Simultaneous Electrokinetic and Hydrodynamic Injection for High Sensitivity Bacteria Analysis in Capillary Electrophoresis

ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2011

Impact Factor: 5.64 · DOI: 10.1021/ac200684t · Source: PubMed

6 AUTHORS, INCLUDING:

Joselito P Quirino
University of Tasmania
102 PUBLICATIONS **4,896** CITATIONS
SEE PROFILE

Laurent Garrelly
colcom
29 PUBLICATIONS **274** CITATIONS
SEE PROFILE

Tao Zou
University of Florida
20 PUBLICATIONS **323** CITATIONS
SEE PROFILE

Hervé Cottet
Université de Montpellier
106 PUBLICATIONS **1,585** CITATIONS
SEE PROFILE

analytical chemistry

ARTICLE

pubs.acs.org/ac

Simultaneous Electrokinetic and Hydrodynamic Injection for High Sensitivity Bacteria Analysis in Capillary Electrophoresis

Farid Oukacine,[†,‡] Joselito P. Quirino,[§] Laurent Garrelly,[‡] Bernard Romestand,[∥] Tao Zou,[†] and Hervé Cottet*[,†]

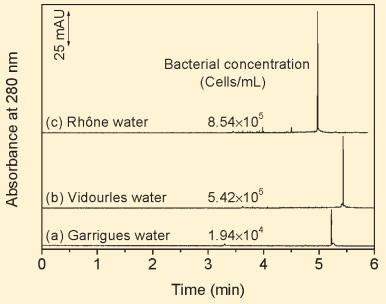
[†]Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS-Université de Montpellier1-Université de Montpellier 2, Place Eugène Bataillon, case courrier 1706, 34095 Montpellier Cedex 5, France

[‡]COLCOM SARL, Cap Alpha Avenue de l'Europe, Clapiers 34940 Montpellier, France

[§]Australian Centre for Research on Separation Science, School of Chemistry, University of Tasmania, Hobart, Australia 7001

[∥]Laboratoire Ecosystèmes Lagunaires, UMR 5119, Université de Montpellier 2, 34095 Montpellier, France

ABSTRACT: A repeatable preconcentration electrophoretic methodology for the analysis of bacteria was developed. This method is based on an isotachophoretic mode coupled with a simultaneous hydrodynamic-electrokinetic injection in conditions of field-amplified sample injection. This electrophoretic method allows the quantification of *Enterobacter cloacae* (studied as a model of Gram negative bacteria) with a limit of detection of 2×10^4 cells/mL. With the optimized conditions, a preconcentration factor of about 500-fold was obtained as compared to a standard hydrodynamic injection. The RSD ($n = 5$) on the migration time and on the peak area were 3% and 5%, respectively. This capillary electrophoretic methodology has been applied for the quantification of microbes in natural water (river and natural spring waters). Filtration of the sample prior to injection was required to remove ions present in the water and to keep the field-amplified sample injection condition at the injection. Filtrated bacteria were then recovered in terminating electrolyte diluted 10 times with water. Good agreements were obtained between cellular ATP measurements and the proposed CE methodology for the quantification of bacteria in waters.



Considerable efforts have been directed toward the development of methods that can rapidly detect low concentrations of pathogens in water, food, and clinical samples. Indeed, the infectious dose of a pathogen can be very low. In some conditions, 10^5 cells of *Vibrio cholerae* are sufficient to cause severe cholera.[1,2] For a pathogen such as *Salmonella*, the infectious dose can be as low as 15−20 cells.[3] The main techniques used for detecting bacteria include immunoassays[4] (e.g., enzyme-linked immunosorbent assay and colony immunoblot assay), DNA-based methods, such as polymerase chain reaction (PCR) and real-time PCR,[5−8] immunomagnetic separation (IMS),[9] and flow cytometry (FCM).[10] While these methods can be sensitive and give both qualitative and quantitative information on the analyzed microorganisms, they are greatly restricted by the assay time.

During the past decade, the analysis of bacteria by capillary electrophoresis (CE) has caused great interest. CE can be used to perform fast analyses with high efficiencies and can be easily automated.[11] Moreover, there is a variety of electrophoretic concentration techniques that can be applied online in the capillary.[12,13] For small molecules, concentration factors of more than 100 000-fold are reported.[14] However, the analysis of bacteria by CE is much more complex than for small molecules due to the size of the bacterial cells (∼micrometer) and due to the formation of aggregates.[15] Hjertén et al. were the first to introduce the idea of microbial analysis by CE.[16] In 1999, Armstrong et al. reported a CE method for highly efficient separation of mixtures of several bacterial species by introducing poly(ethyleneoxide) (PEO) into the background electrolyte.[17] Good peak shapes with extremely high efficiencies were reported (up to ∼600 000 theoretical plates/m),[17] and the corresponding focusing mechanism was explored.[18−20] This method has been widely used for the separation of bacteria and for detection of microbial food contaminants.[17,21−23] However, due to the instability of microbial aggregates, Hoerr et al. reported some repeatability issues.[24] Petr et al. studied microorganisms by using a direct combination of a CE instrument with a microscope.[25] They conclude that the reproducibility of microorganims analysis by CE was also greatly affected by adsorption phenomena. In an effort to control the focusing of bacteria in the capillary, different other approaches by CE have been reported, such as isotachophoretic focusing,[15] large volume sample stacking with polarity switching,[26] capillary isoelectric focusing,[17,27] pH-induced stacking, and normal stacking mode.[28] Different microbial assay applications based on CE techniques have been developed: viability determination of bacteria,[29] quick sterility test,[30] microbial contamination in real

Received: March 17, 2011
Accepted: May 2, 2011
Published: May 02, 2011

biological sample by "sample-self-focusing",[31] and quantification of antibiotic effects.[32]

To our knowledge, there is only one publication relative to the electrokinetic injection modes (EKI) of bacteria by CE.[28] For small molecules, EKI can achieve 100 000-fold sample preconcentrations and can be combined with different stacking modes, such as field-amplified sample injection (FASI),[33] sweeping,[34,35] and electrokinetic supercharging (EKS).[36] The aim of this work was to investigate and compare different injection modes to improve the limit of detection of bacteria in water using capillary ITP.

EXPERIMENTAL SECTION

Chemicals. Boric acid 99.999% (H_3BO_3), TRIS 99.9% $(HOCH_2)_3CNH_2$, hydrochloric acid 99.999%, anthraquinone-1,5-sulfonic acid sodium salt ($C_{14}H_6O_8S_2Na_2$), and hydroxypropyl cellulose (M_w 10^5 g/mol) were purchased from Aldrich (Steinheim, Germany). Ethanol was purchased from Carlo Erba Reagents (Val de Reuil, France). Deionized water was further purified with a Milli-Q system from Millipore (Molsheim, France). ATP biomass Kit HS was purchased from BioThema (Handen, Sweden), and BacTiter-Glo was from Promega (Charbonnières-les-Bains, France). The Nutrient Broth medium was purchased from Difco Laboratories (Franklin Lakes, NJ). The Luria−Bertani (LB) agar plates as well as the bacteria *Enterobacter cloacae* (from IBMC Strasbourg collection) were kindly donated by Lagoon Ecosystems laboratory (University of Montpellier 2).

Bacterial Growth Conditions and Sample Preparation. The bacteria were maintained under strict growth conditions: the starting culture colonies of the bacteria were transferred from LB agar plates into Erlenmeyer flasks containing 8 mL of LB liquid medium. The flasks were incubated for 13 h at 30 °C with constant agitation (160 rpm) on a rotary platform shaker for good aeration. The fresh liquid cultures were prepared daily. To separate the bacteria from the medium, the suspension in the flasks was centrifuged (model: SIGMA 3K12, Larborzentrifugen, Osterode, Germany) at 6000 rpm for 5 min to get the bacteria in pellet form. The supernatant was removed carefully, and the pellet was resuspended in 8 mL of CE buffer by vortexing for 1 min. Next, the bacteria suspension was centrifuged again for 5 min. This washing process was repeated twice. Eight milliliters of CE buffer was added to the washed bacteria cells, and the solution was vortexed until the pellets were resuspended completely. After that, the bacterial suspensions were stored at 4 °C. Cell concentrations of bacteria were determined by cellular ATP measurement.[15] Before use, filtration of the buffer is required to remove any possible bacterial contamination from the buffer. The filtration was performed using MF-Millipore filters with 0.45 μm pore size (Millipore SAS, Molsheim, France).

Recovering of Bacteria after Filtration of Natural Water. Filtration of the natural water sample was required prior to injection to remove ions present in the water, and thus to keep the FASI conditions at the injection. Indeed, the conductivity of leading electrolyte is much lower than the conductivity of tap water or river water due to the presence of many ions such as bicarbonates, chlorides, sulfates, and nitrates. A given volume of natural water was filtered by using a 60 mL capacity syringe (Syringe BD Plastipak Luer-Lok) and a 0.45 μm pore size MF-Millipore filter (Millipore SAS, Molsheim, France). The membrane filters are constituted by biologically inert mixtures of cellulose acetate and cellulose nitrate. After filtration of the water sample, 60 mL of deionized water was introduced in this syringe to wash the bacteria trapped in the filter. Bacteria recovery from the filter was obtained by using a recovery electrolyte (RE) containing TRIS/boric acid buffer (1.36 mM/15 mM) and 1% EtOH. About 1 mL of the RE was introduced by suction in the syringe through the filter. The syringe and the filter attached on it were both placed in ultrasonic bath for 1 min. Next, approximately 4 mL of the RE was again introduced by suction in the syringe through the filter, followed by ultrasonic bathing for 1 min. Finally, 5 mL of the RE was introduced by suction in the syringe through the filter. Consequently, bacteria are finally recovered in about 10 mL of RE before CE analysis.

Capillary Coating. The hydroxypropyl cellulose (HPC)-coated capillaries have been prepared according to a literature protocol.[37,38] The HPC powder (M_w 10^5 g/mol) was dissolved at room temperature in water to a final concentration of 5% (w/w). The polymer solutions were left overnight to eliminate bubbles. The capillary columns were filled with the polymer solution using a syringe pump (KDS100, Holliston, U.S.) for 30 min, and the excess of polymer solution was removed using N_2 gas at 3 bar. The HPC polymer layer was immobilized by heating the capillary in a GC oven (GC-14A, Shimadzu, France) at 60 °C for 10 min and using a linear ramp from 60 to 140 °C at 5 °C/min, and finally 140 °C for 20 min, keeping N_2 pressure at 3 bar. Before use, the coated capillaries were rinsed with water for 10 min. At the end of each day, the HPC-coated capillary was flushed with water for 10 min and with air for 5 min.

Capillary Electrophoresis. CE experiments were carried out with a 3D-CE instrument (Agilent technologies system, Waldbronn, Germany) equipped with a diode array detector. Separation capillaries prepared from bare silica tubing were purchased from Composite Metal Services (Shipley, UK). The temperature of the capillary cassette was maintained constant at 25 °C. Between each run, the capillary was washed by TRIS/boric acid/HCl at (4.5 mM/50 mM/3.31 mM) during 10 min.

RESULTS AND DISCUSSION

Recently, we have developed a highly reproducible method that allows focusing bacteria by isotachophoresis (ITP) with very good repeatability and linearity for the quantification.[15] This method is usable on both fused and coated (hydroxypropylcellulose) silica capillaries. It allows obtaining very short migration times, while maintaining very low electrical currents to minimize the increase of the equilibrium temperature and the lysis of bacteria. The BGE used for the ITP focusing is TRIS/boric acid at 13.6/150 mM (pH 7.9) as terminating electrolyte and TRIS/boric acid/HCl at 4.5/50/3.31 mM (pH 7.4) as leading electrolyte. In this ITP system, boric acid acts as terminating ion and chloride acts as leading ion. To decrease the limit of detection (LOD), we first tried to increase the injected volume.

Effect of the Injected Plug Length of Bacteria Using Hydrodynamic Injection in ITP. Figure 1 shows the isotachophoretic profiles of *E. cloacae* (Gram negative bacteria) diluted in terminating electrolyte (TRIS/boric acid at 13.6/150 mM) for different hydrodynamic injection (HD injection) times at constant pressure (17 mbar). The lowest and the highest injected plug lengths are respectively 1.19 cm (Figure 1a) and 8.33 cm (Figure 1f). As shown in Figure 1, the detection time decreased when the injected plug length was increased. This was due to the decrease of the distance between the sample zone and the detection

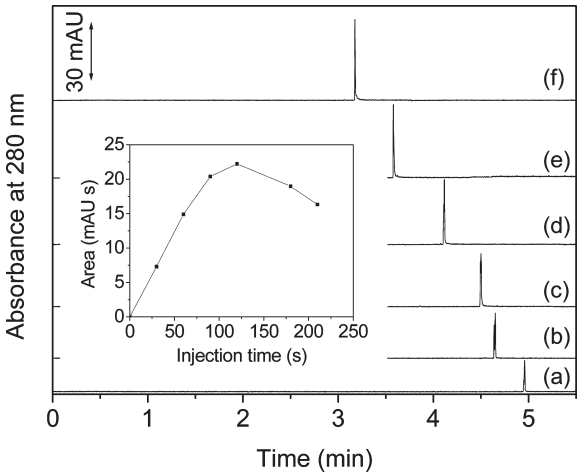


Figure 1. Effect of injection time in hydrodynamic mode on isotachophoretic profile of *E. cloacae*. Experimental conditions: Hydroxypropyl cellulose (HPC) modified capillary 33.5 cm (25 cm to the detector) × 50 μm i.d. Electrolyte: inlet, TRIS 13.6 mM + boric acid 150 mM; outlet and capillary, TRIS 4.5 + boric acid 50 mM + HCl 3.31 mM. HD injection: 17 mbar × 30 s, 1.19 cm (a); 17 mbar × 60 s, 2.38 cm (b); 17 mbar × 90 s, 3.57 cm (c); 17 mbar × 120 s, 4.76 cm (d); 17 mbar × 180 s, 7.14 cm (e); 17 mbar × 210 s, 8.33 cm (f). Applied current intensity: 1 μA. Sample: *E. cloacae* at 8.4 × 10^6 cells/mL diluted in TRIS (13.6 mM) + boric acid (150 mM), pH 7.93. Plain line in the inset is a guide for the eyes.

window. Nevertheless, the velocity was found to be constant (0.82 mm s^{-1} with a RSD values (n = 6) of 3%) for all peaks displayed in Figure 1. The velocity V corresponding to each peak was calculated using eq 1:

$$V = \frac{l - l_{inj}}{t_d} \quad (1)$$

where l is the effective length of the capillary, l_{inj} is the injected plug length, and t_d is the detection time. To monitor the quantity of injected bacteria, the peak area was plotted against the injection time (see inset in Figure 1). The velocity being constant for all peaks, this justifies the use of peak area instead of time-corrected peak area. The peak area of *E. cloacae* increases with the injection time until it reaches a maximum for an injection time of 120 s. The decrease in peak area can be explained if one considers that the detection time becomes too short for the focusing ITP process to be complete. Bacteria are thus detected before being completely focused. It should be noted that even if the bacteria are not completely focused, they do not appear as multiple small peaks usually attributable to irregular clusters and aggregates of bacterial cells.[25,26] This is due to the small concentration of the injected bacteria sample (8.42 × 10^6 cells/mL) and to the higher LOD of *E. cloacae* when no focusing mechanism is used. For a 120 s injection time at 17 mbar, the calibration curve displays relatively good linearity (R^2 = 0.96) with a sensitivity of detection (slope of the calibration curve) of 3.02 × 10^{-6} mAU s mL/cells.

Effect of Injection Mode on Bacteria Analysis in ITP in Conditions of FASI. To further improve the sensitivity of detection, electrokinetic injection (EKI) of bacteria was examined. Figure 2 displays electrophoretic profiles of *E. cloacae* at 1.05 × 10^6 cells/mL for different injection modes. In Figure 2a, only a very small signal, which is not significantly different for the blank, was observed for EKI (−15 kV for 102 s). Whatever the

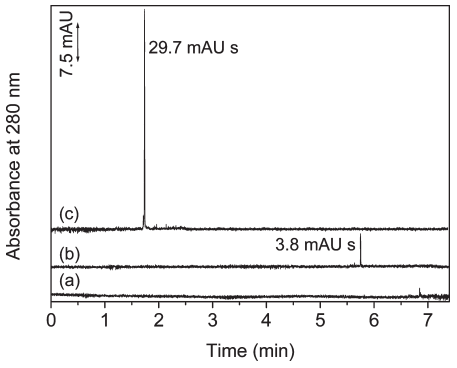


Figure 2. Effect of the injection mode on the peak area of *E. cloacae* in conditions of field amplified sample injection. Experimental conditions: Hydroxypropyl cellulose (HPC) modified capillary 33.5 cm (25 cm to the detector) × 50 μm i.d. Electrolyte: inlet, TRIS 13.6 mM + boric acid 150 mM; outlet and capillary, TRIS 4.5 mM + boric acid 50 mM + HCl 3.31 mM. Injection mode: EKI −15 kV, 102 s (a); HD injection 17 mbar, 102 s (b); SEHI: 17 mbar, −15 kV, 102 s (c). Applied current intensity: 1 μA. Sample: *E. cloacae* at 1.05 × 10^6 cells/mL in a 10 times diluted terminating electrolyte.

concentration of bacteria and the sample electrolyte, it was not possible to inject bacteria using EKI at −15 kV (results not shown). In the experiments presented in Figure 2, the bacteria were suspended in a 10 times diluted terminating electrolyte. The aim of this dilution was to increase the resistivity of the bacteria sample as compared to the leading electrolyte and, consequently, to amplify or enhance the electric field in the sample zone (FASI). In the case of bacteria in FASI conditions, and whatever the bacteria concentration, EKI at −15 kV was not effective to introduce the bacteria into the capillary. Similar results were obtained when the bacteria were diluted in the terminating electrolyte (results not shown).

For the HD injection (Figure 2b), only a small peak was detected (peak area of 3.8 mAU s). This small signal was due to the low concentration of bacteria sample (1.05 × 10^6 cells/mL). When the bacteria sample was injected by simultaneous electrokinetic and hydrodynamic injection (SEHI) (17 mbar, −15 kV for 6 s), the signal was greatly improved (peak area of 29.7 mAU s). This result demonstrates that there was a synergistic effect when both injection modes were used simultaneously. It is worth noting that this synergistic effect was only observed in conditions of FASI with bacteria suspended in a 10 times diluted terminating electrolyte. The RSD values (n = 6) on peak area and migration time for SEHI-FASI of *E. cloacae* (17 mbar, −15 kV for 102 s) are, respectively, 4% and 2%. The slope of the calibration curve using the same injection conditions was 2.66 × 10^{-5} mAU s mL/cells with a correlation coefficient of R^2 = 0.986 (results not shown).

Effect of Injection Mode on Small Molecule and Micronic Latex Analysis in CZE. For a better understanding of the specificity of bacteria analysis, a small molecule (anthraquinone-1,5-disulfonate, AQDS) and a latex (0.91 μm hydrodynamic diameter) were analyzed as model compounds by capillary zone electrophoresis (CZE). Latex can be considered as a good model of synthetic particle having similar size as bacteria. Figure 3A and B displays the electrophoretic profiles using four different injection methods: HD injection at 17 mbar for 6 s (Figure 3A-1 and B-1), EKI at −15 kV for 6 s (Figure 3A-2 and B-2), EKI at −2 kV for 45 s (Figure 3A-3 and B-3), and SEHI at 17 mbar, −15 kV for 6 s (Figure 3A-4 and B-4). In HD injection, the peak area values obtained for AQDS and

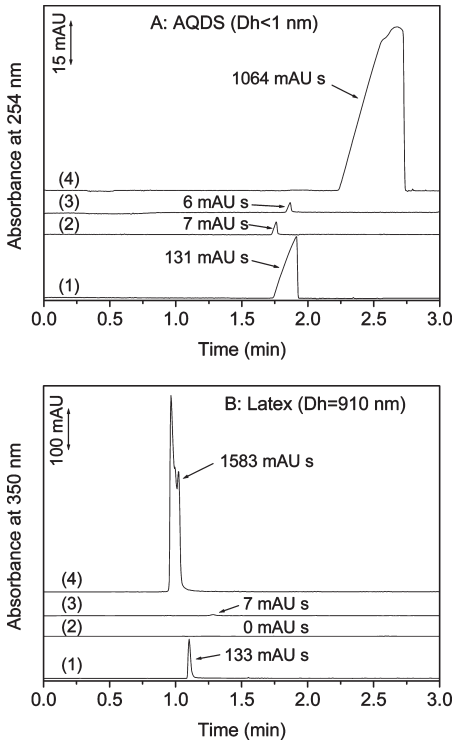


Figure 3. Effect of the injection mode on the analysis of small molecule (A) and micronic latex (B) in CZE. Experimental conditions: hydroxypropyl cellulose (HPC) modified capillary 33.5 cm (25 cm to the detector) × 50 μm i.d. Electrolyte: TRIS 13.6 mM + boric acid 150 mM. Injection mode: HD injection, 17 mbar, 6 s (1); EKI, −15 kV, 6 s (2); EKI, −2 kV, 45 s (3); SEHI, 17 mbar, −15 kV, 6 s (4). Applied voltage: −20 kV. Samples: latex at 1.68 g L^{-1}, anthraquinone-1,5-disulfonic acid at 0.22 g L^{-1}, both diluted in electrolyte.

latex are, respectively, 131 and 133 mAU s. Using EKI (−15 kV for 6 s), the peak area are 7 and 0 mAU s respectively, with the same samples. There is no latex injection by EKI at −15 kV for 6 s, while at −2 kV for 45 s the latex can be injected (7 mAU s peak area). On the contrary, the amount of injected AQDS was similar when the voltage/injected time product was kept constant.

This difference of behavior between the small molecule and the micronic latex is due to the difference in size, and thus in diffusion coefficient. Indeed, during EKI, depletion of sample ions close to the capillary inlet end occurs. This depletion is only partially compensated by molecular diffusion, especially for large solute with low diffusion coefficient, such as the micronic latex ($D \approx 10^{-13}$ m^2 s^{-1}). Dasgupta et al.[39] already reported that for ions of low mobility the amount of injected ions can decrease by increasing the injection voltage. The authors assumed that the reason for this result might be the depletion of the ions near the capillary tip due to the limiting diffusion transport from the bulk of the solution to the capillary inlet end. This effect is more pronounced for ions of low diffusion coefficient and/or if the injection voltage is high.[40] Hirokawa et al.[41] studied the influence of the sample diffusion during EKI by simulations using multiphysics software. In their simulation, the diffusion coefficient was assumed to be equal to 10^{-10} m^2 s^{-1}. For diffusion coefficient 10 times larger, they found there was only negligible change of injection efficiency. The authors suggest stirring or heating the sample to accelerate the sample diffusion during EKI.

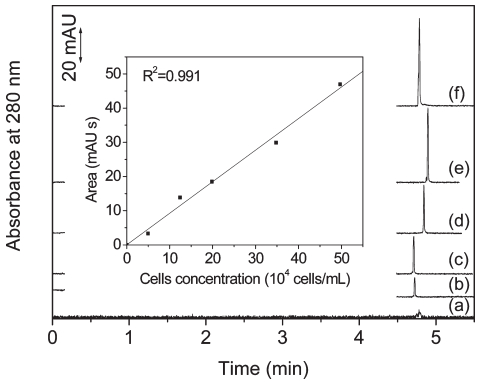


Figure 4. Quantification of *E. cloacae* suspensions by SEHI-FASI-ITP. Experimental conditions: hydroxypropyl cellulose (HPC) modified capillary 67 cm (58.5 cm to the detector) × 50 μm i.d. Electrolyte: inlet, TRIS 13.6 mM + boric acid 150 mM; outlet and capillary, TRIS 4.5 + boric acid 50 mM + HCl 3.31 mM. SEHI-FASI: 17 mbar, −15 kV, 480 s. Applied current intensity: 1 μA. Samples: blank (a), *E. cloacae* at 4.97×10^4 cells/mL (b), 1.24×10^5 cells/mL (c), 1.99×10^5 cells/mL (d), 3.48×10^5 cells/mL (e), 4.97×10^5 cells/mL (f). The sample matrix is a 10 times diluted terminating electrolyte.

Another approach to compensate the sample depletion in the vicinity of the capillary inlet is to implement SEHI. Figure 3A-4 and B-4 displays the electropherograms obtained by SEHI for AQDS and latex, respectively. In this injection mode, the quantities injected for AQDS and latex are, respectively, 8.1 and 11.9 times the quantities injected by HD injection alone in the same conditions. Thus, by using simultaneous injection modes, mass transfer in the vicinity of the electrode/capillary inlet end is facilitated even for solutes with low diffusion coefficients. In the case of a small molecule with high diffusion coefficient, the mass transfer is also accelerated by using SEHI. Thus, as a conclusion of this section, it has been demonstrated that the noninjection of bacteria in the capillary by using the EKI was not specific to bacteria but was related to the size of the analytes.

Linearity and Repeatability of SEHI of Bacteria in ITP. To optimize the sensitivity of detection, a total capillary length of 67 cm was used, allowing longer injection time of bacteria using SEHI (17 mbar, −15 kV for 8 min). Figure 4 displays the linearity curve obtained for *E. cloacae* suspension in a 10 times diluted terminating electrolyte using the aforementioned SEHI-FASI-ITP conditions. The RSD values (n = 5) obtained for *E. cloacae* (4.97×10^5 cells/mL) are 5% and 3% for peak area and migration time, respectively. The slope of the calibration curve was 0.924×10^{-4} mAU s mL/cells with a correlation coefficient of R^2 = 0.991. An enhancement factor of about 500-fold was obtained comparatively to regular hydrodynamic injection (17 mbar, 6 s, using 33.5 cm capillary × 50 μm i.d.). The limit of detection (LOD), defined as 3 times the background noise, obtained by this electrophoretic method for the quantification of *E. cloacae* is 2×10^4 cells/mL.

Quantification of Bacteria in River Water by SEHI-FASI-ITP. The calibration curve represented in the inset of Figure 4 was used for the quantification of bacteria in river water and in tap water. Conductivity of leading electrolyte is much lower than the conductivity of tap water or river water. Indeed, several ions such as bicarbonates, chlorides, sulfates, and nitrates are naturally present in water. Because the preconcentration method used in this work is based on the difference of the resistivity of the

bacteria sample regarding the leading electrolyte (FASI), it is important to remove all ions present in samples by filtration of the bacteria according to the protocol described in the Experimental Section. The filtration can also help in decreasing the minimal detectable concentration of bacteria in water. To improve recovery of bacteria after filtration, a recovery electrolyte (RE) was used to recover the bacteria after filtration. The RE was composed of a chemical dispersant (1% EtOH) in a 10 times diluted terminating electrolyte. It is noted that the presence of EtOH at 1% does not lyse bacteria, even after 1 h contact. The recovery rate (%) was calculated by cellular ATP measurement, before and after filtration process, using eq 2:

$$\text{recovery} = \frac{\text{volume}_{\text{eluate}} \times \text{concentration}_{\text{eluate}} \times 100}{\text{volume}_{\text{sample}} \times \text{concentration}_{\text{sample}}} \quad (2)$$

This filtration procedure allows recovering living *Enterobacter cloacae* (Gram −) and *Micrococcus luteus* (Gram +) with mean recovery percentages of 73% and 82%, respectively. The RSD ($n = 6$) in these recoveries are 9% for *E. cloacae* and 12% for *M. luteus*. Similar recoveries were obtained by filtration for *Listeria monocytogenes*.[42] Figure 5 displays the isotachopherograms obtained by SEHI-FASI of different natural waters. The bacteria concentrations of French Garrigues water (natural spring water), French coastal river (Vidourles), and French rivers (Rhône), determined by ATP cellular measurement, are, respectively, $1.28 \times 10^4 \pm 0.17 \times 10^4$ (95% confidence interval), $3.80 \times 10^5 \pm 0.36 \times 10^4$, and $9.07 \times 10^5 \pm 0.66 \times 10^4$ cells/mL. These values were compared to those obtained by ITP as presented in Table 1. For each water sample, five successive injections were made. Because the concentration of French Garrigues water (1.28×10^4 cells/mL) is below the detection limit of the electrophoretic mode used in the present work (2×10^4 cells/mL), the filtration step was used for a rapid enrichment of bacteria in the analyzed sample. Thus, 100 mL of Garrigues water was filtrated and recovered using only 12.3 mL (preconcentration factor of 8.1) of RE. For the determination of bacteria concentration by SEHI-FASI-ITP, the calibration curve obtained for *E. cloacae* in the same conditions was used (see Figure 4). Because natural water contains a lot of different bacterial strains, at different concentrations, it was assumed that the UV responses for all bacteria strains are approximately the same. It is noted that there is no separation of different bacterial strains in ITP mode.[15] A similar assumption on the response coefficients is also used in the ATP cellular measurements by considering that the mean value of cellular ATP is 1 fg per bacteria cell.[43] The recovery rate of the bacteria from the filters used for the calculation of bacteria concentration in water was 77.5%. This value corresponds to the mean average of the recovery rates obtained for *E. cloacae* (73%) and *M. luteus* (82%). Finally, the bacteria concentration is given by eq 3:

$$[\text{C}] = \frac{A \times V_{\text{RE}}}{\alpha \times R \times V_{\text{sample}}} \quad (3)$$

where A is the peak area (mAU s), V_{RE} is the volume of recovery electrolyte used to recover the bacteria from the filter (mL), V_{sample} is the sample volume of filtrated water (mL), R is the recovery rate (0.775), and α is the slope of the calibration curve obtained for *E. cloacae* (see Figure 4). It should be noticed that very good agreements were obtained between cellular ATP measurement and SEHI-FASI-ITP. For Rhône and Vidourles waters, differences of 6% and 30% were obtained between both methods, while for natural spring water a difference of 34% was obtained. SEHI-FASI-ITP can be easily automated and does not require expensive biochemical reagents such as luciferin/luciferase enzymes used for bacteria quantification via ATP measurement (bioluminescence).

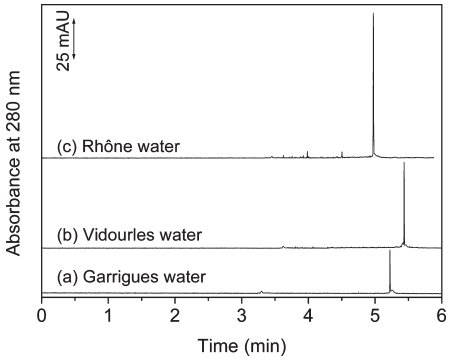


Figure 5. Quantification of bacteria in natural waters by SEHI-FASI isotachophoresis. Experimental conditions: hydroxypropyl cellulose (HPC) modified capillary 67 cm (58.5 cm to the detector) × 50 μm i.d. Electrolyte: inlet, TRIS 13.6 mM + boric acid 150 mM; outlet and capillary, TRIS 4.5 mM + boric acid 50 mM + HCl 3.31 mM. SEHI-FASI: 17 mbar, −15 kV, 480 s. Applied current intensity: 1 μA. Samples: Garrigues water (a), Vidourles water (b), Rhône water (c). The samples were filtered according to the protocol described in the Experimental Section and were recovered in a 10 times diluted terminating electrolyte containing 1% EtOH.

CONCLUSION

A new electrophoretic methodology for the sensitive quantification of bacteria in natural waters was presented. This method that allowed for the analysis of *E. cloacae* with a limit of detection of 2×10^4 cells/mL was based on isotachophoretic focusing coupled to a simultaneous hydrodynamic-electrokinetic injection (SHEI) in conditions of field-amplified sample injection (FASI).

Table 1. Results Obtained by Capillary ITP Coupled with SEHI-FASI for the Analysis of Bacteria in River and Natural Water Samples

water sample	peak area[a] (mAU s)	V_{sample}[b] (mL)	V_{RE}[c] (mL)	cells/mL (ATP)[d]	RSD (%) ($n = 5$) on ATP measurements	cells/mL (ITP)[e]	RSD (%) ($n = 5$) on ITP measurements	difference between methods
Garrigues	11.3	100	12.34	$1.28 \times 10^4 \pm 0.17 \times 10^4$	11	$1.95 \times 10^4 \pm 0.29 \times 10^4$	12	34%
Vidourles	31.6	10	12.27	$3.80 \times 10^5 \pm 0.36 \times 10^4$	8	$5.41 \times 10^5 \pm 0.46 \times 10^5$	7	30%
Rhône	51.2	10	11.95	$9.07 \times 10^5 \pm 0.66 \times 10^4$	6	$8.54 \times 10^5 \pm 1.31 \times 10^5$	12	6%

[a] Average peak area (five successive injections). [b] Volume of filtrated sample. [c] Average volume of recovery electrolyte (RE) used to recover the bacteria from the filter. [d] Cell concentrations determined by cellular ATP measurement (±95% confidence interval). [e] Cell concentrations determined by SEHI-FASI-ITP (±95% confidence interval).

dx.doi.org/10.1021/ac200684t |*Anal. Chem.* 2011, 83, 4949–4954

Hydrodynamic injection prevented sample depletion at the vicinity of the capillary inlet during electrokinetic injection. In the optimized conditions, SHEI-FASI yields to an enhancement factor of about 500-fold as compared to a classical hydrodynamic injection. Very good reproducibility was obtained with RSD values ($n = 5$) on the migration time and peak area of *E. cloacae* of 3% and 5%, respectively. The electrophoretic method was validated and then applied to the bacteria quantification in natural water (river and natural spring water).Very good agreements were obtained between cellular ATP measurement (a classical method for bacteria quantification) and this CE methodology. Differences of only 6−34% are noticed between these two different approaches. Because the electrical current used in this work is very low (1 μA), it would be possible to use SEHI-FASI-ITP with a capillary of higher inner diameter (200 μm) to further decrease the LOD of bacteria.

AUTHOR INFORMATION

Corresponding Author

*Phone: +33-4-6714-3427. Fax: +33-4-6763-1046. E-mail: hcottet@univ-montp2.fr.

ACKNOWLEDGMENT

H.C. gratefully acknowledges the support from the Région Languedoc-Roussillon for the fellowship "Chercheurs d'Avenir". T.Z. thanks the University of Montpellier 2 for a postdoctoral grant from the Scientific Council. We also thank COLCOM for funding a Ph.D. fellowship for F.O., the ANR Dendrimat (grant reference ANR-096MAPR-0022-03), and Yorkshire Forward for support through a Development Grant to Paraytec Ltd (grant reference YHF/02803/RD09). J.P.Q. thanks the University of Montpellier 2 and the University of Tasmania for supporting his research stay at the University of Montpellier 2 and the Australian Research Council for a Future Fellowship (FT100100213).

REFERENCES

(1) Hornick, R. B.; Music, S. I.; Wenzel, R.; Cash, R.; Libonati, J. P.; Snyder, M. J.; Woodward, T. E. *Bull. N. Y. Acad. Med.* **1971**, *47*, 1181–1191.

(2) Sack, D. A.; Tacket, C. O.; Cohen, M. B.; Sack, R. B.; Losonlsky, G. A.; Shimko, J.; Nataro, J. P.; Edelman, R.; Levine, M. M.; Giannella, R. A.; Schiff, G.; Lang, D. *Infect. Immun.* **1998**, *66*, 1968–1972.

(3) Feng, P. *J. Food Prot.* **1992**, *55*, 927–934.

(4) Beecher, D. J.; Wrong, A. C. *Appl. Environ. Microbiol.* **1994**, *60*, 4614–4616.

(5) Nakano, S.; Maeshima, H.; Matsumura, A.; Ohno, K.; Ueda, S.; Kuwabara, Y.; Yamada, T. *J. Food Prot.* **2004**, *67*, 1694–1701.

(6) Yang, I. C.; Shih, D. Y.; Huang, T. P.; Huang, Y. P.; Wang, J. Y.; Pan, T. M. *J. Food Prot.* **2005**, *68*, 2123–2130.

(7) Yang, I. C.; Shih, D. Y.; Wang, J. Y.; Pani, T. M. *J. Food Prot.* **2007**, *70*, 2774–2781.

(8) Manzano, M.; Giusto, C.; Iacumin, L.; Cantoni, C.; Comi, G. *J. Appl. Microbiol.* **2003**, *95*, 1361–1366.

(9) Vali, L.; Hamouda, A.; Pearce, M. C.; Knight, H. I.; Evans, J.; Amyes, S. G. B. *Lett. Appl. Microbiol.* **2007**, *44*, 19–23.

(10) Donnelly, C. W.; Baigent, G. T. *Appl. Environ. Microbiol.* **1986**, *52*, 689–695.

(11) Righetti, P. G. *J. Chromatogr., A* **2005**, *1079*, 24–40.

(12) Quirino, J. P.; Terabe, S. *J. Chromatogr., A* **2000**, *902*, 119–135.

(13) Osbourn, D. M.; Weiss, D. J.; Lunte, C. E. *Electrophoresis* **2000**, *21*, 2768–2779.

(14) Quirino, J. P.; Terabe, S. *Anal. Chem.* **2000**, *72*, 1023–1030.

(15) Oukacine, F.; Garrelly, L.; Romestand, B.; Goodall, D.; Zou, T.; Cottet, H. *Anal. Chem.* **2010**, *83*, 1571–1578.

(16) Hjertén, S.; Elenbirg, K.; Kilar, F.; Liao, J. *J. Chromatogr.* **1987**, *403*, 47–61.

(17) Armstrong, D. W.; Schulte, G.; Schneiderheinze, J. M.; Westenberg, D. J. *Anal. Chem.* **1999**, *71*, 5465–5469.

(18) Schneiderheinze, J. M.; Armstrong, D. W.; Schulte, G.; Westenberg, D. J. *FEMS Microbiol. Lett.* **2000**, *189*, 39–44.

(19) Armstrong, D. W.; Girod, M.; He, L.; Rodriguez, M. A.; Wei, W.; Zheng, J.; Yeung, E. S. *Anal. Chem.* **2002**, *74*, 5523–5530.

(20) Zheng, J.; Yeung, E. S. *Anal. Chem.* **2003**, *75*, 818–824.

(21) Buszewski, B.; Klodzińska, E. *Electrophoresis* **2008**, *29*, 4177–4184.

(22) Jackowski, M.; Szeliga, J.; Klodzinska, E.; Buszewski, B. *Anal. Bioanal. Chem.* **2008**, *391*, 2153–2160.

(23) Garcia-Canas, V.; Cifuentes, A. *Electrophoresis* **2007**, *28*, 4013–4030.

(24) Hoerr, V.; Stich, A.; Holzgrabe, U. *Electrophoresis* **2004**, *25*, 3132–3138.

(25) Petr, J.; Ryparová, O.; Znaleziona, J.; Maier, V.; Ševčík, J. *Electrophoresis* **2009**, *30*, 3863–3869.

(26) Yu, L.; Li, S. M. Y. *J. Chromatogr., A* **2007**, *1161*, 308–313.

(27) Horká, M.; Horký, J.; Matoušková, H.; Šlais, K. *Anal. Chem.* **2007**, *79*, 9539–9546.

(28) Petr, J.; Jiang, C.; Sevcik, E.; Tesarova, E.; Armstrong, D. W. *Electrophoresis* **2009**, *30*, 3870–3876.

(29) Armstrong, D. W.; He, L. *Anal. Chem.* **2001**, *73*, 4551–4557.

(30) Rodriguez, M. A.; Lantz, A. W.; Armstrong, D. W. *Anal. Chem.* **2006**, *78*, 4759–4767.

(31) Tong, M. Y.; Jiang, C.; Armstrong, D. W. *J. Pharm. Biomed. Anal.* **2010**, *53*, 75–80.

(32) Hoerr, V.; Ziebuhr, W.; Kozitskaya, S.; Katzowitsch, E.; Holzgrabe, U. *Anal. Chem.* **2007**, *79*, 7510–7518.

(33) Yang, Y. Z.; Boysen, R. I.; Hearn, M. T. W. *Anal. Chem.* **2006**, *78*, 4752–4758.

(34) Quirino, J. P.; Terabe, S. *Science* **1998**, *282*, 465–468.

(35) Quirino, J. P.; Terabe, S. *Anal. Chem.* **1999**, *71*, 1638–1644.

(36) Dawod, M.; Breadmore, M. C.; Guijt, R. M.; Haddad, P. R. *J. Chromatogr., A* **2009**, *1216*, 3380–3386.

(37) Shen, Y.; Smith, R. D. *J. Microcolumn Sep.* **2000**, *12*, 135–141.

(38) Shen, Y.; Berger, S. J.; Anderson, G. A.; Smith, R. D. *Anal. Chem.* **2000**, *72*, 2154–2159.

(39) Dasgupta, P. K.; Surowicc, K. *Anal. Chem.* **1996**, *68*, 4291–4299.

(40) Krivácsy, Z.; Gelencsér, A.; Hlavay, J.; Kiss, G.; Sárvári, Z. *J. Chromatogr., A* **1999**, *834*, 21–44.

(41) Hirokawa, T.; Koshimidzu, E.; Xu, Z. *Electrophoresis* **2008**, *29*, 3786–3793.

(42) Chen, W. T.; Hendrickson, R. L.; Huang, C. P.; Sherman, D.; Geng, T.; Bhunia, A. K.; Ladisch, M. R. *Biotechnol. Bioeng.* **2005**, *89*, 263–273.

(43) Combrugge, J.; Waes, G. In *Methods for Assessing the Bacteriological Quality of Raw Milk from the Farm*; Heeschen, W., Ed.; The International Dairy Federation: Brussels, Belgium, 1991.