Methods for Rapid Separation and Concentration of Bacteria in Food that Bypass Time-Consuming Cultural Enrichment

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ABSTRACT

The rapid detection of pathogenic organisms that cause foodborne illnesses is needed to insure food safety. Conventional methods for the detection of pathogens in foods are time-consuming and labor-intensive. New advanced rapid methods (i.e., polymerase chain reaction, DNA probes) are more sensitive and selective than conventional techniques, but many of these tests are inhibited by food components, rendering them dependent on slow cultural enrichment. The need for alternative methods that will rapidly separate and concentrate bacteria directly from food samples, thereby reducing the time required for these new rapid detection techniques, is evident. Separation and concentration methods extract target bacteria from interfering food components and/or concentrate bacteria to detectable levels. This review describes several methods used to separate and/or concentrate bacteria in food samples. Several methods discussed here, including centrifugation and immunomagnetic separation, have been successfully used, individually and in combination, to rapidly separate and/or concentrate bacteria from food samples in less time than is required for cultural enrichment.

Foodborne diseases are estimated to cause of 76,000,000 illnesses, 325,000 hospitalizations, and 5,200 deaths in United States each year. Fourteen million of these annual illnesses, 60,000 of these annual hospitalizations, and 1,800 of these annual deaths are caused by known pathogens (13). Over the past decade, reports of foodborne disease outbreaks have become more common (9, 51, 53). Over this same period, federal agencies, including the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) and the U.S. Food and Drug Administration (FDA), began instituting hazard analysis critical control point (HACCP) programs aimed at improving food safety through the improved science-based regulation of food production and quality control.

HACCP programs are designed to identify and prevent hazards, whether biological (e.g., pathogenic bacteria), chemical (e.g., toxins), or physical (e.g., metals), posed by contaminated food. HACCP plans require the monitoring of critical control points in food processing operations into which hazards may be introduced, and these plans also require actions to prevent or eliminate such hazards. The use of HACCP procedures, particularly the monitoring of critical control points for biological hazards, requires the real-time selective detection of small quantities of microorganisms in sizeable quantities (e.g., liters) of food product or raw material in order to ensure food safety. For HACCP procedures to be practical for food processors, the detection of pathogens needs to be achievable with an automated process that can directly monitor food production, reducing the need for time-consuming laboratory procedures.

Traditional microbiological methods for the detection and identification of pathogenic organisms, specifically bacteria, are off-line laboratory procedures. These multistep procedures often consist of several long cultural enrichment procedures, including resuscitation, preenrichment, selective enrichment, and the isolation of bacterial colonies prior to identification by morphological, immunological, or biochemical means. These proven methods have several practical merits, including a low cost for each test. However, the time-consuming and labor-intensive nature of these procedures, which in some cases can take as long as 5 to 6 days (82) to produce results, render them ineffective as a primary means of insuring food safety under HACCP programs. For example, a typical cultural method for detecting Salmonella in both pasteurized and unpasteurized orange juice requires a minimum of 3 days for the confirmation of presumptive positive results, which includes 24 h of preenrichment, 24 h of selective enrichment, and 24 h of selective plating (5).

Advancements in several fields, including molecular biology, immunology, engineering, and microelectronics, have led to the development of detection methods, commonly referred to as “rapid methods,” whose levels of sensitivity, selectivity, and speed are higher than those of conventional methods. These rapid methods include biochemical, nucleic acid–based, antibody-based, and biosensor-based methods as well as modifications of conventional methods (18). Biosensors used as components of sensor arrays, electronic noses, and tongues for the detection of the
presence of biological hazards have also been improved dramatically in recent years owing to advancements in electronics and immobilization technologies. Several biosensors signal the presence of biological hazards by altering continuous electrical or optical signals and therefore may provide the best method for on-line, real-time screening for pathogenic bacteria (57, 68).

Unfortunately, for food analysis, these rapid methods of bacterial detection and identification are still far from being real-time and on-line because most of them still rely on cultural enrichment to enhance sensitivity and selectivity before the analysis is carried out (18). Competitor microorganisms and/or food components such as food debris can interfere with or reduce the sensitivity of rapid methods. Competitor organisms can cross-react with detection systems, producing false-positive results, or can grow to levels that will mask target organisms (6). Minimally processed foods such as fresh fruits, vegetable juices, and homogenate solid food samples contain considerable amounts of food debris, which can also interfere with detection assays, making rapid direct detection without cultural enrichment difficult or impossible. Certain food components, such as collagen, have been shown to inhibit PCR-based identification (30, 70, 88).

Bacterial separation and concentration methods are perhaps the best means of eliminating problems caused by food debris and competitor organisms while reducing the need for time-consuming cultural enrichments. Separation and concentration methods separate target bacteria from food components and/or concentrate bacteria to levels detectable by rapid detection methods without relying on microbial multiplication (6).

Presently, food processors lack commercial on-line, real-time pathogenic bacterium detection equipment similar to industrial pH or conductivity monitors. Progress toward this type of 100% real-time, on-line screening of food for pathogenic bacteria for HACCP programs requires rapid separation and concentration methods combined with rapid detection methods that are able to automatically analyze food in production on a continuous basis.

This review provides an overview of separation and concentration methods that have been used for bacteria in food samples. Information regarding the capabilities, time requirements, and limitations of each method is intended to serve as a basis for research and the development of rapid separation and concentration methods and methods for the overall rapid detection of pathogenic organisms. The existing methods are considered as two main groups: physical methods (such as filtration and centrifugation) and adsorption methods (including immunomagnetic separation). Table 1 presents a brief list of physical separation techniques that have been employed to enrich bacteria from food samples. Table 2 lists adsorption-based techniques that have also been used to enrich bacteria in food samples.

**PHYSICAL METHODS**

**Aqueous polymer two-phase systems.** Aqueous polymer two-phase separation is based on physical and chemical surface properties of bacterial cells (e.g., hydrophobicity and hydrophilicity) and interactions between two incompatible polymers, mainly the attraction of monomer segments of each polymer to other monomer segments of the same polymer. When a mixture of two incompatible polymers and a food sample containing bacteria separates into two phases, bacterial cells within the mixture are separated from the original food sample and concentrated into one of the polymers. In low-ionic-strength (<25 mmol/liter) aqueous systems, ions such as Na⁺, K⁺, Li⁺, Cl⁻, or phosphate can improve the partitioning of a given cell type to a specific phase. The attraction of ions in one polymer phase creates an electrostatic potential difference between the phases, which can attract positively or negatively charged cells (3, 4, 44). The two-phase aqueous system that is most commonly used for biochemical applications is composed of polyethylene glycol, dextran, and water, with the polyethylene glycol being enriched in the top phase and the dextran being enriched in the bottom phase (61). The high water content makes aqueous polymer systems suitable for the separation of biological substances such as bacteria.

Lantz et al. (34) developed a 30-min sample preparation method involving an aqueous polymer two-phase system containing polyethylene glycol and dextran; the method is carried out at room temperature to separate PCR-inhibiting components in homogenized Danish Blue Castello soft cheese in order to improve the detection of *Listeria monocytogenes*. PCR detection confirmed that the aqueous polymer two-phase system successfully partitioned PCR inhibitors to the polyethylene glycol phase and the *L. monocytogenes* to the dextran phase. After separation from the inoculated cheese samples, *L. monocytogenes* was detected only in the dextran phase. Results obtained by Lantz et al. (34) also demonstrated the ability of aqueous polymer two-phase separation to increase PCR’s sensitivity in detecting *L. monocytogenes* in Danish Blue Castello cheese by a minimum of three orders of magnitude. *L. monocytogenes* was detected in cheese samples at levels as low as 1.0 × 10⁷ CFU per ml. In contrast, PCR was successful in detecting *L. monocytogenes* in only half of the cheese samples at a minimum concentration of 1.0 × 10⁵ CFU/ml. One reported limitation of the method described by Lantz et al. (34) was an inability to partition all of the *L. monocytogenes* cells in the cheese samples into a concentrated form within the dextran phase. Levels of *L. monocytogenes* recovered from the dextran phase were 0.2 to 0.4 log units lower than the inoculated level. This loss in bacterial concentration was attributed to the partitioning of some bacteria and other large particles within the cheese sample at the interface between the two phases.

Lantz et al. (35) continued to investigate PCR’s detection of *L. monocytogenes* in different soft cheeses following 30 min of aqueous polymer two-phase separation. Again, the separation of bacteria from a complex food sample was achieved without cultural enrichment. The limits for the detection of *L. monocytogenes* by PCR were reduced one to three orders of magnitude, from 1.0 × 10⁶ to 1.0 × 10⁵.
### TABLE 1. Physical methods for noncultural separation and concentration used to enrich bacteria in food samples prior to detection

<table>
<thead>
<tr>
<th>Separation-concentration method</th>
<th>Description</th>
<th>Sample types</th>
<th>Target bacteria</th>
<th>References</th>
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<tbody>
<tr>
<td>Aqueous polymer two-phase partitioning</td>
<td>Use aqueous solution of two polymers to separate bacteria from small volumes (0.1 ml) of diluted food samples. Time: 5 min with centrifugation; normal separation time, 30–60 min. Limitations: difficult to automate; bacteria are concentrated with food debris at polymer interphase.</td>
<td>Sausage, pork, soft cheese</td>
<td>L. monocytogenes, Y. enterocolitica, Salmonella Berta</td>
<td>3, 4, 33–35, 44, 61</td>
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<tr>
<td>Centrifugation</td>
<td>Bacteria are concentrated by centrifugation so that a majority of the food solution can be removed. Time: 0.5–60 min, depending on number of centrifugation and wash steps. Limitations: Bacteria are concentrated with food debris; difficult to automate for an on-line process.</td>
<td>Beef and minced pork, milk, soft cheese, shrimp, lettuce</td>
<td>L. monocytogenes, Y. enterocolitica, S. flexneri, E. coli</td>
<td>20, 32, 39–41, 80</td>
</tr>
<tr>
<td>Filtration</td>
<td>Bacteria are separated on the basis of their size as food solutions are forced through filters with various pore sizes. Time: minutes, depending on filter area and food sample size. Limitation: food debris tends to clog filters and be enriched with bacteria.</td>
<td>Beef, vegetables, milk</td>
<td>E. coli O157:H7, L. monocytogenes</td>
<td>31, 72, 75, 78, 83</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>Relies on standing acoustic waves to concentrate bacteria into clusters. Bacteria can then be removed from a majority of the food sample by other physical means such as fluid flow. Time: minutes, depending on concentration of bacteria and food debris. Limitations: dilution of food sample; small sample size.</td>
<td>Diluted milk, PBS</td>
<td>E. coli</td>
<td>14, 15, 24, 25, 37, 38, 52</td>
</tr>
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</table>

CFU/ml for Feta cheese and from $1.0 \times 10^6$ to $1.0 \times 10^3$ CFU/ml for Bavaria Blue and Cambozola cheeses.

Again, 100% bacterial recovery was not realized because some bacteria became concentrated in the interphase with food debris. The amounts of *L. monocytogenes* recovered in the dextran phases were 0.9 to 1.5 log units below the inoculated amount. Increases in the cheese sample size and therefore in total bacteria was intended to increase the amount of recovered bacteria and potentially lead to lower detection limits. Unfortunately, detection sensitivity was not increased, indicating that without adequate dilution of the food sample, the additional bacteria cannot be separated by aqueous polymer two-phase separation.

Lantz et al. (33) used a 60-min aqueous polymer two-phase system for sample preparation for the detection of *Yersinia enterocolitica* in pork samples by PCR. *Y. enterocolitica* was not directly detectable in the minced- or whole-pork samples by PCR without prior aqueous polymer two-phase separation. The aqueous polymer two-phase separation was successful for the minced-pork samples. Limits for the detection of *Y. enterocolitica* by PCR were lowered by one order of magnitude, from $1.0 \times 10^7$ to $1.0 \times 10^6$ CFU/ml. However, aqueous polymer two-phase separation was not able to separate enough *Y. enterocolitica* from the whole-pork meat samples, which contained 10 times the amount of pork meat of the minced-pork samples, for detection by PCR. Results of two studies by Lantz et al. (33, 35) indicate that without adequate dilution of food samples prior to the use of aqueous polymer two-phase systems, the separation of bacteria from food samples is hindered or impossible.

The use of three different aqueous polymer two-phase systems to separate both *L. monocytogenes* and *Salmonella Berta* from Cumberland sausage was investigated by Pedersen et al. (61). The aqueous polymer systems and samples were mixed by inversion 20 times and then centrifuged for 5 min at 1,000 $\times g$ for rapid separation. Immediately after centrifugation, the two phases were separated and vortexed, and diluted aliquots were plated on plate count agar (Difco Laboratories, Sparks, Md.). The plates were incubated overnight at 37°C for *Salmonella Berta* and at 30°C for *L. monocytogenes*.

The aqueous polymer two-phase separation was successful; however, as in previous cases, the bacterium recovery rate was not 100%. *Salmonella Berta* recovery rates of 66.6 to 86.1% were achieved with systems at pH 6, and
TABLE 2. Adsorption methods for noncultural separation and concentration used to enrich bacteria in food samples prior to detection

<table>
<thead>
<tr>
<th>Separation-concentration method</th>
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<th>Sample types</th>
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</thead>
<tbody>
<tr>
<td>Immunomagnetic separation</td>
<td>Antibodies bind to specific bacteria; bound bacteria and magnetic particles are then separated from food sample with a magnet. Time: 10–180-min incubation periods. Limitation: food debris may block immobilized antibody, preventing bacterial capture and separation.</td>
<td>Ham, beef, milk</td>
<td><em>L. monocytogenes</em>, <em>E. coli</em>, <em>Salmonella</em> Typhimurium</td>
<td>11, 36, 62, 67, 76–78</td>
</tr>
<tr>
<td>Flow-through immunoseparation</td>
<td>Bacteria are captured by immobilized antibodies as samples are passed through reactor bed. After wash steps to remove unwanted particles, target bacteria can be eluted into cleaner suspension. Time: minutes, depending on flow rate and sample size. Limitations: microporous reactor beds an be clogged by food debris; food debris may block immobilized antibody, preventing bacterial capture and separation.</td>
<td>Meat extract, milk, basil, soft cheese</td>
<td><em>E. coli</em>, <em>P. aeruginosa</em></td>
<td>12, 54, 86</td>
</tr>
<tr>
<td>Bacteriophage-based method</td>
<td>Bacteriophages attach to specific bacteria. Phages are often immobilized on magnetic particles and are able to interact with bacteria in a heterogeneous environment such as a food matrix. Time: 2-h incubation periods are common. Limitations: lack of research on phage-based separation applied to food samples; food debris may block phage-bacterium attachment and subsequent separation.</td>
<td>Buffer</td>
<td><em>Salmonella</em></td>
<td>6</td>
</tr>
<tr>
<td>Dielectrophoresis</td>
<td>Negatively charged bacteria are attracted to the positive electrode and thereby separated from the food matrix. Time: minutes, depending on size of food sample. Limitations: conductivity of complex food matrices may limit bacterial attraction; lack of research on DEP applied to food samples.</td>
<td>Buffer</td>
<td><em>B. subtilis</em>, <em>E. coli</em>, <em>M. luteus</em></td>
<td>10, 45</td>
</tr>
<tr>
<td>Lectin-based method</td>
<td>Lectins immobilized to solid supports adsorb to target bacteria to facilitate separation similar to IMS and FIS with antibodies. Time: 2–3-h incubation periods are typical. Limitations: inability to control the specificity of lectins as can be done with antibodies; releasing agents can inhibit PCR detection.</td>
<td>Beef, eggs, milk, cheese</td>
<td><em>Salmonella</em>, <em>E. coli</em>, <em>L. monocytogenes</em>, <em>S. aureus</em></td>
<td>23, 58–60, 65</td>
</tr>
<tr>
<td>Metal hydroxide-based method</td>
<td>Metal hydroxides attach to bacteria in solution. Bacteria and metal hydroxide complexes may then be concentrated and removed from food matrices by centrifugation. Time: &lt;1 h, but ultimately depends on number of centrifugation steps required. Limitations: difficult to automate; food debris is concentrated with metal hydroxide-bacterium complexes by centrifugation.</td>
<td>Meat, NFDM, whole milk, ice cream</td>
<td><em>E. coli</em></td>
<td>8, 29, 42, 49, 50</td>
</tr>
</tbody>
</table>
rates of 7.6 to 38.6% were achieved with systems at pH 3. *L. monocytogenes* recovery rates ranged from 79.5 to 95% at pH 6 and from 52.2 to 78% at pH 3. Results of the study by Pedersen et al. (61) showed that centrifugation can decrease the time required for aqueous polymer two-phase separation by >90%. The primary focus of the work conducted by Pedersen et al. (61) was the separation of bacteria from food samples. No specific information about the relationships of the different aqueous polymer two-phase separation systems to the bacterial detection limit of a particular detection method (e.g., PCR) was given.

As a method for separation and concentration, aqueous polymer two-phase separation is capable of removing pathogenic bacteria from complex food matrices, thereby lowering the detection limits of rapid detection methods and decreasing the need for time-consuming cultural enrichment. Aqueous polymer two-phase separation is not exceptionally rapid, requiring approximately 30 to 60 min for completion. However, separation time can be significantly reduced (5 min) through the use of centrifugation to expedite polymer separation. Unfortunately, aqueous polymer two-phase separation is limited to small sample volumes (0.1 to 0.4 ml) owing to its dependence on dilution buffers and polymer mixtures. The implementation of aqueous polymer two-phase separation as part of an automated on-line separation and concentration process may also be limited by the use of dilution buffers, polymer mixtures, and prolonged static separation or active separation by centrifugation. Another disadvantage of aqueous polymer two-phase separation is the concentration of some bacteria with large food particles at the interface between phases, which reduces overall bacterial recovery.

**Centrifugation.** Centrifugation is commonly used to concentrate bacterial cells in solution. High-speed rotation pelletizes bacteria. The remaining supernatant fluid is drawn off, and then the bacteria are resuspended in a smaller volume of cleaner solution, effectively concentrating the bacteria while separating them from the original suspension. Furrer et al. (20) used two 10-min centrifugation steps for the preparation of samples for the detection of *L. monocytogenes* in pasteurized milk samples. The detection limit for *L. monocytogenes* in milk with centrifugation separation-concentration and PCR was determined to be as low as 1.0 × 10^2 CFU/liter.

Uyttendale et al. (80) used centrifugation as part of a sample preparation procedure for the detection of *L. monocytogenes* in diluted and homogenized cheese samples by standard plate counts (SPC). This centrifugation-based sample preparation method was able to isolate *L. monocytogenes* from cheese homogenate samples. The levels of *L. monocytogenes* recovered were 0.1 to 1 log unit below the initial inoculum levels. The detection limits of SPC on Oxford media (Oxoid, Basingstoke, Hampshire, UK) were lowered to 0.5 × 10^3 to 1.0 × 10^3 CFU/g of cheese homogenate. The two centrifugation steps took <15 min. Although the need for time-consuming cultural enrichment was avoided, the entire sample preparation procedure required numerous steps and took ca. 45 min. Additional steps included filtering and enzymatic digestion to remove food particles. Enzymatic digestion was the most time-consuming step, requiring a 20-min incubation period.

Another centrifugation method used to separate bacteria from complex food matrices as well as to remove compounds that inhibit rapid detection methods such as PCR is buoyant density centrifugation (BDC). BDC separates bacteria from food particles on the basis of differences in their buoyant densities in a gradient medium.

Lindqvist et al. (41) used BDC with Percoll gradient media (Amersham Biosciences, Uppsala, Sweden) to remove *Shigella flexneri* from blue cheese and milk homogenates. PCR analysis of blue cheese and milk homogenates demonstrated that BDC sample preparation had successfully separated the *S. flexneri* bacteria from the food components. Direct PCR analysis of the cheese homogenate did not detect any bacteria, which supports the hypothesis that BDC separates not only the bacteria but also the PCR inhibitors from the food components. This was not always the case with simple centrifugation (20). Despite only partial recovery of bacteria (25%), the detection limits for *S. flexneri* with BDC were 1.0 × 10^3 and 2.5 × 10^2 CFU/ml in cheese and milk homogenates, respectively. The BDC separation step took <30 min to complete.

Lindqvist (39, 40) employed a similar BDC method with Percoll and BacXtractor (QRAB, Uppsala, Sweden) gradient media as part of a sample preparation method that included 6 h of cultural enrichment for the separation of various strains of bacteria, including *Escherichia coli* O157: H7 and *S. flexneri*, from beef, lettuce, milk, shrimp, and blue cheese samples prior to PCR analysis. The detection limits for *E. coli* by the procedure, which took <8 h, were found to be 1.25 × 10^3 to 2.5 × 10^3 CFU/ml and 1.0 × 10^3 CFU/ml for beef and minced-beef homogenates, respectively. In 1999, Lindqvist (40) successfully detected *S. flexneri* in lettuce, milk, shrimp, and blue cheese samples at arbitrary concentrations of 3.0 × 10^3 CFU/ml. The entire detection procedure, which included cultural enrichment, took <2 days.

Lambertz et al. (32) used BDC and traditional cultural enrichment to pretreat various diluted and homogenized meat samples as part of a method for the overall detection of *Y. enterocolitica*. *Y. enterocolitica* was successfully detected in meat samples at levels of 2.5 × 10^2 to 5.0 × 10^3 CFU/ml. Lindqvist (39) achieved similar PCR detection limits of 1.25 × 10^2 to 2.5 × 10^2 CFU/ml in meat samples with BDC pretreatment. Although bacterial recovery by BDC was not directly investigated, the reported detection limits without BDC, which were 10 times as high at 2.5 × 10^3 CFU/ml, illustrate that BDC is capable of separating and concentrating bacteria from food samples in <10 min.

In recent years, centrifugation has been used in conjunction with cultural enrichment and other separation and concentration methods to achieve rapid separation and concentration of bacteria in food samples. Pedersen et al. (61) used centrifugation to decrease the separation time required for aqueous polymer two-phase separation. Hudson et al. (26) used two centrifugation steps prior to immunomagnetic separation and, ultimately, PCR detection of *L. monocytogenes*.
genes in ham. Lucore et al. (42) also used two centrifugation steps in conjunction with metal hydroxide immobilization of the target bacteria.

Centrifugation is a brief procedure (taking 5 to 30 min) that can reduce the time required for cultural enrichment from days to only hours. The initial removal of supernatant usually significantly reduces the total sample volume by 50 to 90%, thereby concentrating bacteria. The addition of gradient buffers used for BDC has successfully separated and concentrated bacteria from food samples and has been used to improve rapid detection methods (32, 39–41). Unfortunately, centrifugation does have limitations when it is used with food samples. Centrifugation tends to concentrate food debris along with bacteria. Multiple steps alternating between centrifugation and washes are often required to remove food debris, which can reduce bacterial concentration, prolong the sample pretreatment, and limit compatibility with automated on-line systems.

**Filtration.** The separation of bacteria from a food matrix by filtration is based on differences in the size of the bacteria and the sizes of other molecules in the food matrix. The passage of liquid suspensions through a filter via positive or negative pressure traps larger particles (relative to the pore size of the filter membrane). Depending on the filter pore size and the application, target bacteria are either captured on the filter or allowed to pass through while the filter retains larger particles such as food debris.

Bacteria have been washed from filters after capture. Kroll (31) used electropositively charged filters with pore sizes of 2 to 9 µm to separate bacteria from vegetables and milk samples. The filters collected 80 to 90% of the bacteria from the milk and vegetable samples, respectively. However, the rate of recovery of bacteria from the filters was poor and required a change of pH and backwashing techniques. Vacuum filtration has been used to separate bacteria from extracted ATP via the passage of milk samples through 0.8-µm filters (72). Ringer’s solution (600 µl) was used to wash the bacteria from the filters prior to ATP detection.

Starbuck et al. (75) reported on a filtration method that used 0.2-µm-pore-size filters to separate and concentrate L. monocytogenes from milk samples. After the milk samples had been passed through with a syringe, the 13-mm-diameter filters were dissolved in CHCl₃ and then centrifuged (15,000 × g, 1 min) to help concentrate the L. monocytogenes. This sample pretreatment method, which also included 20 min of sample incubation at 50°C in trypsin and Triton X-100, allowed the detection of 1.0 × 10⁻¹ CFU of L. monocytogenes per ml in 30-ml milk samples via PCR in <30 min. Unfortunately, the single use of each filter and the need to dissolve the filter to recover bacteria limits this method’s use in an automated on-line system.

In many applications, simple filtration has been used prior to other separation and concentration steps and/or rapid detection methods to remove large food particles. Tu et al. (78) used simple filtration of a diluted beef sample through glass wool to remove large fat globules and meat particles prior to the immunomagnetic separation of E. coli O157:H7 and ATP detection. Unfortunately, filtration for the removal of large food particles may also remove some bacteria, decreasing the amount of bacteria available for detection.

Venkateswaran et al. (83) employed a two-step filtration technique to remove inhibitory components in homogenized beef samples prior to PCR detection. Following cultural enrichment for 0 to 18 h, two filtration steps were used to remove PCR-inhibitory substances from inoculated beef homogenate samples. The first filtration step, involving a 5-µm-pore-size Ultrafree filter tube (Millipore, Bedford, Mass.), removed food particles and other particulate matter, along with 8 to 10% of the bacteria in the sample. The filtrate from this first filtration step was then filtered through a 0.2-µm-pore-size Ultrafree filter tube (Millipore) to capture bacteria. The bacteria captured on the 0.2-µm-pore-size filter were resuspended in 400 µl of sterile phosphate-buffered saline (PBS) and detected by PCR amplification.

PCR detection was not possible after the first filtration step. Although filtration with the 5-µm-pore-size filter tube removed food debris, the 0.2-µm-pore-size filtration step was required to separate the target bacteria from PCR-inhibiting compounds and significantly improved the sensitivity of the PCR assay. The two-step filtration process allowed the detection of E. coli at levels as low as 1.0 × 10² cells per g after only 6 h of enrichment. Following an 18-h enrichment period, E. coli levels as low as 1.0 × 10⁰ CFU/g could be detected by PCR.

Filtration has commonly been used to effectively enrich bacteria in short periods, greatly improving the sensitivity of rapid methods. Filtration steps take 1 to 10 min, depending on the size of the sample to be filtered and the rate at which the sample can be pumped through the filter. Unfortunately, filtration as a means of separating bacteria from food samples has disadvantages. Food debris clogs filters porous enough to allow bacteria to pass through or becomes concentrated along with bacteria on filters with pores smaller than bacteria. Extensive cleaning and back-flushing of filters can remove clogged food debris so that filters may be reused. However, these additional steps also remove any trapped bacteria and increase processing time, diminishing the practical use of filtration in automated rapid separation and concentration methods.

**Ultrasound.** When cells in suspension are placed in an ultrasonic standing wave, the cells experience forces that move them to preferred regions within the field at half-wavelength intervals. The cells also experience ultrasonically induced attractive or repulsive interparticle forces (15, 56). These forces and the subsequent movement of cells in suspension lead to the formation of high-concentration cell clusters. Relative motion between the fluid and the field (via fluid flow) or electrical modulation can be used to separate or fractionate the cell clusters from the suspension.

Coakley (14) reviewed the ultrasonic concentration and separation of cells from suspension, as well as the use of ultrasound to enhance aqueous polymer two-phase separation and latex-agglutination tests. Researchers (24, 25, 37, 38, 52) have carried out ultrasonic separation of microor-
organisms from suspensions. Unfortunately, the aim of their research was the concentration and ultimate separation of bacteria in food samples with the use of ultrasound. Therefore, no specific information on the ability of ultrasound to lower the bacterial detection limit of a particular detection method was reported.

Hawkes and Coakley (24) achieved collection efficiency levels exceeding 99% by using a continuous-flow ultrasonic sedimentation filter to remove yeast from suspension. Limaye and Coakley (37) used ultrasound to separate and concentrate both Saccharomyces cerevisiae yeast cells and E. coli cells from bacterial suspensions in PBS. Cells were concentrated in vertical planes parallel to the transducer face at half-wavelength intervals after 30 s of continuous ultrasound exposure. The ultrasound was then pulsed to allow the concentrated cells to settle prior to separation from the supernatant.

The ultrasound concentration-separation method was rapid and efficient. More than 99% of both yeast cells and E. coli cells were collected from suspensions with initial concentrations of $3.0 \times 10^6$ and $1.3 \times 10^{11}$ CFU/ml, respectively. Yeast collection required only 4.5 min, while E. coli separation took 11.5 min. Miles et al. (52) used ultrasound to concentrate E. coli K12 cells in diluted milk samples. A resonant chamber was constructed from a clear plastic cuvette. Manipulation of the ultrasound allowed standing waves to form through the resonant chamber and allowed suspended particles to be concentrated into bands at half-wavelength intervals along the length of the cuvette.

Ultrasonic bacterial separation and concentration have been accomplished in as little as 5 to 20 min and provide excellent bacterial recovery. For past applications, nearly complete (99%) bacterial recovery from PBS solutions has been reported (37). Ultrasonic concentration of bacteria is a simple procedure that can easily be automated, requiring only a sonication chamber, an ultrasound source, and a reflector. However, the study and use of ultrasonic separation and concentration of bacteria from food samples to improve rapid detection methods has been limited. Initial results suggest that ultrasonic techniques could be developed to separate and concentrate bacteria from food in order to improve the sensitivity and overall speed of rapid detection methods. Automated ultrasound separation and concentration apparatuses that can handle large-volume samples containing food debris need to be investigated.

** ADSORPTION METHODS **

Adsorption methods for the separation and concentration of bacteria from food samples rely on some type of biosorbent matrix. Biosorbent matrices consist of an affinity agent (i.e., an antibody, bacteriophage, or lectin) that is immobilized on a solid support and adsorbs to particular antigens (Ag) (i.e., Salmonella or E. coli). Numerous affinity agents, including antibodies (26), bacteriophages (6), and lectins (65), have been used to adsorb bacterial Ag. Several types of solid supports, including magnetic particles (17, 84), plastic or glass microspheres (12, 54), and agarose gels (60), have been used for biosorbent matrices. Metal hydroxides, which have also been used to rapidly enrich bacteria from food samples (42, 49), serve as both affinity agents and solid supports. Dielectrophoresis is a technique that captures bacteria on electrodes that also serve as both affinity agents and solid supports. The operation of a particular adsorption-based separation and concentration method is dependent on the affinity agent and solid support used. Therefore, the discussion of adsorption-based methods is divided into sections based on affinity agents.

** Antibody-based methods. ** Antibodies (Ab) are proteins that are produced in response to the presence of an Ag as part of an immune response. An Ab is specific to a particular Ag and has the ability to bind to the specific Ag that stimulated its production (55). Ab are commonly immobilized on the surfaces of magnetic particles by passive adsorption (62). Passive adsorption is often conducted with a basic (pH 9.5 to 9.6) buffer solution and involves non-covalent hydrophobic interaction to bind antibodies to the solid support. Antibodies can also be covalently bonded to the solid surface with the use of groups already present on the solid surface (e.g., NH$_2$ or COOH) or preactivated solid phases. Ab-coated magnetic particles bind to target bacteria in a sample through reversible noncovalent interactions including electrostatic forces, hydrogen bonds, Van der Waals forces, and hydrophobic forces. Both the magnetic particles and the bound target organisms are then concentrated and separated from the sample and any potential food debris and/or background organisms with a magnet. The Ab-based method of separation and concentration is commonly referred to as immunomagnetic separation (IMS) (11, 62, 67, 77).

Tu et al. (78) detected pathogenic E. coli O157:H7 in beef hamburger with the use of IMS and ATP measurement. After 6 h of enrichment at 37°C, inoculated beef samples (0.9 ml each) were filtered to remove large particles prior to 10 min of incubation with ca. $10^5$ magnetic beads coated with anti–E. coli O157:H7 (Dynabeads, Dynal Inc., Oslo, Norway). Following incubation, the magnetic beads were collected with a Dynal magnetic concentrator and resuspended in 0.9 ml of Trypticase soy broth for ATP analysis. Results indicate that the method demonstrated by Tu et al. (78) is capable of detecting $1.0 \times 10^2$ CFU/ml of E. coli O157:H7 in beef hamburger samples in <8 h.

Li et al. (36) achieved a detection limit of $4.0 \times 10^5$ CFU/ml for L. monocytogenes and Salmonella Typhimurium in milk samples by combining IMS with PCR amplification and a slot blot assay. IMS was successfully used to separate and concentrate bacteria from inoculated milk samples prior to PCR amplification. IMS reduced the detection limit for L. monocytogenes and Salmonella Typhimurium from $1.0 \times 10^3$ CFU/ml to level as low as $4.0 \times 10^3$ CFU/ml. Similar sensitivity (a detection limit of $4.0 \times 10^1$ CFU/ml) was achieved for both PBS and milk samples, indicating that the IMS method was capable of removing bacteria from PCR-inhibiting substances known to be present in milk (88).

Hudson et al. (26) used IMS to directly separate and concentrate L. monocytogenes from ham samples prior to detection by PCR. IMS was used on the basis of the com-
commercial availability of anti-*Listeria* magnetic beads (Dynal Inc.) to avoid time-consuming cultural enrichment steps and to remove PCR inhibitors.

Results indicate that bacteria recovery levels were reduced by 50% with each wash cycle; therefore, only a single wash step was used after the magnetic concentration of the bead-bacteria complexes. Recovery levels of only 10 to 20% were realized, but these levels provided enough bacteria for the PCR detection method to be successful. PCR detection together with IMS was capable of detecting as little as $1.1 \times 10^0$ CFU/g in a 25-g sample of inoculated ham. Although bacterial recovery levels were low for IMS, PCR detection without the IMS step, with the use of only double centrifugation of homogenized ham samples, was not successful owing to the presence of PCR inhibitors.

Another promising method for direct bacterial separation and concentration involves the pumping of a suspension of target Ag through a solid support matrix that is coated with immobilized Ab. Agarose gel or plastic microspheres that can be affixed inside a column or flow cell are commonly used as biosorbent solid supports. Ag bind to Ab immobilized on the surface of the solid support as the liquid sample is passed through the solid phase, which often takes the form of a pack bed reactor (immunoreactor). Any remaining portion of the liquid sample is removed with a washing step. Subsequent washing steps remove any unwanted particles from the immunoreactor bed before Ag is eluted into a clean solution, effectively separated and concentrated from the food sample and ready for analysis. These flow-through immunoseparation (FIS) systems offer accelerated binding kinetics due to very high surface area-to-volume ratios in the reactor bed and the use of flow streams to bring Ag into contact with Ab. These factors enhance the Ag-Ab encounter rates during the short residence time (min) samples spend in an immunoreactor (66).

FIS systems have been used mainly for the detection of micromolecules such as pesticides and/or proteins in small ($\leq 100$-$\mu l$) sample volumes containing no interfering particles (e.g., food debris). These methods use pack beds made of micron-diameter beads or porous membranes that offer great surface area-to-volume ratios for the promotion of Ag-Ab encounters and subsequent binding (1, 2, 7, 19, 43, 81). However, many of these same FIS immunoreactors foul rapidly in the presence of food debris without any sample preparation (i.e., filtration or centrifugation) (1, 2, 48, 64, 71, 74, 87).

Bouvertre and Luong (12) employed FIS in conjunction with an enzymatic reaction to selectively detect *E. coli* in different homogenized food samples (powdered milk, basil, ham sandwich, and Camembert cheese samples) diluted in a nutrient broth. *E. coli* was successfully detected in all four food samples, indicating that the Ab separation-concentration step was successful in removing bacteria (provided the initial concentration of bacteria in the sample was $>1.0 \times 10^7$ CFU/ml) from interfering food components and background microorganisms. A significant feature of this work was the repeated use of the Ab column, which differs from most applications of IMS, in which Ab-coated solid phases are only used once (36, 86). The Ab column was reused $>300$ times as a result of the successful desorption of the bound *E. coli* by 0.1 M glycine and hydrochloric acid (pH 2.5) buffer. The acidic buffer reverses the weak forces binding Ab to Ag, thereby reclaiming the active Ag-binding sites on the Ab. Molloy et al. (54) used an FIS method involving Ab fragments against *Pseudomonas aeruginosa* as an affinity agent to construct an Ab column capable of separating *P. aeruginosa* from milk samples. The modification of Ab fragments through protein engineering can increase their stability in nonphysiological environments (22, 69), which may result in more robust proteins for immobilization on solid supports and in food samples of various compositions and pHs.

SPC results for collected bacteria demonstrated excellent binding of *P. aeruginosa* to the solid phase of the Ab column. Seventy-five percent of $2.4 \times 10^6$ cells were captured by the Ab columns. Only 20% of the *P. aeruginosa* cells were nonspecifically bound to the control columns, which did not contain immobilized Ab against *P. aeruginosa*. The *P. aeruginosa* capture rate for buffer solutions was higher (95%) than that for milk, with only 10% nonspecific binding occurring in control columns. Molloy et al. (54) postulated that the reduction in the number of *P. aeruginosa* cells captured in the milk samples was due to the coverage of Ab-binding sites by the larger protein molecules in milk samples. Unfortunately, as shown by Molloy (54), food components can cause problems with Ab-based separation and concentration methods, interfering with Ab capture and thus leading to a reduction in the amount of target bacteria directly recovered from food. Despite the presence of native protein in the milk samples (milk is 9% soluble protein), the Ab columns were able to effectively separate a majority of the bacteria from the food sample and produce an enriched bacterial sample without the need for time-consuming microbial growth.

Weimer et al. (86) used their patented ImmunoFlow system, which consists of a high-flow-rate (1, 2, and 7 liters/min) fluidized bed of large glass beads (3 mm) coated with Ab. Each fluidized solid phase (reactor bed) can handle larger-volume samples but is effective for only a single use. The ImmunoFlow system has been effectively used to remove and detect *E. coli* from PBS–TWEEN 20 solution (PBST) and meat extracts via existing enzyme-linked immunosassay protocols. The capture of *E. coli* in the bead cartridge was demonstrated for both the PBST and the meat extract samples. The enzyme-linked immunoassay response increased with an increase in the initial number of cells inoculated into the PBST and meat extract samples up to levels of $1.0 \times 10^3$ and $1.0 \times 10^4$ CFU/ml, respectively.

Ab-based methods have become very popular means of rapid separation and concentration. Specifically, IMS, which has been shown to separate bacteria from food samples in as little as 10 min (36) and reduce detection limits to approximately $1.0 \times 10^0$ CFU/ml or $1.0 \times 10^0$ CFU/g of sample (26, 78), is widely used. FIS systems can also achieve separation and concentration in minutes. The use of active diffusion to promote encounters between Ab and target bacteria significantly reduces incubation periods. FIS systems are also able to handle large sample volumes,
which are pumped through an immunoreactor instead of merely being mixed with the biosorbent material.

However, food debris in a sample can interfere with the Ab capture of bacteria by blocking or fouling Ab-binding sites on magnetic particles or other solid supports. For FIS systems, food debris can foul microporous immunoreactor beds, resulting in the need to replace the immunoreactor after only one use. Immunoreactors that can handle large-volume food samples without fouling and can be used repeatedly are important for the development of automated on-line rapid separation and concentration. Unfortunately, the development of immunoreactions possessing these attributes is just beginning (86).

**Bacteriophage-based methods.** Bacteriophages (phages), viruses whose hosts are bacterial cells, are well used in strain-specific methods for the detection and identification of bacteria (79). Bioluminescent detection of bacteria has been achieved with the use of recombinant phages containing lux gene constructs that infect target bacteria and induce bioluminescence (73). Similarly, ice nucleation gene constructs have also been used in recombinant phages to detect target bacteria (89, 90). A phage amplification assay based on the phage lytic cycle has been used to selectively detect bacteria with cultural media (76).

Phage-based separation and concentration exploits the ability of a phage to identify and bind to a specific strain of bacteria. The binding of a phage to a bacterial cell is achieved by tail fibers, or by some similar structure on phages without tail fibers. The tail fibers attach to specific receptor sites on the bacterial cell (e.g., proteins on the outer membrane surface of the bacterium). The ability of a phage to absorb to specific bacteria makes possible phage-based biosorbents, which can be used in separation-concentration systems similar to Ab-based biosorbent systems. Upon introduction into a food sample, the phage-based biosorbent would capture any bacterial cells via the phage-cell attachment (6). The biosorbent could then be extracted from the food sample via centrifugation or magnets, depending on the biosorbent construction material, and the target bacteria would be removed with it.

Bennett et al. (6) constructed a phage-based biosorbent specific to *Salmonella* with the use of the sapphire lytic phage (Amersham International, Amersham, UK). The sapphire lytic phage (sapph) was immobilized on both polystyrene microtiter plates and dipsticks. *Salmonella* captured in the microtiter plates was detected by a BAX (Qualicon LLC, Wilmington, Del.) PCR system. Epifluorescent microscopy was used to detect captured *Salmonella* on the dipstick biosorbent after the dipsticks had been immersed in acridine orange (Difco) for 5 min. The total detection time for the dipstick method required <2 h 15 min, while the microtiter plate method took <15 min. The time difference between the two methods was due to incubation periods of 2 h for the dipsticks and 10 min for the microtiter plates.

The microtiter phage biosorbent was successful in capturing *Salmonella* from bacterial cultures even in the presence of a competitor organism (*E. coli*). Unfortunately, initial concentrations of *Salmonella* needed to be ≥1.0 × 10^7 CFU/ml for post-PCR detection to be successful. Nonoptimized passive immobilization of the phage and the low surface area of the microtiter plates were two reasons suggested for the low collection efficiency level for the phage-based microtiter biosorbent. The dipstick biosorbent also demonstrated the phage-based capture of *Salmonella* in bacterial cultures. Phage dipsticks were consistently found to contain more absorbed *Salmonella* cells than the control dipsticks (free of immobilized phage).

The use of a phage biosorbent to selectively separate specific bacteria in the presence of competitor microorganisms in bacterial cultures has been demonstrated. The overall time to complete separation was 10 min to 2 h depending on the type of solid-phase biosorbent used. Bennett et al. (6) discussed some of the anticipated benefits phage-based separation-concentration has in food applications, including the high specificity of phage–target bacteria attraction. The ease and cost of producing phages and their ability to attach to specific bacteria in natural heterogeneous environments is another beneficial phage characteristic discussed by Bennett et al. (6). Nontarget substances such as food debris in the sample can affect performance. Given that phages exist naturally in such environments, their stability and resistance to food components should provide an enhanced ability to capture and directly separate bacteria as part of an automated rapid separation-concentration method. Unfortunately, the application of phage-based adsorption systems to food samples needs to be examined further, along with the identification of specific phages that are limited to only the pathogenic strains of a certain bacterium (e.g., *E. coli* O157:H7).

**Dielectrophoresis.** Dielectrophoresis (DEP) can be used to separate and concentrate bacterial cells in suspension with the use of a nonuniform electrical field polarization to induce forces on the cells (63). The forces induced on bacterial cells by the nonuniform electrical field cause the bacterial cells to migrate toward and adhere to the electrodes used to generate the nonuniform electrical field. DEP forces are dependent on the frequency and degree of nonuniformity of the electric field as well as the dielectric properties of the cells and the suspension medium.

Betts (10) discussed the potential of DEP for the rapid separation and concentration of bacteria in food samples. Specifically, this author discussed the advantage of using cells already present in food samples to improve the speed of new rapid detection methods, which are often dependent on time-consuming cultural enrichment to increase concentrations of bacteria to levels above detection limits and to remove interfering food components.

Markx et al. (45) carried out DEP separations of *Bacillus subtilis*, *Micrococcus luteus*, and *E. coli* from deionized water solutions with the use of a DEP chamber mounted below a microscope to facilitate real-time observation of the collection of bacteria. A typical DEP application would concentrate bacteria from a fluid (of relatively low conductivity) passing through the DEP chamber with the use of microelectrodes energized to capture the bacteria by
positive DEP (45). Following the capture of bacteria on the microelectrodes, the medium conductivity would be increased to elute the bacteria from the chamber, effectively separating the bacteria from the initial suspension. Results obtained by Markx et al. (45) suggest that E. coli can be separated from M. luteus or B. subtilis with the use of various medium conductivities. Suspensions of both B. subtilis and M. luteus were selectively enriched but not necessarily completely separated from one another.

In several other studies, DEP has been used to concentrate and separate various types of cells, including mammalian cells (21, 85) and microorganisms (46, 47). However, DEP has not been readily applied to bacteria in complex food matrices, for which rapid separation and concentration is critical to reducing the need for time-consuming cultural enrichment. DEP separation-concentration of bacteria from food matrices is more complicated than that for bacterial suspensions in homogeneous buffered solutions. Complex food matrices present potential problems that need to be addressed for DEP to be effective as a separation-concentration method. The high conductivity levels for food matrices and food debris interfere with the efficiency of DEP, limiting its expanded use as a means of separating and concentrating bacteria in foods (10).

Lectin-based methods. An alternative biological agent with affinity for Ab or phage is lectins. Lectins are sugar-binding proteins of nonimmune origin with affinity for particular cell membranes. A lectin contains at least two sugar-binding sites that allow it to bind to sugars (monosaccharides or oligosaccharides) on cell membranes. Numerous researchers have used lectin-based biosorbents, with magnetic beads commonly being used as solid supports, to separate and concentrate bacteria from bacterial cultures, food samples, and environmental samples (23, 58–60, 65).

Payne et al. (60) investigated four lectins for their ability to bind to various bacterial strains immobilized on different solid supports. The lectins were from Helix pomatia, Canavalia ensiformis, Agaricus bisporus, and Triticum vulgaris. Solid supports used included magnetic particles (2.8 \( \mu \)m in diameter; Tosyl-activated M280 Dynabeads, Dynal) and 10- to 40- \( \mu \)m magnetic particles with T. vulgaris lectin preattached (BioMag particles, Metachem Diagnostics, Northampton, UK). The other solid supports used to create lectin-based biosorbents were 6-mm polystyrene beads (Receptor Technologies, Leamington Spa, UK), 3-\( \mu \)m-pore-size 47-mm-diameter filter disks (Receptor Technologies), and Sepharose 4B and 6B gels (Amersham Biosciences) in mini columns.

All four lectins bound to Staphylococcus aureus, E. coli, Salmonella spp., and L. monocytogenes over a wide range of pHs (5.0 to 9.0). Binding was more rapid at room temperature (21°C) than at 4°C, and the use of competing substrates (i.e., glucose, mannose, N-acetyl glucosamine) demonstrated that lectin binding was specific and could be reversed. Binding between bacteria and lectins immobilized on a membrane filter, 6-mm polystyrene beads, and sepharose gels was achieved. However, reproducibility was very poor. The method involving lectins immobilized on magnetic particles was the most efficient, practical, and reproducible method for separating and concentrating bacteria from buffers in an overall time of ca. 3 h.

SPC results revealed that T. vulgaris lectin immobilized on magnetic particles bound 87 to 100% of L. monocytogenes cells and 80 to 100% of S. aureus cells in buffer solutions over a range of concentrations (1.0 \( \times \) 10\(^3\) to 1.0 \( \times \) 10\(^6\) CFU/ml). Incubation with a competing substrate (N-acetyl glucosamine) resulted in the release of 51 to 60% of the bound bacteria. Similar results were obtained by SPC with gram-negative bacteria. Thirty-three to 45% of bound Salmonella spp. and 77% of bound E. coli were released. A. bisporus lectins bound 31 to 63% of L. monocytogenes cells and 83% of S. aureus cells but only 2 to 5% of Salmonella cells. Lectin from H. pomatia immobilized on magnetic beads bound 92% of S. aureus cells and 64% of L. monocytogenes cells. H. pomatia was also poor at binding to gram-positive bacteria.

Payne et al. (60) also used a lectin-based method to separate bacteria from skim milk, low-fat milk, whole milk, egg, and ground beef samples. Samples were incubated with lectin-coated magnetic particles for 3 h at 4°C. SPC were carried out for supernatant fluid, wash fluids, and release substrates to quantify bacterial capture. The results obtained illustrate the value of lectin-based magnetic separation of bacteria from food samples. T. vulgaris lectin-coated magnetic particles separated 43% of L. monocytogenes cells and 26% of S. aureus cells from diluted milk samples and 31 to 54% of Salmonella cells from raw egg samples. A. bisporus lectin-coated magnetic beads separated 10 to 47% of L. monocytogenes cells from undiluted milk and 32 to 50% of L. monocytogenes cells from ground beef samples.

Payne et al. (59) expanded on this work involving food samples by using lectins from T. vulgaris and A. bisporus immobilized on magnetic particles to improve the detection of Salmonella Enteritidis, L. monocytogenes, and S. aureus in enriched samples of inoculated ground beef, raw egg, braising steak, and Camembert cheese by SPC. The lectin-coated magnetic particles were effective in separating bacteria from the food samples, yielding cleaner bacterium samples that were free of food debris. However, the detection limits for the bacteria were not lowered (indicating a lack of bacterial concentration) except that for Salmonella Enteritidis in beef samples. Lectin magnetic separation increased the detection level for Salmonella Enteritidis in beef samples by 2 log units in ca. 2 h. Overall detection times were 6 to 18 h. The use of time-consuming cultural enrichment was reduced but not eliminated with the use of lectin-based separation.

Grant et al. (23) isolated Brochothrix from beef homogenate with the use of lectins from A. bisporus immobilized on magnetic beads (Dynabeads). PCR products were not obtained directly from beef homogenate samples. Lectin-based separation was used to remove Brochothrix from the beef samples to allow PCR detection.

The lectin-coated magnetic beads captured 78% of the Brochothrix cells in the beef samples, and 92% of the captured cells were successfully eluted with fetuin. Visible
PCR products were generated with the use of the lectin-concentrated cells from beef samples. However, fetuin appeared to inhibit PCR, and results were less intense than those for control samples (bacterial cultures instead of food samples) with similar cell concentrations.

Lectin-based separation-concentration has been shown to be a very effective means of rapid separation and concentration. The typical incubation periods required to bind bacteria to lectin-coated magnetic particles (2 to 3 h) are two to six times as short as those for cultural enrichment, which normally requires a minimum of 8 h. Unfortunately, lectins need to be selectively isolated for their affinity for specific bacteria, which makes them more difficult to work with than antibodies. The solutions used to release bound bacteria from lectin-coated magnetic beads (fetuin) have also been shown to inhibit PCR detection.

**Metal hydroxide-based methods.** An alternative to a solid matrix, which can be manipulated magnetically, involves particles (i.e., metal hydroxides) that are readily separated by centrifugation after binding between the affinity agent and Ag has occurred owing to the density of these particles (42, 49). Metal hydroxides serve as both an affinity agent and a solid support; immobilization of a separate affinity agent is not required. First described by Kennedy et al. (29), the immobilization of bacteria on metal hydroxides is believed to be caused by covalent bonds between hydroxyl groups of the metal hydroxide and amino acid ligands on the surfaces of bacterial cells. Kennedy et al. (29) also established that some bacterial and yeast cells (E. coli and S. cerevisiae) remain viable, continuing to respire, while immobilized on titanous hydroxide or zirconium hydroxide particles.

Immobilization with metal hydroxides was applied as a pretreatment method for the detection of bacteria in culture media, foods, and clinical samples by Ibrahim et al. (27, 28). This method was successful in separating the bacteria from interfering compounds that caused nonspecific binding in the immunoassays and in reducing the detection limit 100-fold. The ability of metal hydroxide to concentrate bacteria in food samples has been exploited by several researchers in recent years. Berry and Siragusa (8) used metal hydroxides (specifically, hydroxyapatite) to concentrate bacteria in meat slurries. McKillip et al. (49) investigated metal hydroxide immobilization for the concentration of both viable and nonviable E. coli O157:H7 cells from skim milk samples.

McKilip et al. (50) expanded on this work with metal hydroxides through research comparing pretreatment methods for the PCR detection of E. coli O157:H7 in various dairy products. The pretreatment methods consisted of solvent-based DNA extraction directly from food samples and bacterial concentration with titanous hydroxide followed by DNA extraction with guanidinium isothiocyanate solution. Bacteria were then detected in the bacteria-hydroxide pellet by DNA extraction and PCR amplification or by cultural enrichment on Trypticase soy agar pour plates and sorbitol MacConkey agar streak plates. Overall, the five-step procedure took approximately 25 min to complete.

Bacterial recovery rates were >50% of the initial concentrations in all cases after bacterial concentration with titanous hydroxide. The highest recovery rates were those for samples of whey powder (93% ± 1.8%). Recovery rates for skim milk and cheese samples were lower (71% ± 1.4% for skim milk, 68% ± 1.2% for Cheddar cheese, and 59% ± 2.1% for Brie cheese). The results obtained indicate that the final detection limits for the titanous hydroxide concentration method were 1.0 × 10^{3}, 1.0 × 10^{2}, 1.0 × 10^{1}, and 1.0 × 10^{4} CFU/ml for skim milk, Brie cheese, Cheddar cheese, and whey powder, respectively. McKillip et al. (50) listed the reduced number of steps, the limited use of organic solvents, and the ability to assess bacterial recovery and viability by plate counts as advantages of the titanous hydroxide concentration method over direct DNA extraction.

Lucore et al. (42) also demonstrated the ability of metal hydroxide immobilization to concentrate bacterial cells from dairy foods prior to detection by cultural and molecular methods. Nonfat dairy milk (NFDM), whole milk, and ice cream were used for food samples.

Bacteria were concentrated from the food samples and pure cultures with two steps. The first concentration step consisted of clarification (hand mixing with 1.4% sodium citrate for 5 min at room temperature) and high-speed centrifugation (10,000 × g, 10 min, 7 °C). The bacterial pellet was then resuspended in 3 ml of sterile saline buffer. The second concentration step consisted of immobilization with metal hydroxide (10 min of incubation) and low-speed centrifugation (500 × g for zirconium hydroxide and titanous hydroxide and 300 × g for hafnium hydroxide, 10 min, 7 °C).

The two-step concentration method was able to reduce the 25-ml samples of NFDM 50-fold (to 500 μl) in ca. 40 to 45 min. Bacterial recovery rates after the primary concentration step ranged from 78 to 98%, and recovery rates after the second concentration step ranged from 51 to 98%. The overall metal hydroxide concentration method was considerably more efficient and consistent in concentrating bacteria. The low-speed centrifugation results alone were variable and managed to recover at most only 36% of the bacterial cells. Bacterial recovery rates for the whole milk and ice cream samples were 92 to 97% and 61 to 95%, respectively. Again, low-speed centrifugation alone yielded erratic and low bacterial recovery rates of <50%.

In a second phase of the study by Lucore et al. (42), the specificity levels of different metal hydroxides (zirconium hydroxide, titanous hydroxide, and hafnium hydroxide) for different strains of bacteria were investigated with the use of several representative strains of bacteria (E. coli, L. monocytogenes, Salmonella Enterica, P. aeruginosa, Lactococcus lactis, and Bacillus cereus) in growth media. Bacterial recovery rates for zirconium hydroxide, titanous hydroxide, and hafnium hydroxide were 88 to 100%, 89 to 98%, and 70 to 97%, respectively. A comparison of recovery rates showed that all three metal hydroxides were capable of immobilizing various levels of all eight strains of bacteria; however, zirconium hydroxide provided the most consistent results.
The immobilization of bacteria with metal hydroxides has been shown to improve the recovery of bacteria from food samples, with bacterial recovery rates being as high as 100% for small diluted samples. Metal hydroxide concentration requires \(<1\) h and can be applied for the concentration of a variety of pathogenic bacteria prior to the detection of these bacteria by rapid methods. However, one drawback of this method, as discussed by Lucore et al. (42), is its inability to separate the bacteria entirely from food components because of its reliance on centrifugation. Food containing large particles would be concentrated along with the metal hydroxides during the centrifugation steps and could still interfere with or inhibit several types of rapid detection methods, including PCR.

**CONCLUSIONS**

Conventional methods for the detection and enumeration of bacteria in foods are time-consuming and labor-intensive, rendering them ineffective as primary means for ensuring food safety under HACCP programs. Modern advanced detection techniques are more sensitive, selective, and rapid than conventional techniques. Unfortunately, these modern techniques still have many limitations, including inhibition caused by food constituents, which renders these methods dependent on slow cultural enrichment and vulnerable to food debris interference. The need for alternative separation and concentration methods that will rapidly separate and concentrate bacteria from food samples, thereby reducing the time required for advanced diagnostic techniques, is evident.

Progress toward the real-time, on-line screening of food for pathogenic organisms requires a system that can automatically separate and concentrate bacteria in seconds from sizeable food samples (liters) without damaging the food samples. Several methods discussed here have successfully been used, individually or in combination with other methods, to rapidly separate and concentrate bacteria from food samples in far less time than is required for cultural enrichment. Typically, cultural enrichment requires at least 8 h. Some noncultural enrichment methods described here require as little as 5 to 10 min, which is a significant improvement over time-consuming cultural enrichment.

Unfortunately, many of the separation and concentration methods described in this review have limitations that render them unsuitable in their present form for use in an automated HACCP program requiring the real-time detection of pathogenic organisms in food. For example, filters are clogged by large particles in food matrices, while the application of DEP can be limited by high-conductivity food matrices. Centrifugation has limited compatibility with on-line systems and large sample volumes (16), and ultrasound has been applied only in limited research and only to very small sample volumes. Aqueous polymer two-phase separations concentrate some bacteria with food particles at the polymer interface and require the use of dilution buffers and polymer mixtures, which limits compatibility with on-line systems. Adsorption methods also have limitations. Lectins and phage-based separation-concentration systems require the selective isolation of these adsorption agents for their affinity for specific bacteria, which makes their application difficult. Metal hydroxide–based methods have relied on centrifugation, which precludes 100% separation from food debris and limits compatibility with on-line systems. Antibody-based methods are also limited by food debris, which can block affinity binding sites and clog microporous solid support matrices. Therefore, there is still a need for further research on and development of rapid separation and concentration methods in order to progress toward real-time detection of pathogenic organisms in food to improve food safety.

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**REFERENCES**


