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Modified Immunoliposome Sandwich Assay for the Detection of *Escherichia coli* O157:H7 in Apple Cider

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ABSTRACT

Detection of *Escherichia coli* O157:H7 in fruit juices such as apple cider is necessary for diagnosis of infection and epidemiological investigations. However, inhibitors in the apple cider, such as endogenous polyphenols and acids, often decrease the sensitivity of PCR assays and immunoassays, thus routinely requiring laborious cell separation steps to increase the sensitivity. In the current study, polyethylene glycol (PEG)–derivatized liposomes encapsulating sulforhodamine B were tagged with anti–*E. coli* O157:H7 antibodies and used in an immunoliposome sandwich assay for the detection of *E. coli* O157:H7 in apple cider. Even without prior separation, this assay can detect *E. coli* O157:H7 in apple cider samples inoculated with as few as 1 CFU/ml after an 8-h enrichment period. The lower limit of detection in pure cultures without enrichment was 7×10^3 CFU/ml (280 CFU/40-μl sample). PEGylated immunoliposomes are suitable as an analytical reagent for the detection of *E. coli* O157:H7 in fruit juices containing polyphenols.

Escherichia coli O157:H7 *(17)*, an emerging foodborne pathogen causing hemorrhagic colitis and hemolytic uremic syndrome, has been found in highly acidic fruit beverages such as unpasteurized apple juices *(3)* and apple cider *(4)*. Conventional culture methods for the detection *E. coli* O157:H7 typically involve use of indicators for enzyme activities, such as sorbitol fermentation, and may take more than 48 h to complete. Rapid detection using PCR and immunoassays in fruit juices requires prior separation of the pathogen from the fruit juice *(5, 22)* because polyphenols endogenous to fruit juices can form complexes with analytical reagents such as proteins and nucleic acids that are used in immunoassays and PCR, respectively *(13)*, interfering with the detection. However, inclusion of an immunomagnetic or other separation step into these detection methods increases the overall workload and analysis time. Lateral flow assays based on enzyme-substrate reactions and porous filter-membranes have been developed for the detection of specific pathogens *(1, 10)*. By using capillary migration of a mixture of the wicking reagent and a sample on the membranes, the lateral flow assays accelerate the antigen-antibody binding reaction and simplify cleaning procedures, thereby significantly decreasing the assay time.

We previously reported the use of immunoliposomes, i.e., antibody-tagged liposomes, encapsulating sulforhodamine B (SRB) in a field-portable immunomigration strip assay for the rapid screening of *E. coli* O157:H7 in buffer *(16)*. The assay could be completed in as little as 8 min and had a sensitivity comparable to that of standard enzyme-linked immunosorbent assays. The speed and sensitivity of the assay were based on the use of capillary migration on a nitrocellulose (NC) strip in combination with signal amplification and detection based on the instantaneous measurement of SRB in the immunoliposomes in the antibody capture zone rather than a time-dependent enzyme-substrate reaction.

In this study, the applicability of this assay system was tested using *E. coli* O157:H7–inoculated apple cider. For this purpose, we modified the immunoliposome preparation method and the design of the immunomigration test strip. First, succinimidyl-*S*-acetylthioacetate (SATA) was used to thiolate antibodies to make stable immunoliposomes *(23)*. Second, polyethylene glycol (PEG)–derivatized lipids were inserted to coat the surface of the lipid bilayers of immunoliposomes, thereby protecting the liposomes from the nonspecific adsorption of polyphenols endogenous in apple cider. A new immunomigration strip was designed to have two complementary measurement zones (Fig. 1) to assure the test results. In the improved assay system, a mixture of the wicking reagent and a sample containing *E. coli* O157:H7 migrates along the NC strip by capillary action until it reaches the top of the test strip. During this migration, *E. coli* O157:H7 cells if present are retained in the analyte capture (AC) zone, in which goat anti–*E. coli* O157:H7 antibodies have been immobilized, and immunoliposomes containing SRB in turn bind to the captured *E. coli* O157:H7, giving rise to an intensely colored band.

MATERIALS AND METHODS

Materials. Distearoyl phosphatidylcholine (DSPC) and distearoyl phosphatidylglycerol (DSPG) were purchased from Avanti Polar Lipids (Alabaster, Ala.). Distearoyl phosphatidylethanolamine–polyethylene glycol (2000)–maleimide (DSPE-PEG-maleimide) was purchased from Shearwater Polymers (Huntsville, Ala.). Cholesterol, novobiocin, 4-methylumbelliferone-β-D-glu-

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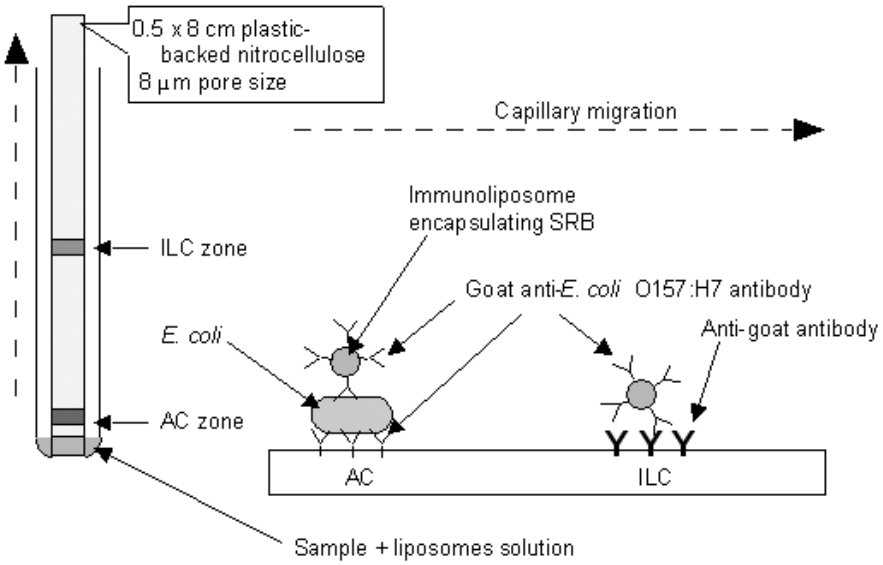

FIGURE 1. *Immunoliposome sandwich assay.*

curonide (MUG), *N*-ethylmaleimide, bile salts, and K_2HPO_4 were from Sigma (St. Louis, Mo.). SATA and hydroxylamine hydrochloride were purchased from Pierce (Rockford, Ill.). *N*-((4-maleimidylmethyl)cyclohexane-1-carbonyl)-1,2-dihexadecanoyl-1-sn-glycero-3-phosphoethanolamine and SRB were from Molecular Probes (Eugene, Oreg.). NC membranes (mean diameter of pores, 8 μm) were purchased from Sartorius AG (Goettingen, Germany). Polycarbonate syringe filters of 3-, 0.4-, and 0.2-μm pore sizes were obtained from Poretics (Livermore, Calif.). Affinity-purified polyclonal antibodies (anti–*E. coli* O157:H7) were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, Md.). Rabbit anti-goat IgGs were purchased from Rockland (Gilbertsville, Pa.). *E. coli* O157:H7 strain 43889 was purchased from the American Type Culture Collection (ATCC; Rockville, Md.). Trypticase soy broth, modified EC medium (mEC), MacConkey sorbitol agar (MSA), and novobiocin were purchased from BD Diagnostic Products (Sparks, Md.).

Preparation of dye-encapsulated liposomes. Liposomes were prepared by the reverse-phase evaporation method from a mixture of DSPC, cholesterol, DSPG, and DSPE-PEG-maleimide in a molar ratio of 5:5:0.5:0.25 *(6, 14, 20)* as previously described *(16).*

Conjugation of antibodies to liposomes. SATA was used to acetylthioacetylate affinity–purified anti–*E. coli* O157:H7 antibodies from goat as described previously *(6).* The antibodies (2 mg/ml) were incubated with SATA at a molar ratio of 1:10 for 20 min at room temperature under a nitrogen atmosphere. The derivatized antibodies were separated from unreacted SATA by gel filtration (Sephadex G-25) in phosphate-buffered saline (PBS, pH 7.5) containing 1 mM EDTA. The acetyl groups on the resulting antibodies were removed with hydroxylamine, and the extent of thiolation on the antibodies was estimated by the use of Ellman's reagent *(18).* Thiolated antibodies were covalently conjugated to the preformed maleimide-PEG–tagged liposomes encapsulating SRB by incubating at a ratio of 100 μg of antibodies to 1 μmol of maleimide-tagged liposomes for 2 h at room temperature and pH 7.5 (Fig. 2). Unreacted sulfhydryl groups were blocked by adding 100 μl of *N*-ethylmaleimide (8 mM in PBS) to 1 ml of the reaction product and incubating for 30 min at room temperature. Unconjugated antibodies and unreacted *N*-ethylmaleimide were removed by gel filtration on a Sepharose CL-4B equilibrated with Tris-buffered saline (TBS; 150 mM Tris, 100 mM NaCl, pH 7.0). The concentration of lipid in the resulting immunoliposome solution was determined using the Bartlett phosphorus assay *(2),*

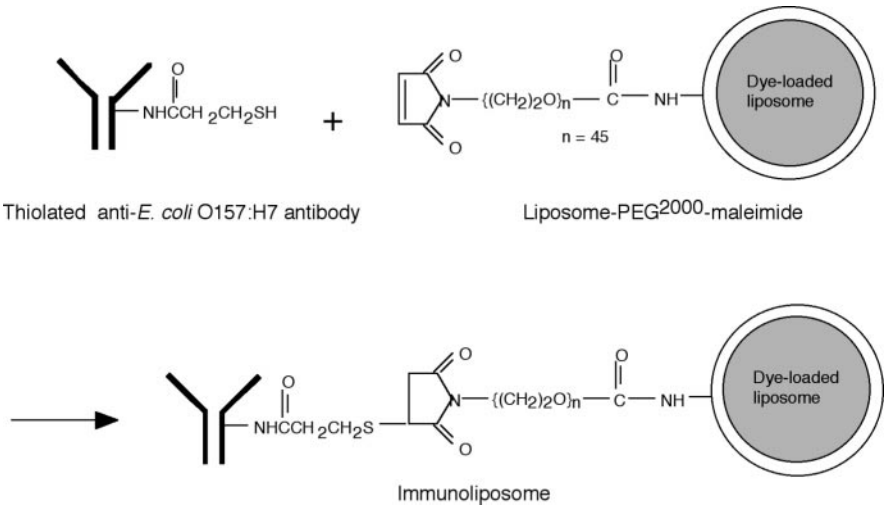

FIGURE 2. *Coupling of thiolated antibodies to maleimide-PEG–tagged liposomes encapsulating SRB.*

and the concentration of protein in the liposome solution was assayed using a modified protein assay *(9).* The average diameters of liposomes and immunoliposomes were measured by laser diffraction particle size analysis in an instrument with a microvolume module (LS-130, Beckman Coulter, Fullerton, Calif.) according to the manufacturer's instructions. The integrity of the immunoliposomes was determined by measuring SRB leakage during storage, whereas retention of the functional affinity or avidity of the immunoliposomes was assessed by measuring the amount of immunoliposomes capable of binding to the immunoliposome capture (ILC) zone in the presence of *E. coli* O157:H7 (10^6 CFU/ml in PBS).

Preparation of test strips. Test strips were prepared as previously described *(16, 20)* except for the configuration of the detection zones and the blocking solution. Acid-treated goat anti–*E. coli* O157:H7 antibodies (1 mg/ml in PBS, pH 7.0) were applied to NC membranes at a distance 0.5 cm from the bottom of the sheet (AC zone) using a microprocessor-controlled Linomat IV TLC sampler (Camag Scientific, Wrightsville Beach, N.C.), whereas anti-goat antibodies (0.7 mg/ml in PBS, pH 7.0) were applied to the membranes at a distance 2.4 cm from the bottom of the NC sheet (ILC zone) (Fig. 1). The membrane was treated with a blocking solution (0.33% polyvinlypyrrolidone, 0.06% nonfat milk, 0.03% gelatin in TBS, pH 7.0) as previously reported *(16).*

Assay protocol. The immunoliposome sandwich assay (ILSA) was performed by dispensing 40 μl of a sample solution and 30 μl of a diluted liposome solution (7×10^7 immunoliposomes per μl for pure cultures of *E. coli* O157:H7 or 2.1×10^8 immunoliposomes per μl for apple cider samples diluted in PBS, pH 6.8) into a glass test tube (10 by 75 mm), gently mixing the contents, and immediately inserting a test strip into the test tube. After the solution reached the end of the strip by capillary migration (about 8 min), the test strips were removed and air dried. The color intensity of each zone on the test strip was estimated visually and quantified by grayscale densitometry as described previously *(20).*

Assay sensitivity. The specificity of the ILSA was assessed by using pure cultures of *E. coli* O157:H7 strain ATCC 43885 grown at 37°C for 16 h in modified Trypticase soy broth supplemented with novobiocin (20 mg/liter), K_2HPO_4 (1.5 g/liter), and bile salts (1.5 g/liter). The culture suspension was centrifuged at $1,500 \times g$ for 10 min, and the cell pellet was resuspended in 0.01 M PBS (pH 6.8) at an optical density (at 640 nm) of 0.5 ($\sim 10^8$ cells per ml) *(15).* The *E. coli* cell suspension was serially diluted (1:10) with PBS to 10^3 cells per ml, and 40 μl of each serial dilution was added in triplicate to ILSA test tubes. Negative controls contained PBS in lieu of *E. coli.* The cell number in each dilution was confirmed by culturing 100 μl of each serial dilution on MSA culture plates containing MUG (0.1 g/liter) at 37°C for 16 h.

ILSA of apple cider. Triplicate samples (25 ml each) of pasteurized unfiltered (turbid) apple cider (pH 3.7) purchased from a local supermarket in Geneva were inoculated with *E. coli* O157:H7 at levels of 1 to 10^5 cells per 25 ml. The concentration of *E. coli* O157:H7 in the inoculum was confirmed by the procedure described above. Uninoculated samples of apple cider were included as negative controls. Apple cider samples (25 ml each) were added to 225 ml of mEC medium supplemented with novobiocin (20 mg/liter) in culture flasks and incubated at 37°C on a rotary shaker (120 rpm) for 8 h. Aliquots (40 μl) of the resulting cultures were subsequently tested according to the assay protocol described above. In addition, 0.1-ml decimal dilutions of the 8-h cultures were prepared in 0.1 M PBS and plated onto MSA-MUG culture plates. The plates were incubated at 37°C for 16 h, and the number of colonies that were sorbitol negative and MUG negative were recorded.

TABLE 1. *Characteristics of liposomes*

Characteristic	Value
Diameter of liposomes (mean ± SD)	266 ± 66.2 nm
Diameter of immunoliposomes (mean ± SD)	269 ± 77.5 nm
Liposome concentration	1.7×10^9/μl
Concentration of SRB in liposomes	150 mM
SRB content	8.5×10^5 molecules/liposome
Maleimide surface density	$\sim 2.3 \times 10^5$ molecules/liposome
Antibody surface density[a]	~170 molecules per liposome
Stability and retention of binding avidity[b]	≥6 months

[a] Measured by the method of Kaplan and Pederson *(9).*
[b] Stored at 4°C in the dark.

RESULTS

Immunoliposome characterization. DSPE-PEG-maleimide liposomes prepared by the reverse-phase evaporation method had an average diameter of 266 nm and encapsulated $8.5 \pm 1.9 \times 10^5$ (mean ± SD) molecules of SRB per liposome ($n = 3$).

Anti–*E. coli* O157:H7 antibodies were thiolated by the sequential use of SATA and hydroxylamine. The molar ratio of SATA to the antibodies was adjusted to yield an average of 1.8 thiol groups per antibody molecule based on the assumption that the antibodies consist mainly of immunoglobulin Gs. The reaction product of the thiolated antibodies with DSPE-PEG-maleimide liposomes did not show any detectable nonspecific binding, as indicated by the lack of spontaneous aggregation of immunoliposomes on the meniscus of the strip. The size distribution and average diameter of liposomes were not significantly changed after the conjugation to the antibodies. Anti-*E. coli* O157:H7 antibody–conjugated liposomes retained 98% of their original SRB and 97% of their original avidity for up to 6 months when stored at 4°C in the dark. These and other characteristics of the anti-*E. coli* O157:H7–conjugated liposomes are summarized in Table 1.

Sensitivity of ILSA with pure cultures. ILSA results using pure cultures of *E. coli* O157:H7 are shown in Figure 3. Because the color intensity on the AC zones is directly proportional to the amount of *E. coli* O157:H7 in the culture, it is preferable to evaluate the assay based on the AC zones rather than the ILC zone. Increasing amounts of *E. coli* in the test samples (7×10^3 to 7×10^7 CFU/ml) led to increasing color intensity in the AC zone and decreasing color intensity in the ILC zones. In the absence of *E. coli,*

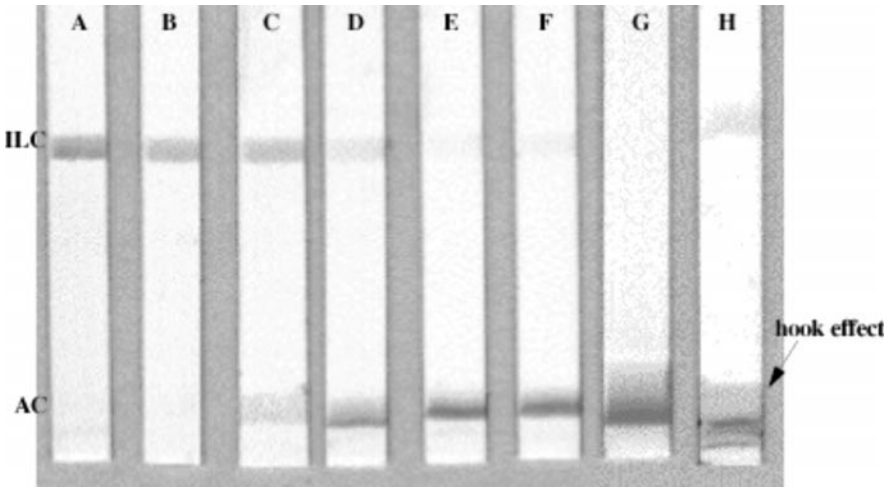

FIGURE 3. *ILSA of pure cultures containing various amounts of* E. coli *O157:H7. Control contained only culture medium (A). Inoculated cultures contained* 7×10^2 *CFU/ml (B),* 7×10^3 *CFU/ml (C),* 7×10^4 *CFU/ml (D),* 7×10^5 *CFU/ml (E),* 7×10^6 *CFU/ml (F),* 7×10^7 *CFU/ml (G), or* 7×10^8 *CFU/ml (H).*

the immunoliposomes are not retained in the AC zone and migrate further to bind in the ILC zone, in which anti-goat antibodies have been immobilized. Because the color intensity of the AC zone obtained at 7×10^2 CFU/ml (lane B, Fig. 3) was not distinguishable from that in the negative control (lane A, Fig. 3), the detection limit of the ILSA was determined to be 7×10^3 CFU/ml (280 CFU/40 μl of test sample volume). Because of an evident immunological prozone or hook effect *(21)* at concentrations $>7 \times 10^7$ CFU/ml, the upper level for quantitation was established as 7×10^6 CFU/ml.

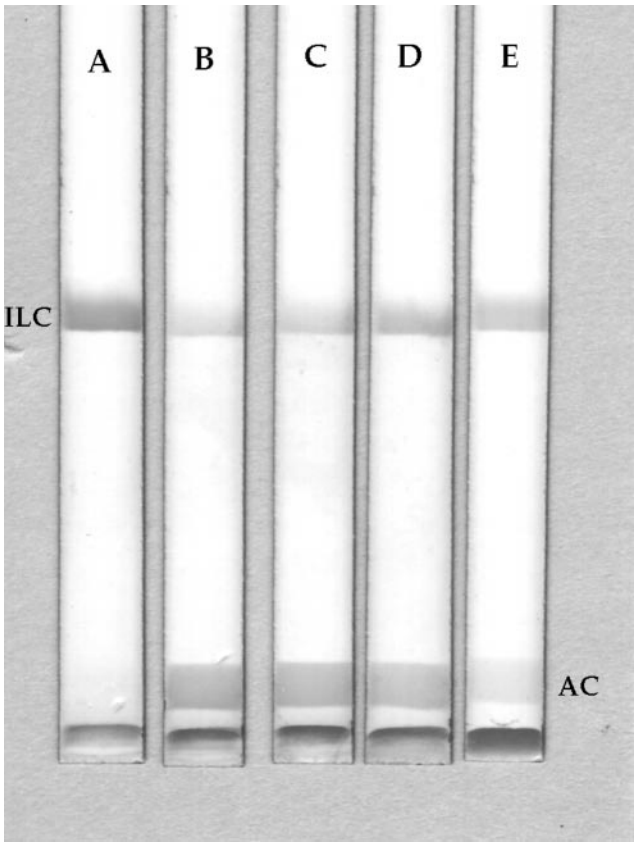

FIGURE 4. *ILSA with apple cider samples after an 8-h enrichment culture. Control consisted of apple cider with no O157:H7 (A). Other apple cider cultures were inoculated with* E. coli *O157: H7 at ca. 1 CFU/ml (B), ca. 10 CFU/ml (C), ca.* 10^2 *CFU/ml (D), or ca.* 10^3 *CFU/ml (E).*

ILSA in apple cider. Pasteurized apple cider samples were inoculated with 1 to 10^5 CFU/ml of *E. coli* O157:H7 grown for 8 h in mEC medium, and aliquots of the enriched cultures were subjected to ILSA. The ILSA was able to detect *E. coli* O157:H7 in apple cider samples inoculated with as few as 1 CFU/ml (equivalent to 7×10^4 to 7×10^5 CFU/ml after the enrichment culture) (Fig. 4). A band was detected at the meniscus of all test strips, including the negative control, most likely representing nonspecific binding of immunoliposomes induced by polyphenols present in the apple cider. However, this band did not overlap with the AC zone and was easily distinguished from the AC zone based on the shape of the band.

DISCUSSION

In this study, we used immunoliposomes encapsulating SRB as an analytical reagent for the detection of *E. coli* O157:H7 in food. To meet the criteria for an analytical reagent, the loading of liposomes with SRB must be simple and reproducible, and the conjugation chemistry should achieve sufficient antibody density without a significant liposomal size change or leakage of the vesicles during long-term storage.

The resulting immunoliposomes should be compatible with the food samples to be tested, i.e., they should be stable in food samples and should bind only to the analyte of interest without nonspecific binding. For this purpose, we improved our immunoliposome preparation method in several ways. DSPE-PEG-maleimide liposomes prepared by the reverse-phase evaporation method encapsulated higher loadings of SRB compared with our previous liposomes *(16).* Huwyler et al. *(8)* showed that liposomes could be readily tagged by coupling antibodies to the distal end of the PEG^{2000}-lipid on the liposomes. In this study, immunoliposomes prepared by this coupling strategy carried a higher antibody surface density of about 170 molecules per liposome. In addition, the immunoliposomes prepared by SATA were able to retain their integrity and avidity at 4°C for up to 6 months, which is twice as long as was

possible for our previous immunoliposomes prepared with 2-iminothiolane *(16).* The reason for the increased stability is related to the surface charge (zeta potential) on the liposomes and its electrostatic effect on aggregation, as described by Vingerhoeds et al. *(23).*

Decreased color intensity in the AC zone caused by the apparent hook effect suggests that after an 8-h incubation of apple cider with mEC culture medium, the small number of *E. coli* O157:H7 cells initially inoculated into apple cider can reach a magnitude that saturates both the solid phase and the antibody-tagged liposomes of the ILSA. Fortunately, one can easily detect this effect by observing a wider band (i.e., extending beyond the band limits) in the AC zone, and the assay can be adjust back ''on scale'' by diluting the sample prior to the assay.

Ogunjimi and Choudary *(13)* reported that the antigen-binding activity of capture antibodies on microplates was completely inhibited during a 2-h incubation in the presence of fruit juices because of nonspecific binding of polyphenols to the antibodies of the analytical reagents. In the ILSA system described here, endogenous polyphenols did not interfere significantly with the performance of the assay because of the PEG coating on the liposomes and the relatively short time required for the assay. Polyphenols bind to the lipid membranes of vesicles and to proteins *(12, 19).* Although antibodies at the end of PEG-lipids are inevitably exposed to the binding of polyphenols endogenous in apple cider, the lipid membranes of immunoliposomes are protected from the nonspecific adsorption of polyphenols by the presence of the inert PEG on the outer surface of the liposomes. Bare (non–PEG protected) immunoliposomes have shown extensive nonspecific binding to the immunomigration strip (data not shown). Other potentially interfering effects, including low pH (3.7) and high osmolarity (880 mmol/kg), of apple cider were eliminated by mixing and dilution of samples with culture media and buffer (420 mmol/kg).

The following studies could improve the performance of the ILSA. Although the affinity-purified antibodies used in this study are highly specific for *E. coli* O157:H7, they were not tested for cross-reactivity to other *E. coli* strains. Further investigation is needed to comfirm the specificity of this assay for the O157:H7 serotype *(10).* Extended storage time for the immunoliposomes and immunomigration strips may be achieved by freeze-drying in the presence of cryoprotectants. Such reagents would be ideal for field screening tests *(7, 11).*

This improved ILSA has several advantages. The colorimetric detection assay, which has a user-friendly configuration with two correlated measurement zones, can provide results that are easily visible to the naked eye. The assay system allows for the detection of *E. coli* O157:H7 in pure culture in less than 8 min, and as few as 1 CFU of *E. coli* O157:H7 per ml of apple cider can be detected without having to separate the analytes from the apple cider matrix. The speed, simplicity, and sensitivity of this ILSA make it a practical method for the detection of *E. coli* O157:H7 in food matrices such as apple cider.

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