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ANALYTICAL TECHNOLOGIES IN THE BIOMEDICAL AND LIFE SCIENCES

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Miniaturised free flow isotachophoresis of bacteria using an injection moulded separation device

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ABSTRACT

A new design of miniaturised free flow electrophoresis device has been produced. The design contains a separation chamber that is 45 mm long by 31.7 mm wide with a depth of 50 μm and has nine inlet and nine outlet holes to allow for fraction collection. The devices were formed of polystyrene with carbon fibre loaded polystyrene drive electrodes and produced using injection moulding. This means that the devices are low cost and can potentially be mass produced. The devices were used for free flow isotachophoresis (FFITP), a technique that can be used for focussing and concentrating analytes contained within complex sample matrices. The operation of the devices was demonstrated by performing separations of dyes and bacterial samples. Analysis of the output from FFITP separations of samples containing the bacterium *Erwinia herbicola*, a biological pathogen, by cell culturing and counting showed that fractionation of the output was achieved.

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1. Introduction

There are many applications which require the monitoring of biological agents such as environmental monitoring, biological weapon monitoring and medical diagnosis. Some applications involve the analysis of atmospheric samples. This would necessitate the sampling of air and would be likely to produce large volumes of samples containing the potential harmful organism amongst other things. Thus, for a suitable monitoring system there is a requirement to extract the target analyte(s) from the samples for subsequent analysis using an appropriate technique such as spectroscopy [1] or an immunoassay [2].

To allow for the detection and identification of bacteria within air samples there is usually a requirement to initially carry out some sample pretreatment. To obtain concentrated bacteria samples from complex samples there are numerous preparative methods that can be used such as solid phase extraction [3] or centrifugation [4], both of which need to be performed in a batch-wise manner. An alternative approach is to use a cyclone-based air sampler [5] that can deliver, for a given period, a continuous output in the form of a liquid, containing bacteria. A potential way of handling such samples is to use free flow preparative electrophoretic techniques. In free flow electrophoresis the sample is continuously supplied to the separation device using pressure driven flow and then subjected to a perpendicular electric field to perform an electrophoretic separation based on mobility differences between the sample components. This process produces a continuous output which, if the appropriate instrumentation is used, can be collected as a series of fractions. The electrophoretic separation stage can employ a number of separation mechanisms such as zone electrophoresis (FFZE), isoelectric focussing (FFIEF) and isotachophoresis (FFITP). Such techniques have seen widespread use for sample preparation in fields such as protein analysis [6] and biological pathogen analysis [7]. Electrophoretic techniques should allow for separations of bacteria to be made based on the overall surface charge exhibited; which will be a function of the proteins, lipids and sugars found in the bacterial membrane.

In recent years free flow electrophoretic techniques have been shown to be suitable for miniaturisation. One of the earliest miniaturised electrophoresis devices was that developed for FFZE by Raymond et al. [8]. Subsequently other forms of electrophoresis have been miniaturised including FFIEF [9] and FFITP [10]. Since the earliest developments a considerable number of miniaturised devices, in particular those that employ FFZE and FFIEF, have been developed. Such devices have been the subject of a number of comprehensive reviews [11,12]. The use of miniaturised devices offers a number of potentially advantageous features for the analysis of airborne biological hazards. These include the ability to produce portable devices so that monitoring can be carried out in the field, reducing sample turnaround times and the ability to produce low

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cost devices if polymer materials are used for fabrication. This latter advantage means that disposable, single-use devices can be produced, thereby eliminating cross contamination and negating the need to clean devices which have been exposed to hazardous materials.

Whilst the use of miniaturised FFZE and FFIEF with bacteria samples has been reported [13–16], for the work described in this paper miniaturised FFITP was perceived as being the most useful technique to employ. This belief was based on the fact that FFITP cannot only be used to focus samples into zones but can also be used to pre-concentrate dilute samples, whereas with FFZE for example some additional process, such as trapping at a gel interface [14,15], is required for pre-concentration. To date there has only been a limited amount of research carried out involving miniaturised FFITP. This group has previously produced polystyrene devices [17] whereas the group headed by Janasek at ISAS in Dortmund have produced glass [18,19] and poly(dimethylsiloxane) [10] devices. These studies have primarily involved the analysis of dye samples to evaluate device performance, however, they have also been used with myoglobin samples [10,19]. No previous use of miniaturised FFITP with bacteria samples is known. Indeed, it is not believed that capillary scale FFITP has been used for the analysis of bacteria. However, capillary scale FFITP has been used for a wide variety of applications including samples such as peptide fragments [20], enzymes from cell extracts [21] and proteins [6]. The use of capillary zone electrophoresis (CZE) for analysing bacteria has been reported by numerous groups [22–24]. Recently Oukacine et al. [25] has reported the use of the pre-concentration effect of isotachophoresis to assist with conventional capillary isotachophoresis and coupled capillary isotachophoresis-CZE separations of bacteria.

In this paper we report on the novel design of a miniaturised chip device suitable for performing free flow electrophoretic separations. The device, which incorporated drive electrodes and multiple outlets for fraction collection, was produced using injection moulding and as such was fabricated entirely of polymeric materials. In this work the device was used for FFITP on a number of samples including those containing the bacterium *Erwinia herbicola*.

2. Materials and methods

2.1. Device fabrication

The miniaturised devices were produced in-house using injection moulding. This process involved, firstly, making a solid model of the device using AutoCAD 3D software (Autodesk, San Jose, CA, USA). This solid model was then converted to toolpaths using EdgeCAM 10.5 (Pathtrace, Reading, UK), to allow mould cavities to be made. These were produced by directly milling aluminium blocks using a CNC milling machine (CAT3DM6, Datron Technology, Milton Keynes, UK). The separation devices incorporated three injection moulded pieces: the drive electrodes, a top plate (which was overmoulded on top of the electrodes), and a bottom plate that defines the separation chamber. The electrodes were formed of 40% carbon fibre filled high impact polystyrene (RTP Company UK, Bury, UK). These were moulded using a Babyplast 6/6 injection moulder (Cronoplast, Barcelona, Spain). The other parts were moulded using a Babyplast 6/10 injection moulder (Cronoplast). The top plates and bottom plates were formed of crystal polystyrene (Northern Industrial Plastics Limited, Oldham, UK). The top plate included two oval recesses into which connector blocks were moulded. These connector blocks were formed of thermoplastic elastomer (TPE) (THERMOLAST K®, Kraiburg, Waldkraiburg, Germany). To form a complete device an ion permeable membrane was placed between each of the electrode chambers and the separation chamber. The membranes used were 0.1 μm pore size polycarbonate (Whatman, Maidstone, UK) bonded in place with superglue. Finally, all of the parts of the devices were put together and then sealed using an ultrasonic welder (2000aed, Branson Ultrasonics Corporation, Slough, UK).

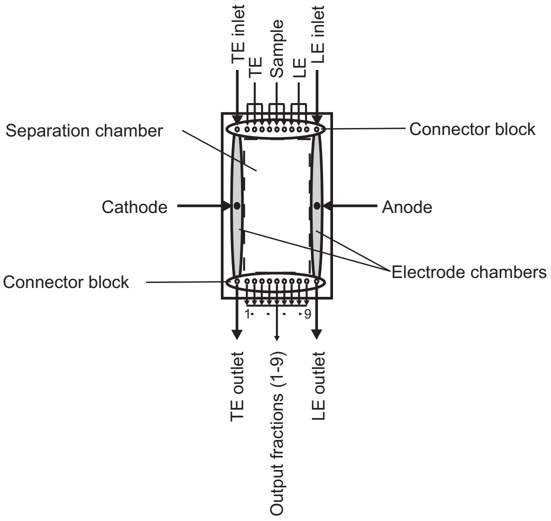

Fig. 1. Schematic diagram of the miniaturised free flow electrophoresis separation device. The separation chamber is 45 mm long by 31.7 mm wide and had a depth of 50 μm.

The basic layout of the free flow separation devices comprised a rectangular separation chamber. Electrode chambers were located along the two long sides of the chamber and inlet and outlet holes along the two short sides of the chamber. A schematic diagram of a device is shown in Fig. 1. The separation chamber was 45 mm long by 31.7 mm wide and had a depth of 50 μm. The electrodes were 45 mm long by 6 mm wide with a 2 mm wide chamber for electrolytes to flow through. Connector blocks were positioned along the short sides of the device. These blocks incorporated 0.9 mm diameter inlet and outlet holes to the separation chamber and the electrode chambers. This arrangement was designed so that fluidic connections to the device could be rapidly achieved as the flexible nature of the TPE allowed for push-fit sealing. Each electrode chamber had a single inlet and outlet hole and the separation chamber had nine inlets and outlets, equally spaced along the width of the chamber.

The device was designed for use with a fluidic interface constructed out of two 78 mm wide by 78 mm long by 6 mm thick blocks of poly (methyl methacrylate) that were bolted together. The bottom block of the interface had two sets of recesses milled into it that were designed to fit the inlet and outlet connector blocks on the separation device. Within the recesses interconnecting tubes were milled that fitted into the inlet and outlet holes. PVC tubing was attached to the top of these interconnects and sealed into the top block of the interface with silicone sealant (RS Components, Corby, UK). The use of the fluidic interface allowed for the easy exchange of the fluidic devices. In practice it was found difficult to collect output fractions from the interface. Thus, in the work performed in this study the device was used with the fluidic interface attached to the inlet holes but not the outlet holes. Instead the outlet holes contained 0.5 mm^2 bootlace ferrules (RS Components) which formed small reservoirs.

2.2. Instrumentation for FFITP

The separations were driven using a PS350 high voltage, 5 kV power supply (Stanford Research Systems, Sunnyvale, CA, USA), configured to supply positive voltages. Electrolytes and sample were fed to the free flow separation chamber using a syringe pump flow system. The syringe pump flow system consisted of three individually addressable, high precision stepper motor driven, computer controllable, syringe pumps fitted with four way distribution valves (VersaPump 6 model number 54022, Kloehn Inc, Las Vegas, NV, USA). The pumps could supply 48,000 steps per stroke and were operated with 250 μL syringes (Kloehn) fitted. Electrolytes were supplied to the electrode compartments from gravity feed solution reservoirs. Flow of these solutions to the device was controlled by means of solenoid actuated valves (LFVA1210120H, The Lee Company, Westbrook, CT, USA). Connections between reservoirs, pumps, valves and the fluidic interface to the device inlets were made using narrow bore polytetrafluoroethylene (PTFE) tubing (i.d. 0.032 in. The Lee Company).

Control of the power supply and gravity feed fluid transport system was achieved using a standard PC with LabVIEW software (version 7.1, National Instruments, Austin, TX, USA). The NIDAQ driver (National Instruments), programmed using LabVIEW, was used to control the hardware interfacing which was made using two National Instruments cards. A PCI-6503 controlled the valves through a DIO-24MX relay box (Goldchip, Wimbourne, UK) and a PCI-GPIB card controlled the power supply. The syringe pumps were interfaced to the computer using RS-232 and controlled with ASCII string commands.

2.3. FFITP method

The separations were carried out using a two step LabVIEW program. In the first step the leading electrolyte syringe was filled for 48,000 steps. The sample and terminating electrolyte syringes were filled for 28,800 steps. The differential filling of the syringes was done because it was found necessary to supply the solutions at different rates when undertaking the isotachophoretic separation. This was because the use of pH indicator dyes as samples gave evidence that there was not always enough leading electrolyte present in the separation chamber to buffer the separations fully. This problem could be lessened by supplying the leading electrolyte at a higher rate than the other solutions. All of the syringes were filled at a rate of 5000 steps s^{-1} which was a flow rate of 1562 $\mu L\,min^{-1}$. For the separation, the leading electrolyte syringe was set to run at 100 steps s^{-1} and the sample and terminating electrolyte syringes at 60 steps s^{-1}. By filling the syringes with different numbers of steps it meant that all the syringes finished in the same position at the end of the second step of the programme. The syringe settings meant that the leading electrolyte was supplied to the device at 31.25 $\mu L\,min^{-1}$ and the sample and terminating electrolyte at 18.75 $\mu L\,min^{-1}$. The leading electrolyte was supplied to the separation chamber through the right hand three inlet holes, the sample through the middle three and the terminating electrolyte through the left hand three, as illustrated in Fig. 1. During the separation step the hydrodynamic valves were opened to allow the leading and terminating electrolytes to flow through the appropriate electrode chambers. For FFITP separations a high voltage with a constant current of 1 mA was supplied between the cathode (ground) and anode (+high voltage).

A Canon IXUS 80 (Canon Inc, Tokyo, Japan) digital camera was used to capture visual images of FFITP separations.

2.4. Chemicals

The following chemicals were used in the production of electrolyte solutions: hydrochloric acid (1.0 M volumetric standard) from Acros (Loughborough, UK); imidazole (>99%) and Tris(hydroxymethyl) aminomethane (Tris) (99%) from Aldrich (Gillingham, Dorset, UK) and N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) (99.5%) from Sigma (Gillingham, Dorset, UK). The dyes used were new coccine (75%) and methyl orange (85%) from Aldrich and bromophenol blue (99%) from Acros. All solutions were prepared using deionised water.

2.5. Bacteria growth

The bacteria species used in this work was *E. herbicola* NCIMB 13953 (NCIMB, Aberdeen, UK). Cultures of *E. herbicola* were produced by growing on nutrient agar plates (Lab008 nutrient agar, LabM, Bury, UK) for 24 h at 28 °C. When samples were required for analysis biomass was harvested from the plates by flooding the plate with sterile physiological saline (0.9% (w/v) NaCl, autoclaved 15 min at 121 °C). A slurry was then produced by mixing using a sterile loop. 1 mL samples of the slurry were then centrifuged for 10 min at 12,000 × *g*. The resultant pellet was resuspended in saline using a vortex mixer and then centrifuged again for 10 min at 12,000 × *g*. The supernatant was drained off and the resulting bacteria cells resuspeneded in sterile saline to produce a sample with a volume of 1 mL. Samples were than analysed using FFITP after dilution as detailed in the text. Samples used had, prior to dilution, optical densities of approximately 2 at 560 nm.

2.6. Bacteria counting

The outputs obtained from the microdevices were analysed using a modified Miles Misra method. Firstly, this involved collecting fractions from the outlet reservoirs using micro-Pasteur pipettes. Then, 10 μL of each collected fraction was taken and diluted with 90 μL of sterile saline. These samples were then subsequently serially diluted seven more times using a similar procedure. Thus, eight samples with dilutions of 1×10^{-1} to 1×10^{-7} were produced. The samples were then analysed on a Petri dish of nutrient agar that had been divided up into eight segments. 10 μL of each of the individual samples was placed in these eight segments. Three plates were produced for each sample to produce triplicate results. The plates were then incubated at 28 °C for 24 h and the number of distinct colonies formed then counted.

3. Results and discussion

The FFITP separations performed in this work were carried out using a novel device design. Whilst this group has previously reported a design of injection moulded free flow chip device [17], the new design contains a number of enhancements compared to that previously shown. The most obvious change is that the number of inlet and outlet holes has been increased from three to nine. This change was made as it should allow for better fraction collection from the apparatus. The size of the separation chamber has also been revised. The new design incorporates a separation chamber that is longer and wider than the previous design but with a narrower depth. This change should improve the performance of the device; the change in geometry should give improved heat dissipation through the walls and the thinner depth should allow lower currents to be used for the separations thereby limiting unwanted electrode reactions, thus reducing the effect of some of the detrimental features of FFITP [26]. A further change that was made to reduce unwanted electrode reactions was that the design of the drive electrodes was altered. In the devices used in this work the electrode forms the base of the electrode chamber, whilst in the earlier design the electrode formed the base and walls of the chamber, an arrangement which caused significant bubble generation to occur during the separations. The longer length of the separation chamber should allow longer residence times and thus improved resolution of complex samples. Connector blocks were incorporated into the new design to improve the operation of the experimental set up as they should enable the rapid exchange of devices to be made.

Table 1
Composition of electrolyte systems used for FFITP separations in this work.

Electrolyte system	A	B
Leading electrolyte	10 mM HCl	5 mM HCl
pH buffer	Imidazole	Tris
pH	6.75	8.0
Terminating electrolyte	10 mM HEPES	10 mM HEPES

Prior to attempting to use the devices with bacterial samples, a series of experiments were carried out using dyes as test mixtures. This was done as such samples could be readily observed visually within the devices; therefore, a rapid assessment of the performance of the devices could be made. Mixtures composed of three dyes, new coccine, bromophenol blue and methyl orange, were analysed using electrolyte system B that is detailed in Table 1. These dyes were selected as they had previously been found to be separable using capillary scale isotachophoresis with similar electrolytes and because they also offered a clear contrast in colours. Using the miniaturised devices it proved possible to separate this mixture using FFITP. An example of one of the separations achieved is illustrated in the photographs shown in Fig. 2. These pictures show half of the separation device (the outlet end) before the application of the electric field, Fig. 2a, and after the electric field has been applied, Fig. 2b. The inlet end of the device could not be clearly observed due to being covered with the fluidic interface block. The results show that prior to the application of the electric field the device only contained a single disperse purple band. This band is positioned close to the centre of the device which shows that there was good hydrodynamic balancing when filling. On the application of the driving current for the FFITP the dye mixture began to separate out into different components, with a full separation into bands of red (new coccine), blue (bromophenol blue) and yellow (methyl orange) taking around 120 s. The photograph shown in Fig. 2b was captured after the separation had been running for 287 s. The individual bands that resulted from the FFITP were all much more sharply defined than the original sample band shown in Fig. 2a. This effect occurred because the process of isotachophoresis causes the individual sample components to form bands that have a concentration that is governed by the concentration of the leading ion used. In this case the sample components were more dilute than the leading ion concentration so a concentration effect was observed. The separation also shows a small amount of evidence of a colourless zone between the red and the blue which is thought to arise from an impurity in one of the samples. The likely source of the contamination was thought to be new coccine, which was the least pure sample, as a similar colourless zone can be seen with mixtures of this dye and bromophenol blue. Whilst not the primary aim of the research, the ability to separate three dyes represented an improvement on the separations that could be achieved using previous designs of injection moulded separation devices [17].

A comparison of the two photographs shows that the position where the dyes exited the device was shifted towards the right hand side of the device after FFITP had taken place. This shift was thought to have occurred due to the mechanisms that take place during FFITP which are hydrodynamic movement from the top to the bottom of the device and a left to right movement due to the applied electric field. The result shown in Fig. 2b also illustrates one of the potential problems with the devices, namely that towards the outlet the zones can become tightly focussed. Therefore, zones could be produced that were smaller than the outlet holes, thus limiting the ability to obtain pure fractions from a single outlet.

Whilst investigating the behaviour of the free flow separation devices using dye samples, attempts were made to see the effect of using a higher separation current than 1 mA. With the previous design of device produced by this group it was found that the best FFITP separations were achieved using a current of 5 mA [17]. Thus, a number of runs were made applying a drive current of 2 mA. However, when this increase was made, whilst a good separation of the dyes was noted, the back edge of the rearmost zone, that of methyl orange, start to exhibit eddying after the FFITP had been running for a whilst. The cause of this effect was thought to be due to Joule heating as previously in conventional scale FFITP distorted profiles have been found to occur due to this phenomenon [26].

After determining that the devices were suitable for performing FFITP separations, experiments were then carried out on using samples containing bacteria. For this work it was decided to use *E. herbicola* as the sample microorganism. This species was selected as it represents a typical Gram-negative rod-shaped prokaryotic microorganism.

The FFITP separations of the bacteria containing samples were made using electrolyte system A, shown in Table 1. This system incorporated a different leading electrolyte to that used for the dye separations. For the bacteria separations a higher strength leading ion concentration of 10 mM was used. Although this change would narrow the width of the zones formed for a particular sample,

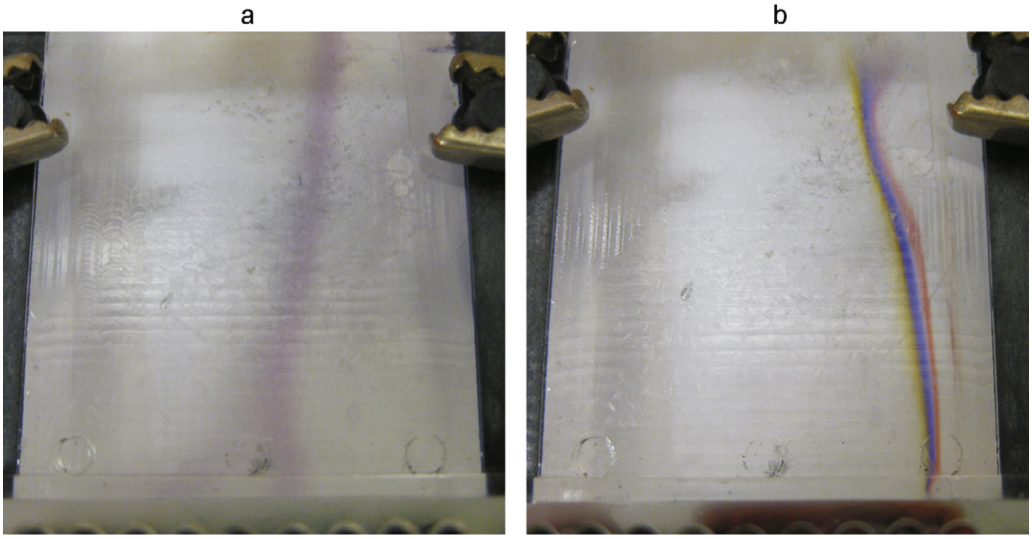

Fig. 2. FFITP separation of a dye mixture achieved in an injection moulded polymer chip. The sample illustrated contained 0.5 mM new coccine (red), 0.35 mM bromophenol blue and 0.5 mM methyl orange (yellow). The separation was achieved using electrolyte system B, detailed in Table 1, with an applied drive current of 1 mA.

which could worsen the problems mentioned above with regard to obtaining pure fractions, it was thought beneficial for other reasons. The higher concentration electrolyte system should help lessen detrimental separation effects which can arise from Joule heating. Whilst this phenomenon was not seen to cause any problems with dye separations when using a drive current of 1 mA, it was thought that detrimental effects may occur if the separations were run for extended periods of time, a situation that may be needed for the bacteria samples. It was also thought likely that the samples to be used would have quite high bacteria concentrations. Possible real samples may contain only low concentrations of bacteria but might contain high concentrations of unwanted species. For handling samples containing species with high concentrations a higher strength electrolyte system is more suitable. The pH used for analysing the bacteria, 6.75 was deemed appropriate. This was because the pI point of *E. herbicola* has been previously suggested to be <4.2 [13]. Therefore, at pH 6.75 the bacteria should exhibit an appreciable anionic mobility.

To see whether the miniaturised flow devices could be used for bacteria separations a series of runs were made using samples of solutions of *E. herbicola*. Prior to analysis the bacteria samples were either diluted with water or with a new coccine solution. Mixing with a dye solution was done to enable visualisation of where the separation was taking place within the separation chamber. This process would also indicate through which outlets the samples were leaving the device. In FFITP visualisation with a dye solution is achieved because in isotachophoresis the separated zones migrate in a stack between the leading and terminating zones. Thus, if one zone, in this case the dye, can be identified, all of the other sample zones will be adjacent. Experiments with the dyes used in the test mixture showed evidence that bacteria co-migrated with bromophenol blue. When mixtures of this dye and the bacteria were analysed the dye zone formed was significantly broader than when the bacteria were absent. This meant the *E. herbicola* had a lower mobility than new coccine under the separation conditions used. Thus, in a mixture of *E. herbicola* and new coccine, the bacteria should migrate behind the dye zone. With mixtures of new coccine and *E. herbicola* no broadening of the dye zone was observed.

With the bacteria samples, the output from the FFITP runs were collected from the nine outlets and then analysed. The fractions were cultured and the number of colony forming units (CFU) counted. Examples of the results obtained with two different samples are shown in Fig. 3. The results shown are for a sample of 40 μL of bacteria diluted with water to give a final volume of 5 mL (Fig. 3a) and 5 μL of bacteria diluted with a solution of 0.5 mM new coccine to a final volume of 5 mL (Fig. 3b). In the results shown it can be seen that in both samples most of the bacteria were recovered in a single fraction, with the majority of the fractions containing no bacteria at all. The results show that more bacteria were found in the sample shown in Fig. 3a compared to that in Fig. 3b which corresponds with the different dilutions carried out on the samples. The fact that bacteria were recovered from the outlet fractions in all samples confirmed that neither the constituents of the electrolyte solutions nor the separation voltage affected the viability of the bacteria.

The results obtained indicated that the samples were being focussed during the separation process. If there was no focussing, the bacteria would be expected to be found in a larger number of outlets from the device. In addition, in the absence of focussing the amount of bacteria found in the outlet fractions would be much more evenly distributed. When samples were flowed through the device in the absence of an applied electric field bacteria were recovered from all of the device outlets, as can be seen in Fig. 3c which shows a result produced under such conditions. The outputs of the miniaturised FFITP separations, in terms of the observed profile across the fraction outlets, have a similar appearance to those that have been achieved using conventional scale equipment [6,27], thus suggesting FFITP was indeed taking place in the miniaturised separation device. If the mobility of the bacteria was very low, it may have migrated zone electrophoretically within the terminating ion zone. Under such circumstances the bacteria would be likely to be found in the outlets positioned towards the left hand side of the device. The spread of the fractions where the bacteria were found would look a little different if this situation had arisen. With conventional scale FFZE less sharp boundaries, in terms of the fractions where the output was collected, were observed compared to the FFITP separations [6].

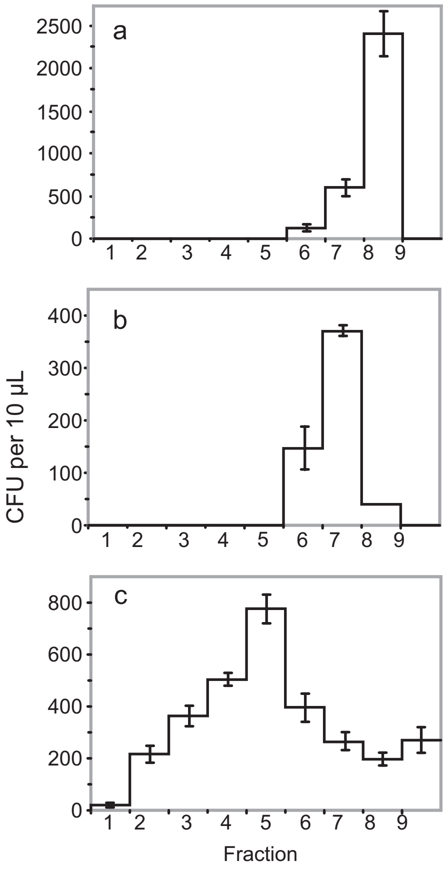

Fig. 3. Examples of the output collected from FFITP separations of bacteria samples. The sample illustrated in (a) was composed of 40 μL of *E. herbicola* diluted with water to give a final volume of 5 mL and that in (b) was composed of 5 μL of *E. herbicola* diluted with a solution of 0.5 mM new coccine to a final volume of 5 mL. The error bars shown represent ±the standard deviation based on three determinations. The separations were achieved using electrolyte system A, detailed in Table 1, with an applied drive current of 1 mA. (c) The output collected after flowing samples through the separation device with no applied electric field. The sample was composed of 5 μL of *E. herbicola* diluted with a 2% (v/v) pH 3–10 ampholyte solution (Fluka, Gillingham, Dorset, UK) to a final volume of 5 mL.

In an ideal situation the FFITP separation would result in the bacteria only being recovered from a single outlet of the separation device. However, in reality such a result was not achieved with any of the samples. Within the experimental procedure used, a number of problems were identified which could prevent such a result being achieved. These potential problems were that removing liquid from the small reservoirs could change the hydrodynamic equilibrium within the device, fractions could be collected before steady state was reached and the syringe pumps might need to be refilled during the course of a separation. It was thought that the most likely reason for seeing the bacteria dispersed in several outlets was due to the procedure used to collect the fractions. With samples containing dyes it was observed that the removal of liquid

Table 2
Collected fraction where the highest concentration of bacteria was found after FFITP separations. Samples 1–4 were composed of 40 μL of *E. herbicola* diluted with water to give a final volume of 5 mL and samples 5–8 were composed of 5 μL of *E. herbicola* diluted with a solution of 0.5 mM new coccine to a final volume of 5 mL.

Sample	Fraction								
	1	2	3	4	5	6	7	8	9
1						X			
2								X	
3						X			
4								X	
5								X	
6							X		
7						X			
8							X		

from the outlet reservoirs sometimes caused the position of the separated zones to move. When such a movement occurred the flows returned to the original position after a short period of time. The hydrodynamic behaviour within the chamber has an effect on the outlets through which the sample was recovered. Thus, changes in the hydrodynamic flow within the device meant that the fraction containing the concentration of bacteria showed some variation between runs. This can be seen in the results shown in Table 2 which shows the fractions in which the highest bacteria concentrations were found. This movement is not a problem if all fractions are collected, but highlights the usefulness of adding a dye to the separation to locate the sample position. Such an approach has also been previously advocated for capillary scale FFITP separations [6]. Collection of samples before steady state was reached could also lead to the samples being recovered from a different location within the device as the leading electrolyte zone would not be fully formed. However, to reduce the chances of this problem occurring sample collection did not start for 120 s. This time delay was selected on the basis of how quickly the dye separations, mentioned earlier, were found to occur. When dye-bacteria mixtures were analysed the dye was observed to have formed a sharp zone within this time. A further possible cause of the sample being spread across a number of outlets was that the syringe pumps needed to be refilled during a separation. This occurred because to ensure enough volume of liquid was collected from all of the fractions to allow subsequent culturing, the separations were run for two cycles of the programme. This meant there was a gap in the middle of the runs of around 40 s when no high voltage was applied whilst the syringe pumps were refilled. During this time there would be a small amount of sample dispersion in the chamber.

The overall aim of the project was to develop a system suitable for the routine analysis of air samples. For such a system, the method used in this work for the detection of the bacteria was obviously not practical for a number of reasons. Disadvantageous features of the counting method include the fact that it is labour intensive, time consuming and is a rather inaccurate measure as only a small population of the overall number of bacteria are counted and performing colony counts assumes that a single colony arose from a single bacterium cell. For a more practical system it will be necessary to couple the FFITP separation to another rapid detection system. The output from an FFITP separation can be used in a wide variety of detection systems because, if a pure zone of a substance can be separated, it will only contain the species of interest and the counter ion from the leading electrolyte. Possible detection systems that could be used with bacteria samples include leaky waveguides [28] and surface enhanced Raman scattering (SERS). This latter method has shown great promise for the detection of very low numbers of bacteria and has been successfully demonstrated for the analysis of pathogens that may be found in airborne samples [29,30]. When SERS has been used with suitable chemometric procedures it has been shown to be capable of discrimination between species and even sub-species [31].

4. Conclusions

A new design of injection moulded polymer miniaturised separation device suitable for free flow electrophoresis has been produced. The refinements made to the design were found to improve the operation of the devices compared to those previously produced as they allowed for the collection of the output in a number of fractions, improved separation performance and reduced unwanted electrode reactions. The devices were successfully used for obtaining FFITP separations of dye samples and bacteria. Analysis of the output from the different fractions after a separation had been carried out showed evidence that fractionation of the bacteria samples was achieved. Thus, the main outcome of this work was that miniaturised FFITP showed promise as a technique for analysis of bacteria. The bacteria used, *E. herbicola*, have dimensions 0.5 μm diameter × 1–2 μm length which are typical of rod-shaped prokaryotic microorganisms. The ability of FFITP to act as a sample preparation technique means it should be useful in many areas that require the analysis of bacteria to be made. This is because it allows for low concentrations of bacteria in complex sample matrices to be extracted in the form of a concentrated solution that can be subjected to further analysis. The devices used were injection moulded using polystyrene with carbon fibre filled polystyrene electrodes and thus can be produced at low cost. They can thus be considered as disposable, a useful feature for many applications as it means cross contamination between samples can be avoided.

There are still a number of challenges to be addressed. The biggest problem with the current method was the procedure used for fraction collection. In this work the output fractions were collected by hand. However, this was a difficult procedure to do as it can disturb the hydrodynamic flow within the separation chamber leading to problems with obtaining the output in a single fraction. Possible solutions to this problem could include allowing the samples to run into larger reservoirs before they were extracted or developing an automated fraction collection system that could be fed constantly during the separations. A device with more, smaller, outlets would also be better as this would mean that it was more likely that pure samples could be collected within a single fraction.

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