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EVALUATION OF A SURFACE PLASMON RESONANCE BIOSENSOR FOR THE IDENTIFICATION OF *SALMONELLA* TYPHIMURIUM AND *ESCHERICHIA COLI* O157:H7 IN THE PORK PRODUCTION CHAIN

By

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ABSTRACT

EVALUATION OF A SURFACE PLASMON RESONANCE BIOSENSOR FOR THE IDENTIFICATION OF *SALMONELLA* TYPHIMURIUM AND *ESCHERICHIA COLI* O157:H7 IN THE PORK PRODUCTION CHAIN

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An off-the-shelf surface plasmon resonance (SPR) sensor was converted to a biosensor to detect *Salmonella enterica* spp. Typhimurium and *Escherichia coli* O157:H7 in contaminated samples. The selectivity and specificity of the SPR biosensor were assayed using pure and mixed cultures of *S.* Typhimurium and *E. coli* O157:H7. Field samples were obtained from swine farms and representative small and medium-sized pork production facilities and analyzed using the SPR biosensor. In addition to the standard plate count, the VIDAS™ immunosensor was used to validate the results of the SPR biosensor.

The optimum antibody concentration for use on the SPR biosensor was 300 μg/ml. The detection limit of the SPR biosensor for *S.* Typhimurium and *E. coli* O157:H7 in pure culture was 10^7 CFU/ml. Inoculated samples reached this limit of detection after 5 1/2 hours of enrichment. Concentrations of non-target bacteria beyond 10^7 CFU/ml caused a decrease in the magnitude of the sensor response to the presence of the target pathogen.

The SPR biosensor has demonstrated potential for rapid, versatile, and accurate pathogen detection. With further studies and refinements, the SPR biosensor shows promise as a complementary pathogen detection system for food safety.

This thesis is dedicated to my family,
Alma, Wayne, Josephine, Sara, Andrew, and Allie Meeusen
for their love and belief in me,
and to
Dr. Evangelyn Alocilja,
for her unswerving enthusiasm and support.
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INTRODUCTION

Contamination of meat products by foodborne pathogens is a major food safety concern. . Foodborne bacterial pathogens are believed to be the most frequently occurring hazard of the nation's food supply. It has been estimated that one in ten persons in the United States experiences bacteria related food poisoning each year, with billions of dollars lost due to medical costs, lost productivity, and product recalls associated with outbreaks of food-borne illness (Hui *et al.,* 1994). In the United States, food-borne diseases cause an estimated 76 million illnesses, 325 thousand hospitalizations, and 5 thousand deaths annually (Buzby *et al.,* 1996). Researchers at the Economic Research Service (ERS) of the United States Department of Agriculture (USDA) estimate the annual cost of human illnesses for just seven food-borne pathogens from all food sources to be $5.6-$9.4 billion (Buzby *et al.,* 1996). Meat and poultry sources account for $4.5-7.5 billion of this total cost (Buzby *et al.,* 1996).

Pork products are growing in popularity among consumers of meat, and continue to expand their share of local and global markets. In 1996, the U.S. pork industry exported 413 thousand metric tons of pork valued at $1.1 billion (U.S. Meat Export Federation, 1997). Every time a pathogenic outbreak occurs in a pork product, consumer health is put at risk, and the viability of the pork industry is threatened.

Escherichia coli O157:H7 has emerged as an important enteric pathogen of considerable public health significance. Illnesses caused by *E. coli* O157:H7 can range from mild, watery diarrhea to life-threatening conditions such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. The combination of the severe consequences of infection, its low infectious dose, and its association with many common foods makes *E. coli* O157:H7 a bacterial pathogen of particular concern. *E. coli* O157:H7 has been identified in many commonly consumed foods, including pork products (Jay, 2000).

Salmonella enterica has been identified as the most prevalent and costly of known foodborne pathogens (Davies, 1997). It is the main cause of documented foodborne illness in most developed countries (Buzby *et al.*, 1996). Adding to the concern about *Salmonella* infection is the existence of several antibiotic resistant strains, especially the multi-drug resistant strain *S.* Typhimurium DT 104. With the emergence of this strain, the incidence and severity of Salmonella-related human illness is increasing. *Salmonella* is of particular concern in the pork industry. Currently 11% of all outbreaks of salmonellosis in humans have been associated with pork (Isaacson *et al.*, 2000). Adding to the problem of *Salmonella* infection in pigs is their ability to become long-term carriers of the organism.

In an effort to reduce the occurrence and numbers of pathogens on meat and poultry products, recent food safety initiatives have emphasized the monitoring and control of foodborne pathogens. Contributing to the prevalence of foodborne disease is the fact that these foodborne pathogens do not

necessarily make the food look, smell, or taste bad. There is no definitive way to tell simply by looking at a food whether or not it is contaminated (Moutville, 1987). Because of this, tests must be performed that are accurate and sensitive to very low levels of microbial contamination. Classical approaches to quality control have relied heavily on microbiological determinations of both raw materials and end products, but the time required for results is too long for many products (Jay, 2000). For these reasons, there is a growing need in the food industry for pathogen detection systems that are sensitive to low levels of bacteria, specific to the target organism, inexpensive, and capable of yielding results at or near real-time.

Surface Plasmon Resonance (SPR) is an optical phenomenon that occurs as a result of total internal reflection of light at a metal film-liquid interface. Although the light is totally reflected, a component of the incident light momentum, the evanescent wave, penetrates into the less dense liquid medium. In a SPR biosensor, the evanescent wave interacts with surface plasmons (free oscillating electrons) in the thin metal film surface. When SPR occurs, energy from the incident light is lost to the metal film, resulting in a decrease in light intensity. The resonance phenomenon occurs only at a precisely defined angle of incidence, which is dependent on the refractive index of the medium adjacent to the metal surface. The refractive index changes in direct proportion to the mass and the make-up of the medium present. When antibodies are affixed to the metal surface, the angle of incidence that causes SPR depends on the amount of antibody-antigen substrates present. By using antibodies specific to

pathogens of interest, it is possible to utilize the SPR phenomenon to measure the amount of pathogenic bacteria present in a sample by measuring the change in refractive index.

The goal of this research is to assess the feasibility of using a SPR biosensor to detect *Salmonella* Typhimurium and *E. coli* O157:H7 in environmental and meat samples from representative pork packing facilities. The objectives of this investigation include determining the sensitivity and specificity of the SPR biosensor assay, and comparing the use of the SPR biosensor as a diagnostic tool to the VIDAS™ immunoassay for detecting *Salmonella* spp. and *E. coli* O157:H7 in samples obtained during pork production.

CHAPTER 1

REVIEW OF THE LITERATURE

I. The Pork Industry

Foodborne bacterial pathogens are believed to be the most frequently occurring hazard in the nation's food supply. It has been estimated that one in ten persons in the United States experiences a bacterial foodborne illness each year (Hui *et al.,* 1994). More than 200 known diseases are transmitted through food, causing an estimated 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths annually (Mead *et al.,* 1999). Of the confirmed cases of human foodborne illness and deaths reported to the Centers for Disease Control and Prevention (CDC), over 90% are attributed to bacteria (Buzby *et al.,* 1996). Worldwide, diarrheal illnesses are the leading cause of childhood death and the second leading cause of death in general, behind cardiovascular disease (Morse *et al.*, 1994). One estimate of the annual U.S. cost for human illness from all food sources is $5.6-$9.4 billion. Meat and poultry sources account for $4.5-7.5 billion of this total cost (Buzby *et al.,* 1996).

Pork products are growing in popularity among consumers of meat, and continue to expand their share of local and global markets. In 1996, the U.S. pork industry exported 413 thousand metric tons of pork valued at $1.1 billion (U.S. Meat Export Federation, 1997). Every time a an outbreak involves a pork product, consumer health is put at risk, and the viability of the pork industry is threatened.

It is generally agreed that the internal tissues of healthy slaughter animals are free of bacteria at the time of slaughter (Jay, 2000). However, husbandry conditions during transport to slaughter may favor the growth or shedding of a particular pathogen, leading to the pathogen becoming an important component in the feces of pigs (Letellier *et al.,* 1999). During evisceration, fecal bacteria may contaminate the meat, and eventually cause diseases in humans. Other primary sources and routes of microorganisms to fresh meats include the stick knife, animal skin or hide, the gastrointestinal tract, the hands of handlers, containers, the handling and storage environment, and/or lymph nodes (Jay, 2000).

When many animals are slaughtered in a single time period, there is a tendency for any bacteria present in the animals to be spread among the carcasses (Jay, 2000). The pork processing chain is a multi-staged process, (Figure 1.1), with the potential for contamination by pathogenic bacteria at each stage.

Pork products have been implicated in a number of outbreaks of foodborne illness. Fecal shedding and growth of pathogens in the farm environment can lead to subsequent contamination of the slaughterhouse environment and the finished food-product. Most outbreaks are associated with intensively reared weaned pigs (Ryser, 2000).

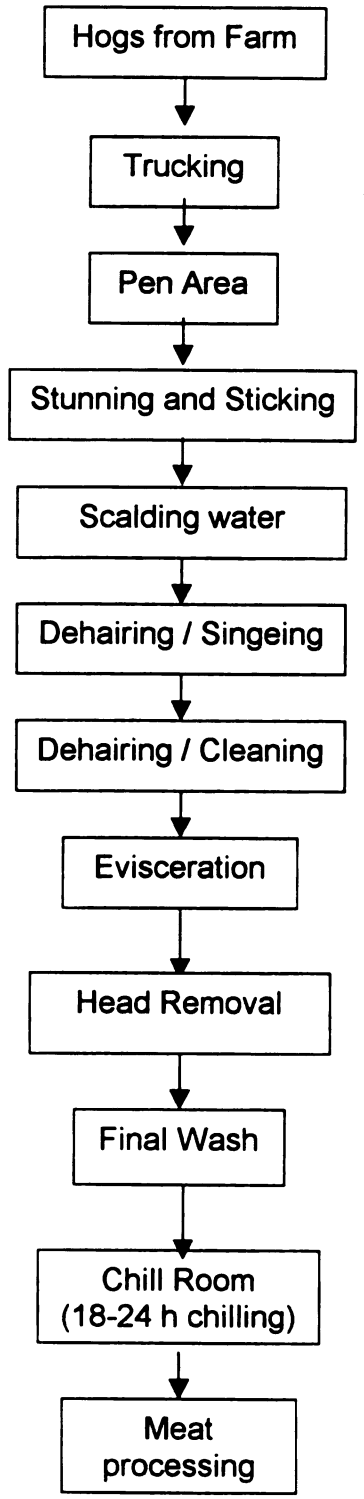


Figure 1.1. Pork processing flowchart (Ryser, 2000).

Recent outbreaks of foodborne illness caused by *Salmonella* have caused pork to be more closely analyzed as a source of bacterial pathogens (Isaacson *et al.,* 1999). Currently 11% of all outbreaks of salmonellosis in humans have been associated with pork (Isaacson *et al.,* 2000). In one study of pork and beef carcasses, salmonellae were recovered from 27% of 49 pork samples (Jay, 2000). One hundred twenty-one *Salmonella* strains were isolated from 225 pork carcasses in northeast Georgia (Epling and Carpenter, 1990).

Adding to the problem of *Salmonella* infection in pigs is their ability to become long-term carriers of the organism. Shedding of *Salmonella* by these animals may contaminate the environment, instruments, equipment, and meat at the slaughterhouse (Letellier *et al.,* 1999). Carrier pigs do not have any clinical signs of infection, which makes them difficult to identify (Isaacson, 2000).

Escherichia coli O157:H7 is a slightly less well-known pathogen in pork products, but because of its potential virulence, it is still important. Enteric bacteria are common meat contaminants, due to their presence in the gastrointestinal tract. Of 442 meat samples examined, 86% yielded enteric bacteria. The mean number for 94 pork sausage samples tested was 7.9 CFU/gram (Jay, 2000). *E. coli* was one of the most commonly found, with 29% of meat samples testing positive (Jay, 2000).

Because of growing consumer demand for pork and pork products, more research is focussing on the pork industry chain as a potential source of human foodborne illness. As a whole, foodborne pathogens are a major food safety concern. Any pathogenic outbreak associated with a pork product puts

consumer health at risk, and threatens the vitality of the pork industry. Improvements in monitoring the pork industry chain for pathogenic bacteria would save time and money, and most importantly, lower public health risks for the consumer.

II. *Escherichia coli* O157:H7

E. coli O157:H7 is a facultative, non-sporing rod-shaped facultative member of the *Enterobacteriaceae* family, which has emerged as an important enteric pathogen of considerable public health significance in the United States, Canada, and Europe. It has caused many outbreaks and numerous sporadic cases of hemorrhagic colitis, hemolytic-uremic syndrome, and diarrheal illness occurring in day-care centers, schools, nursing homes, and the community (Ratnam *et al.,* 1988). Raw or under-cooked hamburger and beef, raw milk, unpasteurized apple cider, contaminated water, mayonnaise, and unwashed vegetables have all caused outbreaks (Monk *et al.,* 1995; Jay, 2000). The Center for Disease Control and Prevention (CDC) estimates that there may be 20,000 illnesses a year due to *E. coli* O157:H7 infection (Jay, 2000).

E. coli in its non-pathogenic forms is common in warm-blooded animals (Buzby *et al.,* 1996). A German pediatrician, Dr. Theodore Escherich, first described what was then called *Bacterium coli commune* in 1885 (Neill *et al,* 1994). Use of the suffix *commune* recognized that this organism is distributed widely in the intestinal flora of many animals, and is the predominant facultative anaerobe in the human bowel, helping to maintain normal physiologic function of

the intestine (Neill *et al.*, 1994; Varnam and Evans, 1991). As a human pathogen, the organism, now called *Escherichia coli*, was recognized as a cause of infant diarrhea as early as the 1700s (Jay, 2000). *E. coli* was established as a foodborne pathogen in 1971 when imported cheeses contaminated with an enteroinvasive strain turned up in 14 American states, causing illness in nearly 400 individuals (Jay, 2000).

Five virulence groups of *E. coli* are recognized: enteroaggregative (EAggEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and enterohemorrhagic (EHEC) (Jay, 2000; Neill *et al.*, 1994; Varnam and Evans, 1991). The five main strains each have a distinct clinical pattern, and differences in epidemiology, pathogenicity, and O:H antigens.

Enteroaggregative (EAggEC) E. coli

Enteroaggregative *E. coli*, also designated enteroadherant, is composed of strains that have been epidemiologically linked to diarrhea disease, but lack properties typical of the other four groups (Neill *et al.*, 1994). EAggEC strains are related to EPEC but display unique aggregative adherence (Jay, 2000). Some EAggEC strains produce heat-stable enterotoxin (ST), designated EAST1. The distinguishing clinical feature of EAggEC strains is a persistent diarrhea that lasts more than 14 days, especially in children. It is unclear whether members of this group are foodborne pathogens (Jay, 2000).

Enterotoxigenic (ETEC) E. coli

Enterotoxigenic *E. coli* strains produce ST enterotoxin and/or heat-labile (LT) enterotoxin (Neill *et al.*, 1994). ETEC strains colonize the small intestine by

means of fimbrial colonization factor antigens (CFAs). Once attached, the ETEC strains produce enterotoxins. Unlike EPEC strains, which produce diarrhea primarily in the very young, ETEC strains cause diarrhea in both children and adults (Jay, 2000). ETEC infection resembles cholera with a severe dehydrating diarrhea and high mortality, most commonly in children (Neill *et al.,* 1994). Approximately 10^8-10^{10} colony forming units (CFU) are necessary for diarrhea by an ETEC strain in an adult human (Jay, 2000). In areas of poor sanitation, transmission is often caused by fecal shedding of ETEC strains from asymtomatic adults (Neill *et al.,* 1994).

Enteroinvasive (EIEC) E. coli

Enteroinvasive *E. coli* strains derive their virulence from their ability to invade epithelial cells (Neill *et al.,* 1994). They do not generally produce enterotoxins as do ETEC strains, but they enter and multiply in colonic epithelial cells and then spread to adjacent cells in a manner similar to the shigellae (Jay, 2000). Members of this group are most often found in the colon, and cause voluminous bloody or non-bloody diarrhea. Although foods are a proven source of this strain, person-to-person transmission is known (Jay, 2000; Neill *et al,* 1994).

Enteropathogenic (EPEC) E. coli

Enteropathogenic *E. coli* strains have been epidemiologically linked to diarrheal illness, produce neither the LT or ST enterotoxins, and are non-invasive (Neill *et al.,* 1994; Jay, 2000). These strains possess adherence factor plasmids that enable adherence to the intestinal mucosa, and the development

of attachment-effacement (A/E) lesions in which surrounding microvilli are destroyed (Neill *et al.*, 1994). The A/E lesions in the intestine appear to be the most important virulence factor of EPEC strains, which do not produce detectable quantities of Shiga toxin (Jay, 2000). EPEC strains cause diarrhea in children generally under one year old. Severe disease resulting in prolonged diarrhea or death is rare (Neill *et al.*, 1994).

Enterohemorrhagic (EHEC) E. coli

Enterohemorrhagic *E. coli* are similar to EPEC strains in that they both possess the chromosomal gene *eae* (*E. coli* attaching and effacing gene), resulting in the production of attachment-effacement lesions that disrupt microvilli on the intestinal wall of the host (Kudva *et al.*, 1997). In contrast to EPEC, however, EHEC strains affect only the large intestine. All EHEC strains produce Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2), also referred to as verotoxin 1 (VT1) and verotoxin 2 (VT2), because of their toxicity to African green monkey kidney tissue cells (Vero cells) (Jay, 2000; Buchanan and Doyle, 1997). EHEC strains produce a clinical illness of severe bloody diarrhea ('hemorrhagic colitis') and possess certain virulence determinants such as SLT production, possession of a 60-Mda plasmid, and eliciting attaching –and-effacing histopathologic lesions in an animal mode. Serotype O157:H7 is considered the most well characterized, 'prototypic' EHEC strain (Neill *et al.*, 1994).

Escherichia coli O157:H7

The H7 antigen type was initially isolated in 1944 from a human diarrheal specimen, whereas the O157 type was first isolated and named from diarrheal swine feces (Orskov *et al.,* 1977). The first O157:H7 strain was recovered in 1975 from a woman with bloody diarrhea (Jay, 2000; Neill *et al.,* 1994). *E. coli* O157:H7 was next recovered in 1978 from diarrheal stools in Canada (Jay, 2000). *E. coli* O157:H7's recognition as a human pathogen was finalized in 1982, following two outbreaks of severe bloody diarrhea in Oregon and Michigan, transmitted through consumption of fast-food hamburgers (Neill *et al,* 1994; Ratnam *et al.,* 1988; Buchanan and Doyle, 1997).

Following a Pacific Northwest outbreak in 1993, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) undertook a multistate study of the prevalence and incidence of *E. coli* O157:H7 in both beef and dairy herds. The largest number per gram found was 15, and the average was approximately 4 CFU/g of fresh beef. In a nationwide survey conducted between 1994 and 1998, the USDA found *E. coli* O157:H7 in 23 of 23,900 ground beef samples (Jay, 2000). Doyle and Schoeni (1987) found *E. coli* O157:H7 in 3.7% of 164 beef, 1.5% of 264 pork, 1.5% of 263 poultry, and 2.0% of 205 lamb samples.

One distinguishing feature of serotype O157:H7 is its lack of rapid (less than 48 hrs) fermentation of sorbitol. The most common screening procedure for this organism is based on this feature, because 93% of *E. coli* isolated from humans do ferment sorbitol (Neill *et al.,* 1994).

Toxins

The toxins produced by EHEC strains of *E. coli* (verotoxin, verocytotoxin) have been referred to as Shiga-like toxins. Shiga toxin is a potent toxin that is produced by *Shigella dysenteriae*. The terms 'Shiga-like toxin' and 'Verotoxin' are considered synonymous, although specific toxins (e.g. SLT-II and VT-2) may not be identical (Neill *et al.*, 1994).

Epidemiology

An estimated 2.8 cases of *E. coli* O157:H7 related illness per 100,000 people were reported in 1998 (USDA-FSIS, 1998). Human infections with *E. coli* O157:H7 are usually linked to consumption of contaminated and improperly cooked beef, unpasteurized milk, or fresh fruits and vegetables, water, or apple cider (Besser *et al.*, 1993; Griffin and Tauxe, 1991; Hofmann, 1993).

People of all ages, from 1 to 80 years old, have developed *E. coli* O157:H7 infection (Neill *et al.*, 1994). The mean incubation period for this serotype is generally 1-2 days after eating contaminated foods (Buchanan and Doyle, 1997), although longer periods of 3.1 to 8 days have been reported (Neill *et al.*, 1994). Illness typically begins with a short prodrome of mild non-bloody diarrhea that progresses within a day to grossly bloody diarrhea, often associated with severe abdominal pain and cramping (Neill *et al.*, 1994). The period of overtly bloody diarrhea accompanied by severe abdominal pain and moderate dehydration typically lasts 4-10 days (Buchanan and Doyle, 1997). Because of these symptoms, cases frequently have been misdiagnosed as having a noninfectious cause such as mesenteric infarction, inflammatory bowel

disease, Meckel's diverticulum, intussusception, and anatomic gastrointestinal bleeding (Neill *et al.* 1994).

E. coli O157:H7 can be carried asymptotically, like many other enteric pathogens such as *Salmonella, Shigella,* and *Campylobacter* (Neill *et al.,* 1994). The most common method of transmission is the fecal-oral route, and illness can result from very low levels of bacteria (Buchanan and Doyle, 1997).

Several complications may arise in patients with hemolytic colitis, of which hemolytic uremic syndrome is the most common. Other potential complications include thrombotic thrombocytopenic purpura, and acute renal failure (Buchanan and Doyle, 1997).

Hemolytic Uremic Syndrome

E. coli O157:H7 has been blamed for 85-95% of all hemolytic uremic syndrome (HUS) cases (Griffin, 1995). First described in 1955 (Neill *et al.,* 1994), HUS consists of an acquired Coombs' negative hemolytic anemia, thrombocytopenia, and acute renal failure. The onset of HUS is typically 7 days after the onset of gastrointestinal symptoms (Buchanan and Doyle, 1997). Approximately 10% of patients with hemorrhagic colitis develop HUS (Simmons, 1997). This proportion varies among reported series and outbreak investigations, most likely due to the presence of various risk factors, such as extremes in age, antimicrobial use, antimotility agents, and toxin type of the infective strain (Neill *et al.,* 1994).

Symptoms of HUS include pallor, intravascular destruction of red blood cells (microangiopathic hemolytic anemia), thrombocytopenia, lack of urine

formation (oligo-anuria), edema, and acute renal failure (Buchanan and Doyle, 1997). HUS is a highly morbid disease, with a fatality rate of 3-5%, and a considerable risk of long-term morbidity such as hypertension, chronic renal failure, and disability (Neill *et al.,* 1994; Buchanan and Doyle, 1997).

Thrombotic Thrombocytopenic purpura

A second complication associated with *E. coli* O157:H7 infection is thrombotic thrombocytopenic purpura (TTP). TTP consists of the cardinal features of HUS (hemolytic anemia, thrombocytopenia, and renal failure) along with fever and altered mental status (Neill *et al.,* 1994). TTP, however, generally causes less renal damage, and is restricted primarily to adults (Buchanan and Doyle, 1997).

III. *Salmonella enterica* serotype Typhimurium

The salmonellae are gram-negative, non-sporing, motile facultative members of the *Enterobacteriaceae* family (Jay, 2000). The USDA has identified *Salmonella enterica* as the most prevalent and costly of the known foodborne pathogens (Davies, 1997). *Salmonella* is a main cause of documented foodborne human illnesses in most developed countries (Buzby *et al.*, 1996). Each year in the United States an estimated 8-18 thousand hospitalizations, 2,400 cases of septicemia, and 500 deaths are associated with *Salmonella* infections (USDA, 1999). There are more than 2 thousand serotypes (or serovars) of the genus, of which 50-150 have been linked to disease outbreaks (Ziprin, 1994). The term 'serotype' refers to a group of related microorganisms

distinguished by its composition of antigens (Buzby *et al.,* 1996). Of the *Salmonella* serotypes, salmonellae have been placed in two species, *S. enterica* and *S. Bongori* (Jay, 2000). The serotypes are divided into five sub-species, most of which are classified under the *S. enterica* type species (Jay, 2000; Buzby *et al.,* 1996). The strains are classified by their serotype designation. For example, the serotype *typhimurium* is found among the *S. enterica* species, but is referred to as *Salmonella enterica* serotype Typhimurium, or *S.* Typhimurium (Jay, 2000; Buzby *et al,* 1996).

Salmonella was first cultured by Gaffky in 1884 (Jay, 2000). The same year, T. Smith and D. E. Salmon first described it in the environment, and the genus was subsequently named in Salmon's honor (Ziprin, 1994; WVA, 1997). Until 1949, *S.* Typhi was the predominant strain found to cause typhoid fever in humans (Tauxe, 1991). As typhoid fever was nearly eliminated due to advances in medicine and food safety, the non-typhoid strains, including *S.* Typhimurium, became leading sources of *Salmonella* infection in humans (Tauxe, 1991). The major *Salmonella* serotypes causing salmonellosis and human gastrointestinal disease are *S. enterica* spp. Typhimurium, Enteritis, and Typhi (Lin and Tsen, 1999, WVA, 1997). A survey of *Salmonella* isolates obtained from patients in Taiwan revealed that *S. enterica* spp. Typhimurium is the most common serotype causing foodborne disease in humans (Wang *et al.,* 1994). Due to the resistance of *S.* Typhimurium strain DT 104 to a range of antibiotics, it is a pathogen of increasing concern (WHO, 1997).

The salmonellae can be placed into three epidemiological groups: those that infect humans only, the host-adapted serotypes, and unadapted (no host preference) serotypes (Jay, 2000; WHO, 1997).

Salmonella infecting humans only

Salmonellae that cause enteric fever only in humans and higher primates include *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi C (Jay, 2000; WHO, 1997). This group includes the agents of the most severe of all diseases caused by salmonellae; typhoid and paratyphoid fevers (Jay, 2000).

Host-adapted Salmonellae serotypes

The host-adapted salmonellae serotypes include *S.* Gallinarum (poultry), *S.* Dublin (cattle), *S.* Abortus-equi (horses), *S.* Abortus-ovis (sheep), and *S.* Choleraesuis (swine). As the name suggests, these are serotypes of *Salmonella* that have been isolated from only one host-species. These serotypes are infrequently human pathogens (Jay, 2000; WHO, 1997). However, when these strains do cause disease in humans, it is often invasive, and can be life-threatening (WHO, 1997).

Unadapted Salmonellae serotypes

Unadapted *Salmonella* serotypes can be pathogenic for humans and other animals, and include most foodborne serotypes, including *S.* Typhimurium (Jay, 2000). These strains typically cause only gastroenteritis that is mild and self-limiting, but can be severe in the young, the elderly, of those with weakened immune systems (WHO, 1997).

***Antibiotic-resistant* S. *Typhimurium* strain DT 104**

Adding to the virulence of salmonellae infection is the recent discovery of several antibiotic-resistant strains, especially the multi-drug resistant *S.* Typhimurium strain Definitive Type (DT) 104 (Foster, 1997; WHO, 1997; WVA, 1997). With the emergence of this strain, the incidence and severity of *Salmonella*-related human illness is increasing, with some countries seeing a 20-fold increase in incidence in the past 10-15 years (WHO, 1997). Today *S.* Typhimurium strain DT 104 is a leading cause of Salmonellosis in the United States (Tauxe, 1991; IFST, 1997).

Strain DT 104 is a subpopulation of *S.* Typhimurium that reacts in a specific way when tested against a battery of bacteriophages. An additional characteristic of *S.* Typhimurium strain DT 104 is that isolates, known as resistance type (R-type) ACSSuT, are commonly resistant to several antibiotics, including ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (Foster, 1997; WVA, 1997; Karpiskoval *et al.*, 1999).

S. Typhimurium DT 104 was first identified in 1970, and was sensitive to antimicrobial agents. Multi-drug resistant strains of *S.* Typhimurium DT 104 emerged in cattle in 1988 in England and Wales (WHO, 1997; Foster, 1997). It has since been isolated from sheep, pigs, horses, goats, emus, cats, dogs, elk, mice, coyotes, ground squirrels, raccoons, chipmunks, and birds (Jay, 2000). *S.* Typhimurium DT 104 infection in humans has been associated with the consumption of beef, chicken, lamb, pork, sausage, raw milk, and meat paste as well as with the handling of sick animals (Foster, 1997; IFST, 1997).

This drug resistant strain is of particular concern, because antimicrobial therapy is used extensively to control *S.* Typhimurium infection in animals (WHO, 1997). The emergence of an antibiotic-resistant strain has made infections with *S.* Typhimurium in food animals difficult to control. Over the past three decades, many countries have reported sharp rises in salmonellosis (WVA, 1997). Between 1990 and 1995, the number of *S.* Typhimurium DT 104 isolates from humans in Britain increased from 259 to 3,837 per year—a 15-fold increase. The percentage of drug-resistant isolates increased from 39% in 1990 to 97% in 1995 (Foster, 1997).

Serotypes that are resistant to antimicrobial agents seem to depend more heavily on characteristics of the host (e.g. extremes in age, and/or strength of the immune system) than do those serotypes that are sensitive to antimicrobial agents (Buzby *et al.,* 1996). Infection with multi-drug resistant *S.* Typhimurium DT 104 has been associated with hospitalization rates twice that of other zoonotic foodborne *Salmonella* infections, and with ten-fold higher case-fatality rates (WVA, 1997). More than one-third of patients have required hospitalization, and 3% have died (Foster, 1997).

Epidemiology

Each year in the United States, 840 thousand to 4 million people become sick, and up to 4 thousand people die as a result of infection with *S. enterica* (Buzby and Roberts, 1996; Tauxe, 1991). *S. enterica* is one of the most costly of foodborne pathogens, with estimated medical costs associated with treatment of salmonellosis ranging from $0.69 to $3.8 billion per year (Isaacson *et al.,* 1999).

Salmonellae are widespread in animals, especially in poultry and swine, and the animal frequently shows no symptoms of the disease. Eggs, poultry, meat, and meat products are the most common vehicles for foodborne salmonellosis (Jay, 2000), however environmental sources, including water, soil, insects, factory surfaces, kitchen surfaces, animal feces, fresh produce, raw meats, raw poultry, and raw sea foods have also been implicated in human illness (Doyle *et al,* 1997; Jay, 2000). Additionally, thousands of cases of human salmonellosis in the United States and other industrialized countries have been transmitted by ice cream, chocolate, potato salad, cheddar cheese, raw milk, black pepper, pâté, aspic, ham, pasteurized milk, and drinking water (Foster, 1997).

Characteristics of Disease

Normally, *S. enterica*-caused human illness is limited an acute gastroenteritis (Buzby *et al.,* 1996). Humans vary in their susceptibility to *Salmonella*, depending on the virulence of the serotype, the individual's immune system, and the quantity of *Salmonella* ingested (Buzby *et al.,* 1996). Typically, 10^7-10^9 CFU/g are necessary for salmonellosis (Jay, 2000). However, the infectious dose may be as low as one cell for some *Salmonella* serotypes (CAST, 1994; Buzby *et al.,* 1996). Individuals most susceptible to *Salmonella* infection include the very young, the elderly, and those with weakened immune systems (Buzby *et al.,* 1996).

Symptoms of salmonellosis typically develop in 12-36 hours after ingestion (Jay, 2000; Buzby *et al.,* 1996), and include dehydration, nausea, fever, vomiting, abdominal pain, headache, chills, and diarrhea, usually

accompanied with prostration, muscular weakness, moderate fever, restlessness, and drowsiness (Jay, 2000; WHO, 1997; Buzby *et al.,* 1996). The incidence is particularly high in children and the elderly; accounting for up to 60% of all laboratory confirmed cases (WHO, 1997). Symptoms persist for an average of 2-3 days. The average mortality rate is 4.1%, higher in the very young (under 1 year old) and up to 15% in people over the age of 50 (Jay, 2000). Up to 5% of patients may become carriers of the organism.

Salmonella infections can cause secondary-disease syndromes, and sometimes chronic illnesses (Buzby *et al.,* 1996). Many *Salmonella* serotypes can penetrate the intestinal lining in humans. Infrequently, the organism may invade the bloodstream, causing septicemia (Buzby *et al.,* 1996). Complications of septicemia include endocarditis, meningitis, and pneumonia. Although an enterotoxin and a cytotoxin have been identified in pathogenic salmonellae, they seem to play a minimal role in infection (Jay, 2000).

Control of salmonellae requires reducing infection in food animals and lowering the risk of contamination at all stages in the food production chain (WHO, 1997). It is very unlikely that the eradication of salmonellae in domestic animals is possible in the foreseeable future (WHO, 1997). For this and other reasons, a method of detection that is rapid, accurate, and reliable is needed in the food industry to control and monitor the incidence of these bacteria.

IV. Food Safety and Current Control Methods

Bacterial pathogens are a major growing concern in both the food industry and the public eye. Microorganisms can enter the human food chain on the farm, during transportation, processing, at retail food outlets, and during food preparation both in homes and public venues (Table 1.1). Bacterial foodborne illness can result when a significant amount of living bacterial pathogens are ingested.

Table 1.1. Leading Causes of Foodborne Illness in the United States (Bryan, 1990)

Factors	1961-1982
Improper Cooling	44%
Lapse of 12 or more hours between preparation/eating	23%
Contamination by handlers	18%
Addition of uncooked raw ingredient	16%
Inadequate cooking/canning/heating	16%

Although it may not be possible, or even desirable, to achieve a zero tolerance for all such organisms, the production of foods with the lowest possible numbers is the desired goal. In an effort to reduce the occurrence and numbers of pathogens on meat and poultry products, reduce the incidence of foodborne illness associated with consuming these products, and provide a framework for modernization of the meat and poultry inspection system, the USDA-FSIS mandated new requirements on July 25, 1996. The new regulations required that all slaughter facilities and plants producing raw ground products conduct regular microbial testing to verify the adequacy of a plant's process controls for the prevention and removal of fecal contamination and associated bacteria, and

that they develop and implement HACCP (Hazard Analysis and Critical Control Points) programs (IDEXX, 1998).

HACCP is a system of science-based process controls designed to identify and prevent biological, chemical, and physical hazards. The system is intended to lead to the production of safe foods by minimizing the hazards in raw materials. It is designed to prevent problems before they occur, and to correct deviation as soon as they are detected (Anderson, 1994). The HACCP approach is used extensively in the meat, poultry, and seafood industries to produce products in compliance with health and safety requirements. By using a HACCP system, control is transferred from end product testing into the processing and manufacturing of foods – that is, from testing for failure to preventing it.

The HACCP system has seven principles:

1. Conduct a hazard analysis, identifying where significant hazards can occur, and describe preventative measures.
2. Identify the critical control points (CCPs) in the process – points at which controls can be put into place to reduce food safety hazards.
3. Establish critical limits for preventative measures associated with each identified CCP.
4. Establish CCP monitoring requirements. Establish procedures for using the results of monitoring to adjust the process and maintain control.

5. Establish corrective actions to be taken when monitoring indicates that there is a deviation from an established critical limit.
6. Establish effective record-keeping procedures that document the HACCP system.
7. Establish procedures for verification that the HACCP system is working correctly.

The HACCP system is a proactive, systematic approach to controlling foodborne hazards. Rather than the traditional approach of end-product testing, HACCP places emphasis on the safety of all ingredients and all process steps, on the premise that safe products will result if these are controlled.

V. Current Detection Methods of Microorganisms

In addition to the new federal regulations for process control, consumers are increasingly aware of the risk to human health due to foodborne pathogens and as such demand a safe, high-quality, and nutritious food supply. This means that having a reliable system for monitoring the quality and safety of foods is of increasing importance, and fast, reliably sensitive screening methods are needed by the food industry.

Although some methods of analysis are better than others, every method has its associated inherent limitations. None permit the determination of exact numbers of microorganisms in foods (Jay, 2000). It is not feasible to test an entire batch of food, as the sampling methods often destroy or contaminate the product, and take time during which the food is losing its freshness and

commercial value. Because of the small sample size used for testing, food analysis methods must be sensitive to low numbers of pathogens. They must be specific, as a product recall based on faulty identification presents enormous losses of time and money. This factor is quite important, as there are often many similarities between pathogenic organisms and non-pathogenic strains. A pathogen detection procedure must be able to differentiate between these microbes. Additionally, it is very advantageous for the detection method to be quick. In the world of food processing, the product is by nature unstable, and must move through the food processing plant quickly in order to maintain product quality. If a detection method takes too long, the product could have already reached shelves. This can result in large-scale product recalls that are enormously expensive, not only in monetary terms but also in the public name recognition of the company. Finally, it is advantageous for pathogen detection methods to be relatively easy to perform. Many smaller food processing plants cannot afford to have an full-time laboratory technician on-site. Therefore, if a detection method could be easily taught to a non-microbiologist, it could increase the quantity of product tested while saving the company money.

Common current types of pathogen detection in food products include conventional plating, molecular, and immunological methods. Each of these are described briefly with their respective advantages and disadvantages.

Conventional Plating Methods

Conventional plating methods are by far the most widely used procedures for determining the numbers of viable cells in a food product (Jay, 2000). These

traditional approaches to microbial assays require enrichment and cultivation, isolation, morphological examination, and biochemical testing to identify human pathogens. These assays often require 4-6 days (Deshpande, 1994).

In the conventional Standard Plate Count (SPC) method, a portion of the food is homogenized, serially diluted, plated onto suitable agar media, and incubated for a given time. Once colony growth has been established, visible colonies are counted by hand or by use of an electronic counter. Variation of the SPC method includes membrane filtration, microscope colony counts, use of dry films such as Petrifilm, dye reduction, and contact plating (Jay, 2000).

An advantage of conventional plating methods for identification and enumeration of pathogenic bacteria in food products is the accuracy and reproducibility of the results. Such methods have been refined and perfected over many years, and microbiologists that perform them have typically had years of laboratory experience performing them.

Drawbacks to conventional plating methods, however, are many: the cost of equipment and consumables, slow sample throughput, the laborious nature of the techniques, and the level of experience and skills required for the analysis, and probably most significantly the length of time necessary to achieve results. It is not always feasible to hold an entire 'batch' of processed food until the microbial plating has been completed. Rather, random samples are taken and tested, and the rest of the batch allowed to continue through. If the samples reveal the presence of pathogenic microorganisms, the product have most likely

already been packaged and shipped. This can lead to large product-recalls, which are expensive both in terms of money and the reputation of the company.

Molecular Methods

Most methods of detecting and characterizing pathogenic bacteria based on molecular or immunological methods were developed since 1960 (Jay, 2000). Molecular methods for detection of pathogenic bacteria are based on some combination of the metabolic activity of microorganisms on specific substrates, genetic typing, measurements of growth response, and/or measurements of certain parts of cells (Jay, 2000). Molecular methods for detecting and characterizing foodborne pathogens include nucleic acid probes, polymerase chain reaction (PCR), restriction enzyme analysis, random amplification of polymorphic DNA (RAPD), pulsed field gel electrophoresis, restriction fragment length polymorphism (RFLP), and ribotyping (Jay, 2000). These methods are all relatively new technologies, and will most likely grow in importance as more foodborne organisms are reclassified by nucleic acid analyses.

Molecular methods based on genetic typing of the pathogen of interest often use a DNA probe to detect homologous DNA or RNA sequences, and/or some combination of restriction endonucleases. Advantages of these methods are their high specificity and selectivity – using the DNA fingerprint of an organism as a target is a good assurance of finding an exact match to your target of interest.

Disadvantages of the molecular methods are the time and expertise required to perform the assays. Most of these methods include several steps, many with extended wait-periods, and although DNA characterization techniques have rapidly advanced in the past few years, they still requires a laboratory technician with extensive training and expertise (Jay, 2000).

Immunological Methods

Immunological methods rely on antibody-antigen interactions to detect pathogens in foods. Methods include the use of fluorescent antibodies for photodetection or fluorescence microscopy of the antibody-antigen complex. The direct fluorescent antibody (FA) technique employs a specific antibody to which is coupled the fluorescent compound (Figure 1.2).

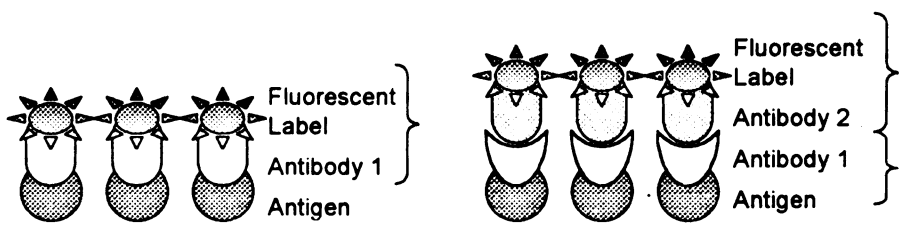


Figure 1.2. (A) Direct method and (B) indirect method of fluorescent labeling

The indirect method uses a second antibody (Antibody 2) that is specific to Antibody 1. The fluorescent label is coupled to Antibody 2, and when this couples to Antibody 1, the antigen is indirectly fluorescently labeled. Although more complicated, the use of the indirect method eliminates the need to prepare FA for each organism of interest (Jay, 2000).

Radio-immunoassay is another technique in which a radioactive label is added to an antigen, allowing it to react to its specific antibody, then measuring the level of antigen-antibody complexes by use of a radioactivity counter (Jay, 2000).

Enzyme linked immunosorbent assay (ELISA) is another popular method of detection. It consists of an enzyme coupled to either an antigen or an antibody. The enzyme remaining in the sample after rinsing is assayed to determine the amount of antibody-antigen coupling, and thus the amount of antigen present in the sample. A commonly used enzyme is horseradish peroxidase, and its presence is measured by the addition of peroxidase substrate. Colorimetric determination of enzyme substrate is then used to measure the presence of the antigen. A 'sandwich' ELISA is a variation of this technique in which the antigen has at least two binding sites. The antigen reacts first with excess solid phase antibody, and then with labeled antibody.

A disadvantage of ELISA is that in most cases, sample enrichment takes about 48 hours, and detection sensitivity is in the range of 10^5 or 10^6 CFU/ml (Alocilja, 2000).

The Vitek Immunodiagnostic Assay System™

The Vitek Immunodiagnostic Assay System (VIDAS™) is an automated enzyme immunoassay for the detection of antigens using the Enzyme Linked Fluorescent Assay (ELFA) method.

A Solid Phase Receptacle serves as the solid phase as well as the pipetting device for the assay. The Solid Phase Receptacle is coated with

antibodies. Reagents for the assay are ready-to-use and pre-dispensed in the sealed reagent strips (bioMerieux, 1998).

The assay is completely automated by the VIDAS instrument. An aliquot of the heated enrichment broth is placed into the reagent strip and the sample is cycled in and out of the Solid Phase Receptacle for a specific length of time. Antigens present in the sample will bind to the monoclonal antibodies coating the interior of the Solid Phase Receptacle. Unbound components are eliminated during washing steps. Alkaline phosphatase labeled antibodies are cycled in and out of the Solid Phase Receptacle and bind to any antigen captured on the Solid Phase Receptacle wall in a sandwich reaction (Verozny-Rozand *et al.*, 1997). A final wash step removes unbound conjugates.

The final substrate (4-Methyl-umbelliferul phosphate) is cycled in and out of the Solid Phase Receptacle. The conjugated enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methylumbelliferone). Fluorescence is measured at 450 nm and expressed as a relative fluorescent value (RFV) (Verozny-Rozand *et al*, 1997; bioMerieux, 1997).

When the VIDAS Assay is completed, the results are analyzed automatically by the computer, a test value is generated, and a report is printed for each sample. Test values are compared to a set of thresholds and each sample is interpreted as positive or negative (bioMerieux, 1997).

VI. Biosensors

Biosensors are analytical instruments that allow direct measurement of biological materials, including foodborne pathogens (Kress-Rogers, 1997). Many of these technologies offer potential in assuring the safety of food products without interrupting the manufacturing process.

A biosensor is made from a biological sensing element attached to a signal transducer. The total effect of a biosensor is to transform a biological event into an electrical signal (Canh, 1993). The sensing element can be enzymes, antibodies, deoxyribose nucleic acid (DNA), or microorganisms, either integrated with, or in intimate contact with a physicochemical transducer (Turner and Newman, 1998; Scott, 1998; Jonsson, 1998). The transducer may be electrochemical, optical, thermometric, or piezoelectric. Electrochemical transducers measure changes in current or voltage; optical transducers measure changes in fluorescence, absorbance or reflectance; thermometric transducers measure change in temperature due to the heat resulting from biological reactions; and piezoelectric transducers measure changes in the resonant frequency of a piezoelectric crystal due to small changes of mass or density at the crystal surface (Rogers and Gerlach, 1996). The transducer signal transforms the specific recognition of the analyte into a signal that can be readily quantified, usually in real or near-real time.

Biosensors were born out of the combination of existing sensors and biological systems. They were first reported by Clark and Lyons in 1962, when biological molecules were coupled to transducers that converted the biological

signal into an electrical signal (Turner and Newman, 1998). These first sensors were amperometric, comprised of glucose oxidase immobilized to oxygen electrodes (Rogers and Gerlach, 1996; Canh, 1993). In 1967, Updike and Hicks prepared an enzyme electrode by polymerizing a gel containing glucose oxidase onto an oxygen electrode (Canh, 1993).

The biological component of the biosensor is used to confer specificity on the device (Turner and Newman, 1998). In a biocatalysis-based sensor, the biological component produces or consumes a component that can be detected by the transducer. In an affinity-based sensor, the biological component binds a molecule that the transducer then measures. The most widely used biomolecules in biosensors are enzymes and antibodies, although other biocatalytic components include whole cells, tissue slices, organelles, lectins, and DNA (Jonsson, 1998).

Medicine has been the strongest driving force in biosensor research to date, but the appeal of biosensor technology to the food industry is becoming more obvious. The potential advantage of using biosensors in food analysis is a rapid, specific quantification without the need for extensive sample preparation (Table 1.2).

Table 1.2: Benefits and features of biosensors for use in the food industry (Turner and Newman, 1998).

Feature	Benefit
Target specificity	Ability to definitively identify single compounds, or a broad range of compounds
Electronic integration	Compact instrument design makes them easy to use
Operation in complex matrices	Lack of need of sample preparation
Fast response time	High throughput in automatic analyzers, and decreased time for spot checks
Continuous signal	Accurate monitoring of fluctuations, and ability to take corrective action
Small size	Portable, inexpensive, many simultaneous assays
Mass producability	Inexpensive, disposable (hygienic), can be widely dispersed

Biosensors are characterized by their *specificity*, and their *sensitivity*. Specificity (or selectivity) is the ability to recognize a single compound among other substances in the same sample. Specificity is achieved through the reaction of an analyte with a specific biological component (Scott, 1998). The specificity of biosensors is determined by both the bioreceptor and the method of transduction.

The sensitivity of a sensor is given by the change in its response as a function of the change in input signal monitored (Canh, 1993). When the variation in the phenomenon ceases to yield an appreciable variation in signal, the detection limit has been reached, which usually corresponds to the limit of the linear range at low concentrations. The linear range of a biosensor is obtained from a calibration curve of its response to different analyte

concentrations (Canh, 1993). For the measurement to be carried out properly, it is necessary to know the response time of the biosensor, or the time taken to reach a steady state from the instant of the variation in the concentration under investigation.

Today, biosensors are powerful tools that allow scientists to monitor biospecific interactions in real time and to derive information about binding kinetics and equilibrium, structure, and function. A key technology to biosensor application for safety monitoring in the food industry is the construction of immunosensors, which have application in microbial identification.

VII. Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a type of optical biosensor that uses the properties of evanescent waves (Canh, 1993). It is widely used in the biosensor, pharmaceutical, and analytical chemistry communities (Salamon *et al.*, 1999). The SPR sensor can be made into a highly specific biosensor to detect biospecific interactions between proteins and biomolecules by forming a functionalized sensor surface that is specific for a particular analyte. For example, coupling an antibody onto the sensor surface converts the sensor into an immunosensor. The SPR biosensor used in this study works by immobilizing antibodies directly on to the metal surface and introducing a quantity of antigen. The sensor is equipped with a thin gold film to which biotinylated antibodies specific to the organism of interest are bound via avidin-biotin interaction. As the antigens bind to the antibodies, the refractive index at the sensor surface

changes and affects the SPR coupling conditions. The change in refractive index can be monitored and displayed as a function of time. Thus, SPR has the ability to measure, in real time, interactions of biomolecules due to the interfacial refractive index changes caused by the antibody-antigen interactions.

Initial applications of SPR involved investigating the optical properties inherent to thin metal films (Earp and Dessy, 1996). Since then, SPR has been used for assessment of antigen-antibody interactions, studies of bio-recognition interactions at surfaces, and screening foodstuffs or other materials for pesticide, antibiotic or drug residues (Fratamico *et al.,* 1998; Cranfield Biotechnology Centre, 2000; Pfaff *et al.,* 1994). Medical applications, such as the human enzyme creatine kinase (CK), the anticonvulsant drug phenytoin, human chorionic gonadotrophin (hCG), and other biological contaminants in matrices, such as blood, serum, plasma, saliva, and urine, have been successfully measured with SPR (Wortberg *et al.,* 1997). Additionally, SPR has been used for characterization of anisotropic biological membranes (Salamon *et al.,* 1999), analysis of protein binding reaction kinetic parameters (Natsume *et al.,* 1994; Malmborg *et al.,* 1992; Karlsson *et al.,* 1991; Faegerstam *et al.,* 1992; O'Shannessy *et al.,* 1993; O'Shannessy *et al.,* 1994; Masson *et al.,* 1994; Shinohara *et al.,* 1994), antibody detection and characterization (Medina, 1997), pesticide analysis (Harris *et al.,* 1996), detection of chemical residues in milk (Sternesjoe *et al.,* 1995; Sternesjoe *et al.,* 1996), chemical analysis of ginseng roots (Kajiwara, 1998), detection of *Clostridium botulinum* toxin (Ogert *et al.,* 1992), detection of Staphylococcal enterotoxin (Tempelman *et al.,* 1996),

analysis of *Bacillus thuringiensis* toxin binding (Masson *et al.*, 1995), protein interactions (He *et al.*, 1997), lectin binding assays (Ozeki *et al.*, 1998), monitoring chemotaxis control in *E. coli* (Schuster *et al.*, 1993), and biosensing techniques (Liedberg *et al.*, 1983). Fratamico *et al.* (1998) used an SPR biosensor to detect *E. coli* O157:H7.

SPR relies on the excitation of the surface plasmon of a thin metal layer covering the surface of the waveguide. The angle at which the incident light best couples to the surface plasmon is sensitive to the refractive index in the vicinity of the metallized surface. Binding of large molecules such as antibodies can thus be monitored as the system goes out of resonance (Turner and Newman, 1998).

Waveguides and Modes

A waveguide is a physical medium through which light can be guided. A common example of this is the use of fiber optics. In an SPR biosensor using the Kretschmann prism arrangement, the optical waveguide is a planar surface coated with a thin metal film. The propagation of light occurs through total internal reflection in the waveguide. When this occurs, the light ray is confined within the waveguide, with very little leakage into the surroundings (Figure 1.3).

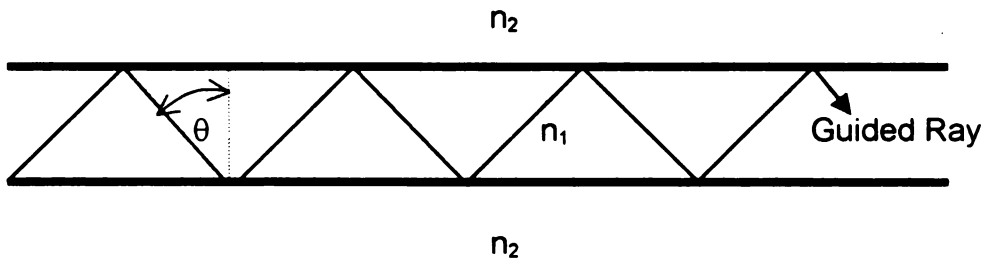


Figure 1.3: Total internal reflectance. $n_1 > n_2$, with angle of incidence θ.

The guiding medium must have a higher index of refraction (n) than the surroundings in order for light to propagate by total internal refraction. As long as the angle of incidence (θ) is larger than the critical angle, θ_c (Figure 1.4), such that

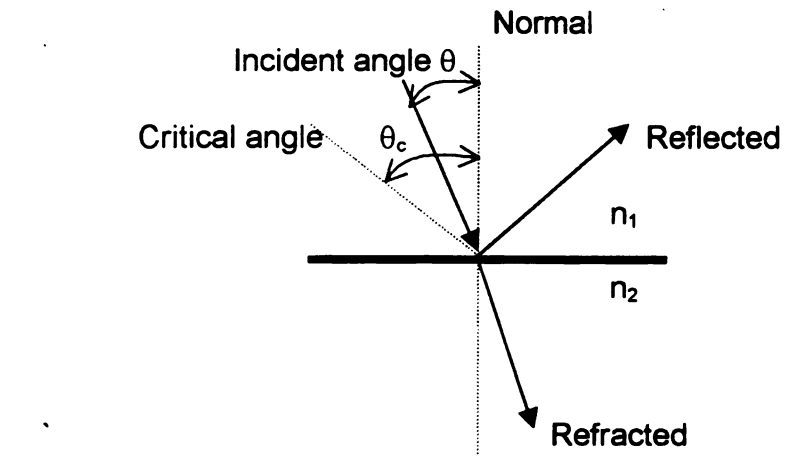
$$\theta_c = \sin^{-1} (n_1/n_2) \qquad (1.1)$$

there will be total internal reflection (Sutherland and Daehne, 1987).

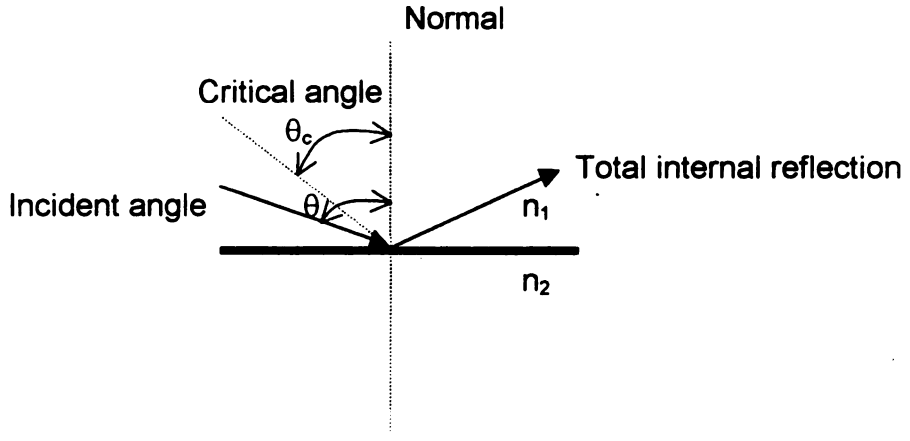
As long as a critical angle is surpassed, total internal reflection can occur at a boundary interface between any two refractive indices. The critical angle defines a minimum angle of incidence for a particular interface, and depends on the ratio of the refractive indices of the media involved (Earp and Dessey, 1996).

Evanescent waves

An evanescent wave is an electromagnetic field that propagates along a surface, but decays exponentially perpendicular to it (Purvis *et al.*, 1998). When light is reflected at an optical interface where refractive index is changing, such as in a waveguide, an evanescent wave develops as energy decays away from the point of reflection into the surrounding medium (Turner and Newman, 1998). At a certain angle of incidence, there is total internal reflectance in the waveguide. Anytime light undergoes total internal reflection, an evanescent field is created. This energy field extends beyond the waveguide boundary into the medium for a distance similar to the wavelength of the light. There is a net flow of energy across the reflecting surface to maintain the evanescent field.



$n_1 > n_2$ **(a)**



$n_1 > n_2$ **(b)**

Figure 1.4: Effect of the critical angle (θ_c) on reflection and refraction. (a) Incident light angle less than θ_c, (b) Incident light angle greater than θ_c, causing total internal reflectance.

This transfer of energy results in attenuation in reflectance, and can be detected with an optical photodetector.

The penetration depth of the evanescent field will depend on the wavelength of the light, the refractive index ratio of the waveguide to the surroundings, and the photon intensity in the plasmon mode. The penetration d_p of the field energy can be estimated as:

$$d_p = \frac{\lambda}{4\pi(n_1^2 \sin^2\theta - n_2^2)^{1/2}} \qquad (1.2)$$

Where λ = the wavelength of light, n_1 = refractive index of the waveguide material, n_2 = refractive index of the surroundings, and θ = the angle of incidence. The evanescent field is only present when there is total internal reflectance in the optical waveguide.

Surface Plasmons

A surface plasmon (SP) is an oscillation of electrons on the surface of a solid, typically a conductor. Under certain conditions, the photon's energy is transferred to the surface of the metal as packets of electrons called plasmons. This energy transfer occurs at a specific wavelength of light, when the quantum energy carried by the photon exactly matches the quantum energy level of the plasmons. Plasmons are electron clouds that behave as if they were single charged particles. Part of their energy is expressed as evanescent waves, which extend about 100 nm above and below the metal surface, decaying exponentially as a function of distance. The interaction between the plasmon's

evanescent wave and the matter within the evanescent field determines the resonance wavelength or angle of incident light that resonates with the plasmon. The magnitude of the change in resonance wavelength or the angle of incidence is directly and linearly proportional to the change in composition at the surface (Purvis *et al.*, 1998).

The main criterion for a material to support SP waves is that the real part of the dielectric permittivity be negative. The dielectric permittivity (ε) is a dimensionless quantity that is proportional to the square of the refractive index (n) of the material within the region of optical wavelengths. The SP is affected by changes in dielectric permittivity of materials in contact with the thin metal film. As these values change, they alter the coupling efficiency of the light into the plasmon mode. To find the coupling efficiency, the angle of incidence of the light beam is scanned through a range of values. A distinct minimum in reflectivity will be observed at a discrete angle, which is labeled the SP coupling angle (θ_{sp}). At this particular angle of incidence, light is most efficiently coupled into the plasmon mode, and the reflection from the metal film is most attenuated. Sensing is done by relating θ_{sp} to changes in the dielectric permittivity, and thus the refractive index of the sample (Earp and Dessy, 1996).

In an SPR biosensor, thin gold or silver films are most often used due to their optical qualities and the ease and accuracy with which they can be deposited onto a substrate. The metal film is deposited onto a glass substrate that will be optically coupled to a waveguide, and the other side of the thin film is exposed to the analyte sample. Surface plasmon resonance occurs as the

evanescent wave propagates through the metal and excites surface plasmons. The resonance wavelength can be determined by measuring the light reflected by the metal surface. At most wavelengths, the metal acts as a mirror, reflecting virtually all incident light. At the SP coupling angle (θ_{sp}), surface plasmons are created and the incident light is almost completely absorbed as SPR occurs (Quantech, 1998).

SPR can be achieved by varying the frequency of the light, or by varying the angle of incidence, as changing the wavelength at a fixed angle is equivalent to changing the angle at a fixed wavelength. In either case, at some point resonance occurs, and the reflected intensity of the light drops significantly. The position of the SPR is extremely sensitive to the refractive index of the sample (Texas Instruments, 1999).

The Kretschmann Geometry

The Kretschmann prism arrangement is the most frequently used geometry in SPR sensor design (Earp and Dessy, 1996). Only transverse magnetic polarized light (the electric field polarized in the plane of incidence) may couple to surface plasmons (Texas Instruments, 1999). The Kretschmann prism arrangement facilitates this coupling of a light wave onto a surface plasmon (SP) (Figure 1.5). In the Kretschmann geometry, the angle of incidence of the light-beam with respect to the metal surface, and the reflected light intensity are measured.

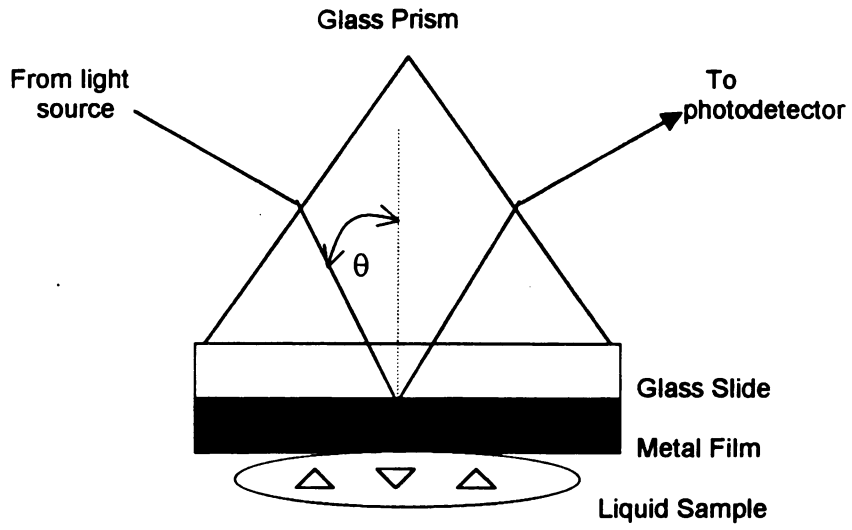


Figure 1.5. The Kretschmann geometry, with angle of incidence θ. When $\theta = \theta_{SP}$, virtually no light is reflected to the photodetector.

Incident light passes through a prism and onto the thin metal film, to which antibodies for the analyte of interest have been bound. The SP coupling angle is sensitive to changes in the refractive index of the biomolecule layer. Light is emitted at varying angles of incidence, and at the SP coupling angle, SPR occurs and the optical photodetector receives almost no light. Both the angle of incidence of the light-beam and the reflected light intensity are measured.

Increasing the concentration of proteins in a given area will create a refractive index change that is directly proportional to mass loading. The sensitivity of the Kretschmann prism device will depend on how accurately the resonance angle can be measured. The response of an SPR instrument due to bimolecular binding is essentially the same for various proteins and biomolecules at similar concentrations. This is because the refractive indices for

many different macromolecules are basically the same, regardless of composition (Stenberg *et al.*, 1991).

In the SPR biosensor used in this research, antibodies are immobilized directly to the metal surface via avidin-biotin interaction, and as the antigen binds to the antibodies, the refractive index at the sensor surface changes. Changes in the analyte layer on the sensor surface affect the effective refractive index of the metal film-antibody interface, which affects the SP coupling angle. Varying the incident angle of light locates the SP coupling angle as the angle at which virtually no light is reflected. This change in the amount of light striking the photodetector, and the corresponding angle of incidence are the sensor outputs.

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CHAPTER 2

MATERIALS AND METHODS

Spreeta SPR Biosensor

The Spreeta Miniature Integrated Surface Plasmon Resonance Liquid Sensing System (Texas Instruments, Inc., Dallas, Texas) was used in this study (Figure 2.1).

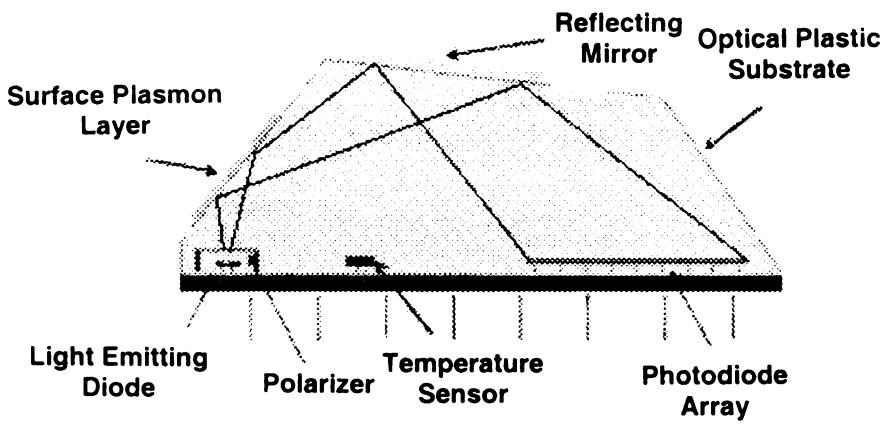

Figure 2.1. Side view of the Spreeta Sensor (courtesy of Texas Instruments, 1999, URL:

Near–infrared light (840nm) from a light emitting diode (LED) is polarized to enhance SPR. The light beam reflects off of the gold sensing film and is directed by the gold mirror onto a linear array of silicon photo-diodes. The active sensing region is the area on the thin gold film that is actively used in liquid sensing. It is a strip approximately 4.5 mm long by 0.1 mm wide on the face of the sensor. Except for the sensing surface, the sensor is coated with an opaque material to block out external light (Texas Instruments, 1999).

The angle at which a ray of light is incident upon the sensing film is directly mapped into a specific point on the photodiode array. Depending on the refractive index of the liquid next to the gold film, at some angle the reflected light intensity will experience a minimum corresponding to where SPR occurs. This minimum is then processed by the Spreeta computational software, and plotted as the change in index of refraction over time (Texas Instruments, 1999).

The SPR biosensor was assembled according to the Spreeta Operation Manual. Neutravidin binding and attachment of biotinylated antibodies to the surface was done following the method of Spreeta's Application Brief 004, with modifications (Texas Instruments, 1999). Table 2.1 enumerates the steps and time to prepare the SPR biosensor.

Table 2.1. Typical preparation of the SPR biosensor.

Step	Action	Time (min)
1	Clean gold surface by immersing in NaOH/Triton X-100 Solution	3
2	Baseline established by immersing in PBS	3
3	Avidinate gold surface by immersing in neutravidin	3
4	Rinse off excess neutravidin by immersing in PBS	3
5	Attach antibody to gold surface via avidin-biotin interaction, by immersing gold surface in biotinylated antibody	10
6	Rinse off unbound antibody by immersing in PBS	2
7	Rinse with PBS-NaOH/Triton X-100 solution	2
8	Immerse in BSA	2
9	Rinse with PBS-NaOH/Triton X-100 solution	2
	Total time:	30

The total time to prepare the SPR biosensor for an assay was 30 min. The Spreeta computational software recorded the index of refraction of each sample approximately every 4.8 seconds. To initialize each sensor, the gold surface was cleaned by immersing it in 10 ml of 0.1 N NaOH in 1% Triton X-100

Solution (NaOH/Triton) (Sigma Chemical Co., St. Louis, MO) for 3 minutes (Figure 2.2-1). The gold surface of the sensor was then immersed in 10 ml phosphate buffered saline (PBS; pH 7.2) (Sigma Chemical Co., St. Louis, MO) for 3 minutes, until a steady PBS baseline was established (Figure 2.2-2). Then the sensor was immersed in 10 ml 100 µl/ml neutravidin in PBS (Figure 2.2-3) (Pierce Chemicals, Rockford, Il), then back into 10 ml of PBS (Figure 2.2-4). Once a new baseline was achieved, the sensor was immersed in 10 ml of 300 µg/ml anti-*Salmonella* spp. or anti-*E. coli* O157:H7 antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) for 10 minutes, to bind the antibody to the avidinated surface via avidin-biotin interaction (Figure 2.2-5). Unbound antibody was rinsed off by immersing the sensor in 10 ml PBS (Figure 2.2-6). The sensor was then immersed in NaOH/Triton in PBS for two minutes (Figure 2.2-7), then Bovine Serum Albumin (BSA)(Pierce Chemicals, Rockford, Il) for two minutes to block any non-specific binding sites (Figure 2.2-8), and finally back into NaOH/Triton in PBS for two minutes, to rinse off any remaining BSA (Figure 2.2-9). The procedures for the assays performed after preparation of the SPR sensor differed depending on the objective of the experiment.

Statistical Analysis

Statistical analysis was conducted using a single factor ANOVA (analysis of variance) function on experiment replications. For all studies, the SPR biosensor average responses were considered statistically different when the P-value was less than 0.05 (95% confidence level).

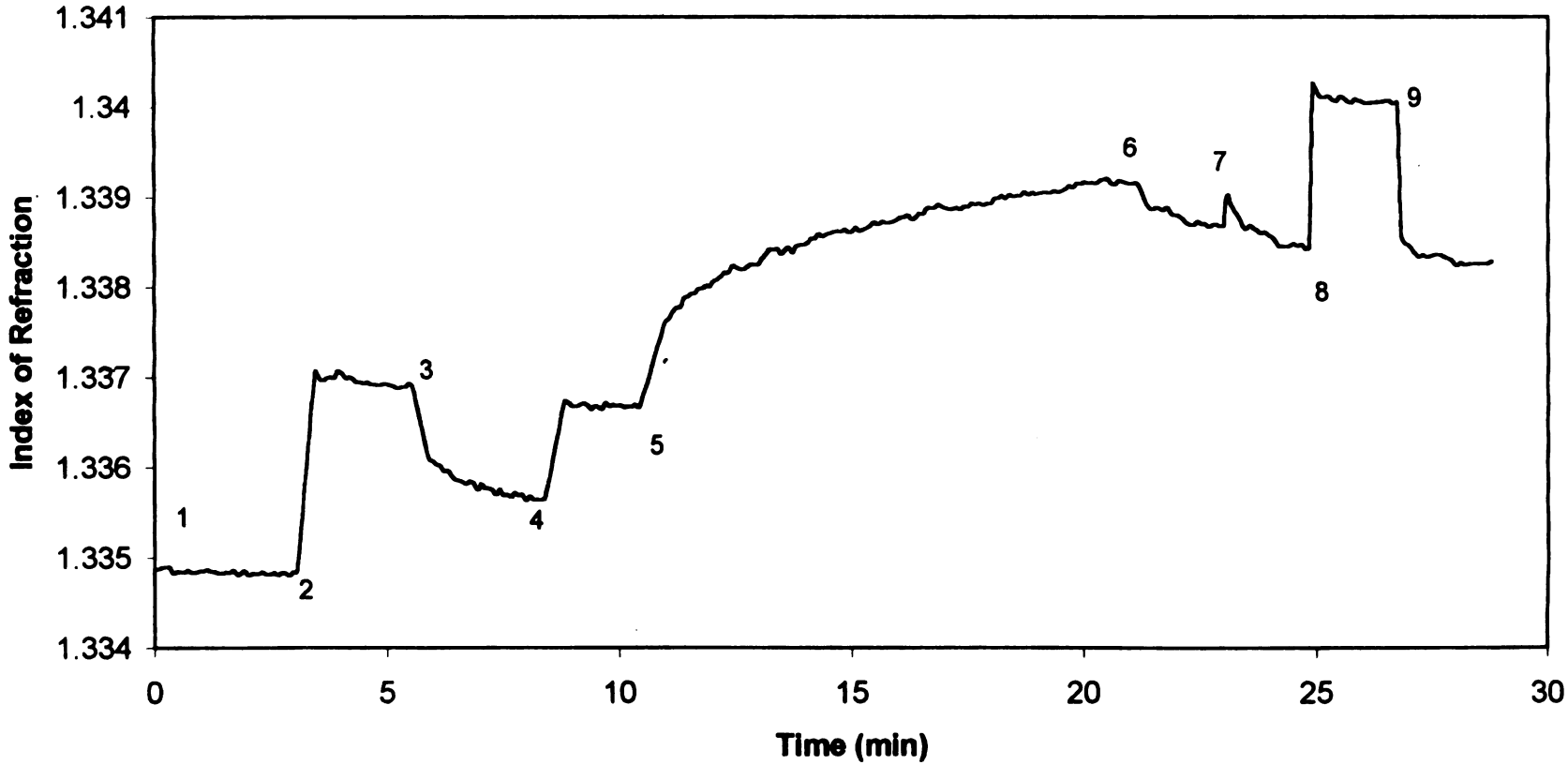

Figure 2.2. A typical SPR Biosensor Preparation. (1) Sensor initially in NaOH/Triton solution, (2) Phosphate Buffered Saline (PBS) solution, (3) Neutravidin solution, (4) PBS solution, (5) Biotinylated antibody, (6) PBS solution, (7) NaOH/Triton in PBS, (8) BSA, (9) NaOH/Triton in PBS.

Standard Plate Counts

Viable plate counts were performed by serially diluting the sample into 0.1% buffered peptone water (BPW; Becton Dickinson and Co., Sparks, MD). One ml of each serial dilution was plated on Petrifilm aerobic plates (3M, St. Paul, MN), and incubated for 18-24 hours at 37°C.

Preparation and Biotinylation of Antibodies

Lyophilized affinity purified antibody to *Salmonella* common structural antigens (CSA-1) and to *Escherichia coli* O157:H7 were purchased (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). All antibodies were stored at 4°C until rehydrated. Antibodies were rehydrated according to the manufacturer's instructions for conjugations in carbonate buffer by adding 0.1 ml of 0.01 M acetic acid (Spectrum Products, Inc., Gardena, CA) to 1 mg of the antibody. Once totally dissolved, 0.1 ml of 0.177 M carbonate-bicarbonate solution was added to the antibody, and mixed thoroughly. The antibody was then heated in a 37°C water bath for 30 minutes, then allowed to cool slowly to room temperature. Biotin labeling was done according to the method of DeMarco *et al.* (1999). Six hundred microliters of PBS was added to the rehydrated antibodies. Two milligrams of succinimidyl-6-(biotinamido) hexanoate (EZ-Link NHS-LC-Biotin; Pierce Chemicals, Rockford, Il) was added to 1 ml of *N,N*-dimethylformamide (DMF; Aldrich Chemicals, Milwaukee, WI), and 75 µl of this solution was added to the rehydrated antibody. The antibody solution was then placed on ice for 2 hours to achieve an insertion of approximately 2 biotins per molecule

Immunoglobulin-G (IgG) (DeMarco *et al.*, 1999). The biotinylated antibody was then serially diluted with sterile PBS, to achieve a concentration of 300 μg/ml.

Bacteria

Characterized strains of *S.* Typhimurium and *E. coli* O157:H7 were obtained from Michigan State University collections. *E. coli* O157:H7 was verified at the Bacteriology Laboratory at the Veterinary Diagnostic Center of the University of Nebraska, Lincoln (Younts, 1999). For verification as *E. coli* O157:H7, the isolates were subjected to PCR for the presence of the *eae* gene, Shiga toxin (Stx) structural gene, and the O antigen biosynthesis (rfb) loci (Younts, 1999). All culturing was done in a certified Biological Safety Level 2 environment at the MSU Meat Microbiology Laboratory. Cultures were maintained in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) at 4°C. Cultures for viable cell counts and SPR assays were grown in TSB at 37°C for 18-20 hours. Cultures were serially diluted in sterile BPW for use in the experiments.

SPR Biosensor Signal Normalization

The output of the biosensor was the index of refraction at which surface plasmon resonance occurred. To normalize the output, the index of refraction for the zero controls was subtracted from the responses. The resulting biosensor output was then the magnitude of the change in index of refraction (the increase in index of refraction above the index of refraction of the zero control).

Comparisons of Antibody Concentration

Anti-*Salmonella* and anti-*E. coli* O157:H7 biotinylated antibodies were prepared at 3 concentrations in sterile PBS; 300 μg/ml, 30 μg/ml, and 3 μg/ml. To analyze the difference in response of the SPR biosensor to differing antibody concentrations, 3 ten-fold dilution series of *S.* Typhimurium and *E. coli* O157:H7 were made in sterile BPW, and run on each of the 3 antibody concentrations. Viable plate counts of the serial dilutions were performed to verify the concentration of the organisms. Results were compared to see if there was any difference in response of the SPR biosensor to increasing concentrations of antibody.

Background Response

The response of the SPR sensor to assays performed without the use of an antibody was monitored to establish the background response of the system. Two separate experimental trials were performed on the sensor using serial dilutions of *S.* Typhimurium, *E. coli* O157:H7, and uninoculated TSB. Viable plate counts of the serial dilutions were performed to verify the concentration of the organisms. The SPR sensor was prepared in the same way as in Table 2.1, with sterile PBS introduced in place of the antibody. For each bacterium, ten-fold dilutions of a 10^8 CFU/ml culture grown in TSB were made in sterile BPW. For the TSB, serial dilutions were made in sterile BPW, and the series run as if they were inoculated samples. Sterile BPW was used as the zero (uninoculated) control. The results of the assays were then compared to find any difference in response of the SPR sensor to inoculated versus uninoculated samples in TSB.

Negative Controls

The negative control used for the serial dilutions of *S.* Typhimurium and *E. coli* O157:H7 was TSB diluted in BPW. From a trial experiment conducted earlier, the SPR biosensor background signal showed that the biosensor was sensitive to the presence of sterile TSB. Since the organism was grown in TSB, this sensitivity could potentially be misleading, so assays of sterile TSB on prepared biosensors were performed to find the contribution of TSB in the sample to the SPR response. Six 10-fold serial dilutions of TSB in sterile BPW were performed, 3 assays using anti-*Salmonella* spp. antibody, and 3 with anti-*E. coli* O157:H7 antibody. The results were analyzed to find the amount of SPR biosensor response that was due to TSB. Results were also compared to the background signal data (assays performed without antibody), to see if there was a statistical difference between the background response and an antibody-prepared SPR biosensor response to sterile TSB. The SPR biosensor response to sterile TSB was then used as the negative control, in order to account for the TSB effect on the biosensor response.

SPR Biosensor Sensitivity Assays

SPR biosensor assays were performed on a series of dilutions of *S.* Typhimurium and *E. coli* O157:H7 in sterile BPW, from starter cultures inoculated to approximately 10^8 CFU/ml (Table 2.2). Concentration was verified by conducting viable plate counts.

Table 2.2. A typical dilution series experiment. The dilution series consists of SPR analysis of 10 varying bacteria concentrations (CFU/ml).

Step	Action	Time (min)
1	SPR sensor preparation (Table 2.1)	30
2	Immerse sensor in sterile BPW (zero control)	2
3	Immerse sensor in sample	2
	a. repeat for 9 additional bacterial concentrations	18
4	Rinse gold surface with PBS	1
	Total time:	53
	Time for one biosensor assay:	35

After the SPR biosensor had been prepared according to Table 2.1, the biosensor was immersed in the zero control (10 ml of sterile BPW) for 2 min. Once a steady reading was established, the sensor was then immersed in 10 ml of the sample for 2 min. This process was repeated for each of the dilutions prepared. Assays were prepared on each sensor with the lowest concentration (0 CFU/ml) first, and the highest concentration (10^8 CFU/ml) last. The index of refraction for each dilution was computed as the average of the readings taken over each 2 min period.

The total time for a dilution series experiment, with 10 samples of increasing concentration (10^0 – 10^8 CFU/ml) was 53 min. The time for one SPR biosensor sample assay (procedure without step 3a) was 35 min.

Growth Curves

Growth activity of *S.* Typhimurium and *E. coli* O157:H7 was monitored by performing a series of three growth curves for each bacterium. In each growth curve, 10 µl of 10^3 CFU/ml stock solution of *S.* Typhimurium or *E. coli* O157:H7 (about 10 CFU) were introduced to 100 ml of sterile TSB. The inoculated solutions were grown at 37°C in a stationary incubator. At half-hour intervals for

ten hours, aliquots of 1 ml were removed from the vials, serially diluted, and plate counts performed. The results from the plate counts were plotted over time to determine the time required by the bacteria to grow to the level of detection of the SPR biosensor.

Specificity Assays

Specificity assays were done to establish the ability of the SPR biosensor to select for the target antigen. The biosensor was prepared as in Table 2.1, then exposed to increasing concentrations of three sample dilutants:

1. Sterile TSB (negative control).
2. Bacteria that the antibody was not specific for:
    2.1. *E. coli* O157:H7 dilutions for anti-*Salmonella* spp. prepped SPR biosensor.
    2.2 *S.* Typhimurium dilutions for anti-*E. coli* O157:H7 prepped SPR biosensor.
3. The bacteria of interest:
    3.1. *E. coli* O157:H7 dilutions for anti-*E. coli* O157:H7 prepped SPR biosensor.
    3.2 *S.* Typhimurium dilutions for anti-*Salmonella* spp. prepped SPR biosensor.

Plate counts of the serial dilutions were performed to verify the concentration of the organisms. Three replications were done for each dilution series for both anti-*Salmonella* spp. – prepped SPR biosensor and anti-*E. coli* O157:H7-prepped SPR biosensor, making a set of 18 assays.

Specificity in Mixed Cultures

Sample cultures composed of mixtures of *S.* Typhimurium and *E. coli* O157:H7 were analyzed on the SPR sensor to establish the ability of the SPR biosensor to select for the target antigen in the presence of other bacteria. The sample cultures were prepared from stock solutions of known concentration, on which viable plate counts were performed to verify the concentration of the organisms.

Field Samples

Samples were obtained from a previous study, in which site visits were made to small-sized (~200 heads/week) and medium-sized (>1000 heads/week) commercial meat packing facilities, as well as to the farms that supplied them with hogs (Tables 2.3-2.4) (Ryser, 2000).

Table 2.3. Environmental samples from slaughterhouse (Ryser, 2000)

Site	Sample	Method and Quantity	Number of samples
Pen Alleyway	Fecal material, floor	Scoop, 50g	1
Prechill	Swab from chillroom wall	Swab	7
Dehairing machine	Hairs scooped from dehairing machine	25g, hair scoop	6
Drain	Drain near evisceration	Swab	3

Table 2.4. Farm samples (Ryser, 2000)

Site	Sample	Method and Quantity	Number of samples
Pen Alleyway	Fecal material, floor	Scoop, 50g	1
Hogs back	Composite	Swab with neutralizing buffer solution	2
Fecal matter	Composite	Scoop, 50g	1
Feed	Composite	Scoop, 50g	2
Water Nozzle	Composite	Swab from nozzle tip	1

Twenty-four samples were analyzed by mini-VIDAS and SPR biosensor for *Salmonella* spp. and *E. coli* O157:H7.

Field Sample Analysis

Salmonella spp. and *E. coli* O157:H7 were assayed using both the mini-VIDAS™ system and the SPR biosensor:

VIDAS™ Salmonella

Recovery of *Salmonella* spp. using the VIDAS™ system was done according to the BAM/AOAC method (bioMerieux, 1998). The samples were pre-enriched in lactose broth, then 1 ml of the pre-enriched sample was incubated in 9 ml of buffered peptone water (BPW) for 18 hours at 37°C. Following this, 1 ml of the BPW enrichment was transferred into 10 ml of selenite cystine broth and 10 ml tetrathionate broth. The selenite cystine and tetrathionate broths were incubated for 8 hours at 37°C and 42°C, respectively. After enrichment, 1 ml of selenite cystine broth and 1 ml of tetrathionate broth were transferred to separate tubes containing 10 ml of M-broth and incubated for 18 hours at 42°C. After incubation, the M-broth enrichments were mixed and 1 ml of each was transferred into a hermetically sealed tube. The tubes were heated for 15 minutes in a water bath at 100°C. After cooling to room temperature, the samples were screened on the mini-VIDAS system (bioMerieux, St Louis, MO). Positive samples were biochemically confirmed by the Food Microbiology Lab of Michigan State University (Ryser, 2000).

VIDAS™ *E. coli* O157:H7

Recovery of *E. coli* O157:H7 was done using the VIDAS™ ECO system (bioMerieux, 1998). One ml of each sample was added to 9 ml m-TSB with novo-biocin, then incubated at 41°C for 6 hours. One ml of the enriched culture was then transferred into 9 ml MacConkey broth with cefixime and potassium tellurite (CT-Mac) (Mast Diagnostics, Merseyside, UK), and incubated for 18 hours at 37°C. After incubation, the CT-Mac solution was mixed, and 1 ml of each suspension transferred into a hermetically sealed tube. The tube was heated for 15 min in a 100°C water bath, and allowed to cool. The samples were then screened for *E. coli* O157:H7 with the VIDAS™ ECO assay (bioMerieux, 1998).

SPR Biosensor *Salmonella* Assays

The same enriched samples from the VIDAS immunoassay were used for SPR analysis. The SPR biosensor was prepared with appropriate antibody, and immersed in sterile BPW for two minutes as a zero control, then into sterile M-broth for two minutes to identify the background response (Table 2.5). The inoculated M-broth sample was then assayed for 3 minutes, followed by a pure culture of *S.* Typhimurium in M-broth (the positive control).

Table 2.5. SPR biosensor procedure for *Salmonella* spp. assays

Step	Action	Time (min)
1	SPR sensor preparation (Table 2.1)	30
2	Sterile BPW (zero control)	2
3	Sterile M-broth (negative control)	2
4	Sample in M-broth	3
4	anti-*Salmonella* spp. antibody (300 µg/l)	2
	Total time:	39

Following the inoculated M-broth sample, the sensor was immersed in anti-*Salmonella* spp. antibody for a second time, to achieve 'sandwich' binding of antibody (Figure 2.3). Two identical assays were performed for each sample.

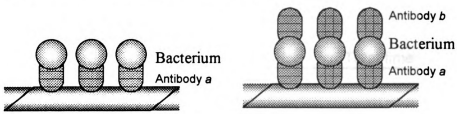

Figure 2.3. (A) Antibody-antigen interaction on the SPR biosensor gold-film surface using one antibody layer *a*. (B) Antibody-antigen interaction on the SPR biosensor gold-film surface in a sandwich-type interaction, with two layers of antibody, *a* and *b*.

The sandwich binding arrangement was designed to increase the molecular mass of the substrate bound to the sensing film of the SPR biosensor. This was done to increase the magnitude of the change in index of refraction of the biosensor.

SPR Biosensor *E. coli* O157:H7 Assays

E. coli O157:H7 was assayed on the SPR biosensor following the same procedure as the *Salmonella* spp. assays, using anti-*E. coli* O157:H7 antibody and sterile CT-Mac broth in place of M-broth for the negative control (Table 2.6).

Table 2.6. SPR Biosensor *E. coli* O157:H7 Assay Procedure

Step	Action	Time (min)
1	SPR sensor preparation (Table 2.1)	30
2	Sterile BPW (zero control)	2
3	Sterile CT-Mac (negative control)	2
4	Sample in CT-Mac	3
4	anti-*E. coli O157:H7* antibody (300 μg/l)	2
	Total time:	39

The sandwich assay was performed in the same manner as for the *Salmonella* spp. assays. Two replicates were performed on each sample.

Because initial testing indicated that the samples contained negligible amounts of *E. coli* O157:H7, five samples were inoculated with both 10 μl of a stock solution of 10^3 CFU/ml *E. coli* O157:H7 (about 10 CFU), and 10 μl of pre-enriched sample from the pork production facilities to create known positives. The samples were prepared in sterile BPW, and enriched according to the method used for VIDAS™ ECO samples.

SPR Biosensor Positive Controls

Positive controls for the *Salmonella* spp. and *E. coli* O157:H7 SPR biosensor assays were pure cultures of *S.* Typhimurium in M-broth, and *E. coli* O157:H7 in CT-Mac broth, respectively. Positive controls were assayed following the SPR biosensor assay procedures used for the samples, with pure culture used in place of the inoculated sample. Two positive controls each were run for *S.* Typhimurium and *E. coli* O157:H7. Viable plate counts were performed on the control cultures.

SPR Biosensor Assays in TSB

To find any difference in response of the SPR biosensor to samples grown according the VIDAS™ enrichment protocols, and samples grown in TSB, the pork industry samples were inoculated in TSB and grown for 18 hours at 37°C. The samples were then analyzed with the SPR biosensor following the same procedure as the assays performed on the samples grown according to the VIDAS™ methods. Sterile TSB was used as the negative control, and pure *S.* Typhimurium and *E. coli* O157:H7 cultures grown in sterile TSB were used as the positive controls. A second rinse of antibody was performed for both anti-*Salmonella* spp. and anti-*E. coli* O157:H7 assays to achieve a 'sandwich'-type antibody binding.

E. coli O157:H7 positive samples were prepared in TSB in the same manner as the *E. coli* O157:H7 positive samples were prepared for the VIDAS™ enrichment method. Five samples were inoculated with both 10 µl of a stock solution of 10^8 CFU/ml *E. coli* O157:H7, and 10 µl of pre-enriched sample from the swine production facilities, to create known positives.

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CHAPTER 3

SENSITIVITY AND SPECIFICITY ASSAYS FOR *SALMONELLA* TYPHIMURIUM AND *ESCHERICHIA COLI* 0157:H7 UTILIZING A SURFACE PLASMON RESONANCE BIOSENSOR

ABSTRACT

The Spreeta™ SPR biosensor was used to detect two foodborne pathogens; *Salmonella enterica* spp. Typhimurium and *Escherichia coli* O157:H7. The selectivity of the SPR biosensor was assayed using a series of antibody concentrations and dilution series of the two organisms. Specificity of the SPR biosensor was demonstrated in pure and mixed cultures of *S.* Typhimurium and *E. coli* O157:H7.

The optimum antibody concentration for use on the SPR biosensor was 300 μg/ml, a result comparable to other immunosensors. The detection limit of the SPR biosensor for *S.* Typhimurium and *E. coli* O157:H7 in pure culture was 10^7 colony forming units (CFU)/ml. A pure culture of these pathogens could be detected after 5 1/2 hours of enrichment. The SPR biosensor was specific to *S.* Typhimurium and *E. coli* O157:H7 in pure cultures at concentrations of 10^7 CFU/ml. In mixed cultures, the detection limit of the biosensor was 10^7 CFU/ml for the target organism, when the non-target bacterial concentration was 10^6 CFU/ml or less. Concentrations of non-target bacteria beyond 10^7 CFU/ml caused a decrease in the magnitude of the sensor response to the presence of the target pathogen.

The SPR biosensor has demonstrated potential for portable, field-based, rapid, and accurate pathogen detection. The SPR biosensor is a versatile instrument. By changing the antibody used in the preparation phase, it can be used to detect many different substrates, including other foodborne pathogens. With further studies and refinements, the SPR biosensor shows promise to provide a complementary detection system to standard lab-based systems currently used in food safety and control systems.

INTRODUCTION

Foodborne bacterial pathogens are believed to be the most frequently occurring hazard of the nation's food supply, causing billions of dollars to be lost in medical costs, lost productivity, and product recalls associated with outbreaks of foodborne illness (Hui *et al.,* 1994). In recent years, the food processing industry has been under increasing pressure to identify and control potential food safety hazards caused by pathogenic bacteria. Although the Hazard Analysis and Critical Control Point (HACCP) system has reduced the need for end-product testing, the demand for rapid and accurate methods to detect foodborne pathogens has increased (Seo *et al.,* 1999).

Escherichia coli O157:H7 has emerged as an important enteric pathogen of considerable public health significance. Illnesses caused by *E. coli* O157:H7 can range from mild, watery diarrhea to life-threatening conditions, such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Jay, 2000; Buchanan and Doyle, 1997). The combination of the severe consequences of infection, its low infectious dose, and its association with many common foods make *E. coli* O157:H7 a bacterial pathogen of particular concern.

Salmonella enterica has been identified as one of the most prevalent and costly of known foodborne pathogens (Davies, 1997). It is a main cause of documented foodborne illness in most developed countries (Buzby *et al.,* 1996). Adding to the concern about *Salmonella* infection is the existence of several antibiotic resistant strains, especially the multi-drug resistant strain *S.* Typhimurium DT 104. With the emergence of this strain, the incidence and

severity of *Salmonella*-related human illness is on the rise (Middleton *et al.*, 1999).

Conventional detection methods, which can take 4 to 7 days to detect and confirm pathogenic bacteria in food, are not acceptable for monitoring critical control points (Seo *et al.*, 1999). A biosensor that could detect pathogens within minutes or hours would allow processors to take quick corrective action when pathogens are detected.

Biosensors are analytical instruments possessing a capturing molecule as a reactive surface in close proximity to a transducer, which converts the binding of an analyte to the capturing molecule into a measurable signal (Fratamico *et al.*, 1998). The SPR biosensor is a biosensor that monitors antibody-antigen interaction in real-time, utilizing surface plasmon resonance (SPR) (Elkind *et al.*, 1998). SPR is an optical phenomenon that occurs as a result of total internal reflection of light at a metal film-liquid interface. A component of the incident light momentum, the evanescent wave, interacts with surface plasmons (free oscillating electrons) in a thin metal film. When SPR occurs, energy from the incident light is lost to the metal film, resulting in a decrease in reflected light intensity. The resonance phenomenon occurs only at a precisely defined angle of incidence, which is dependent on the refractive index of the medium adjacent to the metal surface. The refractive index changes in direct proportion to the mass and the make-up of the media present. When antibodies are affixed to the metal surface, the angle of incidence that causes SPR depends on the amount of antibody-antigen substrate present. By using antibodies specific to pathogens of

interest, it is possible to utilize the SPR phenomenon to measure the amount of pathogenic bacteria present in a sample by measuring the change in refractive index.

The objective of this study was to determine the sensitivity and specificity of the SPR biosensor to *S.* Typhimurium and *E. coli* O157:H7, in order to explore the feasibility of employing an SPR biosensor as a pathogen monitoring technique in the food industry.

MATERIALS AND METHODS

Spreeta SPR Biosensor

The Spreeta Miniature Integrated Surface Plasmon Resonance Liquid Sensing System (Texas Instruments, Inc.) was used in this study (Figure 3.1).

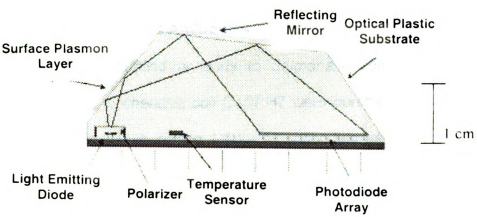

Figure 3.1. Side view of the Spreeta Sensor (courtesy of Texas Instruments, 1999, URL: http://www.ti.com/spreeta).

Near-infrared light (840nm) from a light emitting diode (LED) is polarized to enhance SPR. The light beam reflects off of the gold sensing film and is directed by the gold mirror onto a linear array of silicon photodiodes. The active sensing region is the area on the thin gold film that is actively used in liquid

sensing. Except for the sensing surface, the sensor is coated with an opaque material to block out external light (Texas Instruments, 1999).

The angle at which a ray of light is incident upon the sensing film is directly mapped into a specific point on the photodiode array. The Spreeta™ SPR biosensor is capable of measuring the index of refraction within the range of approximately 1.29 and 1.42 (Elkind *et al.*, 1998). The computational software records the index of refraction of each sample approximately every 4.8 seconds. Depending on the refractive index of the liquid next to the gold film, at some angle the reflected light intensity experiences a minimum corresponding to where SPR occurs. This minimum is then processed by the Spreeta computational software, and plotted as the change in index of refraction over time (Texas Instruments, 1999).

Preparation and Biotinylation of Antibodies

Lyophilized affinity purified antibody to *Salmonella* common structural antigens (CSA-1) and to *Escherichia coli* O157:H7 were purchased (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). All antibodies were stored at 4°C until rehydrated. Antibodies were rehydrated according to the manufacturer's instructions (Kirkegaard & Perry, Inc.). Biotin labeling was done according to the method of DeMarco *et al.* (1999), and the biotinylated antibody was diluted with sterile phosphate buffered saline (PBS) (pH 7.2).

Biosensor Preparation and Antibody Attachment

The total time to prepare the SPR biosensor for an assay was 30 min. The SPR biosensor was assembled according to the Spreeta Operation Manual (Texas Instruments, 1999). Neutravidin binding and attachment of biotinylated antibodies to the surface was done following the method of Spreeta's Application Brief 004 (Texas Instruments, 1999).

Bacteria

Characterized strains of *S.* Typhimurium and *E. coli* O157:H7 were obtained from Michigan State University collections. *E. coli* O157:H7 was verified in a previous study by the Bacteriology Laboratory at the Veterinary Diagnostic Center of the University of Nebraska, Lincoln (Younts, 1999). All culturing was done in a certified Biological Safety Level 2 environment at the Michigan State University (MSU) Meat Microbiology Laboratory. Cultures were maintained in tryptic soy broth (TSB) (Becton Dickinson) at 4°C. Cultures for viable cell counts and SPR assays were grown in TSB at 37°C for 18-20 hours. Cultures were serially diluted in sterile buffered peptone water (BPW) for use in the experiments.

SPR Biosensor Signal Normalization

The output of the biosensor was the index of refraction at which surface plasmon resonance occurred. To normalize the output, the index of refraction for the zero controls was subtracted from the responses. The resulting biosensor output was then the magnitude of the change in index of refraction (the increase in index of refraction above the index of refraction of the zero control).

Comparisons of Antibody Concentration

Anti-*Salmonella* and anti-*E. coli* O157:H7 biotinylated antibodies were prepared at 3 concentrations in sterile PBS; 300 μg/ml, 30 μg/ml, and 3 μg/ml. To analyze the difference in response of the SPR biosensor to differing antibody concentrations, 3 ten-fold dilution series of *S.* Typhimurium and *E. coli* O157:H7 were made in sterile BPW, and run on each of the 3 antibody concentrations. Viable plate counts of the serial dilutions were performed to verify the concentration of the organisms. Results were compared to see if there was any difference in response of the SPR biosensor to increasing concentrations of antibody.

Background response.

The response of the SPR biosensor to assays performed without the use of an antibody was monitored to establish the background response of the system. Two separate experimental runs were performed on the sensor using serial dilutions of *S.* Typhimurium, *E. coli* O157:H7, and uninoculated TSB. Viable plate counts of the serial dilutions were performed to verify the concentration of the organisms. The SPR biosensor was prepared as for a normal assay, but with sterile PBS introduced in place of the antibody. For each bacterium, ten-fold dilutions of 10^8 CFU/ml cultures grown in TSB were prepared in sterile BPW. For the TSB, serial dilutions were made in sterile BPW, and the series run as if they were inoculated samples. Sterile uninoculated BPW was used as the zero control. The results of the assays were analyzed to see if there

was any significant difference in response of the SPR biosensor between inoculated and sterile TSB.

Negative Controls

The negative control used for the serial dilutions of *S.* Typhimurium and *E. coli* O157:H7 was TSB diluted in BPW. From a trial experiment conducted earlier, the SPR biosensor background signal showed that the biosensor was sensitive to the presence of sterile TSB. Since the organism was grown in TSB, this sensitivity could potentially be misleading, so assays of sterile TSB on prepared biosensors were performed to find the contribution of TSB in the sample to the SPR response. Six 10-fold serial dilutions of TSB in sterile BPW were performed, 3 assays using anti-*Salmonella* spp. antibody, and 3 with anti-*E. coli* O157:H7 antibody. The results were analyzed to find the amount of SPR biosensor response that was due to TSB. Results were also compared to the background signal data (assays performed without antibody), to see if there was a statistical difference between the background response and an antibody-prepared SPR biosensor response to sterile TSB. The SPR biosensor response to sterile TSB was then used as the negative control, in order to account for the TSB effect on the biosensor response.

SPR Biosensor Sensitivity Assays

SPR biosensor sensitivity assays were performed on a series of dilutions of *S.* Typhimurium and *E. coli* O157:H7 in sterile BPW, from cultures inoculated to approximately 10^8 CFU/ml (Table 3.1). Three assays were performed for each

organism. Viable plate counts of the serial dilutions were performed to verify the concentration of the organisms.

Table 3.1. A typical dilution series experiment. The dilution series consists of SPR analysis of 10 varying bacteria concentrations (CFU/ml).

Step	Action	Time (min)
1	SPR sensor preparation (Table 2.1, Chapter 2)	30
2	Immerse sensor in sterile BPW (zero control)	2
3	Immerse sensor in sample	2
	a. repeat for 9 additional bacterial concentrations	18
4	Rinse gold surface with PBS	1
	Total time:	53
	Time for one biosensor assay:	35

Assays were prepared on each biosensor with the lowest concentration (10^0 CFU/ml) first, and the highest concentration (10^8 CFU/ml) last. The index of refraction for each dilution was computed as the average of the readings taken over each 2 min period. The total time for a dilution series experiment, with 10 samples of increasing concentration ($10^0 - 10^8$ CFU/ml) was 53 min. The time for one SPR biosensor sample assay (procedure without step 3a) was 35 min.

Growth Curves

Growth of *S.* Typhimurium and *E. coli* O157:H7 was monitored by performing a series of three growth curves for each bacterium. In each growth curve, 10 µl of 10^3 CFU/ml stock solution of *S.* Typhimurium or *E. coli* O157:H7 (about 10 CFU) were introduced to 100 ml of sterile TSB. The inoculated samples were incubated at 37°C. At half hour intervals for ten hours, aliquots of 1 ml were removed from the vials, serially diluted, and viable plate counts

performed. The results from the plate counts were plotted over time to discover the growth curves for each bacterium.

Specificity Assays

Specificity assays were done to establish the ability of the SPR biosensor to select for the target antigen. For each bacteria, the SPR biosensor was prepared with the respective antibody, then exposed to increasing concentrations of three sample dilutants:

1. Sterile TSB (negative control).
2. Bacteria that the antibody was not specific for:
    2.1. *E. coli* O157:H7 dilutions for anti-*Salmonella* spp. prepared SPR biosensor.
    2.2 *S.* Typhimurium dilutions for anti-*E. coli* O157:H7 prepared SPR biosensor.
3. The bacteria of interest:
    3.1. *E. coli* O157:H7 dilutions for anti-*E. coli* O157:H7 prepared SPR biosensor.
    3.2 *S.* Typhimurium dilutions for anti-*Salmonella* spp. prepared SPR biosensor.

Viable plate counts of the serial dilutions were performed to verify the concentration of the organisms. Three replications were done of each dilution series for both anti-*Salmonella* spp.–prepared SPR biosensor and anti-*E. coli* O157:H7-prepared SPR biosensor, making a set of 18 assays.

Specificity in Mixed Cultures

Sample cultures composed of mixtures of *S.* Typhimurium and *E. coli* O157:H7 were analyzed on the SPR biosensor to establish the ability of the SPR biosensor to select for the target antigen in the presence of other bacteria. Five cultures of target bacteria at concentrations 0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/ml were prepared, respectively. Non-target bacteria at concentrations of 0, 10^2, 10^4,10^6, and 10^8 CFU/ml were added to the five initial cultures at each target bacteria concentration. The sample cultures were prepared from stock solutions of known concentration, on which viable plate counts were performed to verify the concentration of the organisms.

Statistical Analysis

Statistical analysis was conducted using a single factor ANOVA (analysis of variance) function on experiment replications. For all studies, the SPR biosensor average responses were considered statistically different when the P-value was less than 0.05 (95% confidence level).

Results and Discussion

The SPR biosensor was both sensitive and specific to the presence of *S.* Typhimurium and *E. coli* O157:H7 in solution. Although the Spreeta™ SPR biosensor has not been used previously as an immunosensor for pathogen detection, initial results show promise for its application for food safety monitoring. The first goal of this study was to establish the optimum antibody concentration for preparation of the biosensor. Anti-*Salmonella* and anti-*E. coli* O157:H7 biotinylated antibodies at concentrations of 300 μg/ml, 30 μg/ml, and 3

μg/ml in sterile PBS were assayed (Figures 3.2 and 3.3). The responses of the SPR biosensor to each of the three antibody concentrations were linear, with R^2 values of 0.998 to 0.999. The magnitude of the biosensor response increased as the antibody concentration increased. ANOVA revealed that the SPR biosensor responses to the different antibody concentrations were significantly different at bacterial concentrations of 10^7 CFU/ml and above for both *S.* Typhimurium and *E. coli* O157:H7. Because the magnitude of the sensor response for an antibody concentration of 300 μg/ml was the most significant, it was chosen as the concentration to be used in all further studies. This was done in an effort to increase the sensitivity of the biosensor, by utilizing an antibody concentration that returned the largest magnitude of response. This antibody concentration is comparable to concentrations used in other immunosensors, which typically range from 50-400 μg/ml (Elkind *et al.*, 1998; Woodbury *et al.*, 1998; Watts *et al.*, 1994; Faegerstam *et al.*, 1992), and is the same concentration used for the detection of *E. coli* O157:H7 by the BIAcore™ SPR biosensor (Fratamico *et al.*, 1998).

Without the attachment of antibodies, the SPR biosensor responds to the incidental contact with any proteins present in solution (Elkind *et al.*, 1998). Therefore, the SPR biosensor without the attached antibody should have a similar response for samples in TSB with or without bacteria present, as the bacteria would not attach to the sensing surface without the antibody. In fact, the responses of the biosensor to dilutions of *S.* Typhimurium, *E. coli* O157:H7, and sterile TSB were not statistically different at any dilution level (Figure 3.4). The

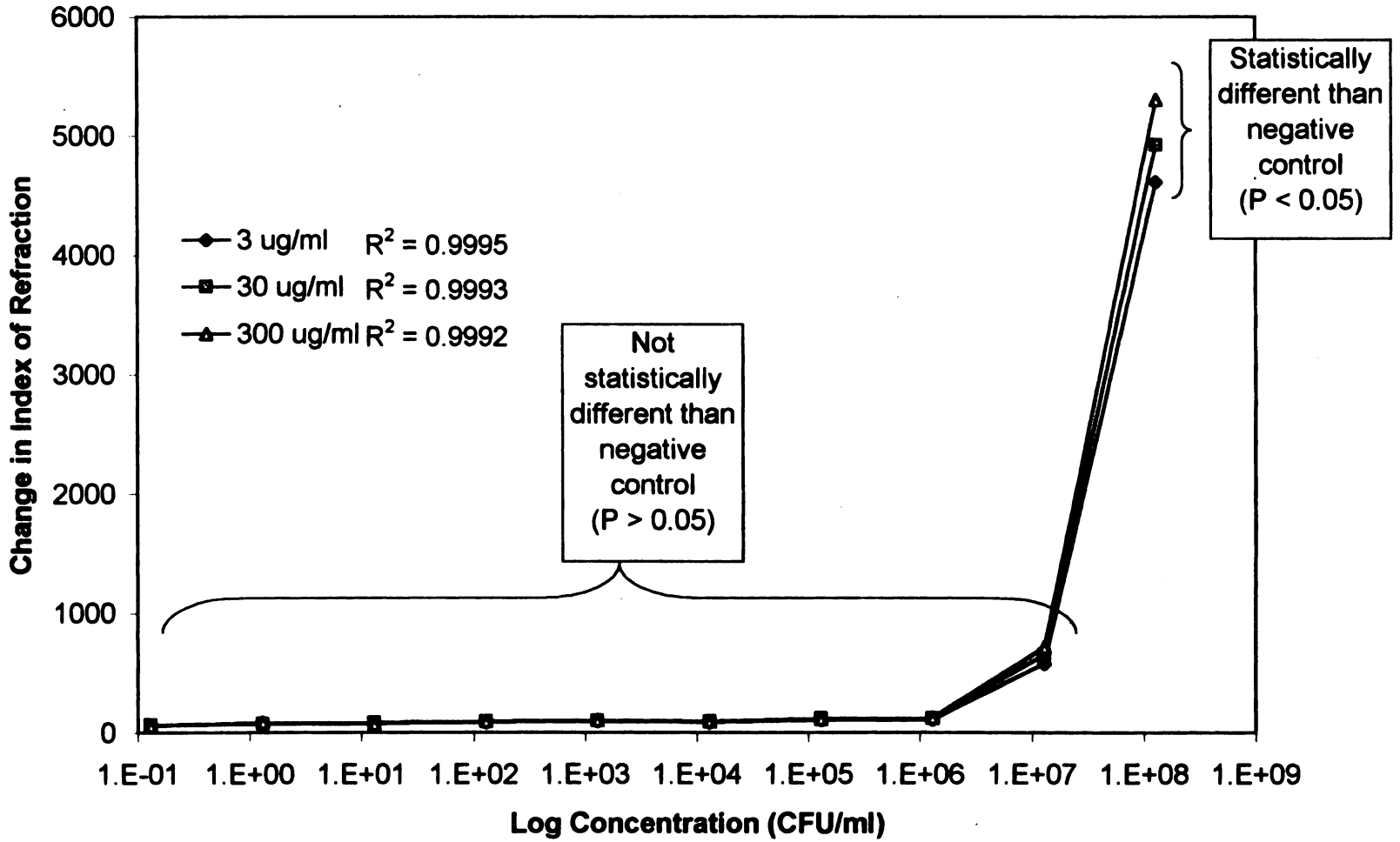

Figure 3.2. Average response of the SPR biosensor to anti-*Salmonella* spp. antibody concentration of 3 ug, 30ug, and 300ug.

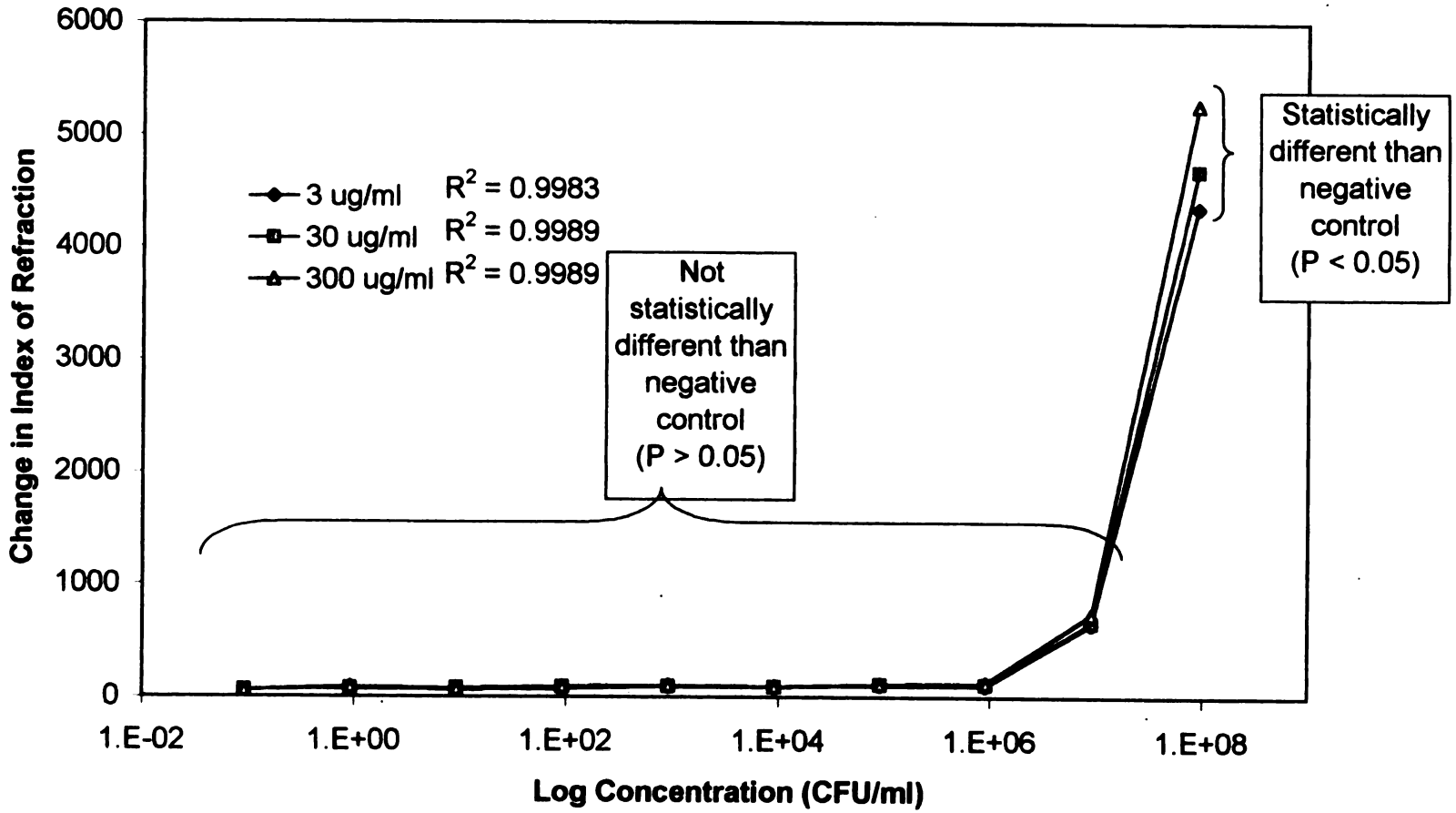

Figure 3.3. Average response of the SPR biosensor to anti-*E. coli* O157:H7 antibody concentration of 3 ug, 30ug, and 300ug.

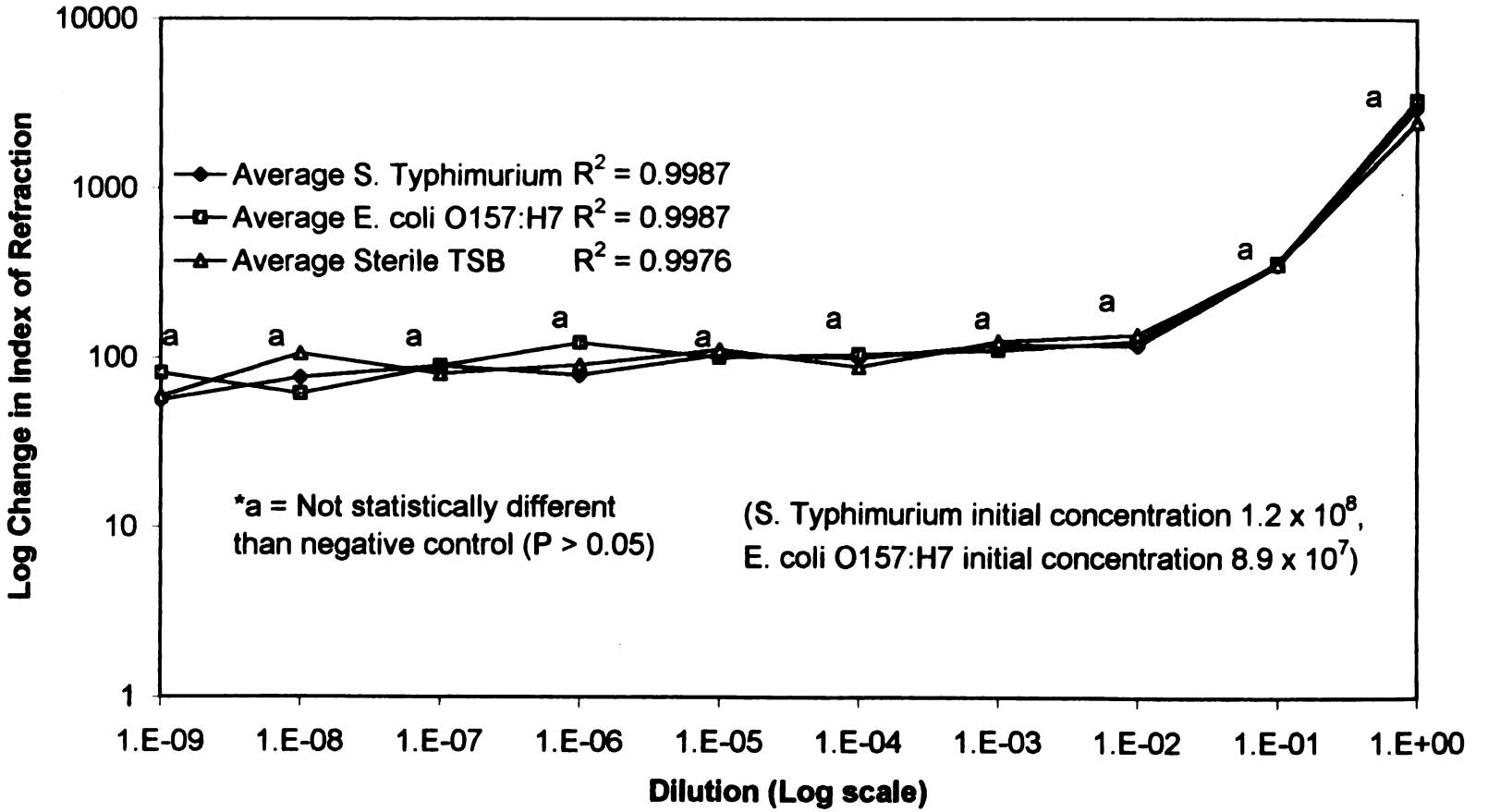

Figure 3.4. Average background responses of SPR biosensor to serial dilutions of *S*. Typhimurium, *E. coli* O157:H7, and sterile TSB without antibody.

similarity of the linear increase of the SPR biosensor to increasing concentrations of samples indicated that it was the concentration of the background matrix of TSB that caused the change in index of refraction, and not the presence of bacteria.

Because sterile TSB had an effect on the response of the SPR biosensor, assays were performed with TSB dilution series on biosensors prepared following the same method as for bacterial assays with antibody attachment. Three assays were completed using anti-*Salmonella* antibody, and 3 with anti-*E. coli* O157:H7 antibody (Figure 3.5). ANOVA was performed on the background signal and negative control data to assess the difference between sterile TSB dilutions assayed with an SPR biosensor prepared without antibody (the background signal) and SPR biosensor prepared with antibody. The response to sterile TSB was not statistically different ($P > 0.5$) from the background response of the biosensor at any dilution. The antibodies used in preparation of the SPR biosensor did not respond to the presence of TSB in the samples, and again it was the protein matrix of the TSB that the SPR biosensor sensed.

To determine the sensitivity of the SPR biosensor to *S.* Typhimurium and *E. coli* O157:H7 in solution, dilution series were assayed. Figures 3.6 and 3.7 show the average SPR response for *S.* Typhimurium and *E. coli* O157:H7 for dilutions between 10^0 and 10^8 CFU/ml. The limit of detection of the SPR biosensor was taken to be the bacterial concentration at which there was a statistically significant variance in mean of the response of the SPR biosensor to the target organism versus a negative control. ANOVA revealed that the level of

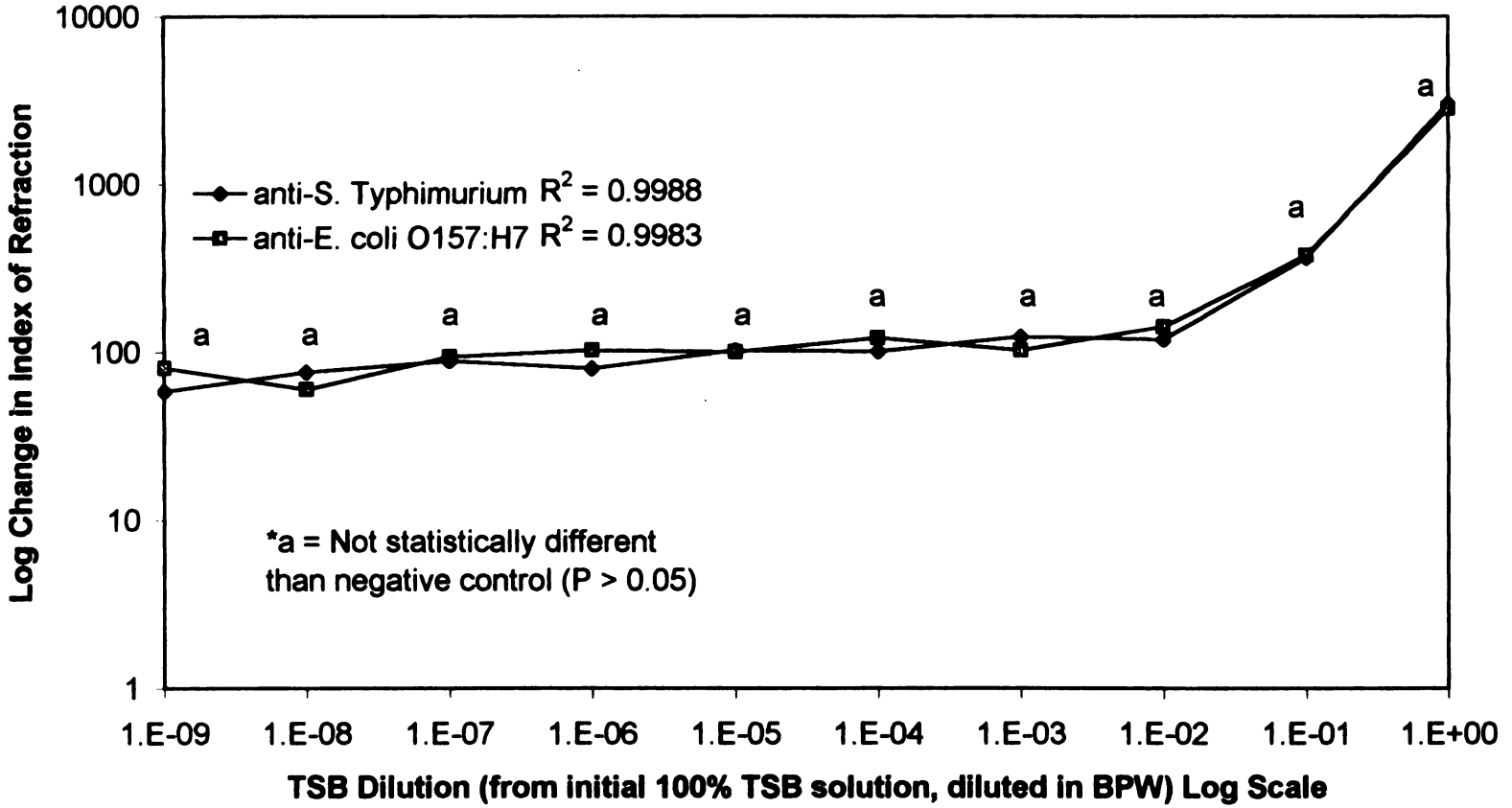

Figure 3.5. Average negative control of SPR biosensor - Response to serial dilutions of sterile TSB using anti-*Salmonella* spp. and anti-*E. coli* O157:H7 antibodies.

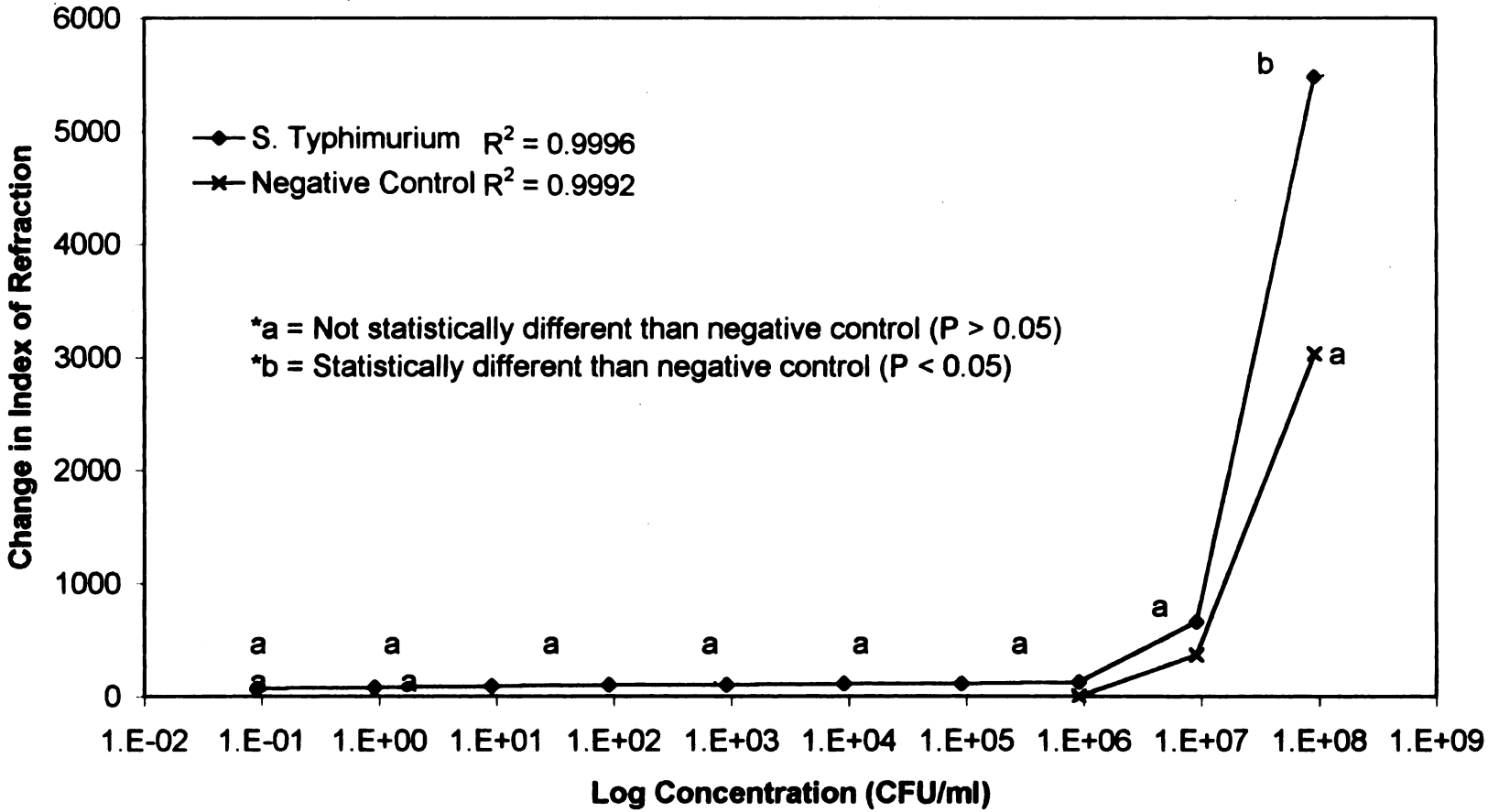

Figure 3.6. Average SPR biosensor response for *S.* Typhimurium.

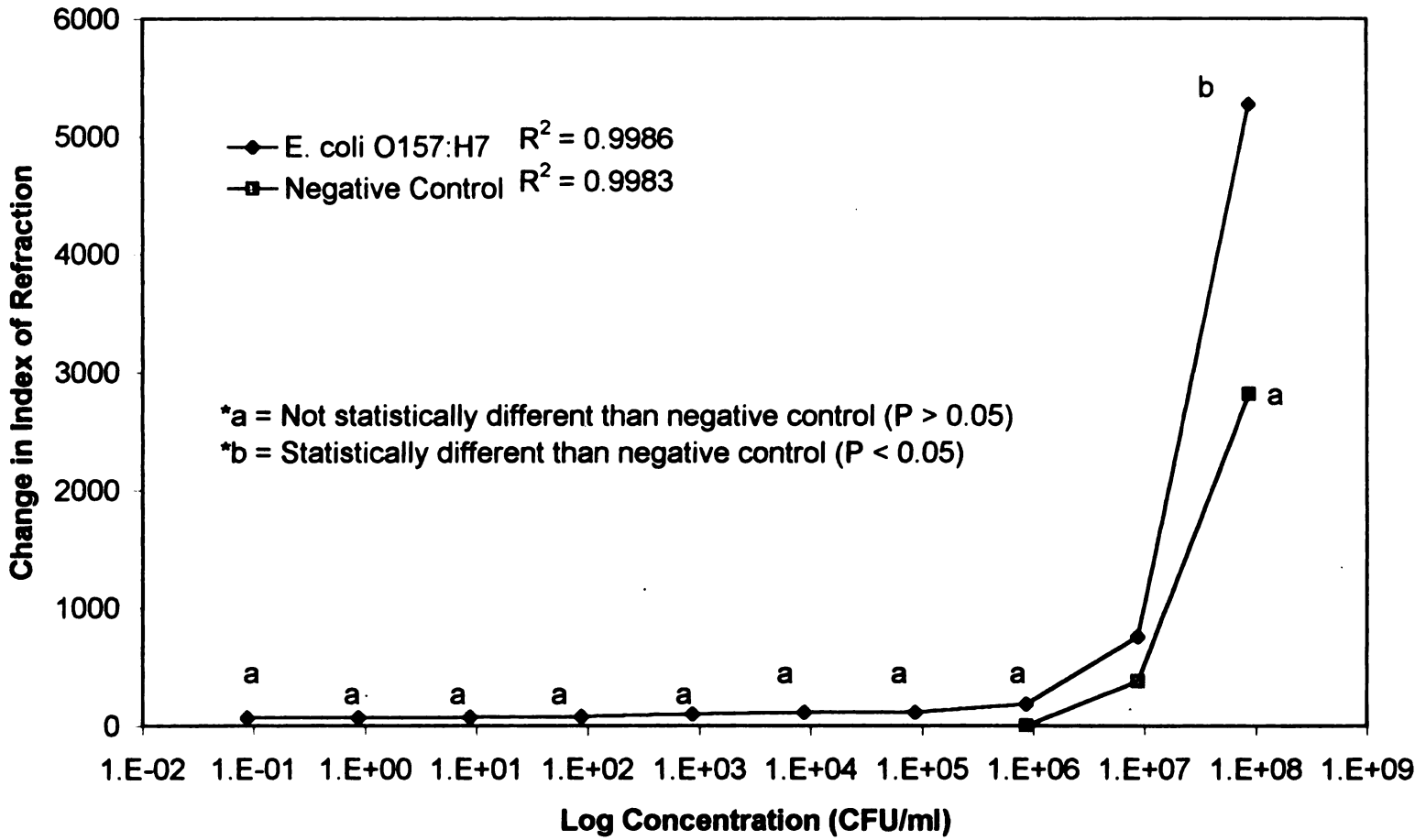

Figure 3.7. Average SPR biosensor response for *E. coli* O157:H7.

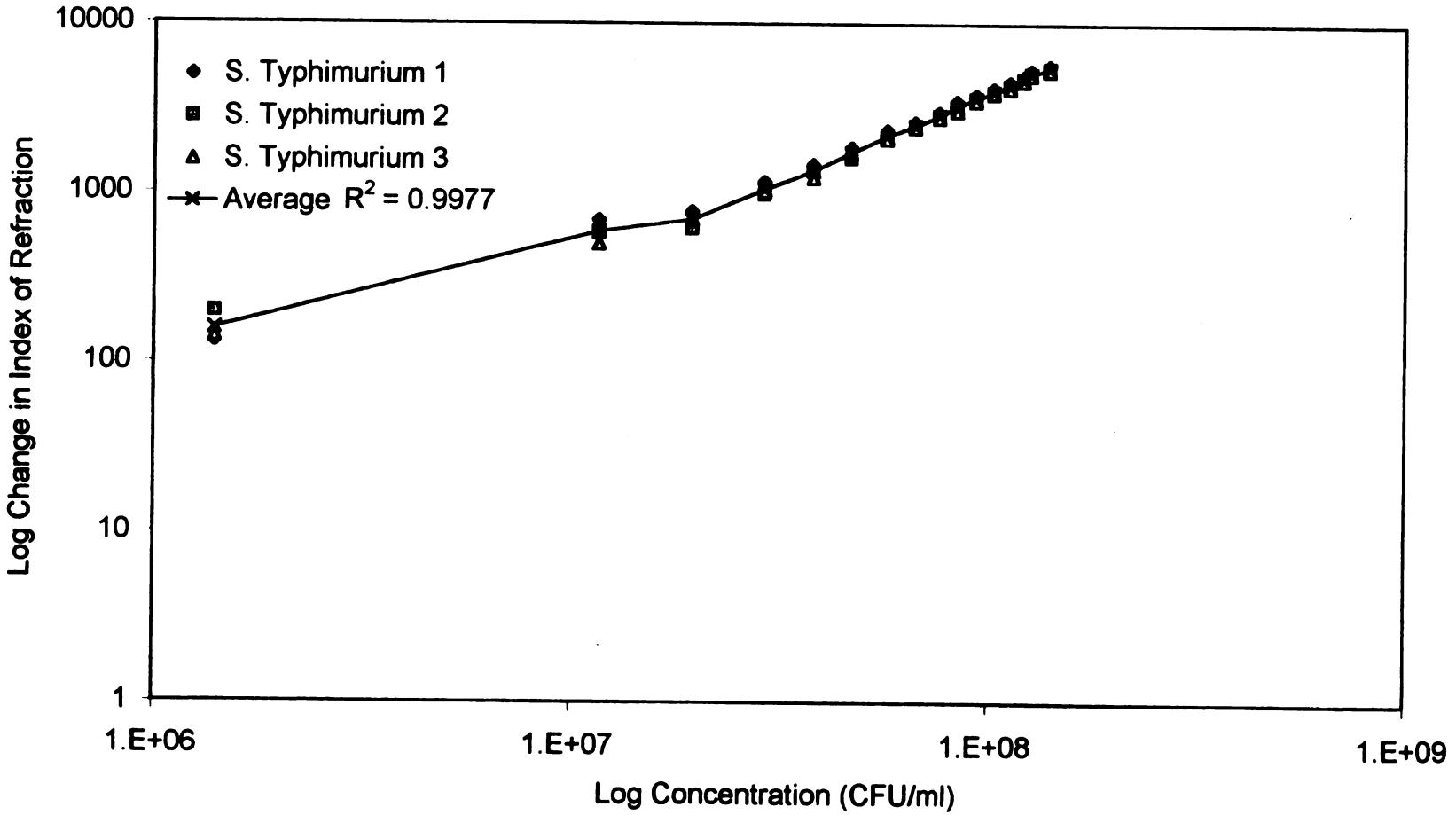

Figure 3.8. Linear region of *S.* Typhimurium Dilution Series.

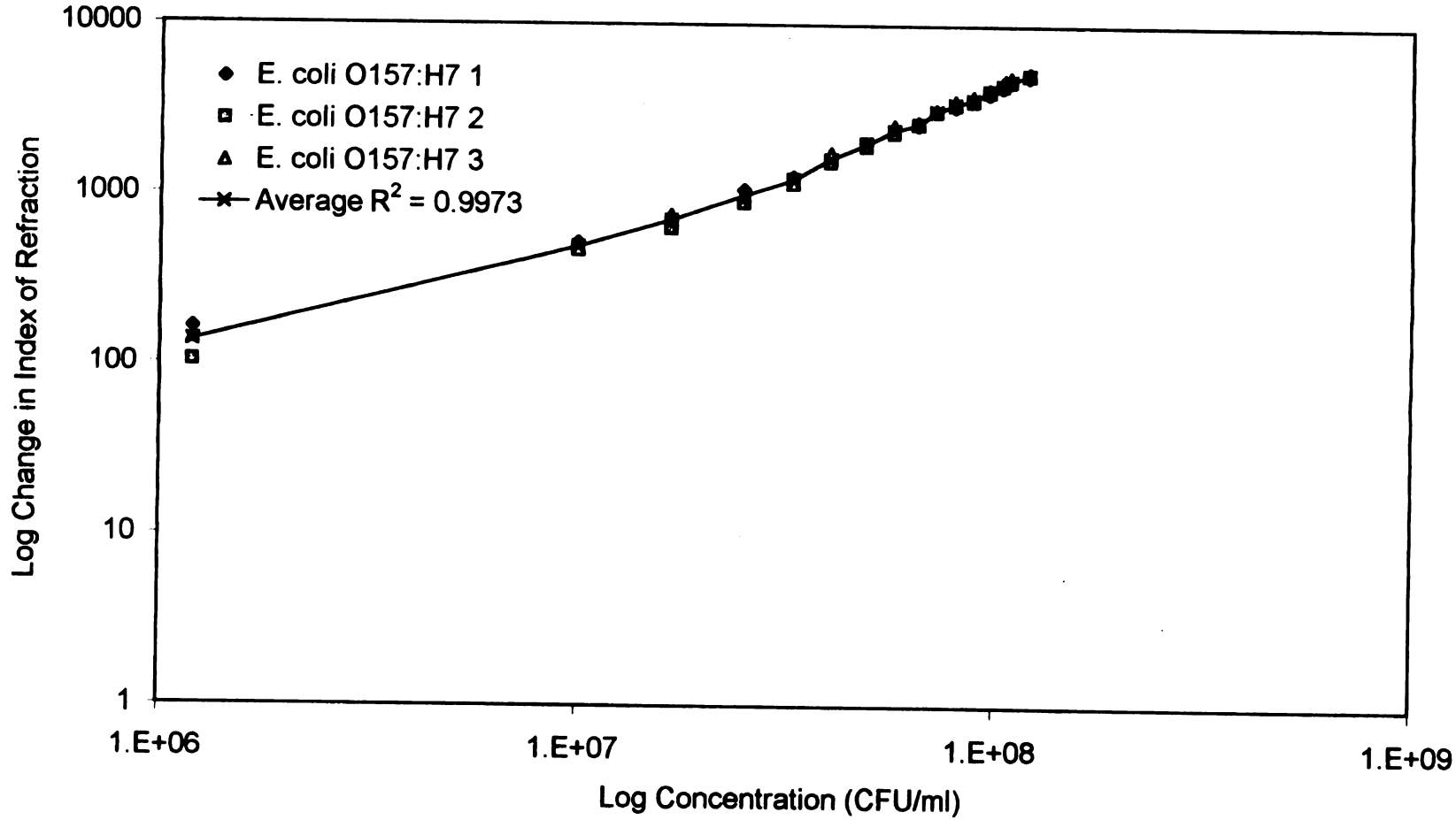

Figure 3.9. Linear Region of *E. coli* O157:H7 Dilution Series.

detection of the SPR biosensor was 9.1 x 10^6 CFU/ml for *S.* Typhimurium, and 8.7 x 10^6 for *E. coli* O157:H7. These limits were rounded to the nearest power, giving a limit of detection for both *S.* Typhimurium and *E. coli* O157:H7 of 10^7 CFU/ml.

Figures 3.8 and 3.9 show the linear correlation between the biosensor response and the bacterial concentration. An R^2 of 0.998 for *S.* Typhimurium, and an R^2 of 0.997 for *E. coli* O157:H7 in the region above the limit of detection indicates the ability of the SPR biosensor to predict the concentration of the target organisms.

A limit of detection of 1 x 10^7 CFU/ml is similar to the detection limits of other SPR biosensors in the literature. Fratamico *et al.* (1998) reported a detection limit of 1.7-2.1 x 10^6 CFU/ml using the BIAcore™ SPR biosensor for *E. coli* O157:H7 detection. Seo *et al.* (1999) reported a detection limit in the range of 1 x 10^5 to 1 x 10^7 CFU/ml for *Salmonella* spp., using an SPR biosensor they constructed. A resonant mirror sensor, similar in concept to the SPR biosensor, had a detection limit of 8 x 10^6-8 x 10^7 CFU/ml (Watts *et al.,* 1994). The ELISA system, another popular immunosensor, has a detection limit of 10^5-10^6 CFU/ml (Seo *et al.,* 1999), and the VIDAS™ immunoassay has a detection limit of 10^5 CFU/ml (Cohen and Kerdahi, 1996). Although several of these other immunosensors report lower detection limits, a limit of 1 x 10^7 CFU/ml is within the range of currently accepted methods, especially for other sensors based on the SPR principle (Elkind, 1998). Additionally, because this was the first study done using the Spreeta SPR biosensor as an immunosensor for pathogen

detection, further experimentation for the refinement of the initiation and sampling protocols could potentially lead to a significant increase in selectivity.

However, it may be argued that there is no need to increase the selectivity of the SPR biosensor. Representative growth curves of *S.* Typhimurium and *E. coli* O157:H7 (Figure 3.10) show that concentrations of *S.* Typhimurium and *E. coli* O157:H7 reach the detectable level of the SPR instrument (10^7 CFU/ml) roughly 5 ½ hours after inoculation. This is much shorter than the time required by many sensing methods that involve pre-enrichment, such as standard plate counts. Currently, other biosensing methods such as VIDAS™ immunoassays and the ELISA protocol require pre-enrichments that take 18-32 hours (Grif and Allerberger, 1998; bioMerieux, 1998). Standard plating methods, moreover, often take 4-7 days for pathogenic detection (Jay, 2000; Seo *et al.,* 1999). If the SPR biosensor can detect pathogens in samples incubated for 5 ½ hours, it is significantly more rapid than these methods.

Specificity assays were conducted to determine whether the SPR biosensor could detect the target organism in samples that have not undergone extensive pre-enrichment. Assays of pure cultures of target and non-target bacteria were first conducted to determine the overall specificity of the SPR biosensor. The average magnitude of response of a SPR biosensor assay on target bacteria was greater than the magnitude of response to a pure culture of non-target bacteria. (Figures 3.11-3.12). In fact, the response of the SPR biosensor to pure cultures of non-target bacteria was not significantly different from the SPR biosensor response to the negative control at all dilution levels.

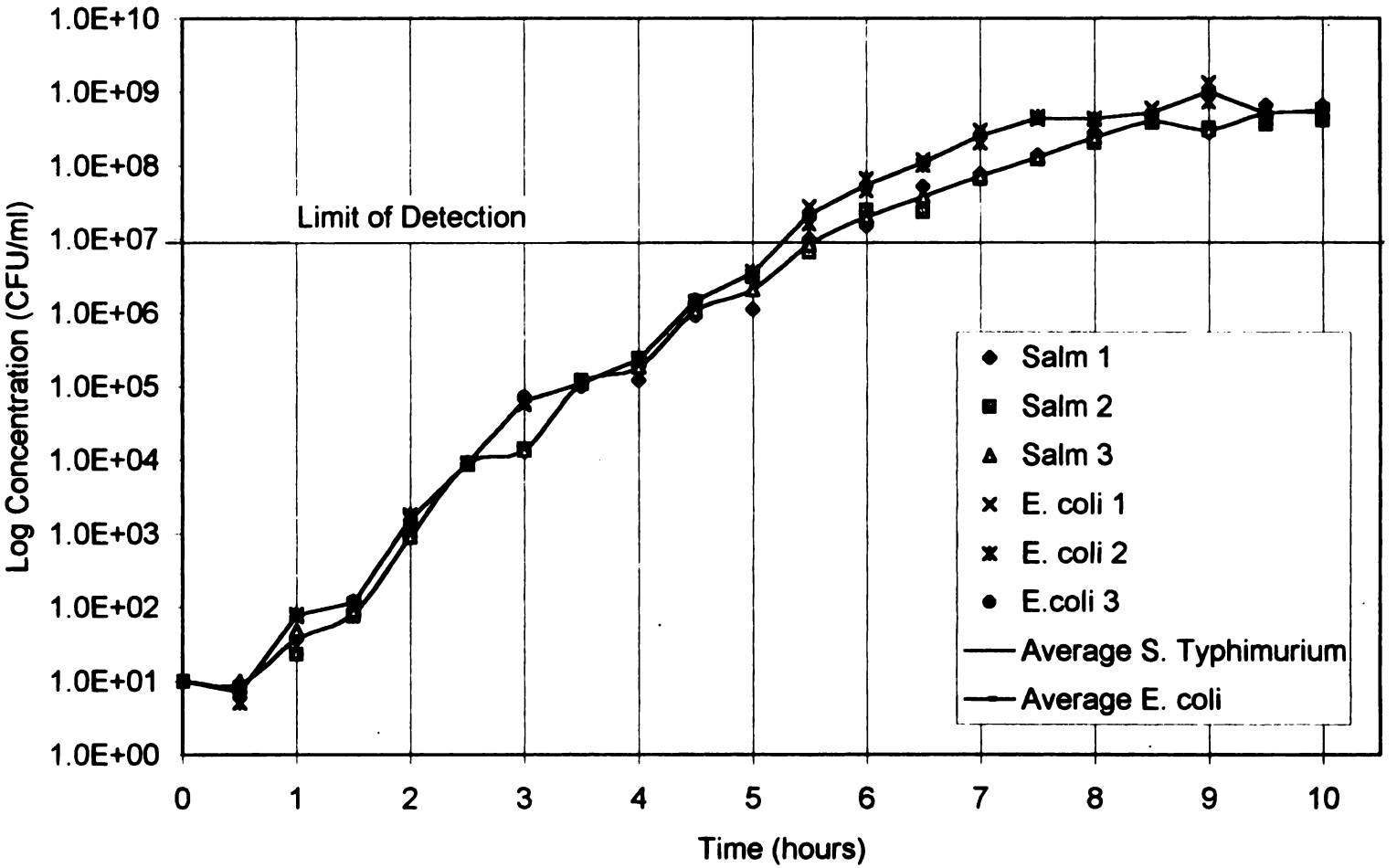

Figure 3.10. Timed growth of *S*. Typhimurium and *E. coli* O157:H7

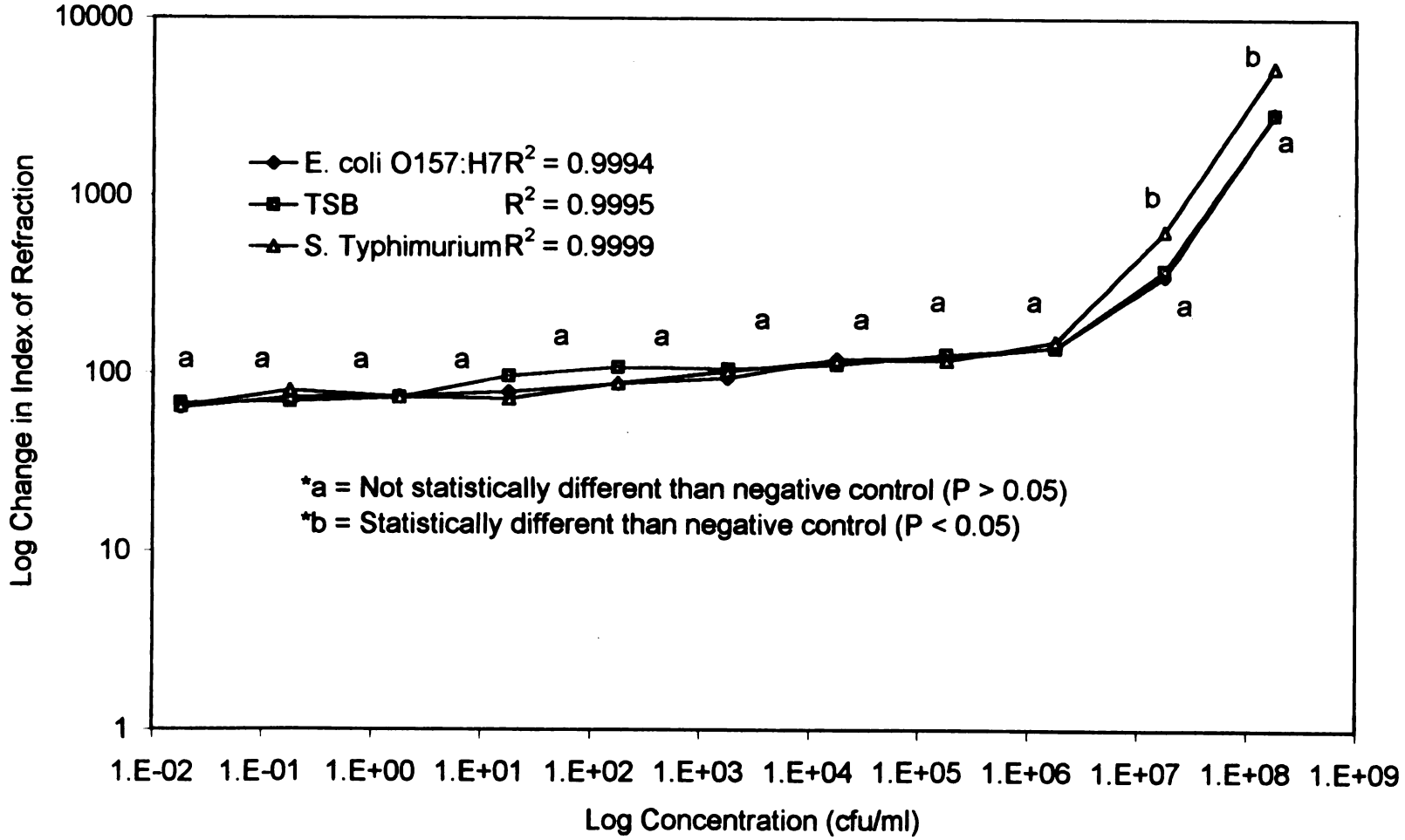

Figure 3.11. Average Specificity for S. Typhimurium.

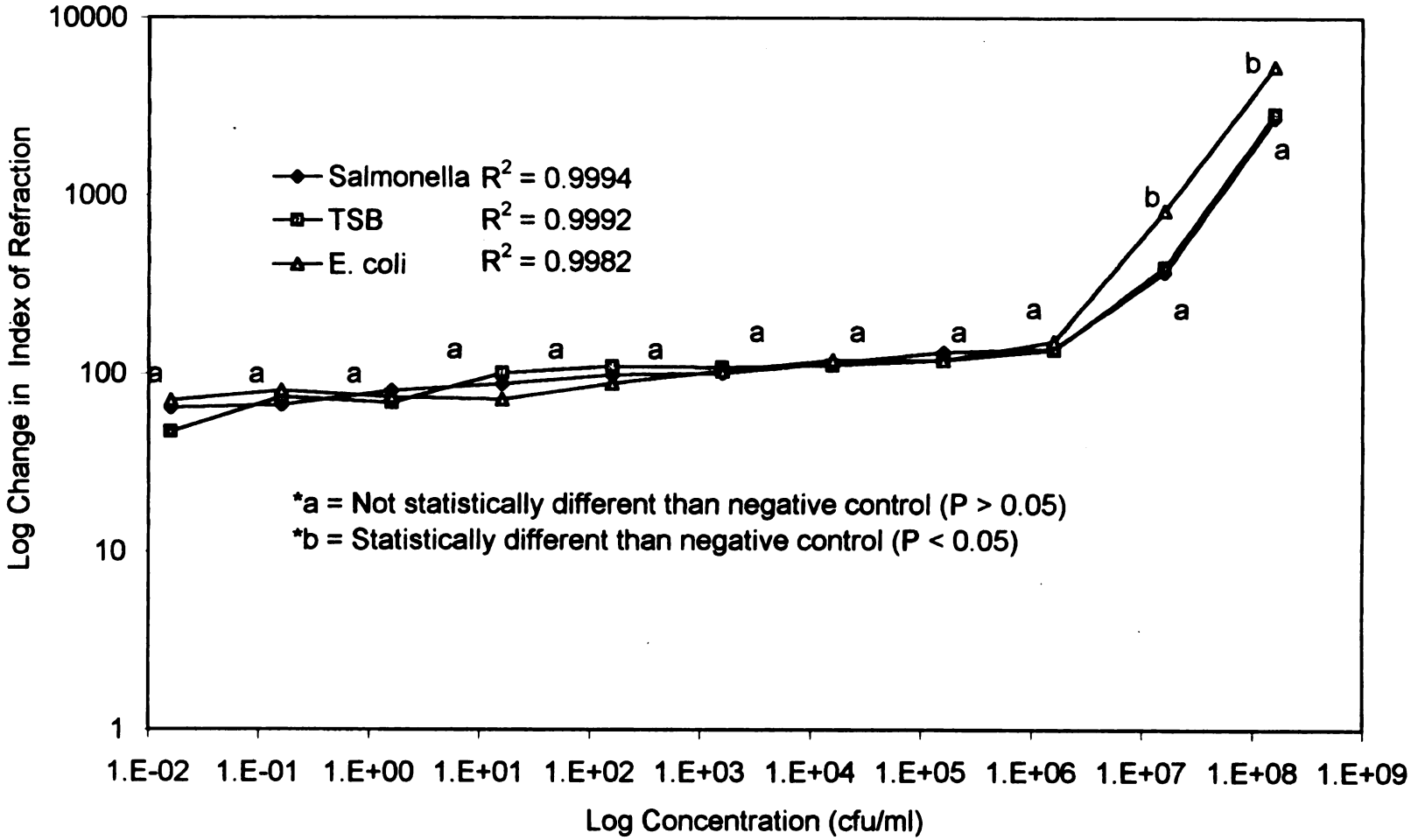

Figure 3.12. Average Specificity for *E. coli* O157:H7.

Conversely, for both anti-*Salmonella* and anti *E. coli* O157:H7–prepared biosensors, the response of the SPR biosensor to target bacteria was significantly different than the negative control at concentrations of 10^7 CFU/ml and above. This result verifies that the detection limit of the biosensor was 10^7 CFU/ml, and indicates that the antibody-prepared biosensor was specific to the target of interest, and not cross-reactive to the non-target bacteria. This may be because *S.* Typhimurium and *E. coli* O157:H7 are not closely related, and have low numbers of similar proteins. Their respective antibodies, therefore, would display little cross-reactivity to the other pathogen.

To assess the specificity of the SPR biosensor in mixed cultures, several assays were conducted on dilution series with varying concentrations of non-target bacteria (Figures 3.13-3.14). The magnitude of the SPR biosensor response increased as the concentration of target bacteria in solution increased. For *Salmonella* spp. specificity assays, at a *S. Typhimurium* concentration of 10^7 CFU/ml, the SPR biosensor response to samples with *E. coli* O157:H7 concentrations of 10^7 CFU/ml and above was statistically the same as the response to the negative control. At *S.* Typhimurium concentrations of 10^8 CFU/ml, adding 10^8 CFU/ml of *E. coli* O157:H7 caused the biosensor response to be statistically the same as the negative control (Table 3.2). Likewise, for *E. coli* O157:H7 specificity assays, ANOVA showed that at an *E. coli* O157:H7 concentration of 10^7 CFU/ml, the SPR biosensor response to samples with *S.* Typhimurium concentrations of 10^7 CFU/ml and above was statistically the same as the response to the negative control. At *E. coli* O157:H7 concentrations of 10^8

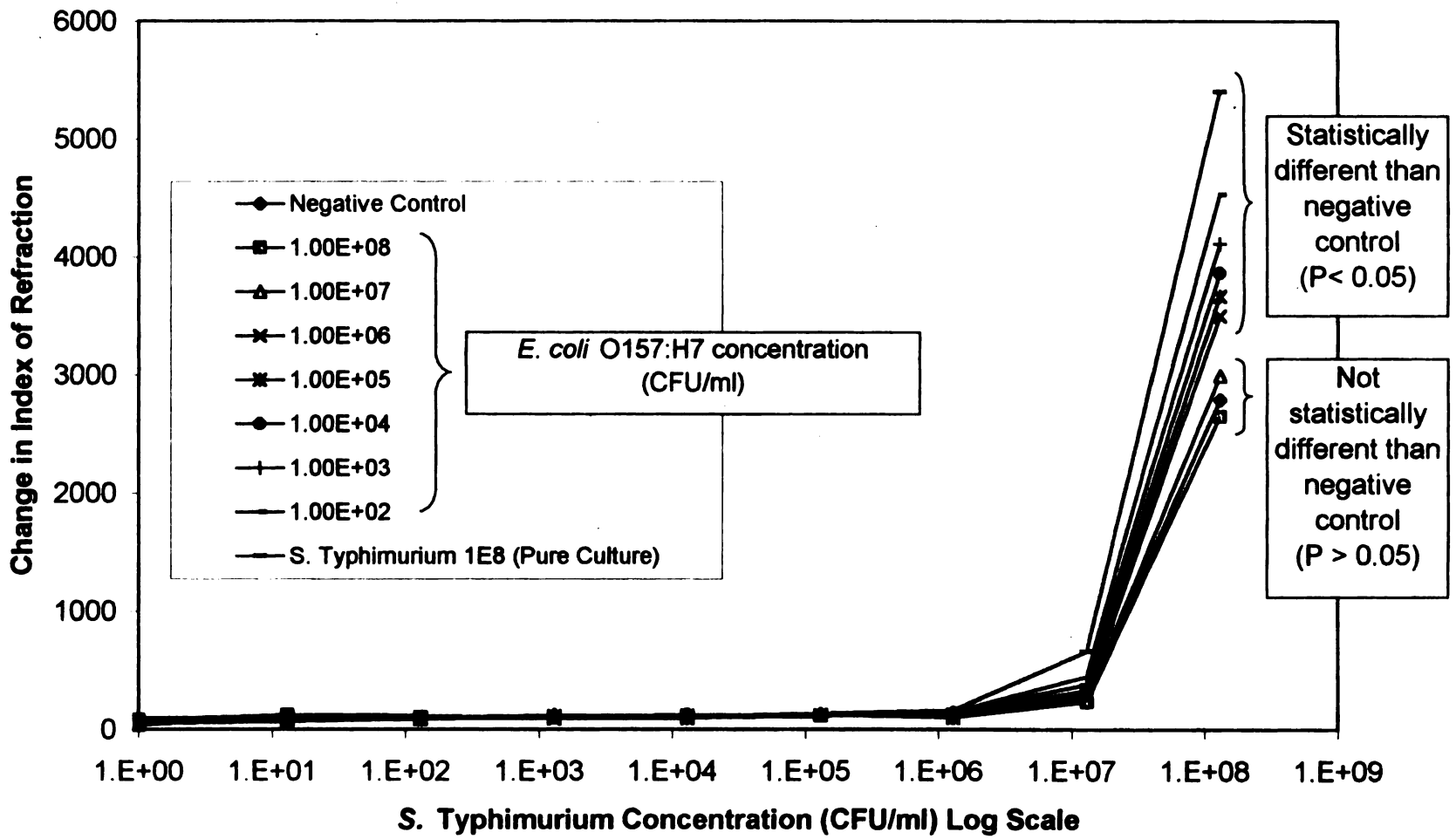

Figure 3.13 . Specificity for anti-*Salmonella* spp. - prepared SPR biosensor in mixed culture.

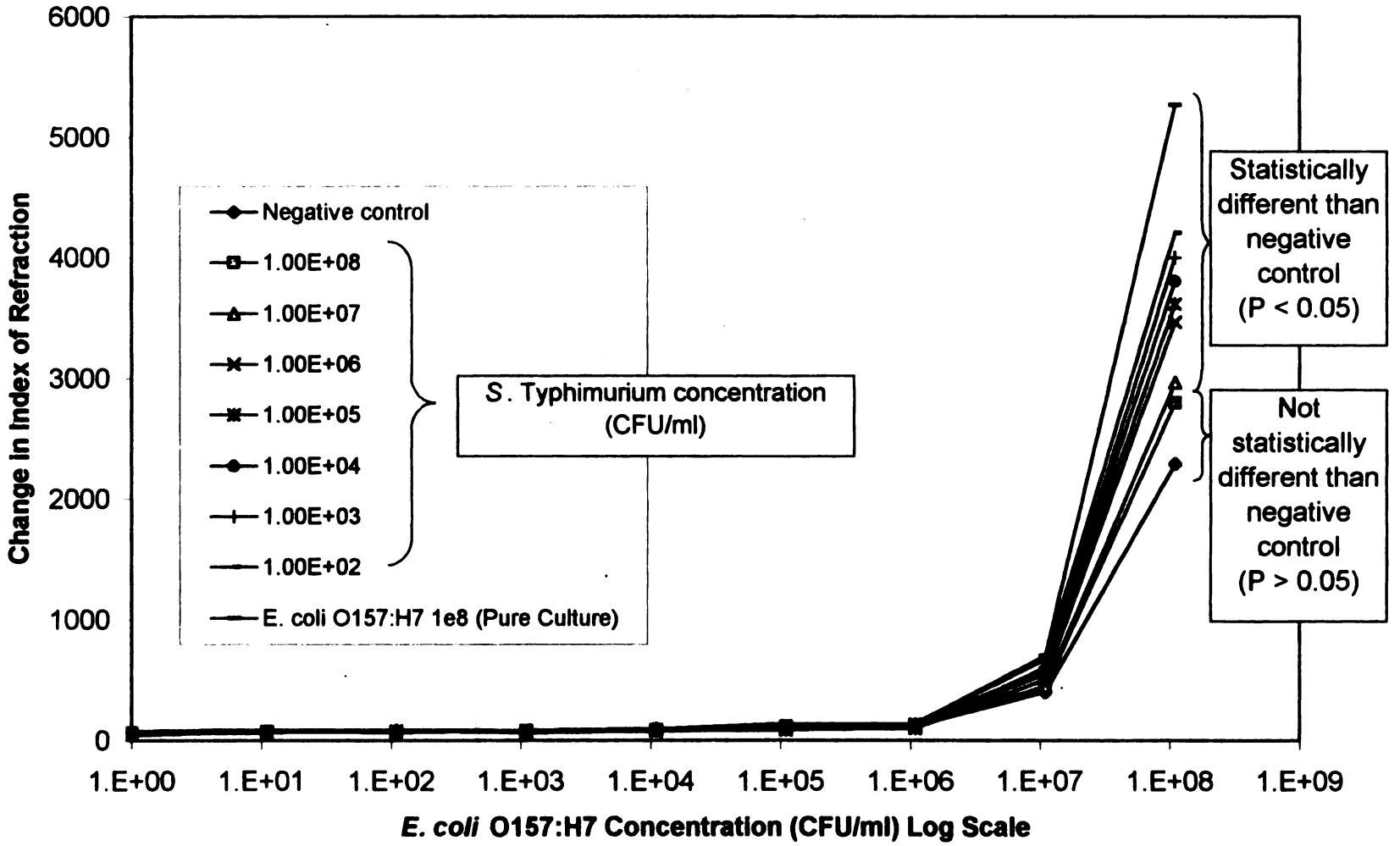

Figure 3.14. Specificity for anti-*E. coli* O157:H7 - prepared SPR biosensor in mixed culture.

Table 3.2. ANOVA results for *S.* Typhimurium specificity in mixed cultures. P values.

E. coli O157:H7 *(CFU/ml)* / S. Typhimurium (CFU/ml)	10^8	10^7	10^6	10^5	10^4	10^3	10^2	*S* Typhimurium 10^8 (Pure Culture)
0	0.650	0.045	0.135	0.362	0.247	0.076	0.214	0.305
1.10E+01	0.077	0.702	0.206	0.292	0.321	0.386	0.165	0.118
1.10E+02	0.149	0.194	0.390	0.137	0.148	0.086	0.170	0.057
1.10E+03	0.181	0.454	0.481	0.094	0.067	0.092	0.188	0.094
1.10E+04	0.353	0.087	0.157	0.053	0.170	0.108	0.092	0.442
1.10E+05	0.089	0.208	0.362	0.119	0.082	0.053	0.334	0.188
1.10E+06	0.445	0.257	0.157	0.257	0.249	0.327	0.239	0.125
1.10E+07	0.099	0.046	0.043	0.038	0.035	0.020	0.014	0.012
1.10E+08	0.189	0.108	0.021	0.018	0.014	0.009	0.006	0.001

Table 3.3. ANOVA results for *E.coli* O157:H7 specificity in mixed cultures. P values

S. Typhimurium (CFU/ml) / E. coli O157:H7 (CFU/ml)	10^8	10^7	10^6	10^5	10^4	10^3	10^2	*E. coli* O157:H7 1.00E+8 (Pure Culture)
0.00E+00	0.201	0.072	0.078	0.048	0.259	0.099	0.194	0.112
1.10E+01	0.829	0.121	0.153	0.180	0.269	0.445	0.304	0.465
1.10E+02	0.350	0.203	0.264	0.431	0.321	0.047	0.009	0.351
1.10E+03	0.992	0.443	0.398	0.365	0.922	0.088	0.802	0.219
1.10E+04	0.321	0.149	0.418	0.391	0.387	1.000	0.181	0.216
1.10E+05	0.165	0.780	0.066	0.175	0.201	0.171	0.353	0.248
1.10E+06	0.566	0.722	0.062	0.326	0.609	0.117	0.092	0.113
1.10E+07	0.337	0.249	0.042	0.018	0.016	0.023	0.006	0.005
1.10E+08	0.059	0.030	0.009	0.007	0.009	0.013	0.004	0.002

Statistically significant response ($P < 0.05$)

CFU/ml, adding 10^8 CFU/ml of *S.* Typhimurium caused the biosensor response to be statistically the same as the negative control (Table 3.3).

These results indicate that the limit of detection of the SPR biosensor can be affected by the presence of non-target bacteria in the sample. The presence of 10^7 CFU/ml or more of non-target bacteria in the mixed samples caused the SPR biosensor to undergo a reduction in selectivity. However, up to levels of 10^6 CFU/ml of non-target bacteria in the sample, the SPR biosensor remained able to detect the target pathogen at 10^7 CFU/ml.

This reduction in sensitivity may be due to physical 'crowding out' of the target bacteria in proportion to non-target bacteria, causing a low number of target antigens to come into physical contact with the antibody. As the sensing surface is immersed into the sample, the antibodies contact only those bacteria closest to them. At higher numbers of non-target bacteria, the target bacteria are spread more thinly through the interfacial layer of bacteria at the sensor surface. This causes the antibody to come into contact with a lower number of bacteria, possibly fewer than the detection level of 10^7 CFU/ml. This would cause a negative response, even though the total number of target bacteria in the sample is above the limit of detection.

CONCLUSIONS

The Spreeta™ SPR biosensor used in this investigation is a new, experimental product that has not before been tested or used for pathogen detection. As this is the first investigation into the use of this sensor as an immunosensor for the detection of *S.* Typhimurium and *E. coli* O157:H7, the

results of this study are promising. This investigation demonstrates that the SPR biosensor has the potential for on-line, rapid, portable, and accurate pathogen detection. The SPR biosensor is capable of reporting the presence of these bacteria at levels of 10^7 CFU/ml, and has shown specificity to the target organism. With additional investigation and refinements, there may be many uses for the SPR biosensor in food safety and control applications.

Another factor adding to the potential value of the SPR biosensor is its versatility. By using antibodies specific to different biochemicals, the biosensor can be made specific for many different target analytes. This expands the potential applications of the SPR biosensor to detection of other forms of pathogenic bacteria in foods, as well as raises the possibility of utilizing the SPR biosensor in applications in fields such as medicine, biochemistry, environmental treatment and remediation, and others.

Finally, the SPR biosensor could have a very positive economic benefit for the food safety industry. According to the Food Safety Research Workgroup, 21 federal agencies are spending $200 million per year on food safety research. State and industry officials match these funds, resulting in a total of at least $400 million spent on food research every year (Forsythe, 1996).

Many current commercial and experimental biosensors, e.g. the BIAcore, IBIS, and Raptor biosensors, cost tens of thousands of dollars to purchase and operate (Alocilja, 2000), while the Spreeta™ SPR biosensor used in this study cost less than $3,000. Furthermore, commercial biosensors are bulky and lab-based, whereas because of its small size, the Spreeta SPR biosensor can be

easily transported to the field. If this biosensor can be optimized for use in the food safety industry, it will provide an affordable, portable, and field-based alternative to other, more expensive and bulky biosensors. This could make food safety inspection much cheaper and accessible, and make it easier for food producers to implement HACCP protocols. USDA's Economic Research Service (ERS) estimates the cost of the HACCP to the meat and poultry industry regulations to be $1.0 to $1.2 billion per year over the next twenty years (Roberts *et al.,* 1996). The SPR biosensor tested in this investigation shows the potential to reduce this cost, by providing an economical alternative or complement to current pathogen monitoring techniques that is sensitive, specific, accurate and easy-to-use.

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CHAPTER 4

DETECTION OF *SALMONELLA* TYPHIMURIUM AND *ESCHERICHIA COLI* 0157:H7 IN THE PORK PRODUCTION CHAIN USING A SURFACE PLASMON RESONANCE BIOSENSOR AND A VIDAS™ IMMUNOSENSOR

ABSTRACT

A Spreeta™ SPR biosensor was evaluated as a diagnostic tool for detecting *Salmonella* spp. and *E. coli* O157:H7 in samples obtained from swine farms and slaughterhouses. A VIDAS™ immunosensor was used as a standard for comparing the accuracy of results obtained from the SPR biosensor. Field samples were obtained from representative small and medium-sized pork production facilities and analyzed using both the SPR biosensor and the VIDAS™ immunosensor. Results of this study indicate that with further refinement the SPR biosensor could be useful as a pathogen monitoring system within the pork industry chain. The average SPR biosensor response was greater in magnitude for *S.* Typhimurium- and *E. coli* O157:H7-positive samples than the average response to negative samples. However, the difference was statistically significant for only one sample. With further modifications of the initiation and sampling protocols, the SPR biosensor has potential to increase its ability to detect target organisms in field-samples. The SPR biosensor shows promise to provide a complementary detection system to standard lab-based systems currently used in food safety.

INTRODUCTION

Salmonella enterica spp. Typhimurium and *Escherichia coli* O157:H7 are foodborne pathogens of major concern in pork production. In the United States, foodborne diseases cause an estimated 76 million illnesses, 325 thousand hospitalizations, and 5 thousand deaths annually, with an associated cost of $5.6-$9.4 billion (Buzby *et al.*, 1996). Pork products are growing in popularity among consumers of meat, and continue to expand their share of local and global markets (U.S. Meat Export Federation, 1997). Every time an outbreak occurs in the pork product category, consumer health is put at risk, and the viability of the pork industry is threatened.

Salmonella enterica has been identified as one of the most prevalent and costly of known foodborne pathogens (Davies, 1997), and currently 11% of all outbreaks of salmonellosis in humans have been associated with pork (Isaacson *et al.*, 2000). Adding to concern about *Salmonella* infection is the existence of several antibiotic resistant strains, especially the multi-drug resistant strain *S.* Typhimurium DT 104. With the emergence of this strain, the incidence and severity of *Salmonella*-related human illness is increasing. *Salmonella* is a particular problem in the pork industry, because of the ability of pigs to become long-term carriers of the organism, with the potential of spreading the pathogen to other hogs through a variety of transmission routes (Letellier *et al.*, 1999).

Escherichia coli O157:H7 is a major cause of serious outbreaks and sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome (HUS)

(Grif and Allerberger, 1998). *E. coli* O157:H7 has been identified in many commonly consumed foods, including pork and pork products (Jay, 2000). The combination of the severe consequences of infection, its low infectious dose, and its association with many common foods make *E. coli* O157:H7 a bacterial pathogen of particular concern (Verozny-Rozand *et al.*, 1998).

Fecal shedding and growth of pathogens on the farm can lead to subsequent contamination of the slaughterhouse environment and finished product. Infected animals and contaminated feed are supposedly the primary routes for introducing these pathogens into herds (Ryser, 2000).

In an effort to reduce the occurrence and numbers of pathogens on meat and poultry products, recent food safety initiatives have emphasized the monitoring and control of foodborne pathogens. The Hazard Analysis Critical and Control Point (HACCP) system has reduced the need for end-period testing, however the demand for rapid and accurate methods to detect foodborne pathogens has increased (Seo *et al.*, 1999).

Because of the 'zero-tolerance' status of *S.* Typhimurium and *E. coli* O157:H7, testing must be performed that is accurate and sensitive at very low levels (Jay, 2000). Classic approaches to microbiological quality control have relied heavily on microbiological determinations of both raw materials and end products, but the time required for results is too long for many products (Jay, 2000). There is a growing need in the food industry for pathogen detection systems that are sensitive to low levels of bacteria, specific to the target organisms, inexpensive, and capable of running at or near real-time.

The properties of surface plasmon resonance (SPR) are used in the SPR biosensor to monitor antibody-antigen interaction in real-time (Elkind *et al.*, 1998). SPR, an optical technique for surface and interfacial studies, is widely used in the biosensor, pharmaceutical, and analytical chemistry communities (Salamon *et al.*, 1999). Surface plasmons in a thin gold film can be propagated by incident light, which in turn excite an evanescent wave that can probe the optical properties of materials in direct contact with the sensing surface (Salamon *et al.*, 1999). When labeled with antibodies, the SPR biosensor can directly analyze the binding of an antiserum to the immobilized ligand. The binding interaction generates a signal resulting in changes in refractive index on the gold surface and the matrix. This change is proportional to the change in adsorbed mass (Medina, 1997). By using antibodies specific to pathogens of interest, it is possible to utilize the SPR phenomenon to quantitate pathogenic bacteria in a sample by measuring the change in refractive index.

Consumers are increasingly aware of the risk foodborne pathogens pose to human health, and as such demand a safe, high quality, and nutritious food supply. This means that having a reliable system for monitoring the quality and safety of foods is of increasing importance. The SPR biosensor shows promise to fulfill the food industry's need for a fast, reliably sensitive screening method for pathogen monitoring. The objective of this research was to explore the feasibility of employing a SPR biosensor to detect *Salmonella* Typhimurium and *E. coli* O157:H7 in environmental and meat samples from representative pork packing facilities. The SPR biosensor was compared to the VIDAS™ immunoassay as a

diagnostic tool for detecting *Salmonella* spp. and *E. coli* O157:H7 in samples obtained from the pork industry.

METHODS AND MATERIALS

Spreeta™ SPR Biosensor

The Spreeta™ Miniature Integrated Surface Plasmon Resonance Liquid Sensing System (Texas Instruments, Inc.) was used in this study. The output of the sensor is the index of refraction at which a ray of light incident upon the sensing film experiences a minimum corresponding to where SPR occurs.

Preparation and Biotinylation of Antibodies

Lyophilized affinity purified antibody to *Salmonella* common structural antigens (CSA-1) and to *Escherichia coli* O157:H7 were purchased (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). All antibodies were stored at 4°C until rehydrated. Antibodies were rehydrated according to the manufacturer's instructions (Kirkegaard & Perry Laboratories, Inc.) Biotin labeling was done according to the method of DeMarco *et al.* (1999). The biotinylated antibody was then serially diluted with sterile phosphate buffered saline (PBS; pH 7.2), to achieve the optimum concentration of 300 μg/ml (Chapter 3).

Biosensor Preparation and Antibody Attachment

The SPR biosensor was assembled according to the Spreeta Operation Manual. Neutravidin binding and attachment of biotinylated antibodies to the surface was done following the method of Spreeta's Application Brief 004, with

modifications (Texas Instruments, 1999). The total time to prepare the SPR biosensor for an assay was 30 min (Chapter 3).

Bacteria

Characterized strains of *S.* Typhimurium and *E. coli* O157:H7 were obtained from Michigan State University collections. *E. coli* O157:H7 was verified in an earlier study by the Bacteriology Laboratory at the Veterinary Diagnostic Center of the University of Nebraska, Lincoln (Younts, 1999). All culturing was done in a certified Biological Safety Level 2 environment at the Michigan State University Meat Microbiology Laboratory. Cultures were serially diluted in sterile buffered peptone water (BPW) for use experiments.

SPR Biosensor Signal Normalization

The output of the biosensor was the index of refraction at which surface plasmon resonance occurred. To normalize the output, the index of refraction for the zero controls was subtracted from the response. The resulting biosensor output was then the magnitude of the increase in index of refraction above the index of refraction of the zero control (Chapter 3).

Field Samples

Samples were obtained from a previous study, in which site visits were made to small-sized (~200 heads/week) and medium-sized (>1000 heads/week) commercial meat packing facilities, as well as to the farms that supplied them with hogs (Ryser, 2000). The samples were pre-enriched in lactose broth within 24 hours, and stored at refrigeration temperatures. Twenty-four samples were

analyzed by mini-VIDAS and SPR biosensor for *Salmonella* spp. and *E. coli* O157:H7 (Tables 4.1 and 4.2).

Table 4.1. Farm samples (Ryser, 2000)

Site	Sample	Method and Quantity	Number of samples
Pen Alleyway	Fecal material, floor	Scoop, 50g	1
Hogs back	Composite	Swab with neutralizing buffer solution	2
Fecal matter	Composite	Scoop, 50g	1
Feed	Composite	Scoop, 50g	2
Water Nozzle	Composite	Swab from nozzle tip	1

Table 4.2. Environmental samples from slaughterhouse (Ryser, 2000)

Site	Sample	Method and Quantity	Number of samples
Pen Alleyway	Fecal material, floor	Scoop, 50g	1
Prechill	Swab from chillroom wall	Swab	7
Dehairing machine	Hairs scooped from dehairing machine	25g, hair scoop	6
Drain	Drain near evisceration	Swab	3

Field Sample Analysis

Salmonella spp. and *E. coli* O157:H7 were assayed using both the mini-VIDAS™ system and the SPR biosensor:

VIDAS™ Salmonella

Recovery of *Salmonella* spp. using the VIDAS™ system was done according to the BAM/AOAC method (bioMerieux, 1998). The samples were pre-enriched in lactose broth, then 1 ml of the pre-enriched sample was incubated in 9 ml of buffered peptone water (BPW) for 18 hours at 37°C. Following this, 1 ml. of the BPW enrichment was transferred into 10 ml of selenite cystine broth and

10 ml tetrathionate broth. The selenite cystine and tetrathionate broths were incubated for 8 hours at 37°C and 42°C, respectively. After enrichment, 1 ml of selenite cystine broth and 1 ml of tetrathionate broth were transferred to separate tubes containing 10 ml of M-broth and incubated 18 hours at 42°C. After incubation, the M-broth enrichments were mixed and 1 ml of each was transferred into a hermetically sealed tube. The tubes were heated for 15 minutes in a water bath at 100°C. After cooling to room temperature, the samples were screened on the mini-VIDAS system (bioMerieux, St Louis, MO). Positive samples were biochemically confirmed by the Food Microbiology Lab of Michigan State University (Ryser, 2000).

VIDAS™ *E. coli* O157:H7

Recovery of *E. coli* O157:H7 was done using the VIDAS™ ECO assay (bioMerieux, 1998). One ml of each sample was added to 9 ml of m-TSB with novo-biocin, then incubated at 41°C for 6 hours. One ml of the enriched culture was then transferred into 9 ml MacConkey broth with cefixime and potassium tellurite (CT-Mac) (Mast Diagnostics, Merseyside, UK), and incubated for 18 hours at 37°C. After incubation, the CT-Mac solution was mixed, and 1 ml of each suspension transferred into a hermetically sealed tube. The tube was heated for 15 min in a 100°C water bath, and allowed to cool. The samples were then screened for *E. coli* O157:H7 with the VIDAS™ ECO assay (bioMerieux, 1998).

SPR Biosensor *Salmonella* Assays

The same enriched samples from the VIDAS immunoassay were used for SPR analysis. The SPR biosensor was prepared with appropriate antibody, and immersed in sterile BPW for two minutes as a zero control, then into sterile M-broth for two minutes to identify the background response (Table 4.3). The inoculated M-broth sample was then assayed for 3 minutes, followed by a pure culture of *S.* Typhimurium in M-broth (the positive control).

Table 4.3. SPR biosensor procedure for *Salmonella* spp. assays

Step	Action	Time (min)
1	SPR sensor preparation (Table 2.1, Chapter 2)	30
2	Sterile BPW (zero control)	2
3	Sterile M-broth (negative control)	2
4	Sample in M-broth	3
4	anti-*Salmonella* spp. antibody (300 µg/l)	2
	Total time:	39

Following the inoculated M-broth sample, the sensor was immersed in anti-*Salmonella* spp. antibody for a second time, to achieve 'sandwich' binding of antibody (Figure 4.1). Two identical assays were performed for each sample.

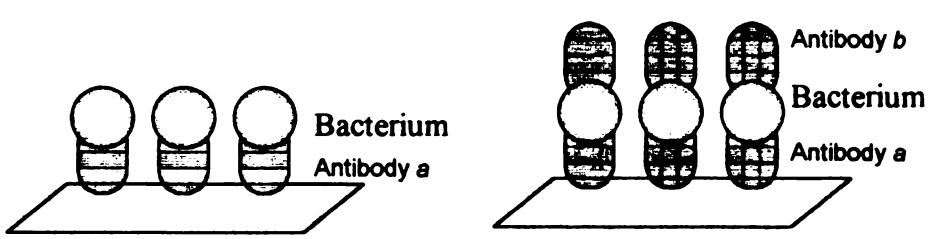

Figure 4.1. (A) Antibody-antigen interaction on the SPR biosensor gold-film surface using one antibody layer *a*. (B) Antibody-antigen interaction on the SPR biosensor gold-film surface in a sandwich-type interaction, with two layers of antibody, *a* and *b*.

The sandwich binding arrangement was designed to increase the molecular mass of the substrate bound to the sensing film of the SPR biosensor. This was done to increase the magnitude of the change in index of refraction of the biosensor.

SPR Biosensor *E. coli* O157:H7 Assays

E. coli O157:H7 was assayed on the SPR biosensor following the same procedure as the *Salmonella* spp. assays, using anti-*E. coli* O157:H7 antibody and sterile CT-Mac broth in place of M-broth for the negative control (Table 4.4).

Table 4.4. SPR biosensor *E. coli* O157:H7 assay procedure

Step	Action	Time (min)
1	SPR sensor preparation (Table 2.1, Chapter 2)	30
2	Sterile BPW (zero control)	2
3	Sterile CT-Mac (negative control)	2
4	Sample in CT-Mac	3
4	anti-*E. coli O157:H7* antibody (300 μg/l)	2
	Total time:	39

The sandwich assay was performed in the same manner as for the *Salmonella* spp. assays. Two replicates were performed on each sample.

Because initial testing indicated that the samples contained negligible amounts of *E. coli* O157:H7, five samples were inoculated with both 10 μl of a stock solution of 10^3 CFU/ml *E. coli* O157:H7 (about 10 CFU), and 10 μl of pre-enriched sample from the pork production facilities to create known positives (Table 4.5).

Table 4.5. Samples inoculated with *E. coli* O157:H7

Site	Number of samples
Prechill	2
Hogs Back	1
Drain	1
Pen Alleyway	1

The samples were prepared in sterile BPW, and enriched according to the method used for VIDAS™ ECO samples.

SPR Biosensor Positive Controls

Positive controls for the *Salmonella* spp. and *E. coli* O157:H7 SPR biosensor assays were pure cultures of *S.* Typhimurium in M-broth, and *E. coli* O157:H7 in CT-Mac broth, respectively. Positive controls were assayed following the SPR biosensor assay procedures used for the samples, with pure culture used in place of the inoculated sample. Two positive controls each were run for *S.* Typhimurium and *E. coli* O157:H7. Viable plate counts were performed on the control cultures.

SPR Biosensor Assays in TSB

To find any difference in response of the SPR biosensor to samples grown according the VIDAS™ enrichment protocols, and samples grown in TSB, the pork industry samples were inoculated in TSB and grown for 18 hours at 37°C. The samples were then analyzed with the SPR biosensor following the same procedure as the assays performed on the samples grown according to the VIDAS™ methods. Sterile TSB was used as the negative control, and pure *S.* Typhimurium and *E. coli* O157:H7 cultures grown in sterile TSB were used as the positive controls. A second rinse of antibody was performed for both anti-

Salmonella spp. and anti-*E. coli* O157:H7 assays to achieve a 'sandwich'-type antibody binding.

E. coli O157:H7 positive samples were prepared in TSB in the same manner as the *E. coli* O157:H7 positive samples were prepared for the VIDAS™ enrichment method. Five samples were inoculated with both 10 µl of a stock solution of 10^8 CFU/ml *E. coli* O157:H7, and 10 µl of pre-enriched sample from the swine production facilities, to create known positives. The same samples from Table 4.5 were used to inoculate the *E. coli* O157:H7- positive samples in TSB.

RESULTS AND DISCUSSION

The results of this study compared the SPR biosensor to the VIDAS™ immunosensor for detection of *Salmonella* spp. and *E. coli* O157:H7 in samples from swine farms and slaughterhouses. Three of 24 (12.5%) samples tested positive for *Salmonella* spp., and 0 of 24 (0%) samples tested positive for *E. coli* O157:H7 (Table 4.6). Confirmation of positive *Salmonella* spp. results was done by the Food Microbiology Lab of Michigan State University (Ryser, 2000). Suspected *Salmonella* colonies were purified and biochemically confirmed using API 20E strips (bioMerieux, St. Louis, MO), followed by serological identification and pulsed field gel electrophoresis (PFGE) analysis by the Michigan Department of Community Health (Ryser, 2000).

All five samples inoculated with a stock solution of *E. coli* O157:H7 tested positive on the VIDAS™ ECO assay. This method of spiking samples to create positive samples for the VIDAS™ ECO assay is commonly used (Grif and

Table 4.6. Incidence of *Salmonella* spp. and *E. Coli* O157:H7 in the pork production chain - Results for the VIDAS™ Immunosensor

	Location	*Salmonella* spp.	*E. coli O157:H7*	Number of Samples
Farm samples	Pen Alleyway	0	0	1
	Hogs back	0	0	2
	Fecal matter	0	0	1
	Feed	0	0	2
	Water Nozzle	0	0	1
		0	0	
Slaughterhouse Samples	Pen Alleyway	0	0	1
	Prechill	3	0	7
	Dehairing machine	0	0	6
	Drain	0	0	3
	Total	3 (12.5%)	0	24

Allerberger, 1998). Positive results of the VIDAS assay indicated that the *E. coli* O157:H7 had grown to a population of at least 10^5 CFU/ml – the detection limit of the VIDAS sensor (Cohen and Kerdahi, 1996). These results were used to verify the presence of *E. coli* O157:H7 in these samples for the SPR biosensor.

Results for the SPR biosensor assays were found by calculating the change of index of refraction of the inoculated sample above the negative control, as well as the change in refractive index of the biosensor reading for the second application of antibody above the negative control. This was done to see if a second application of antibody caused an increase in magnitude of the sensor response due to 'sandwich' antibody binding.

Analysis of the SPR biosensor *Salmonella* spp. assays for samples grown according to the VIDAS™ enrichment method revealed that the average response of the SPR biosensor to the positive samples was greater than the average response to the negative samples. However, ANOVA revealed that there was no statistical difference between the positive and negative samples (Table 4.7). Growing the samples in TSB did not affect these results, again the positive samples were not significantly different from the negative samples both before and after second antibody application (Table 4.8).

The average response of the SPR biosensor to the positive samples was greater than the average response to the negative samples. However, analysis of the positive and negative results for *E. coli* O157:H7 show that response of the SPR biosensor to the forced-positive samples was significantly larger than the negative sample in only one case (Table 4.9). Notably, the significant difference

Table 4.7. P-Values of positive *Salmonella* spp. samples in VIDAS-enriched method, after application of first and second antibody, compared to negative samples.

Sample source	First antibody	Second antibody
Prechill 3	0.56	0.97
Prechill 4	0.36	0.26
Prechill 5	0.46	0.40

Table 4.8. P-Values of positive *Salmonella* spp. samples in TSB, after application of first and second antibody, compared to negative samples.

Sample source	First antibody	Second antibody
Prechill 3	0.97	0.95
Prechill 4	0.26	0.60
Prechill 5	0.40	0.96

*Statistically significant difference when $P < 0.05$

was for the sample before the application of the second antibody. After application of antibody *b*, there was no significant difference of the known-positive sample responses and the negative responses. Fratamico *et al.*, (1998) reported the opposite phenomena: application of a second 'sandwich' antibody caused a significant increase in biosensor response. Other assays, including VIDAS™ and a sandwich-ELISA also use similar sandwich-type binding to increase the magnitude of sensor response (Cohen and Kerdahi, 1996; Jay 2000). Further experimentation needs to be done to analyze the kinetics of the antibody response of the SPR biosensor used in this study, to see if the second antibody is binding adequately.

Results for the SPR biosensor assays in TSB were calculated in the same way as for the assays performed on the VIDAS-method enriched samples. Again, the inoculated *E. coli* O157:H7 samples in TSB were used as positive controls for the *E. coli* O157:H7 SPR biosensor assays. The SPR biosensor responses for the TSB samples designated positive for *E. coli* O157:H7 by the VIDAS™ immunosensor were larger than the responses for the VIDAS™ negative responses. However, ANOVA revealed that the positive sample responses were not statistically different than the negative sample responses, both before and after application of the second antibody (Tables 4.10).

Though the difference was significant in only one case, in a Pen Alleyway sample, the average response of the SPR biosensor to positive samples was larger than the response to negative samples (Table 4.11). This shows the potential of the biosensor for use in pathogen detection. If the magnitude of the

Table 4.9. P-Values of known-positive *E. coli* O157:H7 samples in VIDAS-enriched method, after application of first and second antibody, compared to negative samples.

Sample source	First Antibody	Second Antibody
Prechill 2	0.83	0.08
Hogs Back	0.68	0.87
Prechill 4	0.39	0.27
Drain	0.71	0.10
Alley	*0.04	0.12

Table 4.10. P-Values of known-positive *E. coli* O157:H7 samples in TSB, after application of first and second antibody, compared to negative samples.

Sample source	First Antibody	Second Antibody
Prechill 2	0.17	0.50
Hogs Back	0.34	0.98
Prechill 4	0.25	0.29
Drain	0.08	0.33
Alley	0.26	0.17

*Statistically significant difference when $P < 0.05$

Table 4.11. Average SPR biosensor response for positive and negative samples, comparing the difference of the two with the average standard deviation.

Sample	*VIDAS enrichment method*		*TSB*	
	First Antibody	Second Antibody	First Antibody	Second Antibody
Salmonella positive	4811.98	5450.26	4691.03	5367.08
Salmonella negative	4792.65	5401.70	4663.23	5317.93
Average standard deviation	61.66	34.81	73.58	49.52
Positive response - negative response	27.80	48.56	50.70	13.63
E. coli O157:H7 positive	4779.60	5342.07	4637.40	5211.77
E. coli O157:H7 negative	4728.90	5328.43	4593.48	5202.77
Average standard deviation	78.85	83.79	56.63	59.18
Positive response - negative response	27.80	49.15	43.92	8.23

difference in biosensor response between positive and negative samples could be increased, the SPR biosensor could be used as a diagnostic instrument in pathogen detection in samples of this sort.

There are several possible reasons that the SPR biosensor response is not at this point as of yet. As shown in Chapter 3, the specificity of the SPR biosensor decreases as the amount of background bacteria increase. Early plating of selected samples obtained from the pork production plants revealed a large number of ubiquitous bacteria. These organisms could have caused the 'noisy' response of the SPR biosensor to the target bacteria. The pre-enrichment of the field samples in lactose broth, which is not selective for *Salmonella* or *E. coli,* could have allowed the growth of many types of bacteria (Jay, 2000). Additionally, cross-reactivity of the antibodies with non-target organisms could have led to the decrease in sensitivity of the biosensor. In a similar study of *E. coli* O157:H7, Grif *et al.* (1998) found that the number of ubiquitous organisms other than *E. coli* O157:H7 that adhered to the antibodies of an immunomagnetic biosensor had an affect on the results. Seo *et al.,* (1999) reported that anti-*Salmonella* spp. antibody interaction with non-target bacteria caused a decrease in sensitivity of a rapid response biosensor.

Another factor could have been the decrease in contact between the target organism and the antibody because of the physical presence of other bacteria 'crowding out' the target organism. As the sensing surface is immersed into the sample, the antibodies contact only those bacteria closest to them. At higher numbers of non-target organisms, the target bacteria are spread more

thinly through the interfacial layer at the sensor surface. This causes the antibody to come into contact with a lower number of bacteria, possibly fewer than the detection level of 10^7 CFU/ml.

CONCLUSION

Although statistically significant only in one field sample, the SPR biosensor response was higher for positive samples than for negative, showing the potential for the instrument in real-world applications. Sample preparation involved a one-step process, which took a third or less of the time required by other sensors. This short time required by the SPR biosensor for sample preparation is favorable for field adaptation, as the assays can be easily completed in one working day.

It is evident that modifications must be made to the biosensor. Potential modifications include (1) investigations into increasing the sensitivity and/or specificity of the biosensor, (2) assays to discover a more suitable media for use in growing the sample cultures prior to analysis, (3) investigating new methods of affixing the antibody to the SPR biosensor sensing surface, (4) obtaining antibodies more specific to the target antigens, and (5) investigating a more suitable method of sample preparation, among others.

Should these modifications be implemented, the SPR biosensor shows potential for detecting *Salmonella* spp., and *E. coli* O157:H7 in the pork industry, and shows versatility that may allow it to be used on other foodborne pathogens. With further study, the SPR biosensor shows promise to provide a

complementary detection system to standard lab-based systems currently used in food safety.

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RECOMMENDATIONS FOR FUTURE RESEARCH

The SPR biosensor (Spreeta™) used in this investigation is a new, experimental product that has not before been tested or used for microbial detection. As this was the first investigation along this application, there are many optimizations to be made before the biosensor is perfected. Following are recommendations for future investigations to be conducted on the SPR biosensor to overcome initial limitations.

First, preliminary experiments were conducted to establish operating procedures based upon procedures suggested by the vendor (Spreeta™) manual (Texas Instruments, 1999). Several modifications were made to these procedures, due to the effect of the growth media used for the target pathogens on the SPR biosensor response. Research needs to be done to explore the effect of different media and operating procedures on the biosensor response. Experiments to find a culture medium with a matrix that does not interfere with the biosensor response could increase both sensitivity and specificity of the SPR biosensor. Investigating new sampling methods that minimize the amount of non-target bacteria could also improve the SPR biosensor response.

Results of SPR biosensor assays on cultures composed of a mixture of *E. coli* O157:H7 and *S.* Typhimurium showed a decrease in specificity when compared to assays performed on pure cultures. Future studies need to be carried out using cultures composed of additional types and quantities of bacteria, to establish more precisely the specificity limit of the SPR biosensor.

Another recommended area for future research is in the antibody selection and attachment methods. Research needs to be conducted utilizing other brands and types of antibody, to discover whether the degree of specificity of the antibody to target-antigen is a limiting factor in the SPR biosensor. Also, a new method of affixing the antibody to the sensing surface might improve the biosensor responses. Currently, the antibody is affixed via avidin-biotin interaction. This method may not be optimal for use on the SPR biosensor. Future investigations with other methods of antibody attachment should be done to study the effect of the degree of antibody attachment to the sensing surface.

Another factor to be studied is the incorporation of a flow-cell arrangement with the SPR biosensor. Most other types of commercially available SPR biosensors incorporate a flow-cell arrangement. A constant flow of sample solution over the sensing surface could increase the sensitivity and specificity of the SPR biosensor, because more of the target organism would come into direct contact with the antibody-prepped SPR surface. For this reason, further investigation with this biosensor should be carried out to develop and perfect a flow-cell assembly.

Furthermore, the SPR biosensor is currently an open system, with the samples and preparation solutions open to the air. This can lead to contamination of the sample with any contaminants present in the air or surfaces of the laboratory setting. Creation of a closed system, such as a flow-cell assembly, that brings the sample in contact with the biosensor sensing surface without exposure to background contaminants such as dust, dirt, or other

contaminants could minimize noise in the SPR biosensor response due to contamination of the samples and preparation solutions.

Finally, it is recommended that research be done to investigate whether it would be possible to prepare the SPR biosensor a longer period of time before it is used for sampling. If the SPR biosensor could be prepared 'in bulk' or ahead of time, it would increase the possibility of using the biosensor in the field. Prepared biosensors could be stored until needed, and sampling could be done immediately (2 min), rather than waiting the time needed to initialize the SPR biosensor before sampling (30 min).

Results of this study are promising for converting an off-the-shelf SPR biosensor into a diagnostic tool for pathogen detection. With additional investigation and refinements to perfect the methods and experimental set-up of the SPR biosensor, it shows the potential to fulfill the food industry's need for an on-line, rapid, accurate, and portable pathogen detection system.

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RESEARCH PROCEDURES AND PROTOCOLS

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APPENDIX A.1: Creating stock cultures and determining concentrations

To create stock cultures of *E. coli* O157:H7 and *S.* Typhimurium

1. Vortex sample
2. Aseptically transfer isolate to 10 ml of Tryptic Soy Broth (TSB) (Difco Laboratories) in a sterile 14 ml glass vial
3. Incubate inoculated vial for 18-24 hours at 37°C.

Determination of culture concentrations

1. Vortex sample
2. Prepare 4 vials with 10 ml of sterile Buffered Peptone Water (BPW) (Becton Dickinson and Co., Sparks, MD)
3. Transfer 100 µl of stock culture into first vial (1:100 dilution)
4. Vortex vial and transfer 100 µl to second vial (1:10,000) dilution)
5. Vortex second vial and transfer 100 µl to third vial, and 1 ml to each of 2 plates (3M Petrifilm, St. Paul, MN) (1:1,000,000 dilution)
6. Vortex third vial and transfer 100 µl to fourth vial, and 1 ml to each of 2 plates
7. Vortex fourth vial and transfer 1 ml to each of 2 plates (1:10:000:000 dilution)
8. Incubate plates at 37°C for 18-24 hours
9. Count plates
10. Back calculate 10-fold dilutions for every step, to determine original culture concentrations.

APPENDIX A.2: Antibody Preparation

1. Preparation of Buffers
   1.1. *0.01 M Acetic Acid Solution*
      1.1.1. Mix 25 µl Glacial Acetic Acid (Spectrum Products, Inc, Gardena, CA) with 40 ml reagent quality water
   1.2. *0.177 M Carbonate-Bicarbonate Solution*
      1.2.1. 1.09 g Na_2CO_3 and 0.63 g $NaHCO_3$ dissolved in 100 ml reagent quality water.
2. Antibody Rehydration
   2.1. Add 100µl 0.01 M Acetic Acid to 1 mg of antibody
   2.2. Rotate vial until completely dissolved
   2.3. Add 100µl Carbonate-Bicarbonate Solution
   2.4. Rapidly mix until solution is clear or opalescent
   2.5. Immerse in 37°C waterbath for 30 minutes
   2.6. Allow to cool slowly to room temperature
3. Biotin Labeling
   3.1. Add 600µl of PBS (Phosphate Buffered Saline) (Pierce Chemical, Rockford, IL) to rehydrated antibody
   3.2. Dissolve 2mg EZ-Link NHS-LC-Biotin in 1ml N, N-Dimethylformamide ($HCON(CH_3)_2$ (DMF) (Aldrich)
      3.2.1. Add 75µl of this mixture to the antibody solution
   3.3. Place on ice for 2 hours
4. Antibody Dilution
   4.1. Add 9 ml PBS to Antibody solution
      4.1.1. Antibody concentration is now at approximately 300 mg/ml

APPENDIX A.3: SPR Biosensor Preparation

1. Initialize sensor
   1.1. Immerse gold surface in 10 ml of 0.1 N NaOH in 1% Triton X-100 Solution (Sigma, St. Louis, MO) for 3 minutes.
   1.2. Then immerse in 10 ml PBS for 3 minutes, until a steady PBS baseline is established
   1.3. Immerse in 10 ml 100 μl/ml neutravidin (Pierce Chemicals)in PBS
   1.4. Immerse in 10 ml of PBS
   1.5. Immerse in 10 ml of 300 μg/ml *Salmonella,* or *E. coli* O157:H7 antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 10 minutes, to bind the antibody to the avidinated surface via avidin-biotin interaction
   1.6. Rinse unbound antibody by immersing the sensor in 10 ml PBS
   1.7. Immersed biosensor in NaOH/Triton in PBS for two minutes
   1.8. Immerse in Bovine Serum Albumin (BSA) (Pierce Chemicals) for two minutes
   1.9. Rinse remaining BSA with NaOH/Triton in PBS for two minutes
2. Sampling
   2.1. Immerse sensor in the negative control for 3 min.
   2.2. Immerse in 10 ml of the sample for 3 min.
   2.3. Immerse in positive control (Pure culture) for 3 min.
3. Sensor cleaning
   3.1. Immerse sensor in PBS
   3.2. Immerse sensor in 10 ml 0.1 N NaOH-Triton X-100 Solution

APPENDIX A.4: Buffer Preparation

1. Dilution Blanks
   1.1. 0.1% Buffered Peptone Water (Difco Laboratories)
      1.1.1. Mix 1g of Buffered Peptone in 1 L purified water
      1.1.2. Distribute 9.0 ml or 9.9 ml per test tube
      1.1.3. Autoclave 15 min at 121°C

APPENDIX A.5: VIDAS™ Salmonella Enrichment Preparation

BAM/AOAC Procedure

1. Pre-Enrichment
   1.1. Add 1 ml of sample to 9 ml buffered peptone water
   1.2. Blend and incubate for 18 hours at 37C
2. Enrichment
   2.1. Transfer 1 ml suspension into 10 ml Tetrathionate broth
   2.2. Incubate 6-8 hours at 42C
   2.3. In parallel, transfer 1 ml suspension into 10 ml Selenite Cystine broth.
   2.4. Incubate 6-8 hours at 35-37C
3. Post-Enrichment
   3.1. Transfer 1 ml from Selenite Cystine broth into 10 ml M broth
   3.2. Transfer 1 ml from Tetrathionate broth into 10 ml M broth.
   3.3. Re-incubate both broths for 18 hours at 42C for use in later confirmation
   3.4. Incubate both M-broth samples for 18 hours at 42C
4. After Incubation
   4.1. Mix each M broth and transfer 1 ml from each into a tube.
   4.2. Cap tube tightly, heat for 15 min in a water-bath at 100C
   4.3. Perform VIDAS test
   4.4. Store remaining M broths at 2-8C for confirmation
5. Confirmation of Positive Results
   5.1. Streak positive Selenite cystine or Tetrathionate broths and remaining M-broth onto XLD plate (specific agar for Salmonella) following standard plating procedures
   5.2. Incubate plates at 37C for 18-24 hours
   5.3. Confirm suspect colonies

APPENDIX A.6: VIDAS™ *E. coli* O157:H7 Enrichment Preparation

Sample Preparation

1. Pre-Enrichment
   1.1. Dilute 1 ml sample into 9 ml m-TSB with novobiocin
   1.2. Incubate samples 6-7 hours at 41C
2. Enrichment
   2.1. Transfer 1 ml enriched culture into 9 ml MacConkey broth with cefixime and potassium tellurite (CT-Mac)
   2.2. Incubate 18 hours at 35-37C
3. After Incubation
   3.1. Mix each M broth and transfer 1 ml from each into a tube.
   3.2. Cap tube tightly, heat for 15 min in a water-bath at 100C
   3.3. Perform VIDAS test
   3.4. Store remaining M broths at 2-8C for confirmation
4. Confirmation of Positive Results
   4.1. Make successive 10-fold dilutions of positive samples in tryptone salt
   4.2. Spread 100 µl of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions on the surface of SMAC and CT-SMAC plates
   4.3. Incubate 18-24 hours at 37C

Media preparation

1. m-TSB with novobiocin
   1.1. Mix:
      1.1.1. 30 g dehydrated Trypcase Soy broth base,
      1.1.2. 1.5g Bile Salts #3,
      1.1.3. 1.25 g Anhydrous Na_2HPO_4,
      1.1.4. 10 g Casaminoacids,
   1.2. Autoclave at 121C for 15 min.
   1.3. Add 1 ml of 20 mg/ml novobiocin

2. CT-MAC – MacConkey broth with Cefixime and potassium Tellurite
   2.1. Mix:
      2.1.1. MacConkey broth, prepared and autoclaved at 121C for 15 min.
      2.1.2. 250 µl of 1% potassium tellurite solution / liter of medium
      2.1.3. 1 ml of 50 mg/l stock solution Cefixime / liter of medium

3. CT-SMAC - MacConkey Sorbitol Agar with Cefixime and potassium Tellurite
   3.1. MacConkey sorbitol agar, prepared according to manufacturers instruction
   3.2. Autoclave at 121C for 15min.
   3.3. Cool to 45-50C,
      3.3.1. Add:
         3.3.1.1. 250 µl of 1% potassium tellurite solution / liter of agar

3.3.1.2. 1 ml of 50 mg/l stock solution Cefixime / liter of agar

4. Cefixime stock solution at 50 mg/l
   4.1. Mix
      4.1.1. 5 mg of Cefixim
      4.1.2. 100 ml of 100 mmol/l sodium bicarbonate buffer, pH 8.0
   4.2. Sterilize, store at 2-8C

APPENDIX B

RESULTS FOR CHAPTER 3

SENSITIVITY AND SPECIFICITY ASSAYS FOR *SALMONELLA* SPP. AND *ESCHERICHIA COLI* 0157:H7 UTILIZING A SURFACE PLASMON RESONANCE BIOSENSOR

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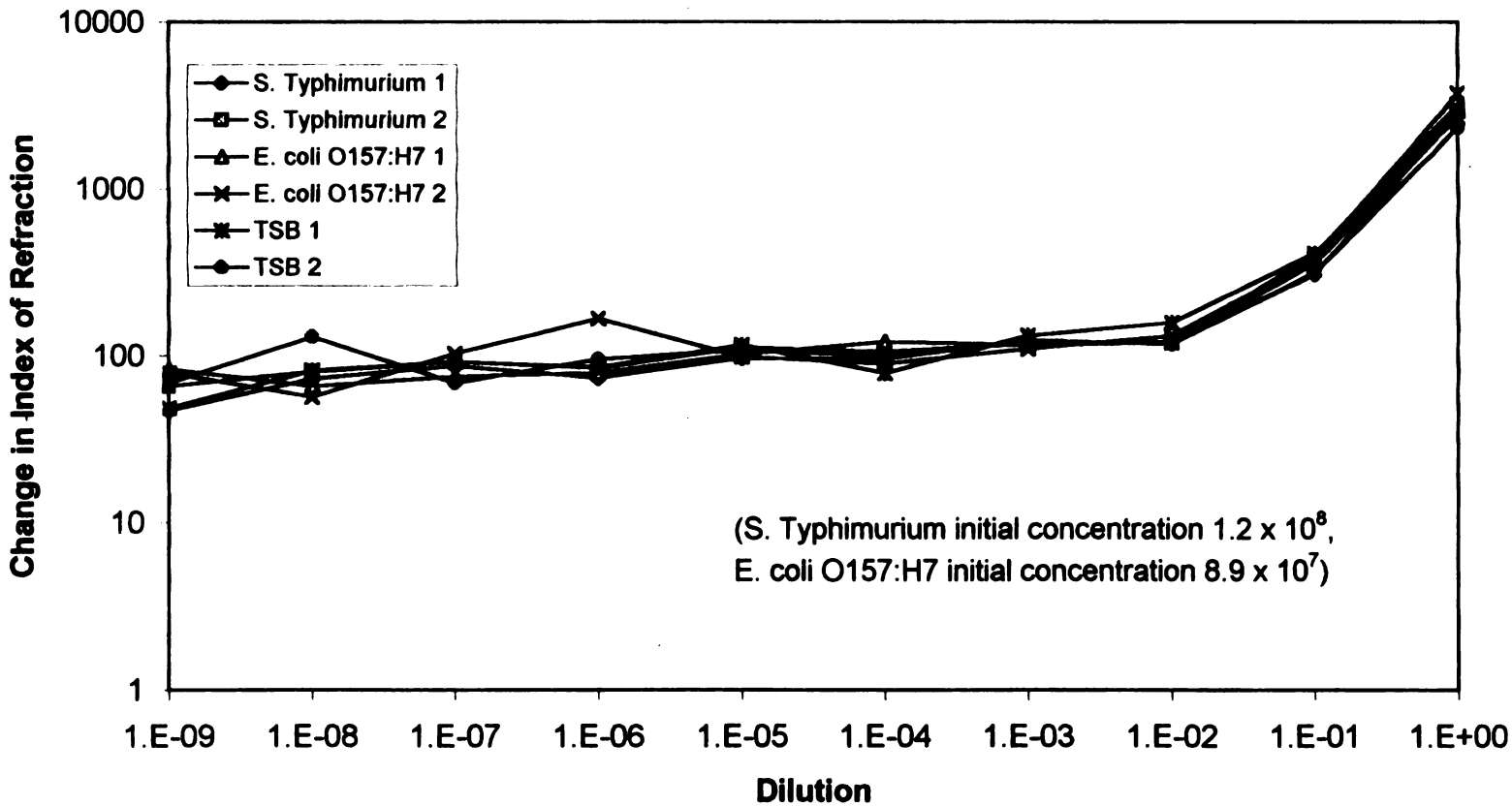

Figure B.1. Background responses of the SPR bioensor to serial dilutions of *S*. Typhimurium, *E. coli* O157:H7, and sterile TSB without antibody. Log-Log scale

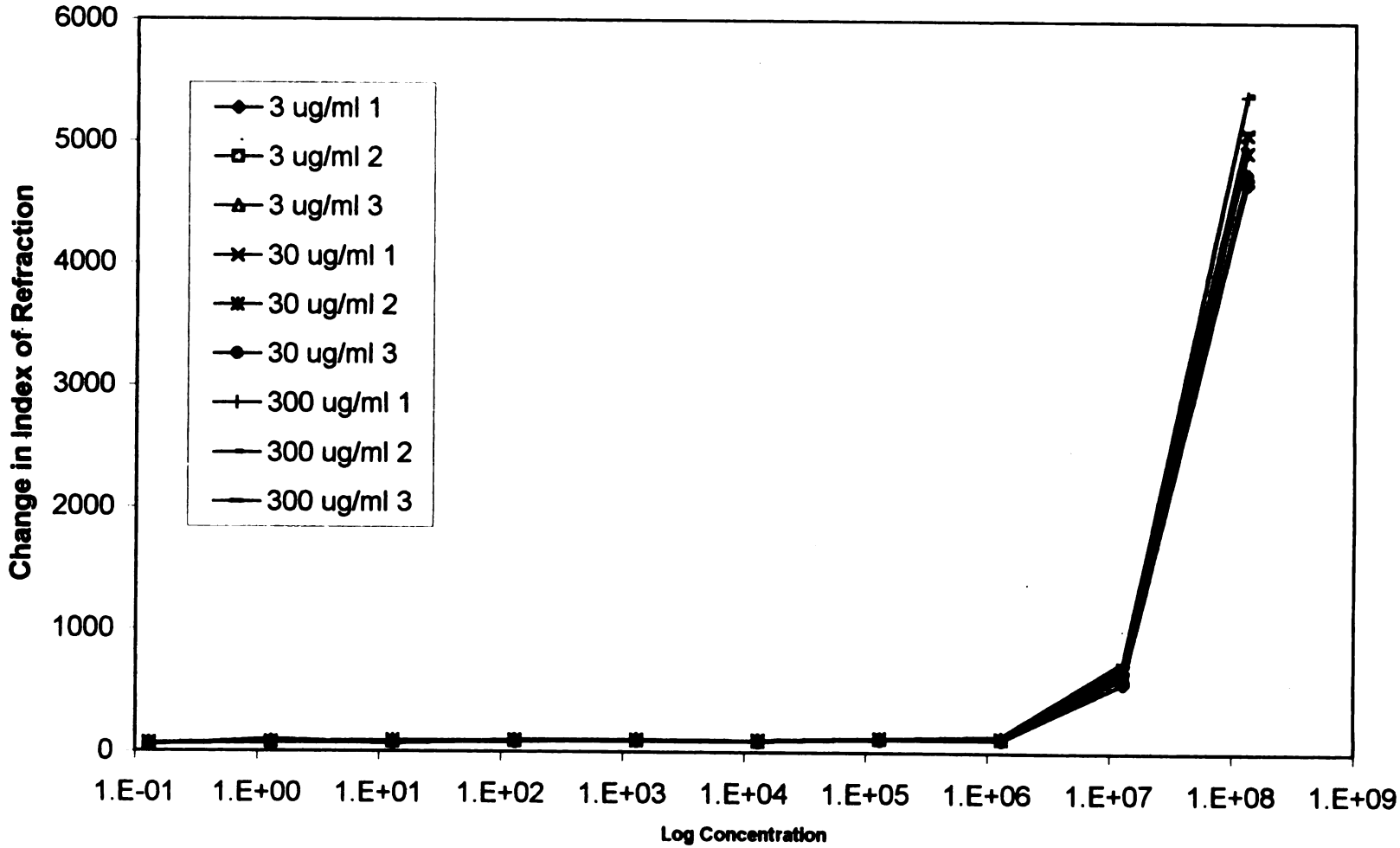

Figure B.2. Anti-*Salmonella* antibody comparisons

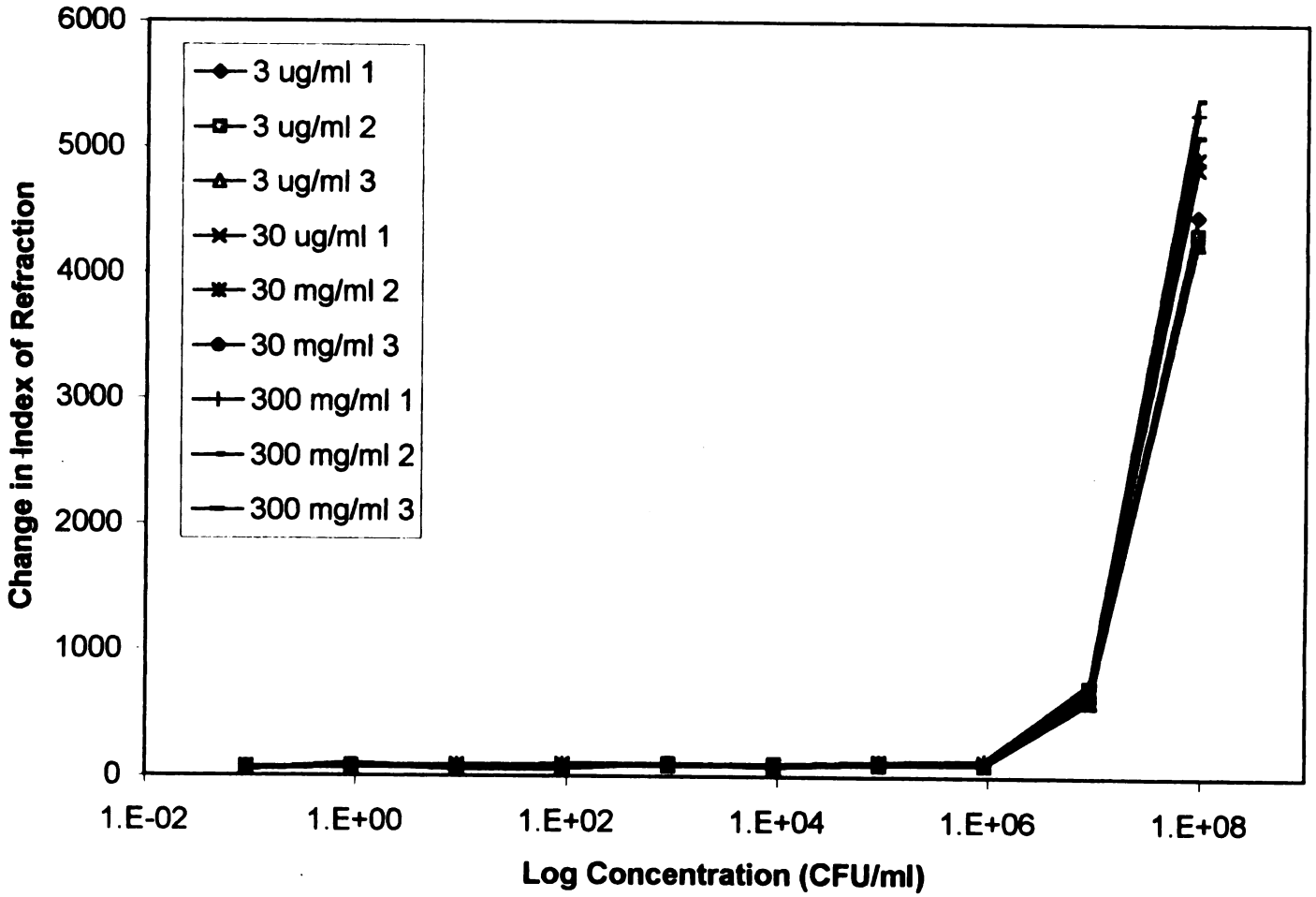

Figure B.3 Anti-E. coli O157:H7 antibody comparisons

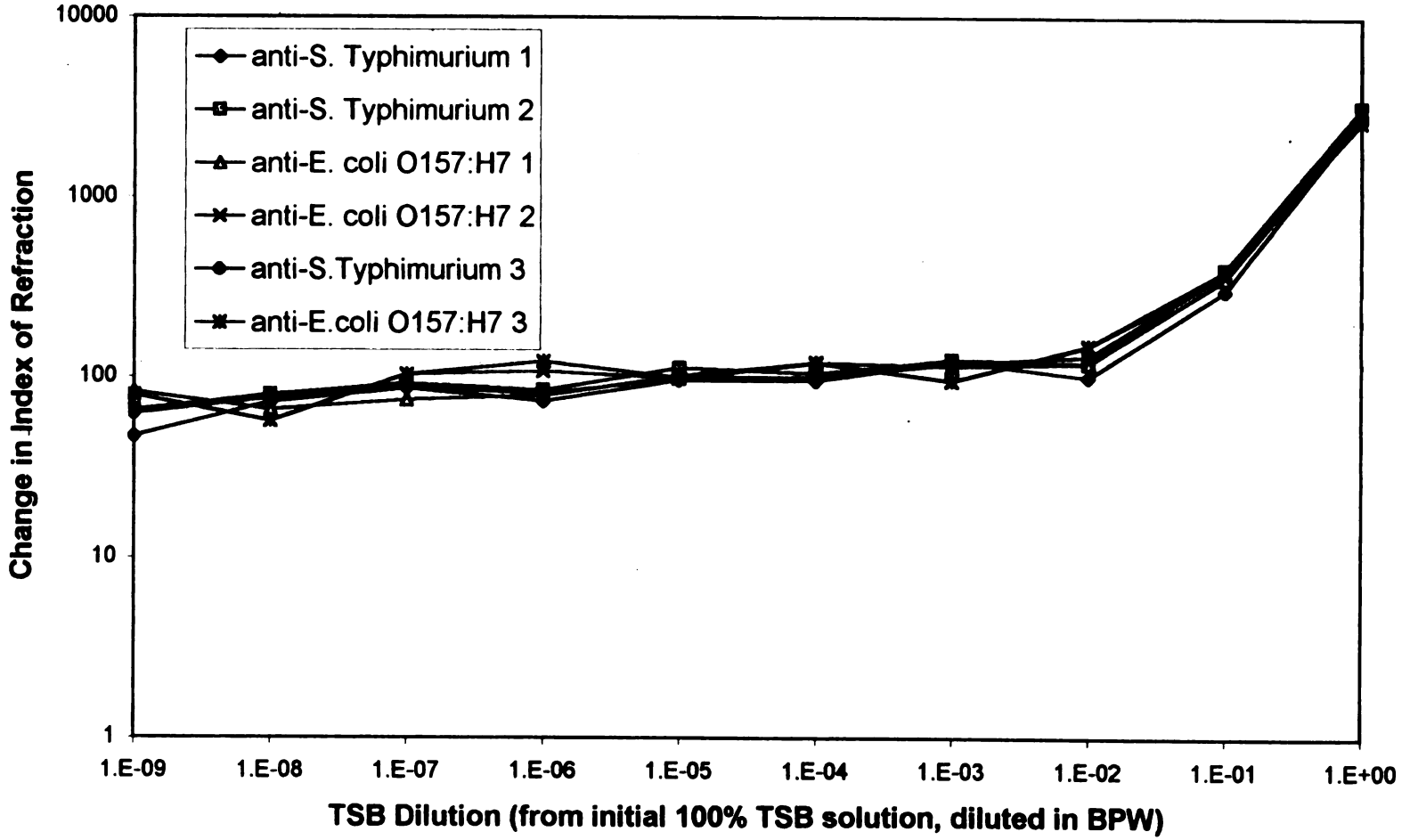

Figure B.4 Negative control of SPR biosensor - response to serial dilutions of sterile TSB using anti-*Salmonella* and anti-*E. coli* O157:H7 antibodies. Log-Log scale

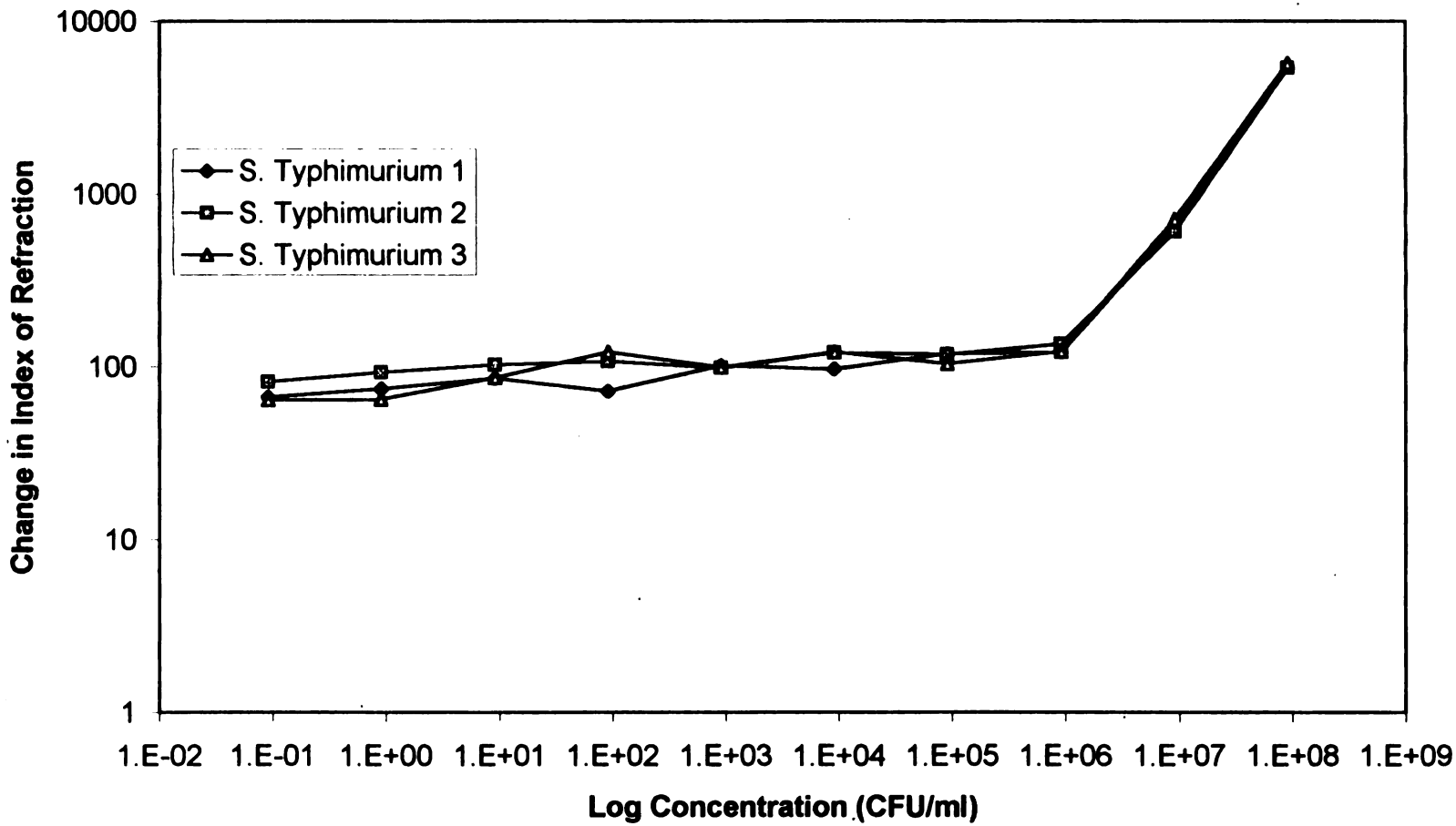

Figure B.5 SPR biosensor response for *S*. Typhimurium. Log scale

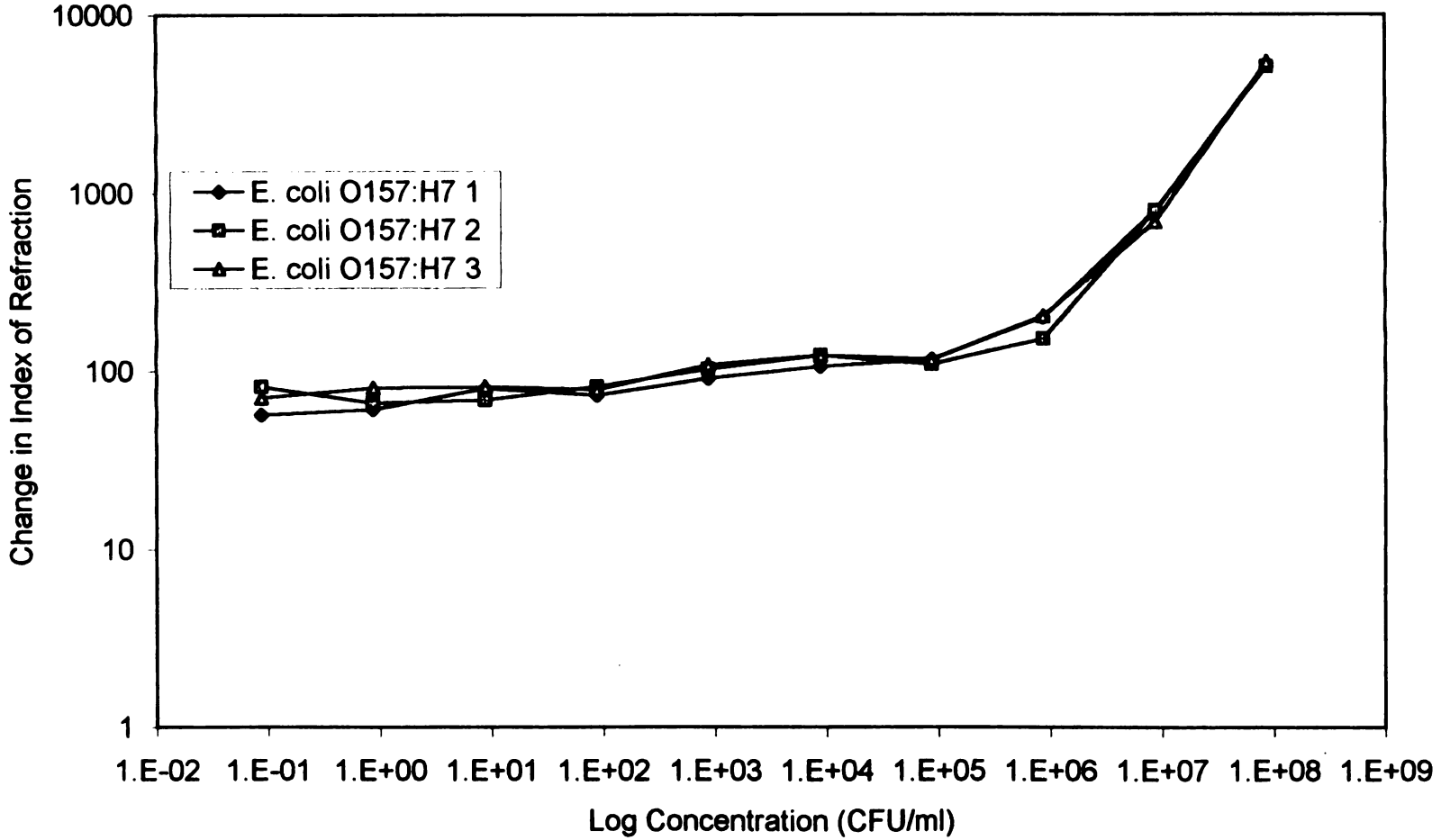

Figure B.6. SPR biosensor response for E. coli O157:H7. Log-log scale

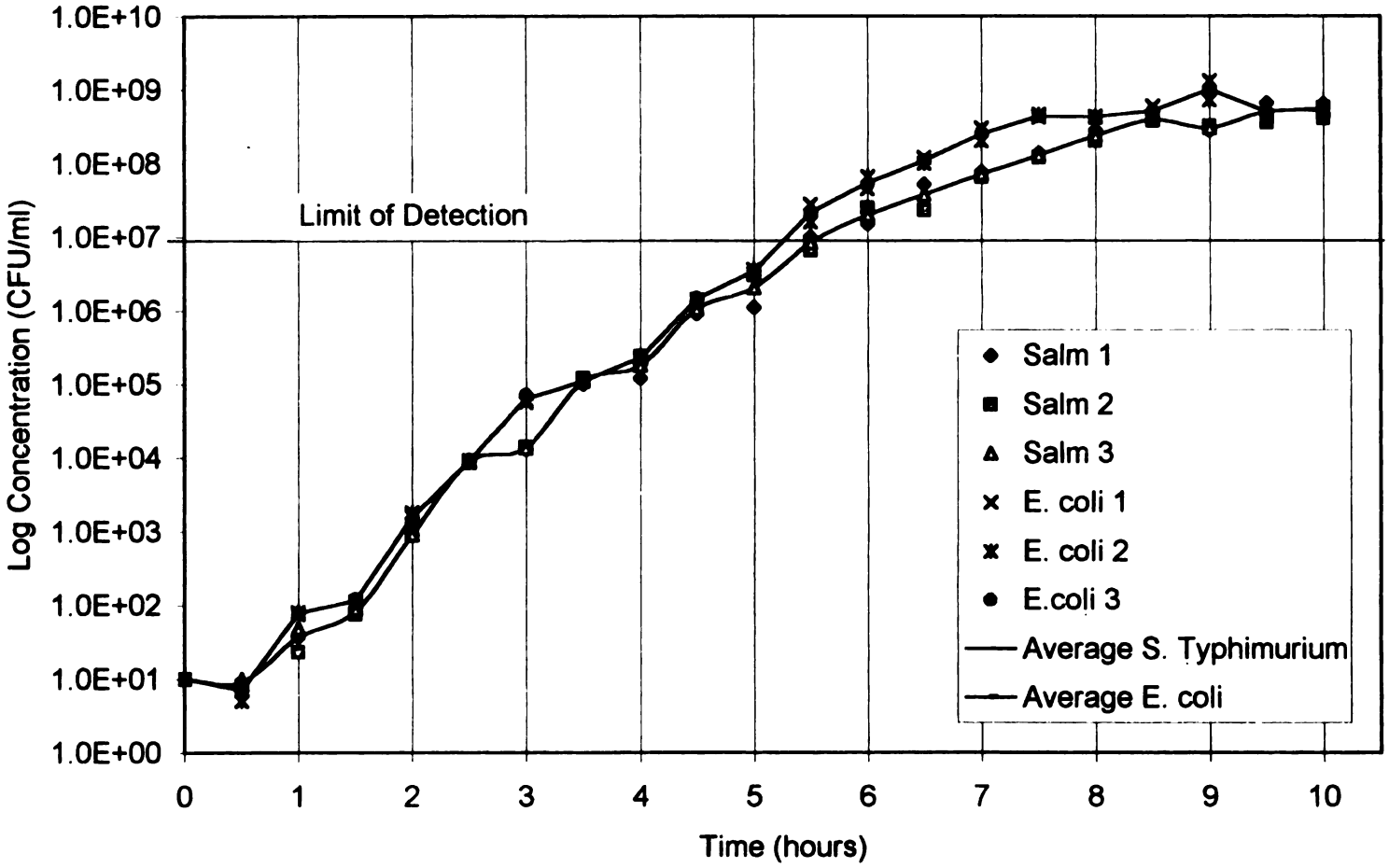

Figure B.7. Timed growth of *S*. Typhimurium and *E. coli* O157:H7

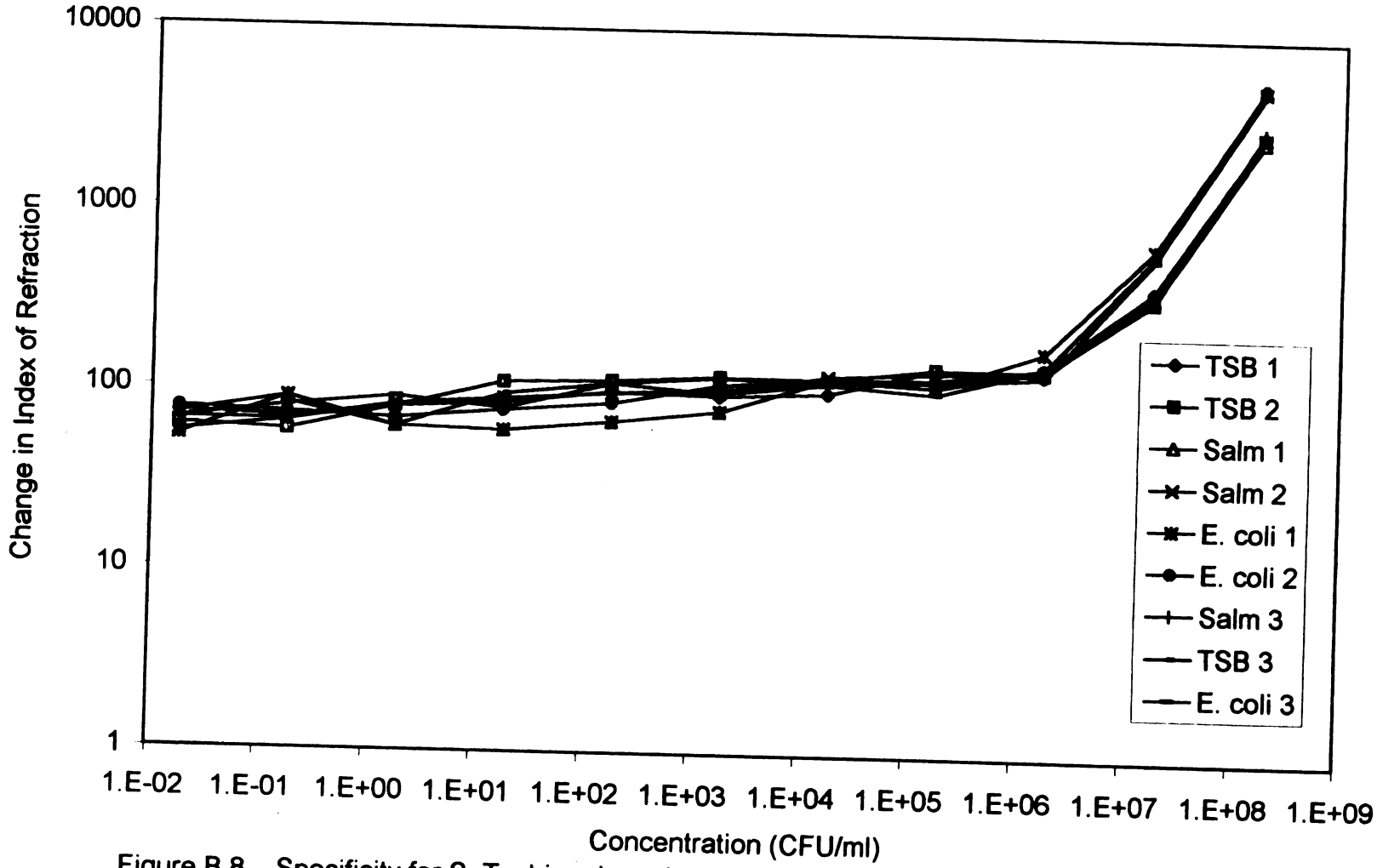

Figure B.8. Specificity for S. Typhimurium. Log-log scale

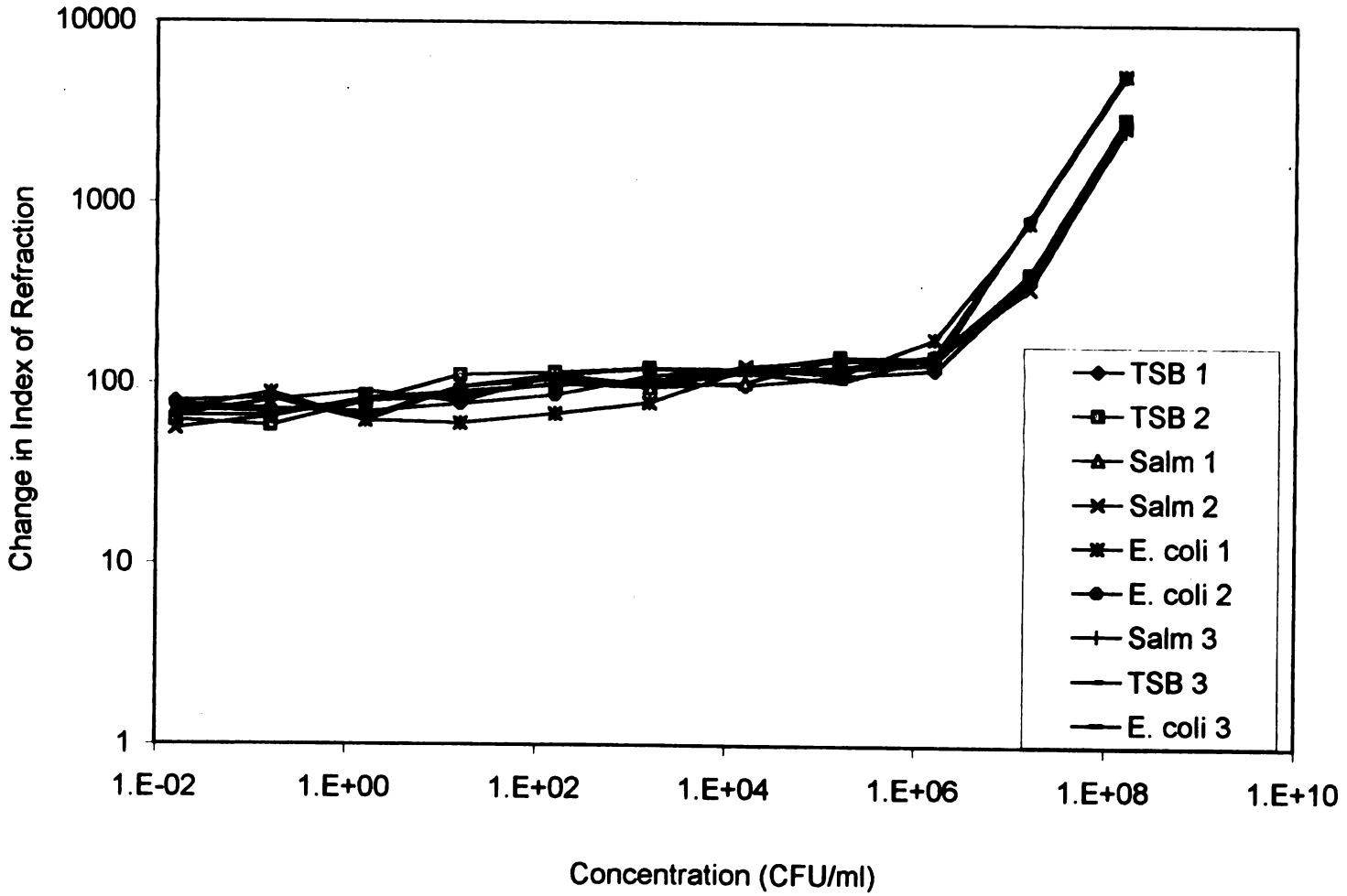

Figure B.9. Specificity for E. coli O157:H7. Log-log scale

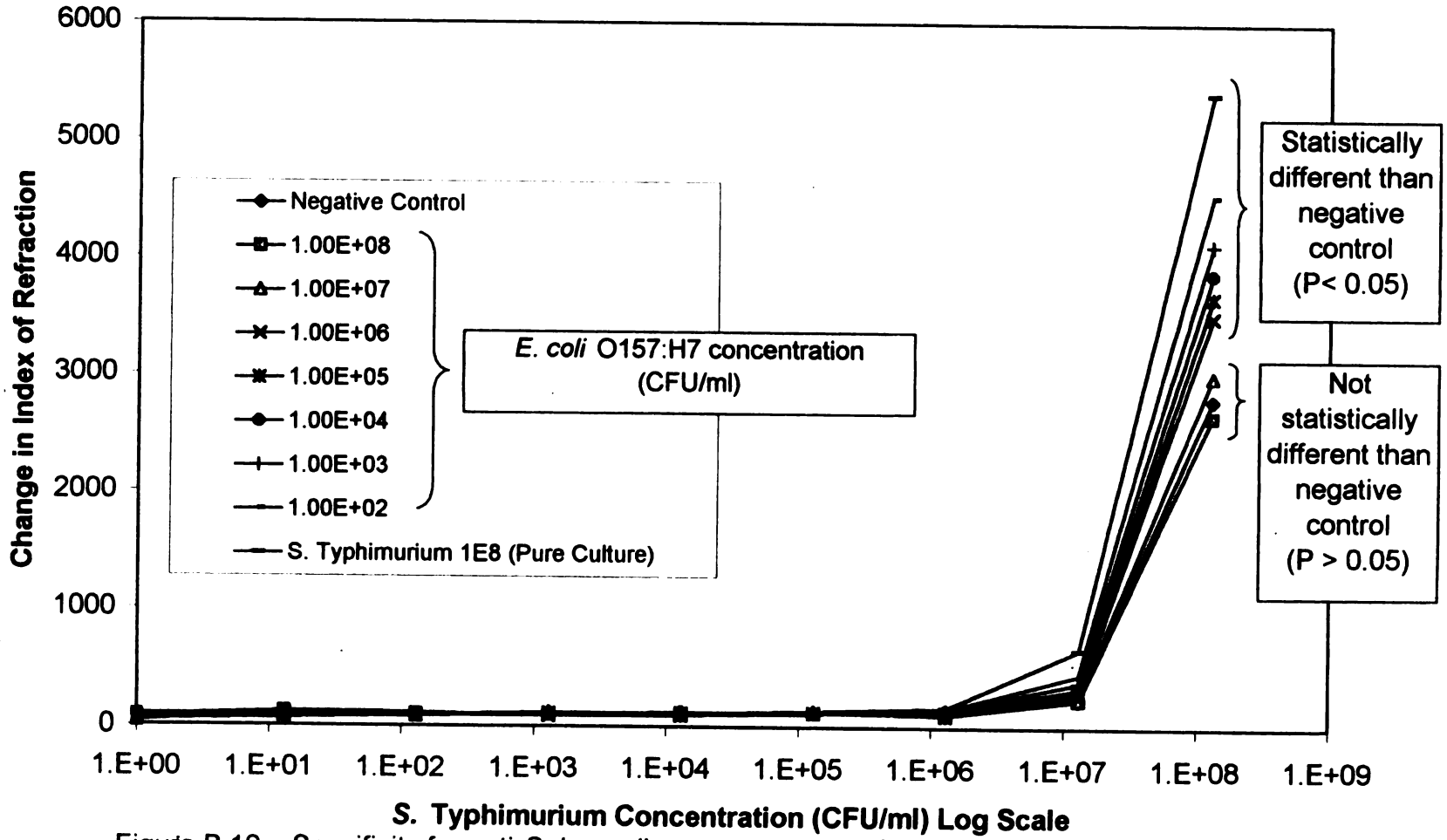

Figure B.10 . Specificity for anti-*Salmonella* spp. - prepared SPR biosensor in mixed culture.

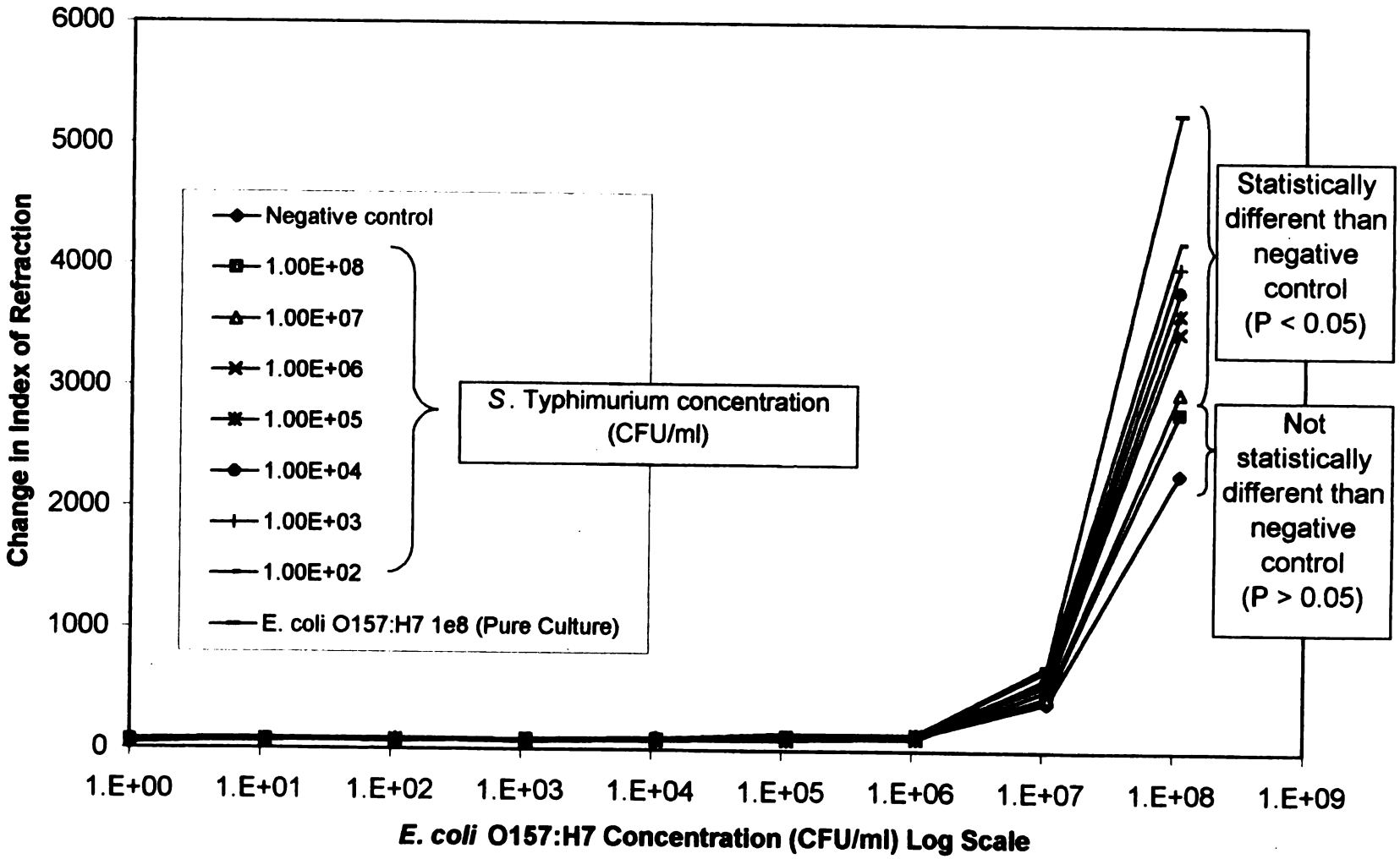

Figure B.11. Specificity for anti-*E. coli* O157:H7 - prepared SPR biosensor in mixed culture.

APPENDIX C

RESULTS FOR CHAPTER 4

DETECTION OF *SALMONELLA* SPP. AND *ESCHERICHIA COLI* 0157:H7 IN THE PORK PRODUCTION CHAIN USING A SURFACE PLASMON RESONANCE BIOSENSOR AND A VIDAS IMMUNOSENSOR

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Table C.1. SPR biosensor results for *Salmonella* spp. assays in samples enriched according to the VIDAS™ protocol

Sample No.	Description	Average Change in Index of Refraction for Sample Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction for Sample Repetition 2 ($\times 10^{-6}$)	Standard Deviation	Average Change in Index of Refraction of Antibody *b* Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction of Antibody *b* Repetition 2 ($\times 10^{-6}$)	Standard Deviation
1	Hogs Back	4720.66	4625.64	67.19	5360.41	5376.70	11.52
2	Fecal Matter	4796.48	4669.64	89.69	5523.88	5552.92	20.53
3	Prechill 1	4878.99	4754.86	87.77	5387.91	5401.52	9.62
4	Prechill 2	4631.85	4575.69	39.71	5325.54	5350.16	17.41
5	Prechill 3	4745.42	4801.39	39.57	5420.42	5512.36	65.01
6	Drain at Evisceration	4908.90	4772.65	96.34	5436.31	5478.19	29.61
7	Carcass Prechill after final wash	4772.92	4655.69	82.89	5414.66	5478.58	45.20
8	Hair from Dehairing machine	4710.55	4572.65	97.52	5390.29	5398.70	5.95
9	Hogs Back	4805.44	4694.64	78.34	5328.77	5344.52	11.14
10	Dehairing Machine	5105.65	4888.96	153.22	5337.37	5403.63	46.85
11	Dehairing Machine	4799.67	4745.11	38.58	5482.82	5528.01	31.96
12	Evisceration 1	4775.30	4691.31	59.39	5407.16	5460.66	37.83
13	Evisceration 2	4713.78	4614.32	70.33	5360.36	5393.45	23.40
14	Prechill 4	4822.69	4791.28	22.21	5412.37	5355.96	39.89
15	Prechill 5	4867.83	4744.32	87.34	5517.98	5491.39	18.80
16	Alley	4792.17	4648.90	101.31	5505.06	5564.91	42.32
17	Chillroom Wall	4745.37	4678.61	47.20	5378.39	5412.35	24.01
18	Hairs from Dehairing machine	4730.35	4672.31	41.04	5351.04	5396.03	31.81
19	Drain	4748.09	4646.01	72.18	5431.07	5459.91	20.39
20	Water Nozzle	4890.07	4775.15	81.26	5418.90	5482.63	45.07
21	Hogs Back	4763.41	4696.65	47.20	5482.82	5585.66	72.72
22	Feed	4736.05	4678.02	41.04	5407.16	5460.66	37.83
23	Fecal Matter	4816.08	4714.00	72.18	5360.36	5393.45	23.40
24	Alley	4803.91	4688.99	81.26	5345.34	5355.96	7.51

Table C.2. SPR biosensor results for *E. coli* O157:H7 Assays in samples prepared according to the VIDAS™ protocol

Sample No.	Description	Average Change in Index of Refraction for Sample Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction for Sample Repetition 2 ($\times 10^{-6}$)	Standard Deviation	Average Change in Index of Refraction of Antibody *b* Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction of Antibody *b* Repetition 2 ($\times 10^{-6}$)	Standard Deviation
1	Hogs Back	4752.91	4701.35	36.45	5279.73	5255.80	16.92
2	Fecal Matter	4728.89	4648.33	56.96	5289.44	5249.59	28.18
3	Prechill 1	4668.26	4789.39	85.66	5274.67	5377.41	72.65
4	Prechill 2	4667.39	4961.36	207.87	5401.94	5483.38	57.59
5	Prechill 3	4751.57	4618.98	93.75	5324.58	5381.08	39.95
6	Drain at Evisceration	4657.53	4631.69	18.27	5267.24	5255.22	8.50
7	Carcass Prechill after final wash	4699.38	4635.81	44.95	5385.66	5290.68	67.16
8	Hair from Dehairing machine	4684.58	4629.60	38.87	5273.50	5266.03	5.28
9	Hogs Back	4654.68	5017.22	256.35	5550.24	5178.60	262.79
10	Dehairing Machine	4781.95	4698.78	58.81	5231.41	5221.96	6.68
11	Dehairing Machine	4704.59	4897.31	136.27	5305.95	5339.85	23.97
12	Evisceration 1	4692.07	4635.23	40.19	5129.37	5282.94	108.59
13	Evisceration 2	4750.17	4670.69	56.20	5286.77	5310.99	17.13
14	Prechill 4	4776.00	4646.04	91.89	5404.48	5527.28	86.84
15	Prechill 5	4675.04	4583.61	64.65	5247.85	5238.22	6.81
16	Alley	4749.76	4626.97	86.82	5275.31	5156.37	84.11
17	Chillroom Wall	4831.07	4710.96	84.93	5276.60	5291.72	10.69
18	Hairs from Dehairing machine	4587.51	4534.38	37.56	5704.17	5996.56	206.75
19	Drain	4691.78	5094.05	284.44	5249.21	5208.60	28.71
20	Water Nozzle	4779.49	5079.55	212.17	5477.83	5353.89	87.64
21	Hogs Back	4733.34	4613.23	84.93	5434.66	5346.56	62.30
22	Feed	4584.50	4531.38	37.56	5252.52	5290.30	26.72
23	Fecal Matter	4710.27	4651.61	41.48	5230.80	5302.52	50.71
24	Alley	5079.19	4987.65	64.73	5224.59	5268.56	31.09

Table C.3. SPR biosensor results for *E. coli* O157:H7 assays of samples inoculated with *E. coli* O157:H7, prepared according to the VIDAS™ enrichment methods

Sample I.D.	Description	in Index of Refraction for Sample Repetition 1 ($\times 10^{-6}$)	in Index of Refraction for Sample Repetition 2 ($\times 10^{-6}$)	Standard Deviation	in Index of Refraction of Antibody *b* Repetition 1 ($\times 10^{-6}$)	in Index of Refraction of Antibody *b* Repetition 2 ($\times 10^{-6}$)	Standard Deviation
4e	Prechill 2	4843.60	4703.64	98.96	5294.78	5313.94	13.55
9e	Hogs Back	4781.16	4717.49	45.02	5348.40	5311.68	25.97
14e	Prechill 4	4756.97	4824.65	47.86	5384.33	5353.62	21.72
19e	Drain	4781.36	4828.57	33.38	5408.33	5321.62	61.32
24e	Alley	4808.69	4749.87	41.59	5370.00	5313.97	39.62

Table C.4. SPR biosensor results for *Salmonella* spp. assays in samples enriched in TSB

Sample No.	Description	Average Change in Index of Refraction for Sample - Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction for Sample Repetition2 ($\times 10^{-6}$)	Standard Deviation	Average Change in Index of Refraction of Antibody *b* Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction of Antibody *b* Repetition2 ($\times 10^{-6}$)	Standard Deviation
1	Hogs Back	4517.64	4681.06	115.55	5190.64	5357.15	117.74
2	Fecal Matter	4734.48	4584.04	106.37	5279.63	5448.67	119.52
3	Prechill 1	4816.99	4669.26	104.46	5316.49	5162.68	108.76
4	Prechill 2	4569.85	4490.09	56.40	5303.56	5282.97	14.56
5	Prechill 3	4575.65	4705.82	92.04	5355.17	5378.98	16.84
6	Drain at Evisceration	4664.65	4605.71	41.67	5716.75	5395.95	226.84
7	Carcass Prechill after final wash	4547.69	4528.72	13.42	5252.10	5422.34	120.38
8	Hair from Dehairing machine	4667.98	4739.37	50.48	5220.70	5351.08	92.19
9	Hogs Back	4586.64	4658.72	50.96	5143.71	5315.35	121.37
10	Dehairing Machine	4780.96	4563.30	153.91	5354.36	5353.85	0.36
11	Dehairing Machine	4637.11	4593.01	31.18	5273.70	5439.42	117.18
12	Evisceration I	4846.90	4586.71	183.98	5372.36	5178.29	137.23
13	Evisceration 2	4710.92	4674.18	25.98	5355.61	5208.00	104.38
14	Prechill 4	4782.36	4724.69	40.78	5332.75	5201.14	93.07
15	Prechill 5	4678.36	4772.76	66.75	5320.60	5378.97	41.28
16	Alley	5043.65	4752.57	205.82	5488.06	5282.14	145.61
17	Chillroom Wall	4567.65	4705.77	97.66	5488.63	5226.04	185.68
18	Hairs from Dehairing machine	4668.35	4590.96	54.72	5207.41	5381.45	123.07
19	Drain	4538.01	4708.49	120.55	5220.99	5386.04	116.71
20	Water Nozzle	4667.15	4850.47	129.63	5397.78	5195.98	142.69
21	Hogs Back	4701.41	4611.05	63.89	5460.70	5273.70	132.23
22	Feed	4674.05	4592.42	57.72	5178.29	5372.36	137.23
23	Fecal Matter	4606.00	4776.48	120.55	5208.00	5355.61	104.38
24	Alley	4580.99	4764.31	129.63	5332.75	5201.70	92.67

Table C.5. SPR biosensor results for *E. coli* O157:H7 assays in samples enriched in TSB

Sample No.	Description	Average Change in Index of Refraction for Sample Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction for Sample Repetition 2 ($\times 10^{-6}$)	Standard Deviation	Average Change in Index of Refraction of Antibody *b* Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction of Antibody *b* Repetition 2 ($\times 10^{-6}$)	Standard Deviation
1	Hogs Back	4570.09	4633.14	44.59	5228.52	5146.61	57.92
2	Fecal Matter	4539.14	4616.70	54.84	5238.24	5140.41	69.18
3	Prechill 1	4463.27	4556.07	65.62	5092.41	5201.46	77.11
4	Prechill 2	4670.87	4825.26	109.17	5219.68	5359.10	98.58
5	Prechill 3	4591.38	4723.50	93.42	5164.39	5263.26	69.91
6	Drain at Evisceration	4497.34	4617.72	85.12	5231.64	5146.03	60.54
7	Carcass Prechill after final wash	4587.20	4526.63	42.83	5264.13	5159.42	74.04
8	Hair from Dehairing machine	4572.40	4520.42	36.75	5200.22	5134.77	46.28
9	Hogs Back	4567.80	4472.42	67.45	5047.34	5310.03	185.76
10	Dehairing Machine	4707.73	4599.69	76.40	5112.77	5195.81	58.72
11	Dehairing Machine	4657.50	4544.41	79.97	5196.77	5263.98	47.53
12	Evisceration I	4579.88	4526.04	38.07	5195.68	5020.19	124.09
13	Evisceration 2	4539.43	4631.52	65.11	5104.51	5171.10	47.09
14	Prechill 4	4514.78	4626.12	78.73	5222.21	5351.33	91.30
15	Prechill 5	4540.78	4452.35	62.53	5106.95	5170.91	45.22
16	Alley	4621.96	4517.79	73.66	5326.69	5225.50	71.55
17	Chillroom Wall	4734.50	4601.78	93.85	5203.81	5145.34	41.35
18	Hairs from Dehairing machine	4425.20	4597.16	121.60	5520.29	5336.85	129.71
19	Drain	4509.52	4565.18	39.36	5175.93	5077.34	69.71
20	Water Nozzle	4597.23	4726.29	91.26	5382.69	5209.82	122.24
21	Hogs Back	4614.69	4481.96	93.85	5296.39	5186.37	77.80
22	Feed	4772.48	4600.51	121.60	5224.16	5092.33	93.21
23	Fecal Matter	4576.00	4520.35	39.36	5121.61	5251.32	91.71
24	Alley	4711.86	4840.92	91.26	5115.41	5181.09	46.45

Table C.6. SPR biosensor results for *E. coli* O157:H7 assays of samples inoculated with *E. coli* O157:H7, prepared in sterile TSB

Sample I.D.	Description	Average Change in Index of Refraction for Sample Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction for Sample Repetition 2 ($\times 10^{-6}$)	Standard Deviation	Average Change in Index of Refraction of Antibody *b* Repetition 1 ($\times 10^{-6}$)	Average Change in Index of Refraction of Antibody *b* Repetition 1 ($\times 10^{-6}$)	Standard Deviation
4e	Prechill 2	4553.64	4600.26	32.96	5242.34	5222.81	13.80
9e	Hogs Back	4597.16	4567.49	20.98	5217.68	5132.91	59.94
14e	Prechill 4	4700.85	4640.36	42.77	5197.38	5191.95	3.84
19e	Drain	4658.29	4735.85	54.84	5164.71	5278.27	80.30
24e	Alley	4624.16	4695.92	50.74	5208.64	5253.29	31.57

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