL tracers for competitive immunoassays, obtained by fusing a BL protein or an enzyme suitable for CL detection with a protein or peptide analyte (Grigorenko, 2001). The latter approach is much more convenient than traditional chemical conjugation because it provides virtually unlimited amounts of a tracer characterized by a constant 1:1 analyte:label molar ratio, which is particularly suited for developing ultrasensitive assays. Fusion proteins have also been used for developing BRET-based open-sandwich BL immunoassays (OS-BLIA), which were demonstrated to be more sensitive than comparable FRET-based assays (Arai, 2001). The assay is based on the antigen-dependent reassociation of the antibody heavy chain and light chain fragments, each one genetically fused either to Rluc or to EYFP.

4.4 Chemiluminescence and high-throughput screening technology

High-throughput screening technology is moving towards miniaturized, high-density analytical formats. Microtiter plate miniaturization has been achieved by increasing the microwell density from 96 to 384 or 1536 wells per microplate. Nanoliter-capacity-well arrays have also been proposed for the design of highly parallel analytical methods, as demonstrated with a BL ATP assay (Young, 2003). Small well volumes often result in fluid handling problems that have been partly overcome by “lab on a chip” approach, which combines the advantages of microwell-based analytical formats with the high density of microarrays (Kanigan, 2003).

Microarrays on glass, silicon, or other surfaces can contain up to thousands of probe molecule spots per square centimeter. In principle, any type of ligand-binding assay that relies on the binding of a target molecule present in solution by an immobilized capture molecule (e.g., nucleic acid–nucleic acid, antigen–antibody, protein–protein, ligand–receptor) can be performed in a microarray format. Microarray-based assays using nucleic acid–nucleic acid interactions (DNA chips) are well established and protein microarray assays are becoming popular (Templin, 2002). As an example, protein microarrays have been used for receptor–ligand assays in order to identify new protein ligands, thus enabling HTS with very low sample consumption. Use of fluorescent and, possibly, BL/CL labels could represent the most suitable detection approach for microarray-based assays. "Lab on a chip" devices, consisting of fluidic channels etched in low density into a glass or plastic solid substrate, are able to process samples and reagents in a fast, serial manner.

These devices have been applied to drug discovery and development, allowing flexible assay formats utilizing small sample and reagents volumes. However, such devices will only work with very sensitive assays due to the short times that reagents dwell in the detection zone and have a relatively low analytical throughput. Moreover, re-using the same device for many reactions can result in sample carryover as well as in degradation of the device components. The Living Chip technology (Kanigan, 2000) combines the advantages of microwell-based analytical formats with the high density of microarrays, and probably represents the most versatile HTS technology available up to now. The Living Chip consists of a bidimensional array of channels and in the current implementation it has more than 10,000 channels per chip, each of them having a volume of 50 nL. The surfaces of the array are hydrophobic, while the interiors are hydrophilic, so that liquid is held in the channels by means of surface tension without cross-talk between adjacent channels. This device allows for performing simultaneously large numbers of chemical, biochemical, or cell-based micro-scale reactions. For example, by aligning and stacking a second chip on top of the first one, mixing of reagents may be performed on 10,000 or more samples, thus accomplishing massively parallel initiation of reactions. The Living Chip technology has been exploited in a variety of applications, such as the screening of compound libraries and enzyme activity assays, and could be applied to both homogeneous and heterogeneous assays. Assay results can be monitored in parallel over the entire chip by different means, including fluorescence and BL/CL imaging techniques.

These new assay formats should dramatically enhance the analysis of large sets of compounds against large numbers of targets. Nevertheless, the exigencies of drug screening will motivate a continued application of state-of-the-art technologies to the process of HTS. In this context, BL and CL techniques are certainly expected to play a more and more important role.

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CHAPTER 5 - Development and validation of an ultrasensitive chemiluminescent enzyme immunoassay for aflatoxin M_1 in milk

A fast and ultrasensitive chemiluminescent enzyme immunoassay for aflatoxin M_1 in milk samples has been developed and validated. The method is an indirect competitive type format involving the immobilization of an aflatoxin M_1-bovine serum albumin conjugate on 384 well black polystyrene microtiter plates and the use of a secondary antibody labeled with horseradish peroxidase detected with a luminol-based substrate. Aflatoxin M_1 standard solutions were prepared in milk-based buffer, and milk samples were analyzed without any cleanup procedure. The limit of quantification was 1 ppt, the coefficient of variation was below 9% for both intra- and interassay precision, and the recovery ranged from 96 to 122%. The method is specific, and other aflatoxins do not significantly cross-react with the antibody. Twenty-four milk samples were analyzed, and a good correlation was observed ($y = 0.98x + 1.71$, $r^2 = 0.98$, $n = 24$) when the data were compared with a reference high-performance liquid chromatography method with a fluorescent detector. The developed method is suitable for an accurate, sensitive, and high-throughput screening of aflatoxin M_1 in milk samples with a reduction of costs and increased detectability, as compared with previously developed immunoassays.

5.1 Introduction

Aflatoxins are highly toxic mycotoxins produced by three species of *Aspergillus* (*Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*), which may contaminate a wide range of foods and animal feedstuffs stored under temperature and humidity conditions favorable to mold growth.

Aflatoxin M_1 is a hydroxylated metabolite of aflatoxin B_1, formed when ruminants ingest feed contaminated with aflatoxin B_1. Aflatoxin M_1 is then excreted in milk (Polan, 1974; Van Egmond, 1989), and because it is relatively stable during milk pasteurization or other thermal treatments, it can also be present in milk-derived dairy products, such as cheese and yogurt (Galvano, 2001; Govaris, 2002).

Because of its hepatotoxic and carcinogenic activity (International Agency for Research on Cancer, 1993), aflatoxin M_1 contamination represents a risk for human health, especially for children, who are the major milk consumers. Aflatoxin M_1 legal limits, ranging from 0 to 1 μg/kg, have been established in various countries worldwide, and in particular, the European Union limit for aflatoxin M_1 in milk is 50 ppt (ng/kg) (European Commission, 2001). Aflatoxin M_1 is frequently present in commercial milk samples and dairy products, and various milk samples containing aflatoxin M_1 levels greater than the legal limit were recently detected, thus causing "alert" among the European Union (Rapid alert system for food and feed, 2003), the World Health Organization, and the Food and Agriculture Organization (World Health Organization, 2002).

Several methods for aflatoxin M_1 determination have been developed, and high-performance liquid chromatography (HPLC) with a fluorescent detector and enzyme-linked immunosorbent assays are mainly used in routine analysis (Stroka, 2002; van Egmond, 2004). For an effective screening and monitoring of aflatoxin M_1 in foodstuffs at ppt levels, analytical methods combining simplicity with high detectability and analytical throughput are required. This can be achieved by means of immunological methods in conjunction with a highly sensitive detection of the label. Previously developed enzyme immunoassays for aflatoxin M_1 analysis using conventional colorimetric detection with chromogenic substrates (Thirumala-Devi, 2002; Sibanda, 1999) were allowed to reach detection limits not lower than 5-10 ppt in milk. In addition, they usually require a 60-120 min incubation time, as well as several analytical steps, which limits their extensive use for a rapid aflatoxin M_1 screening (Badea, 2004; Rodriguez Velasco, 2003).

Enzyme labels detected by chemiluminescent (CL) substrates, such as the

luminol/peroxide/enhancer system for horseradish peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase, represent the most sensitive detection system in immunoassay development (Kricka, 1996; Ito, 2003). In addition, the CL signal detection can be performed immediately after substrate addition, thus shortening the overall analytical procedure when compared with conventional colorimetric assays. In the present work, a fast and ultrasensitive CL enzyme immunoassay for aflatoxin M_1 has been developed, based on the use of an anti-aflatoxin M_1 rabbit polyclonal antibody characterized by a very high affinity constant. An aflatoxin M_1-BSA conjugate was synthesized and immobilized on 384 well black polystyrene microtiter plates for developing an indirect competitive type format immunoassay, in which a secondary anti-rabbit IgG labeled with HRP and a luminol/peroxide/enhancer CL system were used.

5.2 Materials and Methods

Materials. Aflatoxins M_1, M_2, B_1, B_2, G_1, and G_2, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), Freud's complete adjuvant (FCA), goat anti-rabbit IgG-peroxidase conjugate, bovine nonfat-dried milk, certified reference materials BCR 285 and 282 (aflatoxin M_1 in whole milk powder, 0.76 and 0.05 μg aflatoxin M_1/kg, respectively), bovine serum albumin (BSA), and Tween 20 were all obtained from Sigma Chemical Co. (St. Louis, MO).

Black 384 and 96 well or transparent 96 well polystyrene microtiter plates (Labsystems, Helsinki, Finland) were used for the CL or the colorimetric method, respectively. An enhanced (luminol/peroxide/enhancer) CL system (SuperSignal ELISA Femto) (Pierce, Rockford, IL) was used for the measurement of HRP activity. Buffers were as follows: Coating buffer (0.05 M carbonate/bicarbonate buffer solution pH 9.6); blocking solution [0.01 M phosphate-buffered saline (PBS), pH 7.2-7.4, containing 2% nonfat-dried milk]; incubation buffer (PBS, pH 7.2-7.4, containing 3% nonfat-dried milk); washing buffer (PBS with 0.05% Tween 20). HPLC grade acetonitrile was purchased from Carlo Erba (Milan, Italy), and deionized water was purified on a Milli-Q system (Waters, Milford, MA).

Instrumentation. Hapten/protein densities analysis was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS), using a Voyager De Pro (Applied Biosystems, Foster City, CA) instrument equipped with a pulsed-N_2 laser operating at 337 nm (Karas, 1991; Zaluzec 1995). Reagents were dispensed in microtiter plates using a Multidrop 384 dispenser (Labsystems). Microtiter plates were

incubated under shaking at 37℃ using a Wellwarm sh aker-incubator (Labsystems).
A chemiluminescence microtiter reader Luminoskan Ascent (Labsystems), which allows measurement of the CL signal from a whole 96 or 384 well microtiter plate within few minutes, was used. As an alternative, the CL signal was measured using a LB 981 imaging device (EG&G Berthold, Pforzheim, Germany), which employs a highly sensitive, back-illuminated, cooled CCD camera (Roda, 1996). A Multiskan EX spectrophotometric microtiter reader (Labsystems), provided with 492 nm filter, was used for absorbance measurements.
A Varian Star 9010 HPLC system (Varian, Palo Alto, CA) equipped with an isocratic μ-flow pump and coupled to a Varian Fluorichrom scanning fluorescence detector was used for method validation. Data quantification was performed using a Varian Vista 402 integrator.

Aflatoxin M_1-BSA Conjugate (Antigen). Aflatoxin M_1 has no reactive groups for direct coupling to a protein; therefore, it was first converted to aflatoxin M_1-*O*-carboxymethyl-oxime (aflatoxin M_1-oxime) according to the method described by Chu et al. (Chu, 1977a). The antigen was prepared by conjugating aflatoxin M_1-oxime to BSA in the presence of EDAC, using the method previously described by Chu et al. (Chu, 1977b) for the preparation of BSA-aflatoxin B_1-oxime conjugate.
The aflatoxin M_1 to BSA conjugation ratio was determined by MALDI-TOF, by comparing the molecular weight of the standard BSA with that of the aflatoxin M_1-BSA conjugate (Bacigalupo, 2005). The matrix (sinapinic acid) was prepared at a concentration of 10 mg/mL in 1:1 (v/v) water-acetonitrile (+0.1% trifluoroacetic acid). Protein samples were typically 10-50 pmol/μL in water-acetonitrile (2:1, v/v). Sample and matrix solutions were mixed in equal volumes, and then, 2 μL of the resulting solution was transferred to the target plate and left to dry at room temperature. Spectra were recorded at threshold laser irradiance for 50-150 shots in the linear mode at 25 kV, using an external linear calibration with BSA standard solution. Protein conjugate concentration was determined using a Bradford assay.

Antibody Production and Characterization. Polyclonal antibodies against aflatoxin M_1-BSA were produced in rabbit following a previously described protocol (Roda, 1980). Briefly, 100 μg of aflatoxin M_1-BSA conjugate was subcutaneously injected as an emulsion in saline solution and Freund's complete adjuvant for each immunization. Antiserum with adequate titer, affinity, and specificity were obtained 3-4 months after the first immunization. Any anti-BSA present was eliminated by precipitation by adding BSA (0.3%)

to the rabbit sera. The IgG-rich fraction was isolated by salting-out precipitation with sodium sulfate, following a slight modification of the method described by Axen et al. (Axen, 1967). In brief, 180 mg of Na_2SO_4 was added to 1 mL of anti-BSA free rabbit serum, and the mixture was stirred at 25℃ for 45 m in and centrifuged at 4000*g* for 15 min. The supernatant was carefully removed, and the pellet was resuspended in 1 mL of PBS containing 18% Na_2SO_4, and the mixture was vortexed for 40 s and centrifuged at 4000*g* for 15 min. These steps were repeated 2-3 times, and then, the pellet was resuspended in 1 mL of PBS and extensively dialyzed against PBS. The antibody was stored at -20℃ in PBS containing 100 μL/mL trasylol. Antibody specificity was defined by evaluating the extent of cross-reactivity (CR) with structurally related compounds, such as aflatoxins M_2, B_1, B_2, G_1, and G_2, using an indirect competitive binding assay.

Samples. *Control Milk Samples.* Control milk samples were prepared at three aflatoxin M_1 concentration levels: lower (1 ppt), middle (10 ppt), and upper (100 ppt) levels. In particular, 1 mL aliquots of pooled noncontaminated milk samples (i.e., with aflatoxin M_1 content lower than the detection limit of the developed immunoassay) were spiked with the appropriate amount of aflatoxin M_1. Briefly, the standard solution of aflatoxin M_1 (10 μg/mL in methanol) was diluted 1:10000 v/v with noncontaminated milk in order to achieve a 1000 ppt aflatoxin M_1 in milk stock solution. Aflatoxin M_1 fortified milk samples were then obtained by serial dilutions in noncontaminated milk.

Milk Samples. A total of 24 milk samples of different kinds (whole fat, half-skimmed, and light) provided by "Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise" (Teramo, Italy), with aflatoxin M_1 concentration previously evaluated by a validated reference HPLC method, were analyzed. Certified reference materials (aflatoxin M_1 0.76 and 0.05 μg/kg), provided as powdered milk, were suspended in distilled water (0.1 g/mL) and dissolved by stirring.

Milk samples were centrifuged at 3500*g* for 10 min at 4℃, the upper fat layer was completely removed, and the aqueous layer (middle portion) was directly used for the analysis. For an automatic procedure, the sampling system was set up to collect the appropriate middle portion of the centrifuged sample.

Aflatoxin M_1-BSA Immobilization. The developed method is a solid phase indirect competitive immunoassay, involving immobilization of the aflatoxin M_1-BSA on 384 well polystyrene microtiter plates (96 well conventional microtiter plates were also used and the reagent volume was 5-fold higher). In particular, the wells of the microtiter plate were

coated with 20 µL of 150 ng/mL aflatoxin M_1-BSA in coating buffer. The plates were incubated overnight at 4℃. After the plates were w ashed three times with washing buffer, 60 µL of blocking solution was added to each well and the plates were further incubated at 4℃ for 2 h. The solution was discarded, and the pl ates were washed and vacuum-dried, and then, they were either used immediately or sealed with drying materials and stored at 4℃ until use.

Enzyme Immunoassay Procedure. To perform the assay, 20 µL/well of sample or aflatoxin M_1 standard solution (aflatoxin M_1 concentration in the range from 0.2 to 5000 ppt in incubation buffer) was added in duplicate to the antigen-coated plate followed by the addition of 20 µL/well of the anti-aflatoxin M_1 polyclonal antibody diluted 1:60000 (v/v) in washing buffer (primary antibody). The wells were incubated with gentle shaking for 30 min at 37℃. During incubation, a competition took place between the free analyte and the solid phase-bound antigen for binding to antibodies. After they were washed four times with washing buffer, the plate was incubated for 30 min in the dark at 25℃ with 20 µL/well of HRP-labeled anti-rabbit IgG (secondary antibody) diluted 1:4000 (v/v) in washing buffer.
For the colorimetric method, the HRP activity of the tracer bound to the solid phase was measured by adding 100 µL/well of a chromogen [1,2-phenylenediamine (0.5 mg/mL), H_2O_2 30% (0.25 µg/mL) in 0.1 M citrate buffer, pH 5.0]. After incubation in the dark at 25℃ for 20 min, the enzymatic reaction was stopped with 4 N H_2SO_4 (100 µL/well), and the absorbance at 492 nm was recorded.
For the CL method, the peroxidase activity was measured using a CL substrate (30 µL/well). The light emission, which becomes stable within 2-3 min, was measured by means of a microtiter plate luminometer reader using a 1 s integration time. Alternatively, a CCD-based luminograph was used, which allows simultaneously measuring the CL signal from a whole 384 or 96 well microtiter plate within 1 min.

Calculations. Aflatoxin M_1 concentrations were determined by interpolation on the calibration curve, where the bound enzyme activity, expressed as the ratio between signal at each concentration of analyte (B) and the bound activity in the absence of analyte (B_0), was plotted against the log of analyte concentration. For each experiment, a calibration curve was determined by a nonlinear regression analysis of the standards' data using the following equation:

$$Y = Y_O + (100 - Y_O) / (1 + 10^{[(b - x)\,c]})$$

where $Y = B/B_0$, Y_O = bottom asimptote (the dose), x = log of analyte concentration

(ppt), *b* = log of analyte at midpoint of the curve (ppt), and *c* = slope.

Linearization of the calibration curve was performed by plotting the bound enzyme activity, expressed as the logit of the ratio (in percent) between *B* and B_0, against the log of analyte concentration. The best data fit was obtained by linear regression of the standard points. The milk aflatoxin M_1 concentration was expressed as pg/mL (ppt) of milk.

HPLC Analysis. To evaluate the accuracy and to validate the method, a comparative study using both the developed enzyme immunoassay and HPLC was performed. For HPLC analysis, 5 mL of the aqueous layer of the centrifuged milk samples was diluted 1:1 (v/v) with water and submitted to a cleanup procedure using 3 mL C-18 SPE columns (Baker J. T., Phillipsburg, NJ) preconditioned with 2.5 mL of acetonitrile and 5 mL of water. The solution was applied to the cartridge, and then, the column was washed with 10 mL of water, followed by 20 mL acetonitrile/water 10:90 (v/v) and 10 mL of *n*-hexane. Aflatoxin M_1 was eluted with 2 × 3 mL of dichloromethane/acetone 95:5 (v/v), and then, the solvent was evaporated under a nitrogen stream. The fluorescent hemi-acetal aflatoxin M_1 derivative (aflatoxin M_{2a}) was obtained by the addition of 250 μL of trifluoroacetic acid followed by 15 min of stirring in the dark (Orti, 1986; Orti, 1989). The solution was subsequently dried by evaporation under gentle nitrogen stream, and the residue was dissolved with 200 μL of mobile phase and analyzed by HPLC.

Analysis of aflatoxin M_1 was performed on a 250 mm × 4.6 mm i.d., 5 μm, Supelcosil LC-18 column (Superchrom, Milan, Italy). The column was thermostated at 40°C, and the sample injection volume was 20 μL. The mobile phase consisted of acetonitrile/water 20:80 (v/v), and the flow rate was 1.2 mL/min. Aflatoxin M_1 was eluted at a retention time of 5.6 min and detected by setting the fluorescence detector at 353 and 423 nm. All analyses were performed in duplicate. The limits of detection (LOD) and quantification (LOQ) of the HPLC method were 5 and 15 ppt, respectively.

5.3 Results and Discussion

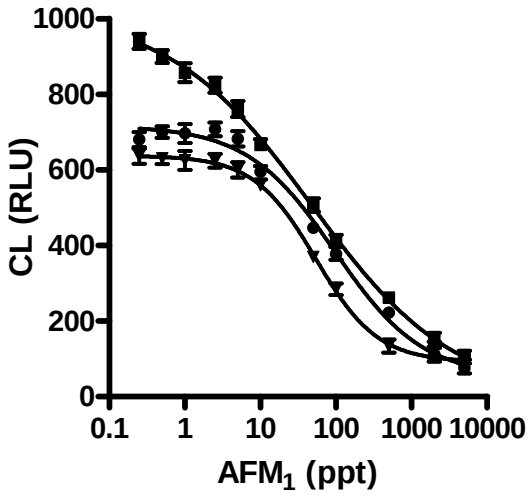
Aflatoxin M_1-BSA Characterization. The average molar ratio between aflatoxin M_1 and BSA in the aflatoxin M_1-BSA conjugate, as determined by the molecular weight obtained by MALDI-TOF mass spectrometry, was 6 mol of aflatoxin M_1 for 1 mol of BSA. This indicated that the aflatoxin M_1-BSA conjugate was characterized by a relative high hapten density, thus by high antigenicity. In addition, mass spectrometry analysis showed the presence of a little amount of unconjugated BSA, suggesting a high reaction efficiency.

The unreacted aflatoxin M_1-oxime has been completely removed by extensive dialysis of the reaction mixture using a cellulose membrane with a cutoff of 14000 Da.

The immobilization of an antigen monolayer on the solid phase is crucial to obtain a good analytical performance of the assay, in particular to minimize the nonspecific and uncontrolled binding. For this reason, the synthesis of the aflatoxin M_1-BSA was optimized by increasing the initial concentration of aflatoxin M_1 in the reaction mixture in order to achieve a high aflatoxin M_1 to BSA molar ratio, thus obtaining a molecule that would work as a powerful immunogen and allow proper antigen immobilization. In fact, at a relatively high conjugation ratio, few BSA and a high number of aflatoxin M_1 molecules are available at the well surface for antibody binding, with low steric hindrance. This facilitates the antibody binding to aflatoxin M_1, thus improving the kinetics of antibody-antigen reaction, being the phenomenon less affected by diffusion of the antibody to the antigen-coated well surface. The aflatoxin M_1-BSA derivative characterization by MALDI-TOF analysis was crucial to achieve the above-reported analytical performance and for a good assay standardization.

Optimization of the Enzyme Immunoassay. *Aflatoxin M_1-BSA and Anti-Aflatoxin M_1 Antibody Concentration.* Preliminary experiments were performed to assess the optimal coating antigen/antibodies ratio. Aflatoxin M_1-BSA and anti-aflatoxin M_1 antibody concentrations were optimized by comparing dose-response curves obtained using different combinations of antigen (50, 100, and 150 ng/mL) and antibodies dilutions (1:30000, 1:40000, and 1:60000 v/v), according to an optimized experimental design. A satisfying compromise between the lowest LOD, the highest sensitivity (slope of the curve), and the widest linear dynamic range was obtained by using a 150 ng/mL aflatoxin M_1-BSA solution in the immobilization step and a 1:60000 (v/v) dilution of the antibody (**Figure 1**).

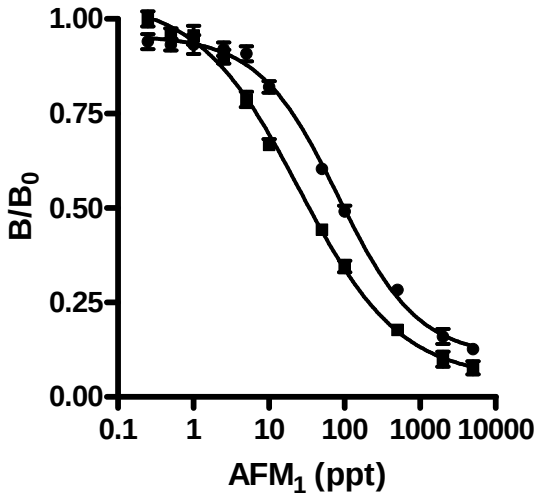
Figure 1: Calibration curves obtained using various dilutions of anti-AFM1 polyclonal antibody (■ 1:60000, ● 1:40000, ▼ 1:30000 v/v). The aflatoxin M1-BSA conjugate was immobilized at the optimal concentration (150 ng/mL), and the curves are expressed as relative light units (RLU) against the log of aflatoxin M1 concentration (ppt).



Comparison between Colorimetric and CL Detection. The optimized CL enzyme immunoassay was compared with a conventional colorimetric method. By comparing the dose-response curves shown in **Figure 2**, it can be observed that the CL method provided a lower detection limit with respect to the colorimetric assay (0.25 and 5 ppt, respectively). In addition, the CL method provided a lower midrange of the calibration curve, i.e., the concentration giving 50% of the response (30 ± 2 and 82 ± 5 ppt, respectively), as well as a higher dynamic range of linearity. The fact that the CL detection offered an improved analytical performance in terms of detectability and dynamic range is not surprising, due to the superior characteristics of the detection system, being the CL signal linear for up to 6 orders of magnitude (Kricka, 1982). Moreover, because the detectability of HRP enzymatic activity is at least 100-1000 times higher, the CL system is particularly suitable for microsystems characterized by very small reading volumes (e.g., 30 μL, as used in the developed method). A further advantage obtained by using the CL detection is the rapidity of the assay, since the CL signal can be measured immediately after substrate addition, while the colorimetric assay requires a 20-30 min incubation step, as well as an enzyme activity blocking step, prior to signal detection. Indeed, thanks to the glow type emission kinetics of the enhanced CL substrate, the steady state light emission is reached 2-3 min after substrate addition and it is maintained for at least 15 min, thus allowing easy handling and standardization of the experimental conditions. The CL signal can be recorded either

with a CCD imaging device or with a conventional photomultiplier tube-based microtiter plate reader. The imaging device allows simultaneously measuring the light signal in the 384 wells of the microtiter plate, and with the same imaging instrument, it is possible to perform simultaneous measurement in up to four 384 well microtiter plates; in addition, thanks to the use of correction optics (flat field correction lenses), no major effects on the light path and geometry have been observed. The light output can be measured using 1 min exposure time. On the other hand, the microtiter plate reader offers better performance in terms of detectability (10 times higher than the CCD imaging), but it requires longer measurement times. Usually a 1 s acquisition time for each well is necessary, which results, also considering the time required to slide the wells under the photomultiplier tube, in approximately a 6 min total measurement time for a 384 well microtiter plate.

Figure 2: Representative calibration curves obtained in milk-based buffer with the immunoassay method for aflatoxin M1 using the colorimetric (●) and CL (■) detection. The curves are expressed as fraction of bound enzyme activity (B/Bo) against the log of aflatoxin M1 concentration (ppt). Each curve point and the SD associated with it are the result of an averaging of 10 standard curves obtained in six different days.



Enzyme Immunoassay Format. CL detection allows the use of 384 well microtiter plates with an assay volume of 20 μL, which is at least five times lower than that used in the conventional 96 well microtiter plate format (Roda, 2000). A 5-fold reduction in antibody,

labeled probe, and CL cocktail volume reduces the costs of the assay, while maintaining the same analytical performance.

Because of the very small volume of samples and reagents used in the analysis and to the high density of wells of the microtiter plate format, the use of an automated system to dispense samples and reagents is essential to control the analytical steps and the reproducibility of the overall analytical procedure. In addition, more compact devices can be used. The combination of a small incubation volume with rapidity and sensitivity of the CL detection of the probe allowed the development of a sensitive and low cost immunoassay, with which more than one hundred samples may be analyzed in the same analytical session, thus representing an attractive method for high-throughput screening (HTS).

Analytical Performance. *Antibody Specificity.* The antibody specificity was defined by evaluating the extent of CR% with aflatoxin M_1 structurally related compounds, such as aflatoxins M_2, B_1, B_2, G_1, and G_2. In particular, for each compound, a calibration curve was produced using the same analytical conditions, and then, the CR% was calculated for each compound as follows:

$$CR\% = \frac{a}{b}100$$

where *b* is the concentration of tested compound corresponding to $B/B_0 = 0.5$ and *a* is the concentration of aflatoxin M_1 corresponding to the same B/B_0 value.

The antibody used is highly specific for aflatoxin M_1. Indeed, all other tested aflatoxins do not significantly cross-react (**Table 1**), since they are able to cause a potential interference only if present at a concentration 10-100 times higher than aflatoxin M_1.

Table 1: CR% of some aflatoxin M_1 related compounds with the anti-aflatoxin M_1 antibody.

AFLATOXIN	CR[a] %
Aflatoxin M_1	100
Aflatoxin M_2	4
Aflatoxin B_1	2
Aflatoxin B_2	1
Aflatoxin G_1	0.5
Aflatoxin G_2	0.5

[a] CR% = (ppt of aflatoxin M_1 that leads to 50% binding inhibition/ppt of aflatoxin that leads to 50% binding inhibition) x 100.

Matrix Effect. To evaluate milk matrix effect, aflatoxin M_1 calibration curves produced in either buffer (PBS), skimmed milk, or full-cream milk were compared. In addition, serial dilutions (2-16-fold with PBS) of a milk sample spiked with aflatoxin M_1 at a high concentration (100 ppt) were assayed by interpolation on a calibration curve produced in buffer and the observed concentration values were compared with the expected concentrations. Because a significant matrix effect was observed by comparing calibration curves and no linearity was observed among different dilutions of the fortified aflatoxin M_1 sample, a calibration curve in milk-based buffer is needed to avoid samples cleanup. Being calibration curves obtained by preparing aflatoxin M_1 standards in buffer containing commercial nonfat dried milk (incubation buffer) overlapping with those prepared in centrifuged milk, we used incubation buffer to prepare calibration curves. The use of this milk-based buffer allowed analyzing all kinds of milk samples without interference and with good reproducibility.

Calibration Curves. **Figure 2** shows a mean standard curve in incubation buffer, obtained by averaging 10 standard curves from six different days. The RSD% of individual points ranged from 4 to 10%. The best fitting of the curve was obtained by a nonlinear regression using a three parameters function. The relative standard deviation (RSD%) calculated for the Y_O, b, and c coefficient values was equal to 3.1, 6.5, and 4.1%, respectively, and the R^2 was 0.998 ± 0.002. The results obtained with 10 standard curves generated either in the same day or on different days demonstrated that the developed assay is reproducible and precise.

LOD. The LOD was calculated by interpolation on the aflatoxin M_1 standard curve, as the

analyte concentration corresponding to the mean signal of B_0 (obtained by averaging the signal of six replicate sets) minus three times its standard deviation (SD) (according to IUPAC, ISO, and AOAC harmonized guidelines) (Thompson, 2002), and it was 0.25 ppt.

LOQ. The LOQ, defined as the aflatoxin M_1 concentration producing a signal corresponding to aflatoxin M_1-free milk minus 10 times its SD (Thompson, 2002), was determined by analyzing six replicate sets of aflatoxin M_1 fortified milk samples ranging from 1000 to 1 ppt. The LOQ of the validated method in milk samples was 1 ppt that is more than 20 times below the legal limit for aflatoxin M_1 in milk. In addition, the LOD and the LOQ of the developed enzyme immunoassay were lower than those of previously developed immunological methods and commercially available kits for aflatoxin M_1 analysis in milk samples (Thirumala-Devi, 2002; Rodriguez Velasco, 2003; Fremy, 1984).

Within Assay and between Assay Precision and Bias. The precision of the method was determined by analyzing replicates of aflatoxin M_1 fortified milk samples, which contained a nominal aflatoxin M_1concentration of 1 (low level), 10 (medium level), and 100 (high level) ppt. The assays were carried out in six replicates on the same day for the within assay precision evaluation and in six different days for the between assay precision evaluation. The values of the mean, SD, and RSD were calculated at each theoretical concentration level and are summarized in **Table 2**. The RSD (%) and recovery (%) values demonstrate that, even at the lower LOQ, the precision and accuracy of the method are suitable for routine screening of aflatoxin M_1 in a very low milk sample volume.

Stability of the Coated Plates. Coated plates were vacuum-dried and stored at 4℃ under vacuum. Stability of the antigen-coated plates was investigated by comparing six calibration curves obtained using plates stored at either 0, 1, 3, 6, or 8 months. Experimental data show that vacuum storage of the antigen-coated plates at 4℃ for up to 6 months did not significantly alter the performance of the assay in terms of LOQ and LOD.

Accuracy. To evaluate the accuracy of the assay, three fortified milk samples, containing a nominal aflatoxin M_1 concentration of 1, 10, and 100 ppt, as well as two certified reference materials, were analyzed. Results are summarized in **Table 2**. Recovery values ranged from 96 to 122%, thus indicating an excellent accuracy of the assay when applied to real samples.

Table 2: Precision and Accuracy calculated by analyzing replicate samples spiked with 1, 10, and 100 ppt of aflatoxin M1, as well as certified reference materials[a].

Expected concentration (ppt)	Found concentration (ppt)	RSD (%)	Recovery (%)
	Within-assay (n=6)		
1	1.10 ± 0.07	6.3	110 ± 7
10	9.72 ± 0.38	3.9	97.2 ± 3.8
100	99.4 ± 2.5	2.5	99.4 ± 2.5
	Between-assay (n=6)		
1	1.22 ± 0.10	8.1	122 ± 10
10	9.63 ± 0.54	5.6	96.3 ± 5.4
100	98.5 ± 3.2	3.2	98.5 ± 3.2
	Certified Reference Materials		
760	745 ± 17.2	2.3	98.0 ± 2.3
50	48 ± 1.2	2.5	96.0 ± 2.4

[a]The report data are the mean values ± SD

Milk Samples Analysis. A total of 24 milk samples were analyzed with the newly developed immunoassay method. Three fortified milk samples prepared at three aflatoxin M_1 concentration levels (1, 10, and 100 ppt) were used in each microtiter plate as control. For each sample, four determinations were performed on different days and the results were compared with those obtained using the reference HPLC method.

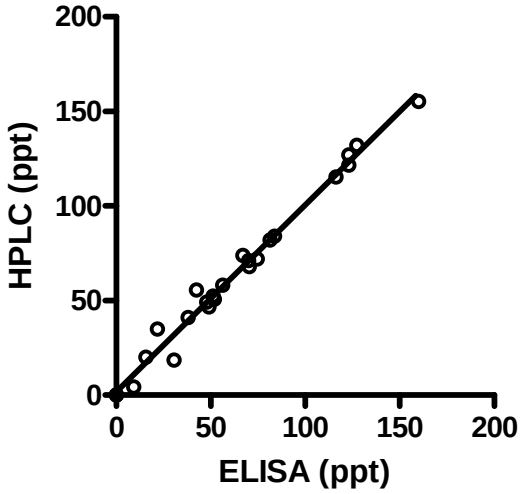
Concordant results were obtained with the two methods: 22 samples were positive for aflatoxin M_1, and only two samples were scored as negative (the aflatoxin M_1 concentrations detected by HPLC were approximately 0.8 and 0.5 ppt). The linear regression analysis (**Figure 3**) yielded a good correlation between methods (y = 0.

98*x* + 1.71, r^2 = 0.98, *n* = 24), even if a higher scattering of the data was observed for samples with aflatoxin M_1 concentration lower than 50 ppt.

These results prove that the developed enzyme immunoassay method can be applied for the analysis of aflatoxin M_1 in milk samples of different kinds at levels of regulatory relevance, with accuracy and precision comparable to those obtained with the reference method. The developed CL enzyme immunoassay is a robust and simple assay characterized by a high sensitivity and analytical throughput, thanks to the combination of the CL detection of enzymatic activity with the use of a 384 well microtiter format.

In addition, the procedure can be easily automated using conventional instrumentation, easily available in laboratories where a continuous screening for aflatoxin M_1 in milk is performed. In the future, the method will also be validated for the determination of aflatoxin M_1 milk-derived dairy products, such as cheese and yogurt. The continuous growth of the production of organic foods potentially increases the risk of aflatoxins production for uncontrolled mold growth when no chemicals and preservatives are used. Therefore, a sensitive, fast, first level control is highly recommended, and this can be achieved with the present HTS method based on a 384 well format. A HTS screening is useful for a routine quality control of milk from different farms before mixing the different milk bulks, especially when the absence of aflatoxin M_1 above the regulatory limit needs to be documented.

Figure 3: Correlation of results obtained by both CL enzyme immunoassay and reference HPLC method on milk samples. The linear regression analysis yielded a good correlation between methods (y = 0. 98x + 1.71, r^2 = 0.98, n = 24).



Abbreviation Used

BSA, bovine serum albumin; LOQ, limit of quantification; LOD, limit of detection; HPLC, high-performance liquid chromatography; CL, chemiluminescent; HRP, horseradish peroxidase; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FCA, Freud's complete adjuvant; PBS, phosphate-buffered saline; aflatoxin M_{2a}, hemi-acetal aflatoxin M_1 derivative; CR%, cross-reactivity; CV%, coefficient of variation; SD, standard deviation; RSD%, relative standard deviation; HTS, high-throughput screening.

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CHAPTER 6 - A rapid multiplexed chemiluminescent immunoassay for the detection of Escherichia coli O157:H7, Yersinia enterocolitica, Salmonella typhimurium, and Listeria monocytogenes pathogen bacteria.

A simple and rapid multiplexed sandwich chemiluminescent enzyme immunoassay has been developed for the simultaneous detection of *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Listeria monocytogenes*. To achieve the multiplexed detection of the four pathogens, a new polystyrene 96-well microtiter plate format has been designed, in which each main well contains in the bottom four sub-wells. The monoclonal antibodies specific for each bacteria were separately immobilized in the bottom of each sub-wells. When the samples were added to the main wells the bacterial able to specifically bind to the corresponding monoclonal antibody were captured in one of the four sub-wells. Subsequently, a mixture of peroxidase-labeled polyclonal antibodies against the four bacteria was added and the peroxidase activity of the bound polyclonal labeled antibodies in each well was measured by an enhanced luminol-based chemiluminescent cocktail using a low-light CCD imaging device. The assay was simple and fast and the limit of quantification was in the order of 10^4-10^5 CFU/mL for all bacterial species. The accuracy of the method, evaluated by comparison the results with a conventional culturing methodology was satisfactory, with recovery values ranging from 90 to 120%. This method can be used as a screening test to evaluate the presence of these pathogen bacteria in different foodstuff.

6.1 Introduction

Bacterial food-borne pathogens are a serious health threat worldwide. The World Health Organization (WHO) estimates that diarrhea-related diseases caused by the consumption of contaminated food or water are the third leading cause of death in the developing countries (Mead,1999; World Health Organization, 2002), and the socio-economic implications of gastroenteritis are a major concern. *Salmonella typhimurium*, *Escherichia coli O157:H7*, *Staphilococcus aureus*, *Clostridium perfringens*, *Campylobacter jejuni*, *Yersinia enterocolitica* and *Listeria monocytogenes* are the major bacterial pathogens commonly involved in foodborne illnesses (Institute of Food Technologists, 2004). Therefore, the production of high quality, pathogen-free food and the assessment of bacteria growth when foods and other biological materials are stored under inappropriate conditions represent priorities for the agro-food industry. In addition, bacteria contamination is a matter of concern related to terrorist attack.

Despite the continuous improvements, conventional microbiological methods for the detection and identification of pathogenic bacteria in food are still labor-intensive and time-consuming (Vasavada, 1997) and therefore, the search for fast, accurate and sensitive alternative methods is an active research task (Feng, 2001; de Boer, 1999; Deisingh, 2004).

Immunological methods for detecting pathogenic microrganisms have been extensively studied since they potentially combine detection specificity and sensitivity with simple and economical assay format. Most of commercially available immunoassay for bacteria detection are based on standard sandwich immunoassay (Chapman, 1997), which involves the formation of an immunocomplex between an immobilized antibody, the target bacteria and a secondary labelled antibody. Generally, the primary antibody is immobilized on beads (magnetic, silica or gold particles), polystyrene microtiter plates, filter membranes or directly on the surface of transducers, and the secondary antibody is labeled with enzymes or fluorescent molecules. The detection limits of immunoassays ranged between 10^3–10^6 colony-forming units (CFU)/mL, with assay times from ten minutes to several hours (Kerr, 2001; Blais, 2004).

More recently, many interesting approaches have been reported in order to improve the immunoassay detectability. For example, Zhao and coworkers (Zhao, 2004) used antibody-conjugated silica fluorescent nanoparticles to develop a fast and ultrasensitive immunoassay for in situ pathogen quantification down to a single bacterium without

amplification or enrichment. Unfortunately, most of these tests are expensive, laborious and often require specialized personnel and instrumentation.

As an alternative, DNA microarray technology has been exploited, which allows simultaneous detection of several bacteria but the construction of DNA arrays are still limited to a restricted number of specialized centers (Garaizar, 2006).

“Multiplex” format immunoassays suitable for the simultaneous evaluation of different bacteria in a sample can be developed to increase the analytical productivity and drastically reduce analysis costs and sample and reagent consumption (Dunbar, 2003; Bhagwat, 2003). Feasibility studies of “multiplex” immunometric methods in microarray format, either “sandwich” or, more recently, competitive type (Delehanty, 2002*;* Kim, 2005) have been already reported, but none of them has been applied for detection of bacteria.

In this work we report the development of a simple, multiplexed sandwich chemiluminescent enzyme immunoassay (CL-EIA) for the simultaneous detection of four of the major food-borne pathogens: *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Listeria monocytogenes*.

Polyclonal and monoclonal antibodies were produced by immunizing rabbit or balb/c mices, respectively, with heat-inactivated and sonicated ATCC bacteria strains of the four pathogens.

To achieve the simultaneous detection of the four pathogens, a new polystyrene 96x4-well microtiter plate format was designed. The microtiter plate, which has a conventional 12.8x8.6 cm frame size, consists of 96 main wells, each of them containing four internal sub-wells.

The developed method involves the immobilization of four monoclonal antibodies, each specific for one bacterium, in the bottom of the different sub-wells of the main well. When the samples, were added to the main well and incubated, the bacterial cells were captured in one of the four sub-wells by the corresponding monoclonal antibody. Subsequently, a mixture of peroxidase (HRP)-labeled polyclonal antibodies was added and the peroxidase activity was determined by means of an enhanced luminol-based chemiluminescent substrate. The light emitted was measured with an ultrasensitive, cooled low-light CCD imaging device. Chemiluminescence is particularly suited for these applications since the chemiluminescent signal is characterized by high detectability and specificity, fast kinetics and low background. In addition, CL detection techniques allow to localize and quantify the light emission on each sub well down to single-photon level (Roda, 2004).

The analytical performances of the method in term of specificity and its preliminary validation will be discussed by comparison with conventional microbiological procedures.

For method application and validation, a total of 32 naturally contaminated faecal and meat samples have been analyzed.

6.2 Materials and Methods

Bacterial strains. *Escherichia Coli O157:H7* ATCC 35150 (American Type Culture Collection Rockville, MD), *Salmonella thyphimurium* ATCC 14028, *Yersinia enterocolitica* ATCC 23715 and *Listeria monocytogenes* 13M ATCC 7644 were used for polyclonal and monoclonal (MAb) antibodies production and as positive control for antibodies screening. The specificity of each antibody was evaluated both against different bacterial species and other wild-type strains of the target bacteria. The bacteria used were either obtained by ATCC or kindly provided by the Istituto Zooprofilattico of Teramo. Stock cultures in 30 % glycerol were maintained frozen at -80°C.

Monoclonal antibody production. Monoclonal antibodies were produced by immunizing Balb/c mice using a slightly modified published protocol (He, 1996). Briefly, heat-inactivated and sonicated ATCC reference strains were diluted in Freud's incomplete adjuvant (IFA) (Sigma Chemical Co., St. Louis, MO) to obtain a protein concentration of 50 μg/mL and inoculated by intraperitoneal injection. The mice received their second injection at two weeks, two inoculations of 25 μg/mL were then administered. A booster injection of 50 μg/mL was given 31 days after immunization, then the mice were sacrificed and the spleen cells were fused with myeloma cells. Cell fusion and hybridoma cloning were performed using Galfre's method (Galfre, 1981) with some modifications. Briefly, splenocytes from the immunized mice were subjected to cell fusion with myeloma cells from Sp2/O-Ag-14 mice (American Type Culture Collection Rockville, MD) using 50% polyethylene glycol (PEG) 1550 solution. Hybridomas were cultured for two weeks in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO) containing 20% foetal bovine serum (Euroclone, UK), 2 mM L-glutamine (HybriMax®, Sigma Chemical Co., St. Louis, MO), amphotericinpenicillin-streptomycin 100x (APS, Sigma Chemical Co., St. Louis, MO), 50 mg/mL gentamicin (Gentamicin Sulphate Solution, Sigma Chemical Co., St. Louis, MO), 10000 UI/mL nystatin (Nystatin suspension HybriMax®, Sigma Chemical Co., St. Louis, MO) and HAT 50x (HAT Media supplement HybriMax®, Sigma Chemical Co., St. Louis, MO). The hybridomas of interest were cloned by limiting dilution method (Campbell, 1987; Goding, 1993). MAbs were produced in vitro

on large scale by serial culturing of antibody-secreting hybridomas and collection of their supernatants.

The cell culture fluids from actively growing hybridomas were screened using an indirect ELISA procedure against other isolated strains of the target bacteria and different bacteria species. Briefly, the hybridoma culture media, properly diluted, was added to well plate previously coated with the immunizing antigen. The plate was then incubated for 30 min at 37℃ and, after washes, HRP-labeled anti mouse anti body (purchased from Sigma, Chemical Co. St. Louis, MO) was added. The HRP activity was measured by adding a chromogenic substrate (TMB, KPL, Gaithersburg, MD). After incubation, the enzymatic reaction was stopped with 2N H_2SO_4 and the absorbance at 450 nm was recorded using a Multiskan EX spectrophotometer microtiter reader (Labsystems, Helsinki, Finland).

MAbs were screened in Immuno Western-blotting (Laemmli, 1970) and the MAb isotype was determinate with an isotyping kit (Pierce, Rockford, IL). IgG MAbs were purified by affinity chromatography using a protein A column (HiTrap rProtein A FF, 5 mL, Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's instructions. Protein concentration was determined by using a Bradford assay.

Polyclonal antibody production. Polyclonal antibodies against the target bacteria were produced in rabbit following a previously described protocol (Harlow, 1988). Briefly, heat inactivated and sonicated ATCC reference strains were diluted in Freud's complete adjuvant (FCA) (Sigma Chemical Co., St. Louis, MO) to a protein concentration of 200 μg/mL and subcutaneously injected six times over 50 days. Antiserum with adequate titre, affinity, and specificity were obtained 3-4 months after the first immunization. The IgG-rich fraction was purified using the same procedure for IgG MAb purification. Polyclonal antibodies were coupled to HRP (Sigma Chemical Co., St. Louis, MO) using the periodate method (NaKane, 1974). Quantitative chemiluminescent enzyme immunoassay. Microtiter plate format. To achieve the simultaneous detection of the four pathogens in the same sample a new polystyrene 96x4-well microtiter plate format was developed (**Figure 1A**). The microtiter plate consists of 96 main wells (volume 100 μL), each of them divided into four internal sub-wells. The presence of several sub-wells within the main well allows the immobilization of different monoclonal antibodies in separate positions (**Figure 1B**). In addition, the plate has a standard frame size (12.8x8.6 cm) and can therefore be used with commercially available automation systems and robotics for reagent dispensing, incubation, and washing steps.

Two different prototypes, in black or white polystyrene, were produced. A similar microtiter

plate but with 7 subwells have been already used by us to develop a PCR-chemiluminescent immunoassay method for the detection of seven most frequent high-risk Papillomavirus DNAs (Roda, 2002).

Calibration Curves. Calibration curves were obtained using a mixture of the four bacteria. In particular, universal peptone broth (UPB) (Nam, 2004; Bailey, 1992) (Difco Laboratories, Detroit, MI) was inoculated with a mixture of the four bacteria, then incubated for 12-18 h at 37℃. An aliquot (2 mL) of cells suspension in U PB was centrifuged at 3000g for 10 min, the supernatant was carefully removed and the pellet was washed twice with phosphate-buffered saline (PBS). Washed bacteria were diluted in PBS to give a 10^9 CFU/mL cell concentration(corresponding to an OD_{600} approximately of 0.8). The bacteria were then serially diluted in PBS to obtain suspensions in the range from 10^8 to 10^2 CFU/mL (total cell concentration), which were used as standards in CL-EIA method. For each dilution, the actual concentration (CFU/mL) of each bacterium was determined by colonies counting on selective media agar plates. The obtained CFU/mL values were used for calibration curve plots. Each calibration curve consist in seven points in the range from 10^3 to 10^7 CFU/mL.

Samples. Spiked human faecal and bovine meat samples were used to evaluate the analytical performance and the applicability of the multianalyte CL-EIA method for the detection of bacteria.

Faecal (obtained from healthy volunteers) and bovine meat (purchased from local food stores) samples were previously assayed with conventional procedures to verify the absence of the four bacteria. Then, they were divided in two portions: one portion was used as negative control and one was artificially contaminated with the four pathogens, alone or in combination, by using previously reported methods (Chandler*,* 2001*;* Ferretti*,* 2001). The samples (25g) were inoculated with the appropriate amount of bacteria (final concentration of each species ranging from 1 to 100 CFU/g), homogenized in a stomacher bag and incubated with 225 mL of buffered peptone water (BPW) for 3h at 37℃. Subsequently, 1 mL of suspension was removed and incubated with 9 mL of UPB for 6 h at 37℃ with shaking,. then assayed using the CL-EI A method. For each sample the bacteria concentration was also verified by using a conventional microbiological reference method.

For validation of the newly developed CL-EIA method, we tested a total of 32 naturally contaminated faecal and meat samples for the presence of the four bacteria, and the

results were compared with those obtained using a reference microbiological method.

Multiplexed chemiluminescent enzyme immunoassay procedure. The four MAbs, specific for each bacteria species, were immobilized in the different sub-wells of the main well. In particular, 10 μL of a 300 ng/mL antibody solution in coating buffer (0.05 M carbonate/bicarbonate buffer solution pH 9.6) were dispensed in each individual sub-well and the plates were incubated overnight at 4℃. After washing with washing buffer (PBS containing 0.05% Tween 20), 100 μL of blocking solution (PBS containing 1% bovine serum albumin) was added to each main well, and the plates were further incubated at 4℃ for 2 h. The solution was discarded and the plates were washed three times with washing buffer and vacuum dried. The plates were either used immediately or sealed in plastic bags together with a 5-g package of silica gel desiccant (Sigma) and stored at 4℃ until use.

To perform the assay, 100 μL of enriched sample suspension or bacteria standard solutions were added to each main well of the microtiter plate and incubated for 30 min at 37℃. During the incubation the bacterial cells in the sample were captured by the corresponding MAb in one of the four sub-wells. After three washing, 100 μL of a mixture of the HRP-labeled polyclonal antibodies specific for the four bacteria species were added and incubated for 30 min at 37℃. The HRP activity of the bound labeled antibodies was measured, after washing, by means of an enhanced luminol-based CL substrate (SuperSignal ELISA Femto, Pierce, Rockford, IL) and an imaging device (LB 981 Night Owl, EG&G Berthold, Pforzheim, Germany), which employs a highly sensitive, back-illuminated, cooled CCD camera. Images were acquired using a 40-s exposure time and the CL signal of each sub-well was measured by integrating the photon emission over the sub-well area.

Before data analysis, the blank signal was subtracted from all the sample and calibration curve signals (CL intensity). The concentration of each bacterium was then determined by interpolation on the calibration curves obtained using bacteria standard solutions.

6.3 Results and Discussion

Monoclonal antibodies screening. The peculiarity of the monoclonal antibodies used in this study was their ability to recognize all the strains of the four target bacteria without any cross-reactivity with the other bacteria species tested.

Approximately 3600 hybridoma supernatants were obtained and tested for each bacteria. Among these, 200-400 clones showed positive reaction to the four target bacteria by indirect ELISA test. Subsequently, a further ELISA screening with other bacterial antigens led to the identification of four MAbs (8B8C3, 54B11, 1B6D9 and 6F12C8) reacting with *Escherichia coli O157:H7, Yersinia enterocolitica, Salmonella typhimurium and Listeria monocytogenes,* respectively, and showing no cross-reaction to any other bacteria used in this study (**Table 1** and **Table 2**). These MAbs, which were identified as IgG1 isotypes, were used for the development of the CL-EIA method. The anti-*Yersinia enterocolitica* monoclonal antibody was also used in a previously published work (Magliulo, 2006).

Table 1: Monoclonal antibody cross-reactions with various bacterial antigens evaluated by indirect ELISA.The percentage of cross reaction (CR%) was calculated by dividing the OD_{450} value of each bacteria by the OD_{450} for the target bacteria.

Bacterium	**Source**	**MAb 8B8C3 CR%**	**MAb 54B11 CR%**	**MAb 1B6D9 CR%**	**MAb 6F12C8 CR%**
Escherichia					
E.coli O14	BGVV	2.1	1.1	2.2	1.1
E.coli	ATCC 25922	1.2	2.1	2.2	1.2
E.coli O157:H7	ATCC 35150	**100**	1.1	2.3	1.3
E.coli O157:H7	Wild type strain	97	1.1	2.4	1.4
E.fergussoni	Wild type strain	1.1	2.4	2.1	1.2
Listeria					
L.innocua	ATCC 33090	3.1	1.3	3.1	4.3
L.ivanovii	ATCC 19119	1.3	2.3	1.1	3.5
L.monocytogenes	ATCC 7644	1.2	1.2	1.1	**100**
L.monocytogenes	Wild type strain	1.3	1.3	1.2	97
Salmonella					
S.bredeney	Wild type strain	2.1	1.1	3.4	2.1
S.derby	Wild type strain	1.2	3.4	3.3	2.1
S.enteritidis	ATCC 13076	1.1	2.2	2.3	2.2
S.enteritidis	Wild type strain	2.1	3.2	1.2	2.2
S.hadar	Wild type strain	1.2	2.3	1.1	2.3
S. muenchen	Wild type strain	1.2	2.3	1.5	2.3
S.panama	Wild type strain	1.2	2.2	1.2	2.3
S. saint-paul	Wild type strain	0	1.2	1.2	2.1
S.typhimurium	ATCC 14028	0	1.1	**100**	2.1
S.typhimurium	Wild type strain	1.5	1.1	99	2.1
Yersinia					
Y.enterocolitica	ATCC 23715	3.1	**100**	1.1	3.2
Y.enterocolitica	Wild type strain	3.1	98	1.1	3.4

Table 2: List of other bacterial stains screened by indirect ELISA. The percentage of cross reaction (CR%) was calculated by dividing the OD_{450} value of each bacteria by the OD_{450} for the target bacteria.

Bacterium	Source	MAb 8B8C3 CR%	MAb 54B11 CR%	MAb 1B6D9 CR%	MAb 6F12C CR%
Bacillus					
B.cereus	ATCC 11778	1.1	1.3	1.2	1.1
B.cereus	Wild type strain	2.2	2.3	1.3	1.2
B.subtilis	Wild type strain	1.1	1.3	0	1.3
Bordetella					
B.bronchiseptica	Wild type strain	1.1	1.2	1.2	2.1
Citrobacter					
C.freundii	Wild type strain	1.1	1.3	1.2	1.2
Enterobacter					
E. agglomerans	Wild type strain	0	2.3	2.2	1.1
E.amnigenus	Wild type strain	2.2	2.3	2.3	1.1
E.cloacae	Wild type strain	1.1	2.2	2.1	1.2
E.faecium	Wild type strain	1.1	1.5	2.2	1.1
Klebsiella					
K. Oxytoca	ATCC 49131	1.2	1.3	0	1.1
K.pneumoniae	Wild type strain	1.2	2.4	0	1.1
Proteus					
P. vulgaris	ATCC 49132	0	1.2	1.2	1.2
Pseudomonas					
P.aeruginosa	Wild type strain	1.4	1.3	1.2	1.2
Shigella					
S. flexneri	ATCC 12022	1.3	1.3	2.2	2.3
Staphylococcus					
S.aureus	ATCC 25923	3.2	2.3	2.1	2.2
S.epidermidis	Wild type strain	1.5	2.4	1.1	2.2
Brucella					
B.abortuss train 99	Wild type strain	1.5	1.2	2.2	1.1
B.melitensisisb biotype 1	Wild type strain	0	1.2	2.1	2.1
B. abortus RB51	Wild type strain	1.5	1.4	2.2	1.1
B.melitensisis biotype 2	Wild type strain	2.2	1.3	2.1	1.2
B.melitensis biotype 3	Wild type strain	0	1.3	2.1	1.2

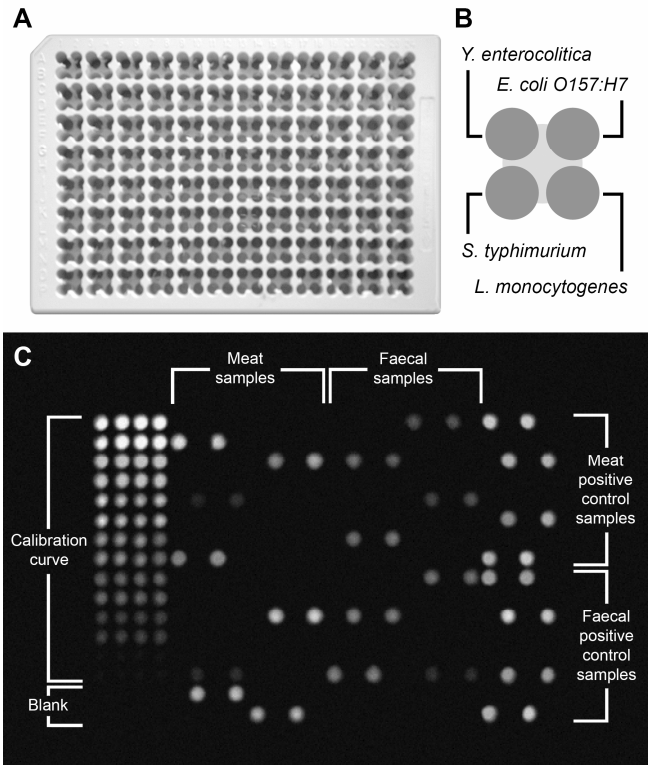
CL-EIA method. *MAbs and HRP-labeled polyclonal antibodies concentrations.* Preliminary experiments were performed to evaluate the optimal concentration of the four MAbs and the HRP-labeled polyclonal antibodies. Antibody concentrations were optimized by comparing dose-response curves obtained using different initial concentrations of MAbs (300, 400 and 600 ng/mL) and different dilutions of HRP-labeled polyclonal antibodies (1:40000, 1:60000, 1:80000 v/v), according to an optimized experimental design. A satisfying compromise in terms of linearity of the dose–response curve and limit of detection was obtained by using a MAb concentration of 300 ng/mL in the immobilization step and a 1:60000 (v/v) HRP-policlonal antibody dilution in the detection step.

Detection system. The developed CL-EIA assay allows to recognize simultaneously the four target bacteria thanks to the specificity of each antibody pair and the use of an imaging detection technique based on an ultrasensitive CCD camera.

A typical CL signal obtained using the new microtiter plate format is shown in **Figure 1C**: the emission signal is well-localized in the sub-wells, thus allowing the quantification of the signal of each sub-well by integrating the CL emission over the entire sub-well area (Roda, 1998).

Thanks to the spatial resolution of the imaging system and the high detectability of the HRP label enzyme by CL, the sensitive and rapid quantification of each bacterial species was performed in low reaction volumes without any interference of the CL signal among adjacent sub-wells even in presence of signals with different intensities. This assay format thus prevents cross-talk phenomena, which have been observed, for example, in microarray systems (Ekins, 1998; Nielsen, 2004). In addition, because of the standard size of the plate, the measurement could be also performed in conventional microtiter plate luminometers with a custom programming of the *xy* position of the wells. However, the sequential reading of all the sub-wells could require as long as 2–5 min, depending on the signal integration time for each well, whereas an imaging device allows the simultaneous measurement of the whole plate. Therefore, in the case of sequential measurements one would have to check the constancy of the light output over the entire period of measurement, especially for high CL signals.

Figure 1: (A) Live image of the new 96x4-well microtiter plate. The plate contains 96 main wells, each of them divided into 4 sub-wells. (B) Position of the immobilized antibodies within each sub-well. (C) Chemiluminescent image of the microtiter plate obtained after a 40-s exposure time (the position of standards and samples within the plate is also shown).



Calibration curve. The calibration curves (**Figure 2**) obtained for the four bacteria were linear in the range between 10^4 and 10^7 CFU/mL and the relative standard deviation (RSD) of individual points ranged from 3% to 9%. The quantification limit (LOQ), calculated as the bacteria concentration giving a signal equal to the mean signal of negative controls plus ten times their standard deviations, were in the range 10^4-10^5 CFU/mL.

Different incubation times and temperatures were also examined and the best performance was obtained when each incubation step was performed for 30 min at 37℃. Longer incubation times did not produce any significant improvement in the performance of the immunoassay.

Within-assay and between-assay precision. The precision of the method was determined by analyzing replicates of meat and faecal samples spiked with the four target bacteria at three different concentrations: 1 (low level), 10 (medium level), and 100 (high level) CFU/g of each bacteria. The bacteria-fortified samples were analyzed after enrichment in six replicates and in four different days to evaluate the within-assay and the between-assay

precision. Standard bacteria suspensions with initial concentrations ranging from 1 to 200 CFU/mL of each bacteria were also subjected to enrichment to obtain calibration curves for assessing the initial concentration of pathogens in the spiked samples. The results obtained for *Escherichia coli O157:H7* (mean and relative standard deviation calculated at each theoretical concentration level) are summarized in **Table 3**. Both the within- and between-assay relative standard deviations were below 15%. Similar results were obtained for all the other bacteria species tested.

Stability of the coated plates. Stability of the antibody-coated plates (vacuum dried and stored at 4℃) was investigated by comparing five c alibration curves obtained using plates stored either zero, one, three, six, or eight months. Experimental data show that storage of the antibody-coated plates up to six months did not significantly alter the performance of the assay in terms of limit of detection.

Method validation. A total of 32 naturally contaminated faecal (16) and meat (16) samples were analyzed for the presence of the four target bacteria with both the developed CL-EIA method and the reference microbiological method. No false negative or false positive results were observed and for all samples the bacteria strains detected using the CL-EIA method corresponded to those revealed by the microbiological reference method. In addition, there was a good agreement between the bacteria concentrations determined with the CL-EIA method and those measured with the microbiological one, as demonstrated by the recovery values ranging between 90 and 120%.

The obtained results prove that the newly developed CL-EIA method allows the simultaneous quantification of four pathogen bacteria in a given sample with accuracy and precision comparable to those achievable with the conventional microbiological methods. In addition the multiplexed assay format allows reducing assay time and sample and reagents consumption with respect to conventional single-analyte immunoassays and classical culture microbiology procedures., Other multiplexed assays could be also developed using the same approach i.e. by customizing the plate layout to divide each main well in the required number of sub-wells.

Figure 2: Representative calibration curves, obtained by averaging ten standard curves obtained in different days, for the determination of (A) *Escherichia coli O157:H7*, (B) *Listeria monocytogenes*, (C) *Salmonella typhimurium* and (D) *Yersinia enterocolitica* using the CL-EIA method. The curves are expressed as relative light units (RLU) against the bacteria concentration (CFU/mL).

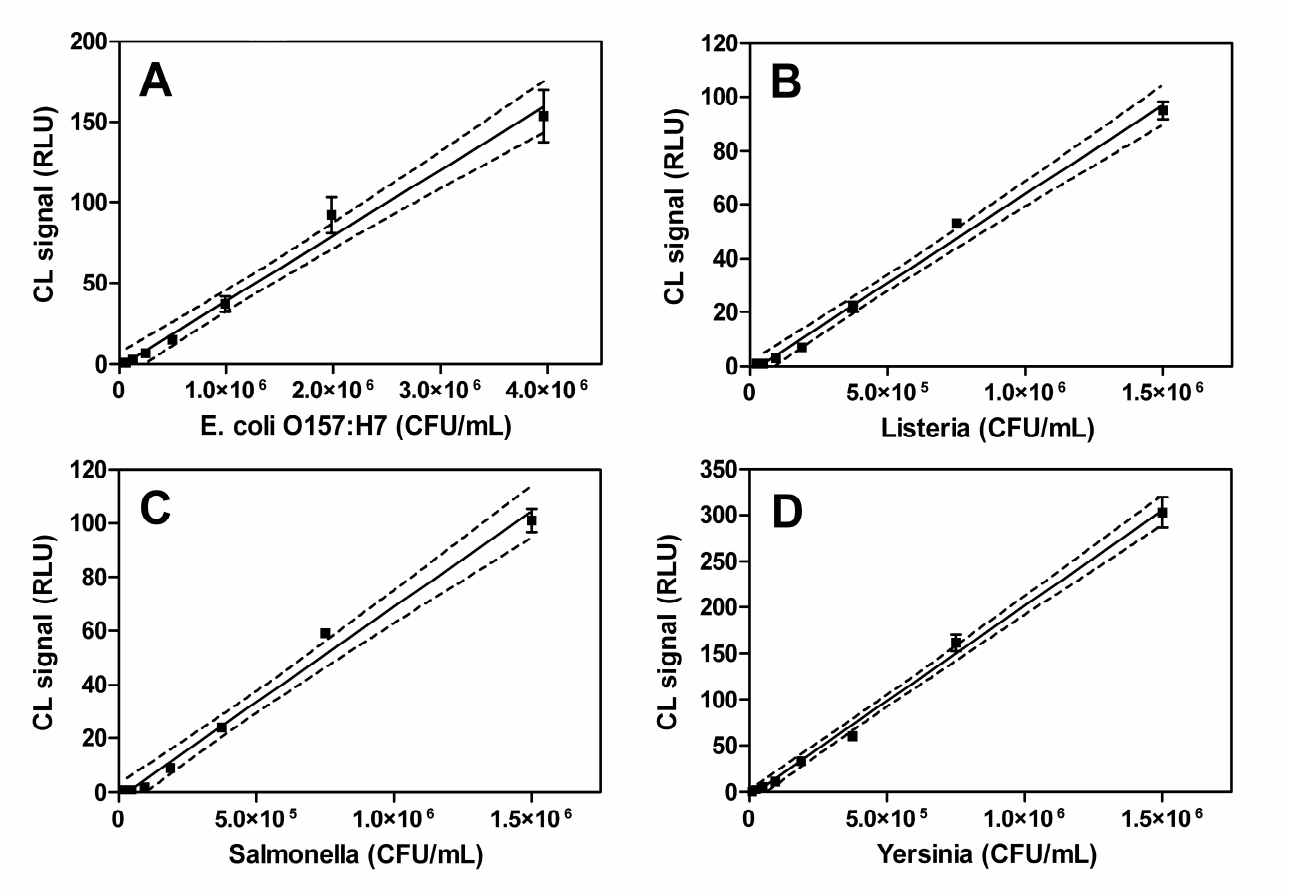


Table 3. Precision of the CL-EIA method. The table reports the results obtained for *Escherichia coli O157:H7* spiked meat samples. The values are expressed as CFU/g.

Sample concentration (CFU/g)	Found concentration (CFU/g) Mean ± SD	RSD (%)
Within-assay (n=6)		
1	1.10 ± 0.09	8.2
10	9.72 ± 0.82	8.4
100	99.2 ± 7.5	7.6
Between-assay (n=6)		
1	1.22 ± 0.16	13.1
10	9.33 ± 1.24	13.3
100	99.5 ± 11.2	11.2

Abbreviations used

LOQ, limit of quantification; CL, chemiluminescent; HRP, horseradish peroxidase; CL-EIA, chemiluminescent enzyme immunoassay; FCA, Freud's complete adjuvant; IFA, Freud's incomplete adjuvant; MAb, monoclonal antibody; UPB, universal peptone broth; PBS, phosphate-buffered saline; BPW, buffered peptone water; SD, standard deviation; RSD%, relative standard deviation; CFU, colony-forming units; RLU, relative light units; CR, cross reaction.

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CHAPTHER – 7 An innovative, flow-assisted, non-competitive chemiluminescent immunoassay for the detection of pathogenic bacteria.

Flow-assisted immunoassays offer several advantages with respect to conventional microtiter solid-phase format immunoassays, including faster kinetic of the immunological reaction and easier automation. This study describes the implementation of gravitational field-flow fractionation (GrFFF) for the development of an innovative, non-competitive CL enzyme immunoassay for the detection of intact pathogenic bacteria in biological samples. *Yersinia enterocolitica* was chosen as model analyte. A peroxidase-labeled monoclonal antibody is added to the sample containing the bacteria, and the mixture is immediately injected into the GrFFF channel where the immunological reaction takes place. After 30 min incubation, the free and bacterium-bound antibody fractions are separated by GrFFF in 15 min. The activity of the peroxidase-labeled bound fraction is online measured by a flow-through chemiluminometer, following post-channel infusion of an enhanced H_2O_2/luminol-based CL substrate. Calibration was performed on standard bacteria suspensions and the method was validated on faecal samples. The detection limit of the method was 10^6 CFU/mL, and calibration curves were linear in the range between 10^5 and 10^7 CFU/mL. Both run-to-run and day-to-day RSD were always ≤ 15%. Accuracy of the method was evaluated by comparing the results with those obtained with a conventional culturing method on selective media, and sample recovery resulted to be in the 90-120% range. The assay shows analytical performance that is similar that of to a microtiter plate format with a cost reduction and a shorter analysis time since the antibody is not immobilized, and only a single monoclonal antibody is needed for the detection of one bacterial species.

7.1 Introduction

Each year, as many as 80 million cases of foodborne illness, caused by the consumption of contaminated food or water, occur in the United States. It is acknowledged that approximately 30% of these cases are caused by bacteria and their related toxic products (Mead, 1999; World Health Organization, 2002). The bacteria species most frequently responsible for food contamination are *Salmonella typhimurium* (meat, milk, eggs), *Escherichia coli O157:H7* (meat), *Staphylococcus aureus* (milk cream, meat), *Clostridium perfringens* (sausages, preserved food), *Campylobacter jejuni* (poultry, eggs), *Vibrio parahaemolyticus* (shellfishes), *Yersinia enterocolitica* (meat, milk) and *Listeria monocytogenes* (dairy products) (Institute of Food Technologists, 2004).

Conventional microbiological methods for the identification of pathogenic bacteria are labor-intensive (several enrichment steps) and time-consuming (two to three days to obtain results) (Vasavada, 1997). Currently, new rapid procedures based on immunological, DNA hybridization or biosensing methods have been proposed (Bohaychuk, 2005; Deisingh, 2004). However, many of these rapid tests are expensive, laborious, and all of them are based on cultural enrichment to enhance sensitivity and selectivity before analysis. Simpler, faster and more sensitive diagnostic techniques are then required to improve food safety (food production, processing, storage and distribution) and to screen for potential bacterial infections in humans. Several immuno-based detection methods for infectious agents have been recently developed (Kerr, 2001; Chapman, 1997). Such methods are of great diagnostic importance because they offer unique detection specificity and sensitivity, with relatively simple and low-cost assay formats. Most commercially available immunological methods for the detection of bacteria are based on conventional sandwich immunoassays, which involve the formation of an immunocomplex with an immobilized antibody, the target bacteria, a second labeled antibody, and the separation of free and bound antibody fractions by washing steps. Unfortunately, these separation steps reduce the assay productivity, so alternative approaches are welcome.

In general, flow-assisted immunoassays offer advantages with respect to conventional microtiter solid-phase format, mainly related to a faster kinetic of the immunological reaction, and to an easier automation (Bao, 1997; Bilitewski, 2003). The kinetic of reaction is fast because the immunological reaction takes place in a pseudo-homogenous phase, that is the antibody is not immobilized on a bulk surface.

Field-flow fractionation (FFF) is an analytical technique suited for the

separation of nano- and microsized dispersed analytes (Shimpf, 2000). Separation is achieved through a relatively simple device, i.e. within an empty capillary channel by the combined action of a transporting laminar flow and a field applied perpendicularly to the flow. According to their physical features (size, density, surface properties, etc.), analyte particles are distributed at different positions within the parabolic flow profile, and are thus swept down the channel at different velocities, i.e. they are eluted at different times (Reschiglian, 2005). Recently, we proposed a flow-assisted, competitive immunoassay format based on flow FFF with chemiluminescent (CL) detection of microsized beads with immobilized antibodies (Roda, 2005). This new format did not require washing steps to separate free and bound tracer, which was quantified from void-peak and retained-peak area measurements, respectively.

In this work we report the implementation of gravitational FFF (GrFFF), the simplest FFF variant that employs Earth's gravity as the applied field, into an innovative whole-cell CL enzyme immunoassay format for the detection of pathogenic microorganisms in biological samples. The method combines the high sensitivity of CL detection with the GrFFF separation capabilities of able to efficiently separate the bound bacterium-antibodies complex from the free antibodies. The immunoassay employs a horseradish peroxidase (HRP)-labeled monoclonal antibody (MAb) produced against the target bacterium *Y. enterocolitica,* which is chosen as model analyte. The immunological reaction takes place on the whole bacterial cells, which constitutes the separate phase.

7.2 Materials and Methods

Monoclonal antibody production. *Yersinia enterocolitica* ATCC 23715 (American Type Culture Collection Rockville, MD) was used for monoclonal (MAb) antibody production and as positive control for MAb screening. The anti-*Y. enterocolitica* MAb was produced by immunizing balb/c mice with heat-inactivated and sonicated ATCC reference strain using a slightly modified published protocol (He, 1996; Harlow, 1998). Briefly, heat-inactivated and sonicated ATCC reference strains were diluted in Freud's incomplete adjuvant (IFA) (Sigma Chemical Co., St. Louis, MO) to obtain a protein concentration of 50 μg/mL and inoculated by intraperitoneal injection. The mice received their second injection at two weeks, two inoculations of 25 μg/mL were then administered. A booster injection of 50 μg/mL was given 31 days after immunization, then the mice were sacrificed and the spleen cells were fused with myeloma cells. Cell fusion and hybridoma cloning were performed using Galfre's method (Galfre, 1981) with some modifications. Briefly, splenocytes from

the immunized mice were subjected to cell fusion with myeloma cells from Sp2/O-Ag-14 mice (American Type Culture Collection Rockville, MD) using 50% polyethylene glycol (PEG) 1550 solution. Hybridomas were cultured for two weeks in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO) containing 20% foetal bovine serum (Euroclone, UK), 2 mM L-glutamine (HybriMax®, Sigma Chemical Co., St. Louis, MO), amphotericinpenicillin-streptomycin 100x (APS, Sigma Chemical Co., St. Louis, MO), 50 mg/mL gentamicin (Gentamicin Sulphate Solution, Sigma Chemical Co., St. Louis, MO), 10000 UI/mL nystatin (Nystatin suspension HybriMax®, Sigma Chemical Co., St. Louis, MO) and HAT 50x (HAT Media supplement HybriMax®, Sigma Chemical Co., St. Louis, MO). The hybridomas of interest were cloned by limiting dilution method (Campbell, 1987; Goding, 1993). MAbs were produced in vitro on large scale by serial culturing of antibody-secreting hybridomas and collection of their supernatants.

The cell culture fluids from actively growing hybridomas were screened using an indirect ELISA procedure against other isolated strains of the target bacteria and different bacteria species. Briefly, the hybridoma culture media, properly diluted, was added to well plate previously coated with the immunizing antigen. The plate was then incubated for 30 min at 37℃ and, after washes, HRP-labeled anti mouse anti body (purchased from Sigma, Chemical Co. St. Louis, MO) was added. The HRP activity was measured by adding a chromogenic substrate (TMB, KPL, Gaithersburg, MD). After incubation, the enzymatic reaction was stopped with 2N H_2SO_4 and the absorbance at 450 nm was recorded using a Multiskan EX spectrophotometer microtiter reader (Labsystems, Helsinki, Finland).

The MAb isotype was determined using an isotyping kit (ImmunoPure Monoclonal Antibody Isotyping Kit, Pierce, Rockford, IL). The antigen-independent method was employed. Microtiter plates were coated with a goat anti-mouse antibody, and the hybridoma supernatant was added to the plate wells. After incubation and washing, subclass-specific anti-mouse immunoglobulins (IgG2a, IgG2b, IgG3, IgA and IgM) were added in separated wells. A horseradish peroxidase conjugated, goat anti rabbit IgG was then added and detected by spectrophotometry. The monoclonal antibody was screened by immuno Western-blotting (Laemmli, 1970), purified by affinity chromatography and coupled to HRP using the periodate method (NaKane, 1974). Protein concentration was determined by using a Bradford assay.

Standard bacterial suspensions. Standard bacterial samples were obtained from *Y. enterocolitica* cultures. For the isolation of bacteria, colonies were transferred from selective solid agar (Herellea Agar, Biolife, Milan, Italy) to 5-7 mL Luria-Bertani (LB) broth

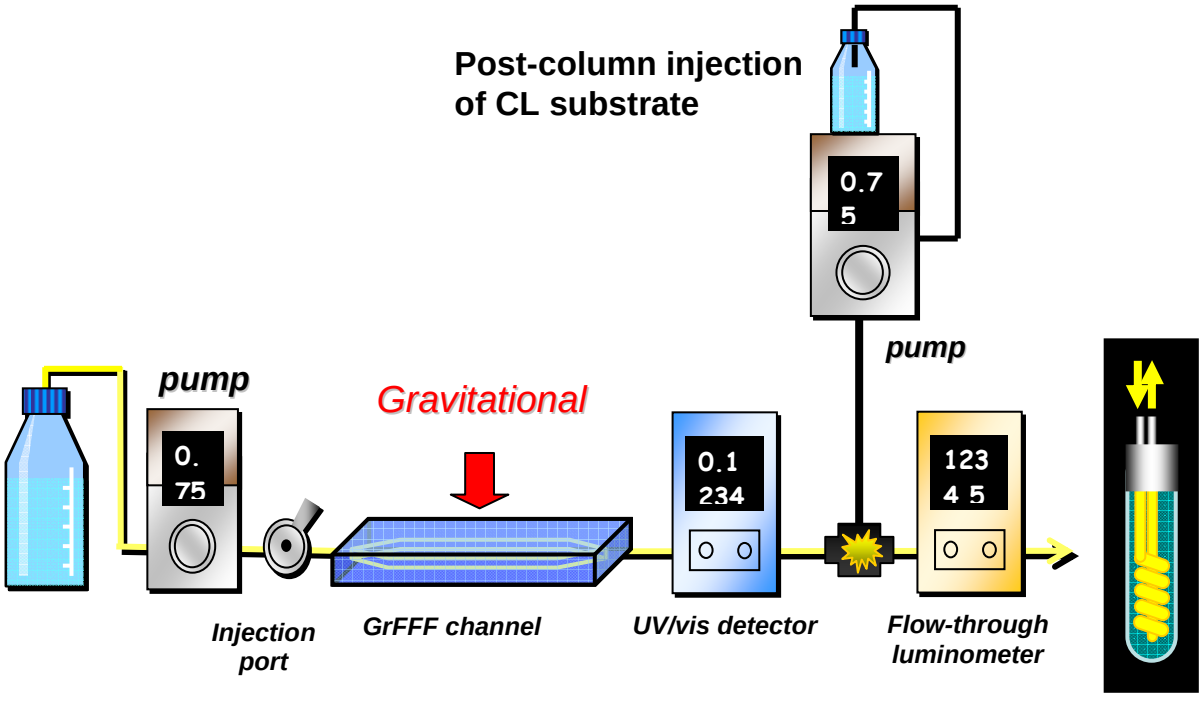
(Bacto® LB BROTH, LENNOX, DIFCO Laboratories, Detroit, MI), and were grown for 12-18 h at 37℃. A 2 mL aliquot of cell suspension in LB broth was centrifuged at 3000 x g for 10 min, the supernatant was carefully removed, and the pellet was washed twice with phosphate buffered saline (PBS). Washed bacteria were serially diluted in PBS to obtain concentrations in the range 10^9 - 10^4 CFU/mL which were used as standards in FFF immunoassay method. For each dilution, the actual concentration (CFU/mL) of each bacterium was determined by colonies counting on selective media agar plates. The obtained CFU/mL values were used for calibration curve plots.

Samples. Human faecal samples were used to evaluate the analytical performance and the applicability of the GrFFF immunoassay method for the detection of bacteria.
Unsterilized faecal samples (obtained from voluntaries) were first assayed to verify the absence of *Y. enterocolitica*, and then they were artificially contaminated with the bacterium. The samples (25 g) were inoculated with the appropriate amount of bacteria (final concentration ranging from 0 to 10 CFU/g), homogenized in a stomacher bag, and incubated with 225 mL of buffered peptone water for 2 h at 37℃. Subsequently, 1 mL of this solution was removed, and incubated with 9 mL Yersinia enrichment broth (Sigma, Chemical Co. St. Louis, MO) for 6 h at 37℃ under shaking. For each sample, the actual bacterium concentration (CFU/mL) was determined by colony counting on selective medium agar plates.
For clinical validation, we evaluated a total of 15 faecal samples (ten positive and five negative) for the presence of *Y. enterocolitica*, and the results were compared with those obtained using a conventional microbiological method.

GrFFF system. The separation device was a home-made, empty and flat capillary channel.The GrFFF channel was built in the laboratory according to a design already reported for fractionation of biological samples (Sanz*,* 1996; Reschiglian, 2002) and the system was assembled as described elsewhere (Roda, 2005*).* The channel volume was cut out from a thin plastic foil that was sandwiched between two plastic walls, the one on top made of polycarbonate (PC; PlastiCenter, Bologna, Italy), and the one on bottom made of polyvinylchloride (PVC; PlastiCenter). Channel dimensions were 2.0 cm breadth, 0.014 cm thickness and 30 cm length. Sample injection was performed by a Model 7125 valve (Rheodyne, Cotati, CA) equipped with a 20 µL PEEK loop. The carrier liquid was PBS at pH = 7.4, added with 0.05% (w/v) bovine serum albumin (BSA). The carrier flow was delivered by a peristaltic pump (Miniplus 3, Gilson, Middleton, WI) at 0.3 mL/min flowrate,

and the channel outlet was connected to a flow-through luminometer (home modified FB12 luminometer, Berthold Detection Systems, Pforzheim, Germany). To generate the CL signal, an enhanced luminol-based CL substrate (SuperSignal ELISA Femto, Pierce, Rockford, IL) was used. In previous work, this substrate was found to give low background. (Roda, 2002; Roda, 2000). The substrate was 1:5 diluted in the carrier, and post-channel injected at 0.03 mL/min flowrate by a syringe pump (KdScientific, KDS100, Holliston, MA, US) through a low-volume "tee" reactor. The injection of the substrate was synchronized with the elution flow. The GrFFF-CL instrumental set-up is reported in **Figure 1**.

Figure 1: GrFFF-CL instrumental set-up.

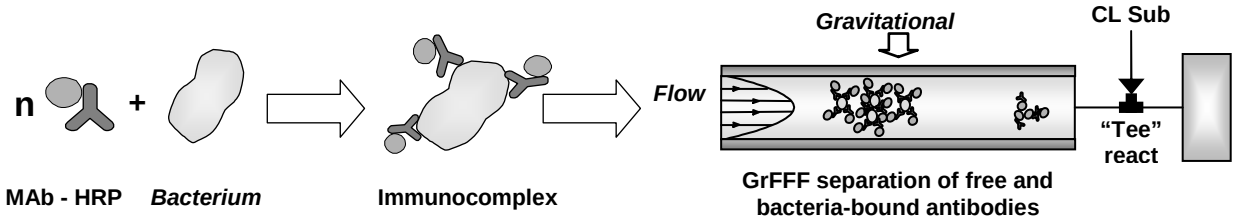


Home-made, flow-through detector cell (50µl)

GrFFF-CL immunoassay method. A schematic representation of the GrFFF-CL immunometric method is reported in **Figure 2**. For the assay, 100 µL MAb-HRP was added to 100 µL standard or faecal sample; the mixture was vortexed for 30 s, and 20 µL was injected in the GrFFF channel. After injection, the flow was stopped for 30 min, during which sample relaxation and the immunological reaction between bacterial cells and MAb took place. After relaxation, elution flow was restored. The free and bacterium-

bound MAb fractions were eluted in ~15 min, with the MAb-bacterium immunocomplex eluted in a relatively broad band, the retention time of which was t_r = 8.3 min. The excess of unbound labeled MAb was eluted within the void volume. The resulting CL fractogram was thus constituted of two resolved peaks: a void peak and a retained peak corresponding to the free and bound MAb fractions, respectively. The activity of the HRP-labeled bound fraction, which is proportional to the bacterial concentration, was measured from the entire area of the eluted immunocomplex band.

Figure 2: Schematic of the GrFFF-CL immunometric method for the determination of *Yersinia enterocolitica*.



7.3 Results and Discussion

Monoclonal antibodies screening. The MAb specificity was evaluated both against other isolated strains of the target bacteria and different bacteria species using an indirect ELISA procedure. As negative control to exclude cross-reactivity, IgG MAbs against *E.coli O157:H7*, *Salmonella thyphimurium*, and *Listeria monocytogenes* were used.

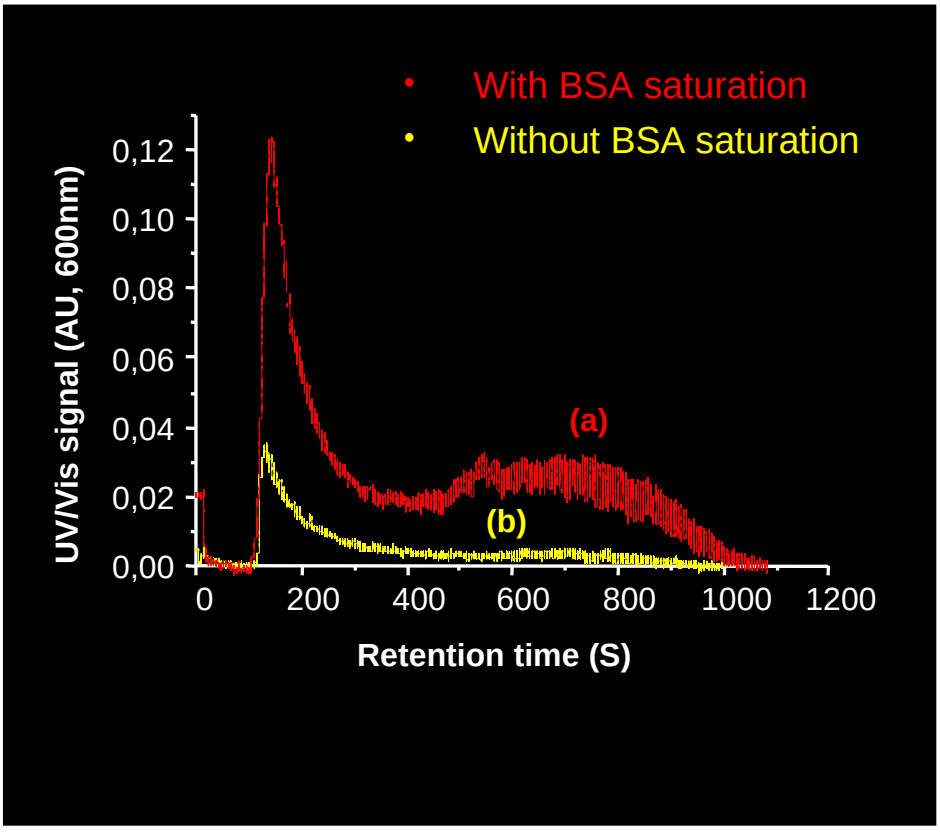
Approximately 1500 hybridoma supernatants against the target bacteria were obtained and tested. Among these, 200-400 clones showed positive reaction to *Yersinia enterocolitica* by indirect ELISA test. Subsequently, a further ELISA screening with other bacterial antigens led to the identification of the MAb 54B11 reacting with *Yersinia enterocolitica* and showing no cross-reaction to any other bacteria used in this study (**Table 1**). The MAb 54B11, which was identified as IgG1 isotypes, was used for the development of the GrFFF-CL immunoassay method. In addition, this monoclonal antibody was also used to develop the multiplexed chemiluminescent immunoassay for the detection of pathogen bacteria described above.

Table 1: Monoclonal antibody cross-reactions with various bacterial antigens evaluated by indirect ELISA.The percentage of cross reaction (CR%) was calculated by dividing the OD_{450} value of each bacteria by the OD_{450} for the target bacteria.

Genus	**Species**	**Source**	**Mab 54B11 CR%**
Bacillus	*B.cereus*	ATCC 11778	1.3
	B.cereus	Wild type strain	2.3
	B.subtilis	Wild type strain	1.3
Bordetella	*B.bronchiseptica*	Wild type strain	1.2
Citrobacter	*C.freundii*	Wild type strain	1.3
Enterobacter	*E. agglomerans*	Wild type strain	2.3
	E.amnigenus	Wild type strain	2.3
	E.cloacae	Wild type strain	2.2
	E.faecium	Wild type strain	1.5
Escherichia coli	*E.coli O14*	BGVV	1.1
	E.coli	ATCC 25922	2.1
	E.coli O157:H7	ATCC 35150	1.1
	E.coli O157:H7	Wild type strain	1.1
	E.fergussoni	Wild type strain	2.4
Klebsiella	*K. Oxytoca*	ATCC 49131	1.3
	K.pneumoniae	Wild type strain	2.4
Listeria	*L.innocua*	ATCC 33090	1.3
	L.ivanovii	ATCC 19119	2.3
	L.monocytogenes	ATCC 7644	1.2
	L.monocytogenes	Wild type strain	1.3
Proteus	*P. vulgaris*	ATCC 49132	1.2
Pseudomonas	*P.aeruginosa*	Wild type strain	1.3
Salmonella	*S.bredeney*	Wild type strain	1.1
	S.derby	Wild type strain	3.4
	S.enteritidis	ATCC 13076	2.2
	S.enteritidis	Wild type strain	3.2
	S.hadar	Wild type strain	2.3
	S. muenchen	Wild type strain	2.3
	S.panama	Wild type strain	2.2
	S. saint-paul	Wild type strain	1.2
	S.typhimurium	ATCC 14028	1.1
Shigella	*S. flexneri*	ATCC 12022	1.3
Staphylococcus	*S.aureus*	ATCC 25923	2.3
	S.epidermidis	Wild type strain	2.4
	S.faecalis	ATCC 29212	2.1
Brucella	*B.abortuss train 99*	Wild type strain	1.2
	B.melitensisisb biotype 1	Wild type strain	1.2
	B. abortus RB51	Wild type strain	1.4
	B.melitensisis biotype 2	Wild type strain	1.3
	B.melitensis biotype 3	Wild type strain	1.3
Yersinia	***Y.enterocolitica***	**ATCC 23715**	**100**
	Y.enterocolitica	Wild type strain	98

GrFFF-CL method optimization. Because it is known that interactions between the analytes and the channel in FFF can cause sample adhesion, specially when samples and wall surface have similar chemistry (particularly polarity), a procedure for conditioning the channel walls was considered in order to increase the surface hydrophobicity of the PVC accumulation wall and to increase sample throughput. This was achieved by adding 0.5% (w/v) BSA to the mobile phase and by continuously flushing it into the GrFFF channel. The BSA is known to reduce cell interactions and then to better preserve cell integrity [Battu, 2001]. It is also commonly used to block plastic surfaces in cell adhesion tests that employ protein-covered plastic plates. **Figure 3** shows the fractograms obtained under identical experimental conditions for *Yersinia enterocolitica* elution without (Figure 3b) and after conditioning (Figure 3a) with BSA-containing isotonic PBS used as mobile phase; retention of bacteria can be observed in both.

Figure 3: GrFFF fractogram obtained for *Yersinia enterocolitica* with and withouth BSA saturation.



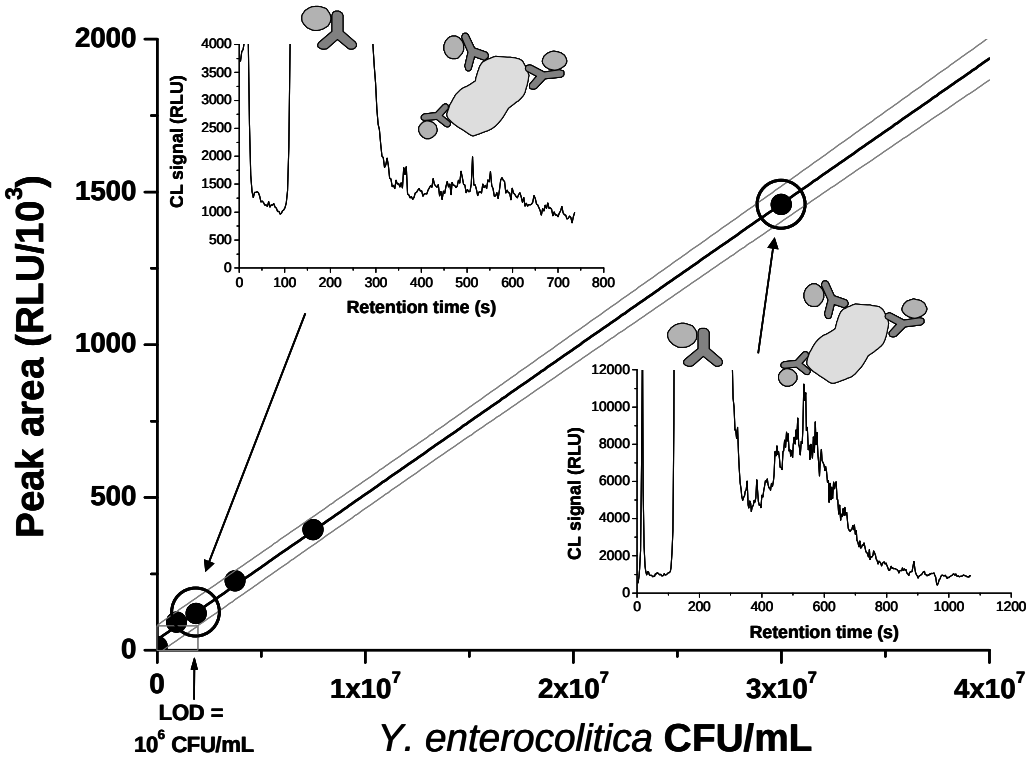
In particular, for the fractogram obtained after the longer conditioning period, the overall sample throughput was quantitative, indicating that all the injected cells were eluted from the GrFFF channel, and recovery of the retained part of the cells was 90%. When the channel was not conditioned, the overall sample throughput was 40% of the injected cells, and the intense void peak compared with the peak of the retained cells indicates lower recovery than when conditioning is longer. These findings suggest that BSA can improve recovery and sample retention, if a long conditioning period is applied to the channel.

To avoid system cross-contamination, a cleaning procedure was performed after each working day by flushing a solution of 30% (v/v) EtOH and 0.5% (w/v) SDS in water. Under these conditions, no run-to-run carry over was observed.

Preliminary experiments were performed to assess the optimal MAb-HRP ratio. Antibody concentration was optimized by comparing the CL signal obtained using different MAb (90-700 ng/mL) concentrations. The best results were obtained by using a MAb concentration of 350 ng/mL.

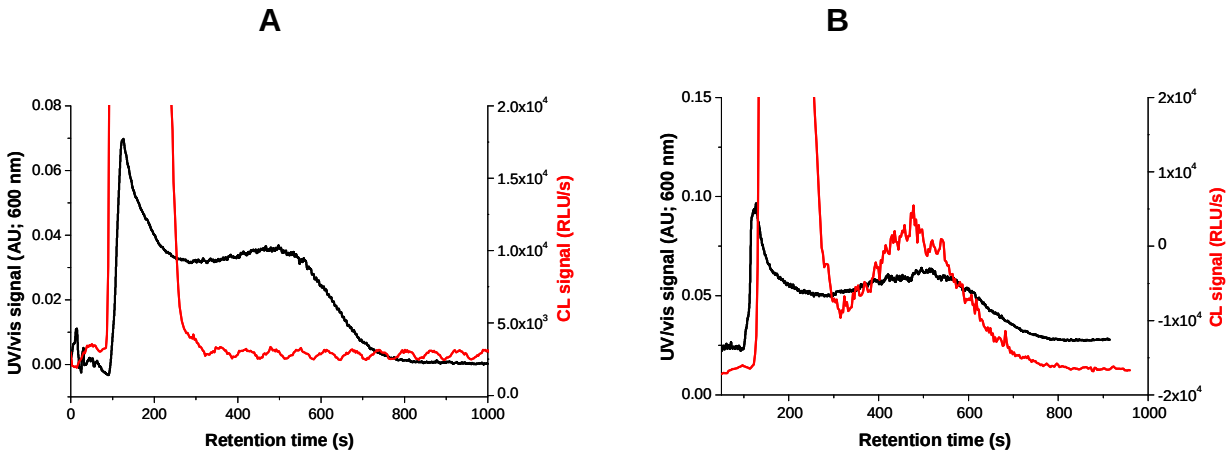
GrFFF-CL method analytical performances. The calibration curve (Figure 4) obtained for *Y. enterocolitica* was linear in the range between 10^5 to 10^7 CFU/mL, and the detection limit (LOD) of the method was 10^6 CFU/mL (P = 95%). Analytical validation of the method was performed by using spiked human faeces. Bacterial concentration in faecal samples was determined by interpolation from calibration curve. The precision was satisfactory being both run-to-run and day-to-day relative standard deviation (RSD), determined by analyzing replicates (n=6) of spiked samples, ≤ 15%. Method accuracy was evaluated by comparing results with those obtained with a conventional culturing method on selective media, and concordance between the two methods was found.

Figure 4: Representative calibration curve, obtained by averaging ten standard curves from different days. The insets show CL fractograms obtained for the highest and lowest concentration level.



Thanks to the MAb specificity, the method proved able to recognize all the wild strains of the target bacteria without any cross-reactivity with other species. In addition, the analysis of bacteria mixtures confirmed that no cross-reaction occurred. No CL signal was detected for negative mixtures, while it was possible to detect the target bacterium in mixture even at low relative concentration (**Figure 5**).

Figure 5: **A)** Fractionation of negative mixture (*E.coli*, *Salmonella typhimurium, Listeria monocytogenes*). **B)** Fractionation of positive mixture (*E.coli*, *Salmonella typhimurium, Listeria monocytogenes, Yersinia enterocolitica*).



For clinical validation, we evaluated a total of 15 faecal samples (ten positive and five negative) for the presence of *Y. enterocolitica*, and the results were compared with those obtained using a conventional microbiological method. An 100% agreement was found between the two methods.

The developed GrFFF-assisted, whole-cell, non-competitive immunoassay shows similar analytical performance, and many advantages with respect to a microtiter plate sandwich-type format. Being a flow-assisted immunoassay, the antibody is not immobilized, and only a single MAb is required for the detection of one bacterial species. Costs and analysis time are then reduced, and the method is suitable for automation. The GrFFF channel could be easily microfabricated for disposable usage or designed in multichannel format for multi-run analysis. Moreover, multiplexed immunoassays can be developed for simultaneous analysis of bacterial mixtures, since pathogenic bacteria present in the same sample with different morphological properties can be efficiently fractionated by GrFFF. For example, bacteria like *Y. enterocolitica*, *E. coli O157:H7*, *S. typhimurium*, and *L. monocytogenes* can be separated, detected and quantified in a single run using a mixture of MAbs, each Ab specific for one bacterium. For diagnostic purposes in clinical analysis, we expect that this method could be applied not only to bacteria but also to eukaryotic cells.

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