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Escherichia coli detection in vegetable food by a potentiometric biosensor

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Abstract

The present work describes the application of an antibody based biosensor for the determination of *Escherichia coli* cells in vegetable food. The presence of *E. coli* as a bioindicator of bacterial contamination—faecal one in particular—was detected using the potentiometric alternating biosensing (PAB) system based on a light addressable potentiometric sensor (LAPS) transducing element, detecting pH variations due to NH_3 production by an urease–*E. coli* antibody conjugate.

Commercial samples of vegetable—lettuce, sliced carrots, and rucola—were washed with peptone water at pH 6.8, blended either in a stomacher or in a sonicator, to detach bacterial cells and to recover them in the liquid medium. This liquid phase was analyzed both by PAB system and conventional colony forming units (CFUs) methods. The proposed PAB system appears to be very sensitive and fast, in comparison with conventional methods: concentration of 10 cells/ml were detected in an assay time of ca. 1.5 h, showing detection time from 10 to 20 times shorter than the conventional CFU procedure.

Keywords: *Escherichia coli*; Potentiometric sensor; Immunoassay biosensor; Vegetable food

1. Introduction

Demand for fresh, minimally processed vegetables has led to an increase in the amount and variety of products readyly available to the consumer. Minimally processed vegetable may consist of trimmed, peeled, sliced/shredded and washed and/or disinfected vegetable. The products are packed and stored at refrigerator temperature [1]. Such ''ready-to-use'' (RTU) vegetable retain much of their indigenous microflora after minimal processing. Vegetables are actually contaminated by various saprophytic environmental microorganisms, the great majority of which are Gram-negative rod-shaped bacteria belonging both to the Pseudomonadaceae and Enterobacteriaceae (coliform bacteria present in soil or water); lactic bacteria, yeast and moulds are also present, but to a lesser extent. Thus, the species of microorganisms present and their quantity are dependent on the populations present in the soil, on the type of irrigation and fertilizers used and on the water utilized in cleaning the vegetable before packing [2]. Failure to implement the hazard analysis and critical control points (HACCPs) during the preparations of RTU vegetable may constitute a hazard, particularly during the warm season.

In most countries criteria has been established whereby RTU products should contain $<10^6$ colony forming unit (CFU)/g or ml of mesophiles at the consumption stage and that pathogenic microorganisms or their toxins which are a risk to health should also be absent [3].

In Italy there is no specific legislation governing these kind of products; in France, the General Executive of the Concurrence of Consumption and Repression of Fraud [2] published a ''Guide to good hygiene standards regarding type RTU'' containing a summary of the legislation and regulations governing safety and essential hygiene standards.

To estimate food sanitary quality, the classic approach is based on the search for not only pathogenic microorganisms, but also for indicator microorganism such as faecal coliforms, whose presence indicates possible pathogens and faecal food contamination of human and/or animal origin [4]. The routinary detection methods for microorganisms are based on CFU count requiring selective culture, or biochemical and serological characterizations. Although, bacterial detection by these methods is sensitive and selective, days to weeks are needed to get a results. Besides, these methods are costly and time consuming.

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The growing market of fresh RTU vegetables, requires a rapid, sensitive and reliable monitoring of bacterial contamination.

The present work describes the application of an antibody based biosensor for the determination of *Escherichia coli* cells in vegetable food. Commercial samples were washed with peptone water to recover bacterial cells in the liquid medium. The presence of *E. coli*—as a bioindicator of bacterial contamination, faecal one in particular—was detected in this phase was using both conventional CFU methods and the potentiometric alternating biosensing (PAB) system, based on a transducing element (LAPS, light addressable potentiometric sensor) detecting pH variations due to NH_3 production by an urease–*E. coli* antibody conjugate [5].

2. Experimental

2.1. The PAB system

The transducer consists of a heterostructure made of silicon n-type, silicon dioxide and silicon nitride positioned into a measuring chamber [5]. The insulator layer is pH sensitive owing to the proton binding capacity of its surface groups over a large pH range. Redox potential measurements are obtained when the solution to be analysed reacts with the biosensor immobilized on the cover slip in contact with the silicon structure. The PAB system's response is implemented by a lock-in technique using a modulated pulsed light from an infrared LED. The light is focused on the back of a silicon substrate and the potentiometric signal in-phase from the metallic electrode is recovered. The apparatus is computer controlled in order to obtain on-line data acquisition and recording.

The redox potential revealed by the transducer system is generated by the biosensor. The change in the redox potential is due to the production of NH_3 by a urease–*E. coli* antibody conjugate linked with the *E. coli* cells present in the water. Urea is enzymatically converted to ammonia proportionately to the amount of *E. coli* present in the sample. The sensitivity recorded is 59 mV per pH units.

The biological component was made by utilizing an environmental *E. coli* strain, DH5α, to produce rabbit polyclonal antibodies. It was chosen out of several *E. coli* strains, environmental, enteric and enteropathogenic, after capsule isolation and SDS-page protein analysis, because of its band pattern typical of this genus. Anti-*E. coli* polyclonal-IgG were purified from the serum of a New Zealand rabbit by protein-A affinity-chromatography column, after ammonium sulphate precipitation. Measurements for quantity and purity of this antibody were done spectrophotometrically at 280 nm and the anti-*E. coli*-IgG were identified by ELISA immunoassay.

The anti-*E. coli*-IgG was coupled to urease (Sigma/Aldrich) by a modified periodate coupling procedure [5,6].

The primary antibody was immobilized on a silanized glass coverslip (2 cm × 2 cm). The silanization was made by immersion on a solution containing 5% aminopropyltriethoxysilane (APTES) and 5% water in isopropanol at 60 °C. As APTES presents NH_3 groups, a bifunctional cross-linking agent like glutaraldehyde was used to create covalent bonds with the amino-groups of the antibody. The coverslip was rinsed with a PBS solution and incubated on a 0.1% casein solution in PBS in order to block the unlinked sites so avoiding non-specific linking of the antigen.

To detect the sensitivity of the PAB system, the coverslip was washed in PBS and kept horizontally for 15 min with 1 ml of *E. coli* solution in PBS at different concentrations (from 10 to 10^7 cells/ml), rinsed again with PBS and incubated with the secondary antibody (this last conjugated to urease at the concentration of 30 μg ml^{-1}) for 1 h.

Finally, the coverslip was introduced inside the measuring chamber of the PAB system, the substrate mixture (urea 50 mM) was introduced by a peristaltic pump, at flow rate of 200 μl min^{-1} and the enzymatic activity was automatically calculated from the signals acquired during flow off periods of about 5 min. For the application described here, a microvolume reaction chamber was utilized with a complete microchannel system near the sensitive area.

Instrument calibration was made by measuring the potential difference between pH 7 and 8 standard titration solutions ($\Delta V_{out} = \Delta pH \times S$, where S is the sensitivity).

2.2. Characteristics of the samples

Six packages of commercial RTU vegetable salad, consisting of rucola (RC), lettuce (LT), carrots (C) and three samples of mixed salad (MS1-2-3) were purchased from chilled cabinets of local retailers 5 days after packing, over the March–June period. The products were packed under ordinary atmosphere in polypropylene bags and stored at 4 °C.

2.3. Samples treatment

Routinary analyses of bacterial contamination—faecal bacteria in particular—in RTU vegetable are generally conducted by a previous sample homogenisation by a stomacher, followed by serial dilutions and plating on agarized selected media. However, the biological component of the biosensor cannot analyze samples containing plant cells such vegetable homogenates, because of their possible interference with the binding of the bacterial cell to the antibody layer. Three washing methods were set up to bypass this problem. To verify which one was the most suitable, the results of microbiological analyses of all methods were compared to those obtained by the standardized stomacher procedure: 25 g of sample were diluted in 225 ml buffered peptone water pH 6.8 (peptone 1 g/l, K_2HPO_4 8.75 g/l, KH_2PO_4 0.5 g/l) and homogenized for 120 s at

regular speed with a Seward Stomacher Laboratory Blender 400 [7,8].

Our sample washing methods were

(a) A 25 g of sample were diluted in 225 ml sodium-bicarbonate solution (5%) and incubated at room temperature for 2 h.
(b) A 25 g of sample were diluted in 225 ml peptone water pH 6.8 and incubated at room temperature for 2 h.
(c) A 25 g of sample were diluted in 225 ml distilled water and put in an ultrasonic Sonomatic bath (Langford Ultrasonics) for 8 min.

Washing solution of RTU vegetable were analysed at the same time by conventional microbiological methods, ELISA and by the biosensor.

2.4. Microbiological analyses

Serial dilutions of the washing solutions were made in test tubes containing 9 ml physiological solution and plated in triplicate onto the following RTU media, all purchased from Oxoid:

- Membrane endo-agar LES was utilized to count total coliforms. Plates were incubated at 37 °C for 26 h.
- Membrane faecal coliform agar [4] and violet red bile agar (VRBA) [9,10] were both used for faecal coliforms counts. Plates were incubated at 44 °C for 22 h. VRBA plates showed the best results.
- Triptone Bile X-Glu (TBX) agar was utilized for counting *E. coli* cells. Plates were incubated at 44 °C for 30 h [11].

2.5. Acquisition curves

Washing solutions of RTU vegetable were also analyzed by the PAB system for the presence of faecal coliforms. Samples were prepared outside the measuring chamber *E. coli* cells suspension in peptone water or PBS (1 ml), and PBS or peptone water without cells as a blanks, were layered on the cover slip for 30 min. Unbound cells were washed out thrice with PBS and the second antibody was added, kept for 60 min and then washed with PBS. The coverslip was put in the measuring chamber and an urea/water solution 50 mM was introduced by a peristaltic pump at a flow rate of 200 μl min^{-1}. Blanks were treated with secondary antibody and urea both together and separately. The amount of released ammonia is a function of the number of cells present in the sample and bound to the coverslip pH variation due to ammonia release is therefore a measure of cell number.

The PAB system was also utilized for the analysis of environmental water samples. Water samples were aseptically collected on sterile bottles and kept at 4 °C. Sampling was done in the following sites: Aterno river, L'Aquila upstream and downstream of the municipal sewage treatment plant, Bracciano Lake, Rome.

3. Results and discussion

3.1. Conventional microbiological analyses

Routinary analyses of bacterial contamination in food are generally conducted by a previous sample homogenisation by a stomacher, followed by serial dilutions and plating on agarized selected media. However, the biological component of the biosensor cannot analyze samples containing plant cells such vegetable homogenates, because of their possible interference with the binding of the bacterial cell to the antibody layer. Three washing methods were set up to bypass this problem.

As expected, the greatest number of colonies was found in the samples treated by the stomacher (Figs. 1 and 2 and Table 1). Sodium-bicarbonate solution was found to be the less rich in CFU and this washing method was no longer

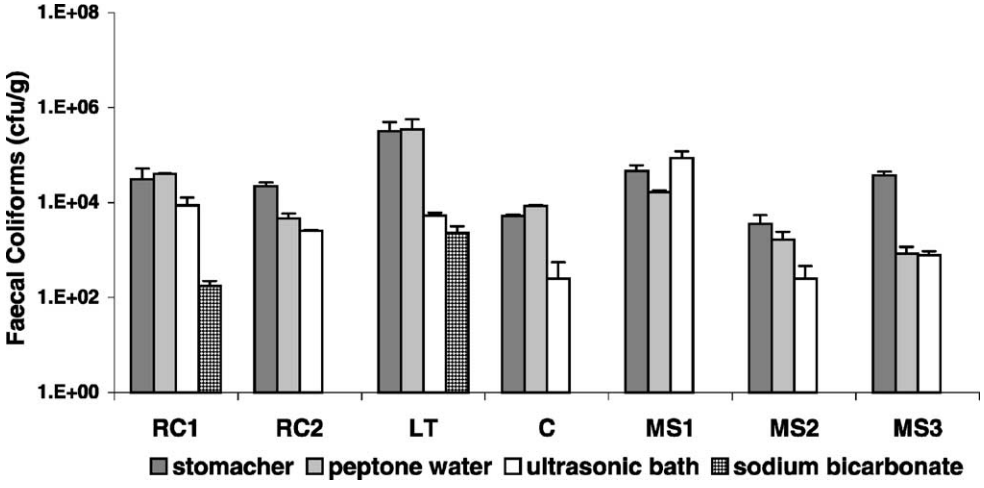

Fig. 1. Number of faecal coliform in RTU vegetable obtained by conventional microbiological analyses.

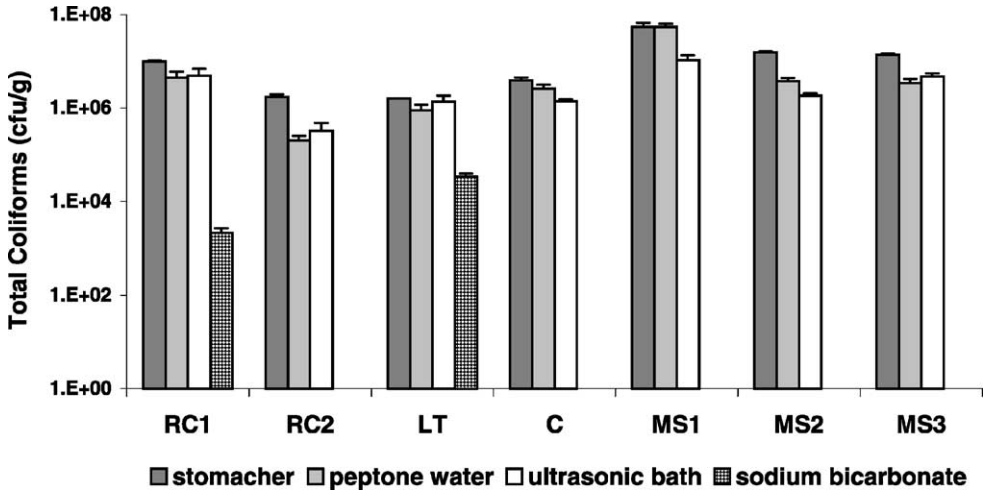

Fig. 2. Number of total coliform in RTU vegetable obtained by conventional microbiological analyses.

utilized. Two hours infusion in peptone water, however, gave acceptable results. The samples treated in this way were chosen to be tested with the biosensor. Cells were also tested by ELISA to check the binding with the antibodies utilized in the biosensor (data not shown). This method, however, was less sensitive because it cannot detect less than 10^3 cells/ml.

Table 1
Counts of *E. coli* cells (CFU/g) in RTU vegetable obtained by conventional microbiological analyses

Sample	Vegetable	Stomacher	Peptone water	Ultrasonic bath
RC1	Rucola	0	0	0
RC2	Rucola	290	90	50
LT	Lattuce	40	33	27
C	Carrots	0	0	0
MS1	Mixed salad	16	4	1
MS2	Mixed salad	3	1	0
MS3	Mixed salad	400	200	16

3.2. Biosensor analyses

Measurements of *E. coli* cell suspension were done for the calibration curve by utilizing attenuated cells at different dilution, from 10 to 10^7 cells/ml [5]. The concentration of bacterial cells in the analysed liquid samples (a) washing water of RTU vegetable and (b) water from the Aterno river (Italy) is read by the instrument as pH variation responsible for the intensity of the signal. When *E. coli* is absent, the acquisition curve is almost flat (Fig. 3) instead, when bacterial cells are present, the signals variate in clear and direct connection with the *E. coli* cell number, so that one can assume that pH variation is influenced by bacterial cell number.

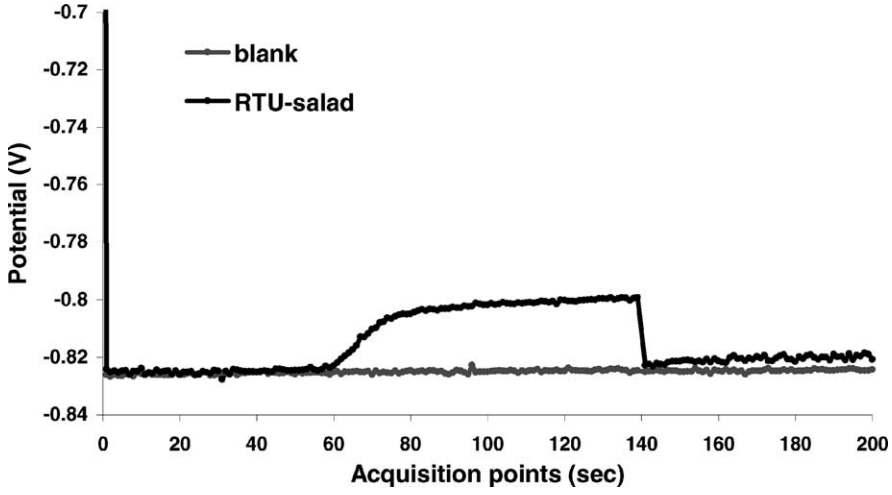

Fig. 3. Acquisition curves of *E. coli* in washing water of mixed salad sample (top line) and blank (bottom line) in the presence of polyclonal *E. coli* antibodies. The same sample analysed by conventional methods revealed the presence of 200 *E. coli* cells/g. Blank acquisition curve obtained by flowing urea on a coverslip treated as the sample but with cell-free.

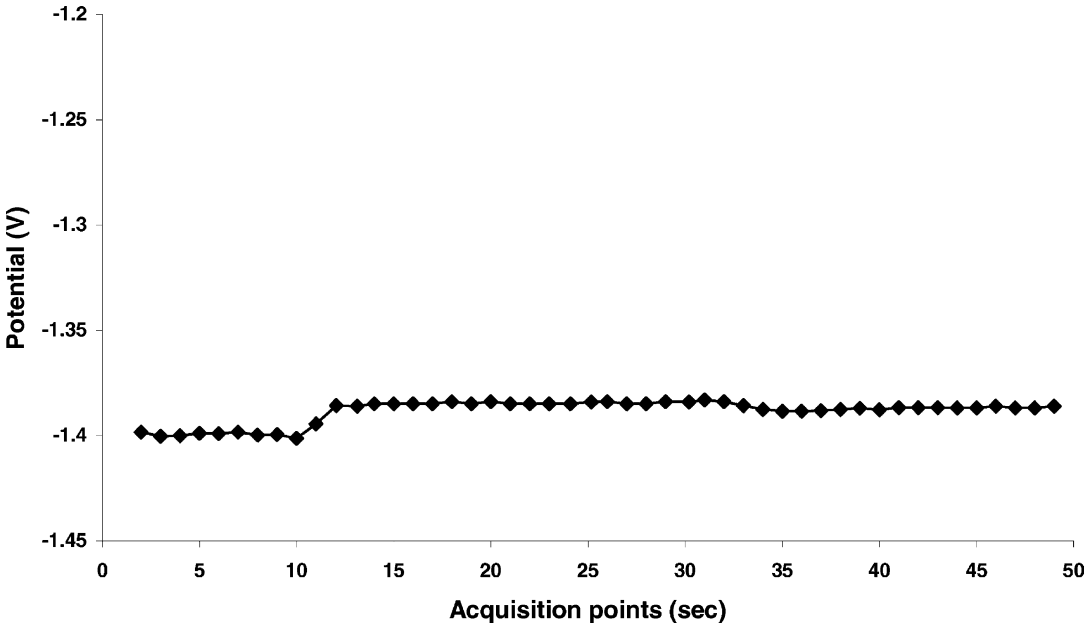

Fig. 4. Acquisition curves of water samples from the Aterno river (L'Aquila) with 17 cells/ml of *E. coli*.

Fig. 3 shows a curve made by a sample of mix salad washing water/peptone water containing around 10^2 cells of *E. coli* per ml over a blank of peptone solution. The system appears to be very sensitive: concentrations of 10 cells/ml are clearly detected over a blank of *E. coli*-free PBS and/or peptone water.

The PAB system was utilized to analyze environmental water samples. These samples were analyzed following the above mentioned procedure and the results obtained by the PAB were compared with the ones obtained by conventional microbiological methods and ELISA (the same antibody utilized for the biosensor). In Fig. 4 instead, is shown a curve made by a sample of water collected from the Aterno river (L'Aquila). Conventional analyses of this sample revealed the presence of 17 cells of *E. coli* per ml. Tap water was utilized as blank.

Bacterial cells at concentrations as low as 10 cells/ml showing detection time from 10 to 20 times shorter than the conventional CFU procedure. These preliminary results indicate PAB as an alternative profitable method to asses quality product and health care standards.

4. Conclusions

An assay for the evaluation of *E. coli* cells has been set up which could be useful for environmental and food control. This bacterium was chosen as typical indicator of faecal contamination. The biological component is based on a polyclonal antibody. The binding constant of the antibody is very high demonstrating that a sensing system based on the recognition antigen–antibody able to specifically detect *E. coli* strains in food and environmental water samples can be assembled. This system is quite flexible, manageable and easy to construct utilizing commercial electronic components although it can be improved, in particular by utilizing monoclonal antibodies.

Work is in progress to obtain a monoclonal antibody and to optimize the immobilization procedure.

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