

**A REVIEW OF METHODS FOR DETECTION OF  
STAPHYLOCOCCAL ENTEROTOXINS AND EVALUATION  
OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY  
APPLIED TO FOODS**

JAMES L. SMITH and MARIANNE M. BENCIVENGO

**ABSTRACT**

*Immunological techniques for the detection of staphylococcal enterotoxins, including gel diffusion, electroimmunodiffusion, passive hemagglutination, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA) are reviewed in regard to their applicability to the routine analysis of foods. An ELISA employing minimal clean-up of food samples was evaluated and was capable of detecting 5 ng of staphylococcal enterotoxin A/g or ml which had been added to food.*

**INTRODUCTION AND REVIEW**

In spite of intensive scientific research by microbiologists and extensive educational efforts directed toward both food processors and consumers, *Staphylococcus aureus* food poisoning remains a major cause of foodborne illness in both the United States and Canada (Smith *et al.* 1983; Todd 1983). Currently, routine evaluation of foods for this pathogen is limited to detection of viable cells. However, because of the extreme heat stability of staphylococcal enterotoxins, it is generally recognized that it would be more appropriate to directly analyze for these toxic components. In the past, this has not been feasible due to the complexity and/or insensitivity of available techniques. Recent advances have largely eliminated these difficulties so that it is now possible for a moderately equipped laboratory to analyze for staphylococcal enterotoxins on a routine basis. The objectives of this publication are, first; to review available techniques for detection and quantitation of staphylococcal enterotoxins with particular

emphasis on applicability for routine evaluation of foods and food products and second; to assess the feasibility of employing an enzyme-linked immunosorbent assay (ELISA) without extensive clean-up procedures to directly analyze foods for staphylococcal enterotoxin A.

The ideal staphylococcal enterotoxin assay would use a test animal. Very few animals react to the enterotoxins in a manner similar to humans. Many animals are refractory to the enterotoxins and others lack the appropriate sensitivity. Tissue culture and microbiological assay techniques are not effective tools for staphylococcal enterotoxin measurement (Bergdoll 1972; Minor and Marth 1976). Buxser and Bonventre (1981) show that staphylococcal enterotoxin A (SEA) and enterotoxin B (SEB) lack cytotoxic activity toward a human intestinal epithelial cell line and could not be assayed in this model system. Utilizing sensitized guinea pigs, Scheuber *et al.* (1983), described a quantitative skin test for SEB. The skin reaction detects as little as 0.1 ng/g SEB in foods and the reaction could be read within 20 min.  $\alpha$ -Toxin and other enterotoxins produced by *S. aureus* did not elicit a skin response in animals sensitized with SEB. The guinea pig skin test appears to have promise as a fast and sensitive technique for quantitation of staphylococcal enterotoxins.

Administration of staphylococcal enterotoxins parenterally to kittens induces vomiting, but the samples must be treated to prevent false positive reactions since *S. aureus* produces other compounds that induce emesis in kittens (Bergdoll 1972; Minor and Marth 1976). The usefulness of kittens, as detection and quantitative tools for staphylococcal enterotoxins, is quite limited. Monkeys are ideal for use in enterotoxin assays. Suspected staphylococcal enterotoxin-containing samples are administered via stomach tube to Rhesus or Cynomolgous monkeys and the animals observed for vomiting (Bergdoll 1972, 1979; Minor and Marth 1976). The emetic dose ( $ED_{50}$ ) for SEA is approximately 5  $\mu$ g/monkey. Unlike kittens, other toxic compounds produced by *S. aureus* do not induce nonspecific vomiting. Monkeys are difficult to obtain and expensive to maintain. Taylor *et al.* (1982) suggest the use of weanling pigs to replace monkeys in enterotoxin assays. Oral administration of SEA to weanling pigs induces an emetic response and neurobehavioral abnormalities which correlate well with the dose level. Higher concentrations of SEA are required to produce a response in pigs than in monkeys. Weanling pigs are less expensive to maintain and more readily available than monkeys and may prove useful when it is necessary to test enterotoxins in animals.

Most foods implicated in staphylococcal food poisoning outbreaks contain low levels of enterotoxin, often less than 1  $\mu$ g/100 g food (Reiser *et al.* 1974). Animals, which require large doses to respond to the presence of

enterotoxin, are not useful in determining the small amounts of toxin found in foods. Therefore, most laboratories will find it more expedient to determine the presence of staphylococcal enterotoxin by serological methods.

### **Production of Antibodies Against Staphylococcal Enterotoxins**

Appropriate culture media for the production of large quantities of enterotoxins by *S. aureus* and purification of enterotoxins for use as antigens have been reviewed by Bergdoll (1972, 1979) and Minor and Marth (1976). Unfortunately, purified enterotoxins are not available commercially.

Antibodies against staphylococcal enterotoxins are usually produced in rabbits but goats have also been used. The enterotoxin used as an antigen should be of high purity to minimize undesirable cross-reactions with other enterotoxins (and antigens) that may be present in *S. aureus* contaminated food samples. Procedures for producing antibodies to various staphylococcal enterotoxins have been described (Casman and Bennett 1964; Bergdoll *et al.* 1965; Casman *et al.* 1967; Langner and Sinell 1975; Bradstreet *et al.* 1977; Notermans 1982; Robbins and Bergdoll 1984).

### **Extraction of Staphylococcal Enterotoxins From Foods**

Little, if any, clean-up is necessary to prepare culture media samples for serological determination of enterotoxin. When a suspect food is examined, an extensive clean-up is necessary to extract the enterotoxin prior to serological assay. Proteins present in the food may obscure positive precipitin lines or give nonspecific reactions. Concentration of the toxin, which is often present at a level of  $<1 \mu\text{g}/100 \text{ g}$  food, is essential to provide adequate material for assay. Procedures for the extraction of enterotoxins from foods have been published by Gilbert and Wieneke (1973) and Reiser *et al.* (1974). The procedure for extraction of toxin consists of the following steps: Separation of the enterotoxin from the insoluble food constituents by centrifugation and solvent extraction followed by binding of the enterotoxin to an ion-exchange resin to remove soluble food constituents. The enterotoxin is then eluted from the resin, concentrated to a small volume, and subjected to serology. A modification of the above procedure in which the use of ion-exchange resins was omitted has been used to extract staphylococcal enterotoxin from a variety of foods (Sinell and Mentz 1978).

A procedure for extracting enterotoxin from foods depending on affinity

chromatography in which immobilized specific antisera is used to remove the enterotoxin from a food slurry was described by Genigeorgis and Kuo 1976. The toxin is then eluted, concentrated, and assayed by serology.

With some of the more recent immunological techniques such as radioimmunoassay and enzyme-linked immunosorbent assay, clean-up of the food samples is minimal.

### Serological Assays for Staphylococcal Enterotoxins

*Single- or Double-Gel Diffusion Tube Assays (Table 1).* When a soluble antigen reacts with specific antibody, a visible precipitate may result. Such a reaction is called a precipitin reaction and is the basis for a large variety of immunoassays which involve agar gel precipitin tests.

Melted agar containing antiserum is poured into test tubes, allowed to harden, and then the agar layer is overlaid with enterotoxin-containing solution. The enterotoxin diffuses downward into the antiserum-agar

Table 1. Use of single- and double-gel diffusion tube assays in determination of staphylococcal enterotoxins

Procedure	Enterotoxin/System	Lower Detection Limit	Reference
Single-gel diffusion tube	SEA/culture medium	2000 ng/ml	Weirether <i>et al.</i> 1966
	SEA/culture medium	400 ng/ml	Donnelly <i>et al.</i> 1967
	SEB/culture medium	600 ng/ml	
	SEA/milk	330 ng/ml	Read <i>et al.</i> 1965b
	SEB/milk	1000 ng/ml	
Micro single-gel diffusion capillary tube	SEA, SEB, SEC/culture medium	1000-2000 ng/ml	Fung and Wagner 1971
Double-gel diffusion tube	SEA/culture medium	200 ng/ml	Donnelly <i>et al.</i> 1967
	SEB/culture medium	200 ng/ml	
	SEA/milk	15-200 ng/ml	Read <i>et al.</i> 1965b
	SEB/milk	30-630 ng/ml	
	SEA/cheese	20 ng/ml	Read <i>et al.</i> 1965a
	SEB/cheese	50 ng/ml	

layer and forms a precipitate. The distance that the precipitin band migrates into the antibody-agar layer plotted against the log of antigen concentration gives a straight line. Hall *et al.* (1965) and Weirether *et al.* (1966) described a single-gel diffusion assay for the determination of SEA and SEB. These workers discuss the effect of various parameters such as temperature, pH, salt concentration, and time on the migration of the band into the antibody-agar layer. Fung and Wagner (1971) developed a capillary tube single-gel system to quantitate SEA, SEB, and staphylococcal enterotoxin C (SEC).

The double-gel diffusion tube assay is a modification of the single-gel system in which the antibody-containing agar layer is overlaid with plain agar; then the enterotoxin solution is placed over the plain agar layer. Both antigen and antibody migrate into the plain agar layer to form precipitin bands. The precipitin band position ( $p$ ) is the distance from the antigen solution-plain agar interface to the center of the band divided by the total plain agar length; a plot of  $p$  versus the log of the antigen concentration gives a straight line. Hall *et al.* (1965) describe the use of the double-gel diffusion tube for the assay of SEA and SEB in foods.

*Single- and Double-Gel Diffusion Plate and Microslide Assays (Table 2).* A single radial immunodiffusion microslide assay was developed by placing enterotoxin in wells cut into an antibody-agar mixture contained on microslides. The enterotoxin and antibody form a precipitate around the wells. When the diameter of the precipitin rings were plotted against the enterotoxin concentration, a straight line resulted. Meyer and Palmieri (1980), by adding a mixture of antisera to the agar layer, produced a polyvalent system that was useful as a screening technique to determine if *S. aureus* isolates produced enterotoxins.

Table 2. Use of single- and double-gel diffusion plate or microslide assays in determination of staphylococcal enterotoxins

Procedure	Enterotoxin/System	Lower Detection Limit	Reference
Single radial immunodiffusion microslide	SEA, SEB, SEC, SED, SEE/culture medium	300 ng/ml	Meyer and Palmieri 1980
Optimal sensitivity plate	SEA, SEB, SEC, SED, SEE/culture medium	500 ng/ml	Robbins <i>et al.</i> 1974
Double-gel diffusion microslide	SEA/cheese	400 ng/ml	Zehren and Zehren 1968
	SEA, SEB/variety of foods	1000 ng/ml	Casman and Bennett 1965

The optimal sensitivity plate is a double-gel diffusion plate developed to detect the production of staphylococcal enterotoxins by isolates of *S. aureus*. Utilizing small petri dishes containing agar, a center well (for antibody) surrounded by wells for enterotoxin are cut into the agar layer. As the antigen and antibody migrate toward each other, precipitin lines develop. The technique is quite useful for detection of the enterotoxigenicity of staphylococcal isolates. The optimal sensitivity plate can be quantitated by plotting the distance that the precipitin band traveled (from the center of the antigen well to the far edge of the band) against the concentration of enterotoxin. For a discussion of the use of the optimal sensitivity plate, see Harvey and Patterson (1982).

In the double-gel diffusion microslide assay, small wells are cut in agar-coated microslides, antiserum added to the center well, and enterotoxin placed in the wells surrounding the antiserum well. The diffusion of antiserum and enterotoxin produces a precipitin band between the wells. The assay can be made semiquantitative by noting the highest dilution of enterotoxin which will form a coalescing line of precipitate with the reference precipitin line multiplied by the lowest concentration of known enterotoxin that will form a similar line. Zehren and Zehren (1968) and Casman *et al.* (1969) have described the use of the microslide test in great detail.

*Electroimmunodiffusion Assay.* Enterotoxin contained in wells cut into antibody-containing gels can be forced to migrate across the antibody gel when an electric field is applied. The resulting precipitin pattern resembles a cone; for a constant antibody dilution and electrical field, the length of the cone plotted against the enterotoxin concentration will give a straight line. Gasper *et al.* (1973) used electroimmunodiffusion to detect SEA and suggests that the technique could be applied to foods with a potential sensitivity of <150 ng/ml. Electroimmunodiffusion has been used to assay SEB present in culture medium; the lower limit of detection was 1000 ng/ml (Iandolo and Shafer 1977).

*Passive Hemagglutination Assays (Table 3).* When enterotoxin and antiserum are allowed to react and then sensitized red blood cells (red blood cells to which enterotoxin has been attached) are added, there is no hemagglutination as long as antibody has reacted with enterotoxin, i.e., hemagglutination is inhibited. When there is unreacted antibody, however, then the enterotoxin-sensitized red blood cells agglutinate. Salomon and Tew (1968) modified this technique by using enterotoxin-coated latex particles instead of red blood cells to produce a passive latex agglutination-inhibition assay. They reported that the minimum detection limit for SEB was 0.2 ng/ml.

By attaching the appropriate antibody globulin to red blood cells, the

Table 3. Use of passive hemagglutination assays in determination of staphylococcal enterotoxins

Procedure	Enterotoxin/System	Lower Detection Limit	Reference
Passive hemagglutination-inhibition assay	SEB/culture medium	400-1600 ng/ml	Morse and Mah 1967
	SEB/culture medium	130 ng/ml	Johnson <i>et al.</i> 1967
Reversed passive hemagglutination	SEB/culture medium and a variety of foods	1.5 ng/ml	Silverman <i>et al.</i> 1968

direct agglutination of sensitized red blood cells by enterotoxin levels are determined by multiplying the reciprocal of the greatest dilution of enterotoxin-containing sample that gives a reaction by the smallest amount of purified enterotoxin that reacts. The tests are only semiquantitative but can be refined by making the dilutions very close together.

*Radioimmunoassay (Table 4).* A radioimmunoassay (RIA) for detection of staphylococcal enterotoxin requires that enterotoxin containing a radioactive level binds with enterotoxin-specific antibody. When a sample containing unlabeled enterotoxin is added, the unlabeled antigen competes with the labeled enterotoxin for combining sites on the antibody. The greater the concentration of unlabeled enterotoxin in a food or sample, the greater the probability that the unlabeled enterotoxin will bind to the antibody. After the competitive part of the assay is complete, it is necessary to separate the unreacted enterotoxin from the antibody-enterotoxin complex. In the double antibody precipitation procedure, a second antibody is utilized which binds with the first antibody. The second antibody is produced in an animal species different from that in which the first antibody was produced. The precipitate which forms consists of the second antibody bound to the first antibody previously complexed with the enterotoxin.

Another separation technique involves the use of Protein A which is obtained from specific strains of *S. aureus*. Protein A specifically binds immunoglobulins from a number of animal species. Adding an excess of Protein A to the radioimmunoassay system leads to precipitation of the antigen-antibody complex. Miller *et al.* (1978) felt that the use of Protein A to remove the antigen-antibody complex in radioimmunoassay is the method of choice at the present time.

Table 4. Use of radioimmunoassay in determination of staphylococcal enterotoxins

Procedure	Enterotoxin/System	Lower Detection Limit	Reference
Double-antibody separation	SEA, SEB, SEC/fermented sausage	~4 ng/ml	Robern and Gleeson 1978
Double-antibody solid-phase (cellulose) separation	SEA/dry sausage and minced meat	2-5 ng/ml	Lindroth and Niskanen 1977
Coated polystyrene tube separation	SEA, SEB, SEC/potato	1 ng/ml	Orth 1977
Coated polypropylene tube separation	SEA/various foods	0.3 ng/ml	Dickie and Akhtar 1982
Solid-phase separation (sepharose 4B column)	SEB/various foods	2.2-6.3 ng/g	Niyomvit <i>et al.</i> 1978
Protein A separation	SEA, SEB, SEC, SED, SEE/various foods	1 ng/g	Miller <i>et al.</i> 1978
	SEA/various foods	0.1 ng/ml	Areson <i>et al.</i> 1980
	SEB/various foods	0.5 ng/ml	
	SEA/cheese	0.5 ng/ml	Ibrahim <i>et al.</i> 1980

A third procedure (solid-phase antibody) is to link antibody either covalently or by adsorption to an inert support such as particles or to the walls of assay tubes. The labeled and unlabeled enterotoxin compete for binding sites on the bound antibody. After the reaction is complete, the free antigen is removed from the immobilized antigen-antibody complex by appropriate means (decantation or centrifugation).

The isotope concentration can be determined in either the free antigen portion or in the antigen-antibody portion. Extrapolation from a standard curve in which the percent isotope bound by the antibody (or percent free isotope) plotted against the concentration of enterotoxin gives the amount of enterotoxin present in the unknown samples.

Bergdoll and Reiser (1980) published a review outlining various aspects of the RIA method for detection of staphylococcal enterotoxins in foods. Iodination of enterotoxin with  $^{125}\text{I}$  and separation methods for the antibody-antigen complex from unbound enterotoxin are thoroughly discussed. A more general review of RIA methodology was published by Bolton (1981).

*Enzyme-Linked Immunosorbent Assay (Table 5).* The enzyme-linked immunosorbent assay (ELISA) utilizes an enzymatic reaction to detect the presence of a specific antigen-antibody reaction. In the double antibody single-sandwich ELISA technique, a solid phase (polystyrene [or another polymer] microtiter plates, tubes, or spheres) is coated with

antibody, and enterotoxin is added and allowed to react. Then, the same specific antibody conjugated to an enzyme is added and allowed to react with the enterotoxin bound to the solid-phase antibody. Between each step, unbound reactants are washed out. Finally, a substrate is added and a chromogenic product is formed by the action of the enzyme. The absorbance (A) of the chromogen is measured at the appropriate wavelength. Plotting the log A against the log of the enterotoxin concentration will give a straight line.

Table 5. Use of enzyme-linked immunosorbent assay in the determination of staphylococcal enterotoxins

Procedure	Enterotoxin/System	Lower Detection Limit	Reference
<b>Single sandwich</b>			
Microtiter plates, peroxidase-antibody conjugate	SEA/ various foods	0.4-3.2 ng/ml	Saunders and Bartlett 1977
Polystyrene tubes, peroxidase-antibody conjugate	SEB/cheese and culture medium	10 ng/ml	Koper <i>et al.</i> 1980
Polyvinyl or polystyrene microtiter plates, peroxidase-antibody conjugate	SEB/vanilla custard	1 ng/g	Büning-Pfaue <i>et al.</i> 1981
Polystyrene microtiter plates or spheres, peroxidase-antibody conjugate	SEA, SEB, SED, SEE/ various foods	≤1 ng/g	Freed <i>et al.</i> 1982
Polystyrene microtiter plates, peroxidase-antibody conjugate	SEA, SEB, SEC, SEE/minced meat	5 ng/g	Notermans <i>et al.</i> 1983
Polystyrene spheres, alkaline phosphatase-antibody conjugate	SEA, SEB, SEC, SED/ various foods	0.1 ng/ml	Fey <i>et al.</i> 1984
<b>Double-sandwich</b>			
Polyvinyl microtiter plates, alkaline phosphatase-antibody conjugate	SEA, SEB, SEC/culture medium	0.5 ng/ml	Berdal <i>et al.</i> 1981
<b>Competitive</b>			
Polystyrene spheres, alkaline phosphatase- or peroxidase-enterotoxin conjugate	SEA, SEB, SEC <sub>1</sub> / various foods	≤0.1 ng/ml	Stiffler-Rosenberg and Fey 1978
Polystyrene tubes, alkaline phosphatase-enterotoxin conjugate	SEA/ various foods	2 ng/ml	Kauffman 1980
Polystyrene tubes, alkaline phosphatase-enterotoxin conjugate	SEA, SEB, SEC/culture medium	2.5 ng/ml	Lenz <i>et al.</i> 1983

In the double-sandwich ELISA, a solid-phase anti-enterotoxin complexed with enterotoxin is reacted with a second anti-enterotoxin produced in an animal species different from that of the first. An anti-IgG-enzyme conjugate is then added. The antibody to which the enzyme is conjugated is produced in an animal different from that of the second antibody and is specific for the proteins present in the second antibody. The chromogen-producing substrate is then added, and after an incubation period, the absorbance of the chromogen is determined. The amount of the second antibody bound in the assay is directly proportional to the amount of enterotoxin and to the absorbance of the chromogen.

Another ELISA method for detection of enterotoxin is a competitive method in which the enzyme is conjugated to the enterotoxin rather than to the antibody. The specific antibody, attached to a solid phase, is allowed to react with labeled enterotoxin added simultaneously with unlabeled toxin. The labeled and unlabeled enterotoxin are allowed to compete for antibody sites; the greater the concentration of the unknown (unlabeled) enterotoxin, the less the binding by enzyme-labeled enterotoxin with the solid-phase antibody. After removal of unbound enterotoxin, a chromogen-producing substrate specific for the enzyme is used to quantitate the amount of enterotoxin, the less the amount of chromogen that is produced. Comparison to a standard curve will indicate the level of enterotoxin in the unknown sample.

A general discussion of the ELISA theory and technique was published by Voller *et al.* (1979) and Monroe (1983). Kuo and Silverman (1980) and Notermans (1982, 1983) reviewed specific details of the ELISA for staphylococcal enterotoxin quantitation. These authors discuss conjugation of enzyme to antibody and the use of the sandwich and competitive ELISA as well as the limitation of the ELISA technique as applied to staphylococcal enterotoxin assay.

Of the methods used to detect and quantitate staphylococcal enterotoxins, the RIA and ELISA techniques are the most sensitive (1-10 ng/ml culture fluid or g food) methods presently available for use in both foods and culture media. In addition to being more sensitive, the RIA and ELISA techniques require minimal sample preparation prior to the assay. Most workers, when using RIA or ELISA, merely blend the food with distilled water and adjust the pH to 7.2 and centrifuge. The supernatant fluid is then adjusted to pH 4.5 and centrifuged again. After the second centrifugation, the extract is adjusted to pH 7.2 and is ready for assay; however, concentration of the extract may be necessary (for procedures, see references listed in Tables 4 and 5). The extraction procedure described by Reiser *et al.* (1974) that is used to prepare food samples for the microslide assay is considerably more tedious and complicated.

While both RIA and ELISA are quite sensitive, the ELISA method offers some distinct advantages over the RIA technique. The enzyme label is not radioactive and is easily available and inexpensive. Handling and disposal of radioactive waste is a growing concern nationwide. RIA requires sophisticated radiocounting systems, but ELISA requires only simple equipment. A simple + or - can be determined visually or the chromogen can be quantitated utilizing a spectrophotometer. Minimal technical training is required to determine enterotoxin with ELISA, whereas, with RIA, the technician must be familiar with radioisotope regulations and must be licensed to use isotopes.

## EXPERIMENTAL

Experience in our laboratory indicated that detection of staphylococcal enterotoxins by ELISA is efficient, sensitive, and capable of being performed with a minimum of equipment and training. The sensitivity and specificity of the technique suggest that it should be feasible to develop an ELISA protocol that can detect staphylococcal enterotoxins in foods without requiring extensive clean-up of the samples. This possibility stimulated our interest in assessing the effectiveness of the assay for detecting SEA in spiked foods using a sampling protocol that involved minimal sample preparation and clean-up.

### Materials and Methods

Food samples were spiked with SEA in the following manner: 1000, 500, 100, and 0 ng SEA was added to each of 100-ml samples of pasteurized skim and whole milk and to soup (cream of chicken with mushrooms condensed soup diluted with distilled water according to the manufacturer's directions). Each sample was blended with the enterotoxin using the Stomacher 400 (Cooke Laboratory Products<sup>2</sup>, Alexandria, VA). 100  $\mu$ l of the milk samples were analyzed by ELISA directly; the soup was centrifuged at 16,000 x g (5 min at 0°C) to remove particulate matter and 100  $\mu$ l of the supernatant fluid was analyzed. 50 g cooked ham were blended (using the Stomacher) with 50 ml phosphate buffer (0.1 M, pH 7.2; solution 3, Table 6). To each 100ml sample, 1000, 500, 100, or 0 ng SEA was added and blended into the meat slurry. Each sample was centrifuged at 16,000 x g (5 min at 0°C) and 100  $\mu$ l supernatant fluid was analyzed for SEA. 50 g chicken-beef frankfurters were blended with 100 ml phosphate

<sup>2</sup>Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 6. Solution used in the ELISA determination

Solution 1	Carbonate buffer, 0.01 M, pH 9.8 136 mg Na <sub>2</sub> CO <sub>3</sub> , 230 mg NaHCO <sub>3</sub> , 400 ml distilled water
Solution 2	Phosphate buffer with saline and Tween 20 (PBS-T), the phosphate buffer is 0.01 M, pH 7.2 0.380 g K <sub>h</sub> 2PO <sub>4</sub> , 2.59 g Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O, 8.0 NaCl, 1.0 g Tween 20, 1000 ml distilled water
Solution 3	Phosphate buffer 0.01 M, pH 7.2 1.15 g K <sub>2</sub> HPO <sub>4</sub> , 0.48 g KH <sub>2</sub> PO <sub>4</sub> , 1000 ml distilled water 0.1 M, pH 7.2 11.5 g K <sub>2</sub> HPO <sub>4</sub> , 4.8 g KH <sub>2</sub> PO <sub>4</sub> , 1000 ml distilled water
Solution 4	ABTS-H <sub>2</sub> O <sub>2</sub> in citrate buffer (prepare fresh) 693 mg citric acid, 500 mg Na citrate, 100 ml distilled water, dissolve all ingredients and then add 22 mg ABTS and 60 µl 30% H <sub>2</sub> O <sub>2</sub>

buffer (0.1 M, pH 7.2; solution 3, Table 6) containing 1000, 500, 100, or 0 ng SEA using the Stomacher. The samples were centrifuged at 16,000 x g (5 min at 0°C) and 100 µl of supernatant fluid was analyzed by ELISA.

The solid support used for ELISA was the 96-well Nunc-Immuno Plate I (Vanguard International, Neptune, NJ). Enterotoxin and antisera were obtained from Dr. Anna Johnson (USAMRIID, Frederick, MD). The procedure used for ELISA was modified from the double-sandwich method of Berdal *et al.* (1981). The various layers of the ELISA were built up as follows:

*Layer 1.* 100 µl of a 1:1000 dilution of rabbit anti-SEA in carbonate buffer (solution 1, Table 6) was added to each well of the plate; the plates were incubated at 4°C for at least 3 h (plates were usable for 2-3 weeks if they are stored at 4°C in plastic bags). Before adding next layer, the plates were washed four times with phosphate buffer containing saline and Tween 20 (PBST; solution 2, Table 6).

*Layer 2.* A stock solution of 0.5 mg SEA in 100 ml potassium phosphate buffer (0.01 M, pH 7.2; solution 3, Table 6) was diluted 1:500 in PBS-T. 50-1000 picograms SEA was added to the wells to prepare the standard curve; total volume in each well was made to 100 µl with PBS-T. 100µl of unknown samples were added to the other wells. The plates were placed in plastic bags and incubated overnight at 10°C. Before adding the next layer, the plates were washed four times with PBS-T.

*Layer 3.* Goat anti-SEA was diluted 1:2000 in PBS-T; 100  $\mu$ l was added to each well and plates were placed in plastic bags and incubated at 37°C for 2 h. Before the addition of the next layer, plates were washed four times with PBS-T.

*Layer 4.* Rabbit anti-goat IgG conjugated with horseradish peroxidase (Sigma) was diluted 1:1000 in PBS-T; 100  $\mu$ l was added to each well. Plates were placed in plastic bags and incubated at 37°C with gentle agitation for 2 h. Plates were washed eight times with PBS-T before addition of substrate layer.

*Layer 5.* The substrate for the horseradish peroxidase was diammonium 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (Sigma; ABTS). ABTS-H<sub>2</sub>O<sub>2</sub> was added to citrate buffer (solution 4, Table 6) and 100  $\mu$ l was added quickly to each well. Plates were incubated at 13°C for approximately 30 min. The reaction was stopped by addition of 50  $\mu$ l NaF solution (1.25%) and absorbancy of the chromogen was determined at 410 nm using a Dynatech Microelisa Minireader (Model MR590). Absorbancy values were compared to a standard curve prepared with purified SEA (0-1000 picograms) and the concentrations of the unknowns determined.

When washing plates with PBS-T, 100  $\mu$ l was added to each well and removed gently. Plates were placed in plastic bags during incubation periods to prevent drying out.

Table 7. ELISA determination of SEA in spiked food samples

samples			
SEA added (ng/ml)	SEA Found (ng/ml)		
	Whole Milk	Skim Milk	Soup
0	1.0	1.2	0.0
1	2.3	3.1	1.4
5	5.5	6.5	3.3
10	8.3	8.8	5.8

  

SEA Added (ng/g)	SEA Found (ng/g)	
	Cooked Ham	Frankfurters
0	0.0	0.5
2	1.6	1.4
10	10.7	8.6
20	17.4	14.1

## Results and Discussion

In food samples that had received minimal pretreatment before ELISA, the presence of >5 ng SEA/ml or g of food could reliably be detected (Table 7). There was some nonspecific binding in the absence of SEA, particularly with milk. Our results, however, indicate that ELISA can detect SEA in a variety of food products with a minimum of sample preparation. A liquid food like milk was assayed directly and soup was merely centrifuged to remove particulate matter before assay. Solid foods like meat were blended with phosphate buffer followed by centrifugation in the cold to remove fat and particulate matter. With meats, only a single type of buffer was used; other types of buffer at different pH values or molar concentrations should be tested to determine the best system for preparation of meat samples for ELISA.

Our preliminary studies do indicate that an extensive clean-up of food samples for ELISA is not necessary. Thus, the use of ELISA to determine the presence of staphylococcal enterotoxins in foods required little sample preparation and is simple, fast, and sensitive.

## REFERENCES

- ARESON, P. D. W., CHARM, S. E. and WONG, B. L. 1980. Determination of staphylococcal enterotoxins A and B in various food extracts, using staphylococcal cells containing protein A. *J. Food Sci.* **45**, 400-401.
- BERDAL, B. P., OLSVIK and OMLAND, T. 1981. A sandwich ELISA method for detection of *Staphylococcus aureus* enterotoxins. *Acta Path. Microbiol. Scand. Sect. B* **89**, 411-415.
- BERGDOLL, M. S. 1972. The enterotoxins. In *The Staphylococci* (J. O. Cohen, ed.), pp. 301-331, Wiley-Interscience, New York.
- BERGDOLL, M. S. 1979. Staphylococcal intoxications. In *Food-Borne Infections and Intoxications*, 2nd ed. (H. Riemann and F. L. Bryan, eds.) pp. 443-494, Academic Press, New York.
- BERGDOLL, M. S. and REISER, R. 1980. Application of radioimmunoassay for detection of staphylococcal enterotoxins in foods. *J. Food Prot.* **43**, 68-72.
- BERGDOLL, M. S., BORJA, C. R. and AVENA, R. M. 1965. Identification of a new enterotoxin as enterotoxin C. *J. Bacteriol.* **90**, 1481-1485.
- BOLTON, A. E. 1981. Radioimmunoassay. In *Immunoassays for the 80's* (A. Voller, A. Bartlett and D. Bidwell, eds.) pp. 69-83, University Park Press, Baltimore, MD.

- BRADSTREET, C. M. P., DIGHERO, M. W., GILBERT, R. J. and WIENEKE, A. A. 1977. Raising antibodies to staphylococcal enterotoxins A and B. *J. Appl. Bacteriol.* **42**, 117-122.
- BÜNING-PFAUE, H., TIMMERMANS, P. and NOTERMANS, S. 1981. Einfache methode für den nachweis von staphylokokken-enterotoxin-B in vanillepudding mittels ELISA-test. *Z. Lebensm. Uters. Forsch.* **173**, 351-355.
- BUXSER, S. and BONVENTRE, P. F. 1981. Staphylococcal enterotoxins fail to disrupt membrane integrity or synthetic functions of Henle 407 intestinal cells. *Infect. Immun.* **31**, 929-934.
- CASMAN, E. P. and BENNETT, R. W. 1964. Production of antiserum for staphylococcal enterotoxin. *Appl. Microbiol.* **12**, 363-367.
- CASMAN, E. P. and BENNETT, R. W. 1965. Detection of staphylococcal enterotoxin in food. *Appl. Microbiol.* **13**, 181-189.
- CASMAN, E. P., BENNETT, R. W., DORSEY, A. E. and ISSA, J. A. 1967. Identification of a fourth staphylococcal enterotoxin, enterotoxin D. *J. Bacteriol.* **94**, 1875-1882.
- CASMAN, E. P., BENNETT, R. W., DORSEY, A. E. and STONE, J. E. 1969. The micro-slide gel double diffusion test for the detection and assay of staphylococcal enterotoxins. *Health Lab. Science* **6**, 185-198.
- DICKIE, N. and AKHTAR, S. M. 1982. Improved radioimmunoassay of staphylococcal enterotoxin A. *J. Assoc. Off. Anal. Chem.* **65**, 180-184.
- DONNELLY, C. B., LESLIE, J. E., BLACK, L. A. and LEWIS, K. H. 1967. Serological identification of enterotoxigenic staphylococci from cheese. *Appl. Microbiol.* **15**, 1382-1387.
- FEY, H., PFISTER, H. and RÜEGG O. 1984. Comparative evaluation of different enzyme-linked immunosorbent assay systems for the detection of staphylococcal enterotoxins A, B, C, and D. *J. Clin. Microbiol.* **19**, 34-38.
- FREED, R. C., EVENSON, M. L., REISER, R. F. and BERGDOLL, M. S. 1982. Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxin in foods. *Appl. Environ. Microbiol.* **44**, 1349-1355;
- FUNG, D. Y. C. and WAGNER, J. 1971. Capillary tube assay for staphylococcal enterotoxins A, B, and C. *Appl. Microbiol.* **21**, 559-561.
- GASPER, E., HEIMSCH, R. C. and ANDERSON, A. W. 1973. Quantitative detection of type A staphylococcal enterotoxin by laurell electroimmunodiffusion. *Appl. Microbiol.* **25**, 421-426.
- GENIGEORGIS, C. and KUO, J. K. 1976. Recovery of staphylococcal enterotoxin from foods by affinity chromatography. *Appl. Environ. Microbiol.* **31**, 274-279.

- GILBERT, R. J. and WIENEKE, A. A. 1973. Staphylococcal food poisoning with special reference to the detection of enterotoxin in food. In *The Microbiological Safety of Food* (B. C. Hobbs and Christian J. H. B., eds.) pp. 273-285, Academic Press, New York.
- HALL, H. E., ANGELOTTI, R. and LEWIS, K. H. 1965. Detection of the staphylococcal enterotoxins in food. *Health Lab. Science* 2, 179-191.
- HARVEY, J. and PATTERSON, J. T. 1982. Optimal sensitivity plate method for the detection of staphylococcal enterotoxins. In *Isolation and Identification Methods for Food Poisoning Organisms, Society for Applied Bacteriology Technical Series*, No.17 (J. E. L. Corry, D. Roberts and F. A. Skinner, eds.) pp. 211-216, Academic Press, New York.
- IANDOLO, J. J. and SHAFER, W. M. 1977. Regulation of staphylococcal enterotoxin B. *Infect. Immun.* 16, 610-616.
- IBRAHIM, G. F., RADFORD, H. M. and FELL, L. R. 1980. Determination of staphylococcal enterotoxin in cheddar cheese produced without starter activity. *Appl. Environ. Microbiol.* 39, 1134-1137.
- JOHNSON, H. M., HALL, H. E. and SIMON, M. 1967. Enterotoxin B: Serological assay in cultures by passive hemagglutination. *Appl. Microbiol.* 15, 815-818.
- KAUFFMAN, P. E. 1980. Enzyme immunoassay for staphylococcal enterotoxin A. *J. Assoc. Off. Anal. Chem.* 63, 1138-1143.
- KOPER, J. W., HAGENAAERS, A. M. and NOTERMANS, S. 1980. Prevention of cross-reactions in the enzyme linked immunosorbent assay (ELISA) for the detection of *Staphylococcus aureus* enterotoxin B in culture filtrate and foods. *J. Food Safety* 2, 35-45.
- KUO, J. K. S. and SILVERMAN, G. J. 1980. Application of enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxin in food. *J. Food Prot.* 43, 404-407, 413.
- LANGNER, H. J. and SINELL, H. J. 1975. Probleme bei der herstellung von staphylokokken-enterotoxin-D-antiserum. *Die Fleischwirtschaft* 55, 973-975.
- LENZ, W., THELEN, R., PICKENHAHN, P. and BRANDIS, H. 1983. Nachweis von enterotoxin in kulturüberständen von *Staphylococcus aureus* mit dem ELISA-test and dem microslide-test. *Zbl. Bakt. Hyg., I. Abt. Orig. A* 253, 466-475.
- LINDROTH, S. and NISKANEN, A. 1977. Double antibody solid-phase radioimmunoassay for staphylococcal enterotoxin A. *European J. Appl. Microbiol.* 4, 137-143.
- MEYER, R. F. and PALMIERI, M. J. 1980. Single radial immunodiffusion method for screening staphylococcal isolates for enterotoxin. *Appl. Environ. Microbiol.* 40, 1080-1085.

- MILLER, B. A., REISER, R. F. and BERGDOLL, M. S. 1978. Detection of staphylococcal enterotoxins A, B, C, D and E in foods by radioimmunoassay, using staphylococcal cells containing protein A as immunoadsorbent. *Appl. Environ. Microbiol.* 36, 421-426.
- MINOR, T. E. and MARTH, E. H. 1976. *Staphylococci and Their Significance in Foods*. Elsevier Scientific Publishing Co., New York.
- MONROE, D. 1983. ELISA, a versatile chemical tool. *Am. Clinical Products Review* 2, (3), 22, 24, 26-27.
- MORSE, S. A. and MAH, R. A. 1967. Microtiter hemagglutination-inhibition assay for staphylococcal enterotoxin B. *Appl. Microbiol.* 15, 58-61.
- NIYOMVIT, N., STEVENSON, K. E. and McFEETERS, R. F. 1978. Detection of staphylococcal enterotoxin B by affinity radioimmunoassay. *J. Food Sci.* 43, 735-739.
- NOTERMANS, S. 1982. Detection of staphylococcal enterotoxins (SE) with special reference to the enzyme-linked immunosorbent assay (ELISA). In *Isolation and Identification Methods for Food Poisoning Organisms, Soc. Appl. Bacteriol. Tech.*, Series No. 17 (J. E. L. Corry, D. Roberts and F. A. Skinner, eds.) pp.199-209, Academic Press, New York.
- NOTERMANS S. 1983. Nachweis von staphylokokkus-enterotoxinen in lebensmitteln mittels ELISA-test. *Deutsch Lebensmittel-Rundschau* 79, 156-159.
- NOTERMANS, S., BOOT, R., TIPS, P. D. and DE NOOIJ, M. P. 1983. Extraction of staphylococcal enterotoxin (SE) from minced meat and subsequent detection of SE with enzyme-linked immunosorbent assay (ELISA). *J. Food Prot.* 46, 238-241.
- ORTH, D. S. 1977. Statistical analysis and quality control in radioimmunoassays for staphylococcal enterotoxins A, B, C. *Appl. Environ. Microbiol.* 34, 710-714.
- READ, R. B., JR., BRADSHAW, J., PRITCHARD, W. L. and BLACK, L. A. 1965a. An assay of staphylococcal enterotoxin from cheese. *J. Dairy Sci.* 48, 420-424.
- READ, R. B., JR., PRITCHARD, W. L., BRADSHAW, J. and BLACK, L. A. 1965b. In vitro assay of staphylococcal enterotoxins A and B from milk. *J. Dairy Sci.* 48, 411-419.
- REISER, R., CONAWAY, D. and BERGDOLL, M. S. 1974. Detection of staphylococcal enterotoxin in foods. *Appl. Microbiol.* 27, 83-85.
- ROBBINS, R. N. and BERGDOLL, M. S. 1984. Production of rabbit antisera to the staphylococcal enterotoxins. *J. Food Prot.* 47, 172-176.
- ROBBINS, R. N., GOULD, R. S. and BERGDOLL, M. 1974. Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Appl. Microbiol.* 28, 946-950.

- ROBERN, H. and GLEESON, T. M. 1978. The use of polyethylene glycol in radioimmunoassay of staphylococcal enterotoxins. *Can. J. Microbiol.* *24*, 765-766.
- SALOMON, L. L. and TEW, R. W. 1968. Assay of staphylococcal enterotoxin B by latex agglutination. *Proc. Soc. Exp. Biol. Med.* *129*, 539-542.
- SAUNDERS, G. C. and BARTLETT, M. L. 1977. Double-antibody solid-phase enzyme immunoassay for the detection of staphylococcal enterotoxin A. *Appl. Environ. Microbiol.* *34*, 518-522.
- SCHUEBER, P. H., MOSSMAN, H., BECK, G. and HAMMER, D. K. 1983. Direct skin test in highly sensitized guinea pigs for rapid and sensitive determination of staphylococcal enterotoxin B. *Appl. Environ. Microbiol.* *46*, 1351-1356.
- SILVERMAN, S. J., KNOTT, A. R. and HOWARD, M. 1968. Rapid, sensitive assay for staphylococcal enterotoxin and a comparison of serological methods. *Appl. Microbiol.* *16*, 1019-1023.
- SINELL, H.-J. and MENTZ, I. 1978. Versuche zur extraktion von staphylokokken-enterotoxinen aus lebensmitteln. *Arch. Lebensmittelhygiene* *29*, 150-155.
- SMITH, J. L., BUCHANAN, R. L. and PALUMBO, S. A. 1983. Effect of food environment on staphylococcal enterotoxin synthesis: A review. *J. Food Prot.* *46*, 545-555.
- STIFFLER-ROSENBERG, G. and FEY, H. 1978. Simple assay for staphylococcal enterotoxins A, B, and C: Modification of enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* *8*, 473-479.
- TAYLOR, S. L., SCHLUNZ, L. R., BEERY, J. T., CLIVER, D. O. and BERGDOLL, M. S. 1982. Emetic action of staphylococcal enterotoxin A on weanling pigs. *Infect. Immun.* *36*, 1263-1266.
- TODD, E. C. D. 1983. Foodborne disease in Canada—a 5-year summary. *J. Food Prot.* *46*, 650-657.
- WEIRETHER, F. J., LEWIS, E. E., ROSENWALD, A. J. and LINCOLN, R. E. 1966. Rapid quantitative serological assay of staphylococcal enterotoxin B. *Appl. Microbiol.* *14*, 284-291.
- VOLLER, A., BIDWELL, D. E. and BARTLETT, A. 1979. The enzyme linked immunosorbent assay (ELISA). Dynatech. Labs., Inc., Alexandria, VA.
- ZEHREN, V. L. and ZEHREN, V. F. 1968. Examination of large quantities of cheese for staphylococcal enterotoxin A. *J. Dairy Sci.* *51*, 635-644.