FOOD SAMPLE PREPARATION AND ENRICHMENT FOR RAPID DETECTION

A. N. Sharpe
FILTAFLEX Ltd
PO Box 1224, Almonte, Ontario K0A 1A0, Canada
Tel: 613 256 3066; Fax: 613 256 8681
e-mail: tsharpe@filtaflex.ca

1 INTRODUCTION

The requirement by food agencies to detect one *Salmonella* cell in 25g of food represents a limit of detection of $1:10^{12-13}$ for the cell itself, and $1:10^{18-19}$ for a nucleotide sequence characterizing the genus. Food microbiologists manage the detection daily, using a few $$ of materials, letting microbial cells to *multiply* (amplify), essentially noise-free, by factors of $\geq 10^7$. The millions of cells in agar colonies or broth suspensions are detectable by eye or by chemical, biochemical, immunological or DNA-based tests. The disadvantage of techniques for enriching pathogens by *incubation* is the 24-96h incubation incurred. To meet demands for shorter analytical times, scientists are developing ways to avoid the lengthy incubations that yield large microbial populations, by substituting physical or chemical enrichment for microbial growth.

2 THE ANALYTICAL PROBLEM OF RAPID MICROBIAL DETECTION

A food microbiologist faced with confirming the *absence* of a microbial pathogen in 25g of food by traditional methods can incubate the whole sample in broth in the assurance that, if there is at least one target cell, even one firmly attached to the sample tissue (biofilm), it will multiply and spread into the broth during incubation and eventually be detectable.

Detecting or quantifying foodborne microbes *without* incubation is a bigger challenge - one must get microbes from a food into the detection system. Currently one must extract whole cells and detect them by serological, enzyme, or other phenotypic property; or extract components (DNA or RNA) to provide a consistent analytical base. Currently there are no sampling methods capable of removing microbes quantitatively from foods; for physical or chemical separation and concentration one has little alternative to first suspending the food in 100-250 ml of diluent, in order to have a modest probability of liberating the target cell for detection.

Microscopy, flow cytometry and other direct techniques currently are useless at regulatory limits of detection. At the high magnification needed to observe bacterial cells one needs 10^{6-7} cells/ml to have any practical probability of getting a target cell into a microscope field. With just one target cell in 250 ml, the limit of detection is far away. The situation is very little better for immunological, DNA-based or other analyses.

The detection problem is not actually in achieving microbial populations of $\geq 10^6$ cells, but of $\geq 10^6/ml$ (i.e., the essence is *concentration* not *number*). Tests *can* be made on microscopic volumes *if* target cells can be persuaded to be in them (i.e., can be shifted into smaller volumes). However, if an initial suspension of 250 ml contains only a single target cell (regulatory limit) the whole volume must be "enriched" physically or chemically in order to detect it. Just *how* distant the limit of detection is, can be seen by considering a single high-power microscope field (with the *potential* for identifying microbial cells on the basis of a phenotypic property). With a (focused) volume of around 10^{-9} ml, to ensure that target cells from 250 ml of suspension arrive in a field of view, one must concentrate the sample through a factor of $10^{11}:1$. Microscopy is an extreme example, but serves as a good illustration of the problem.

Some separation/concentration techniques are: centrifugation; membrane filtration; immunomagnetic particles; ion-exchange; affinity chromatography; biphasic partitioning; electro- or dielectrophoresis; standing-wave ultrasound; and foam flotation. Some are useful in limited situations, but few are practical at volumes greater than 1-2 ml, through cost (e.g., antibodies), short range (magnetic or electric fields), or other limitations. Currently, membrane filtration (Direct Epifluorescent Filter Technique, or DEFT) and immunomagnetic particles are the most useful.

The successful current techniques combine a 6-24 h broth incubation, during which the target multiplies, capture on a selective substrate (e.g., immunomagnetic beads), then detection by, say, polymerase chain reaction (PCR). Such compromise methods are used in several commercial test kits. The *ultimate* goal of instantly detecting microbial pathogens in foods may lie far in the future.

Study is urgently needed on: i) methods to cheaply collapse large primary sample volumes into a few ml so that techniques capable of more specific separations (but currently too costly for routine use) can be applied, and; ii) techniques to produce food sample suspensions that are inherently better suited to separating out the target cells.

3. SEPARATION AND CONCENTRATION TECHNIQUES

3.1 Multiplexed Separations

Practical procedures to achieve large concentration factors might combine a multiplicity of less efficient steps. A simple model suggests multiplexed steps could yield separations dramatically *faster* than single-step processes, at the same time attacking the *volume/cost* problem. The philosophy should be kept in mind by anyone developing rapid detection procedures.

The argument (Sharpe 1991) is quite general, one could consider any separation process (centrifugation, membrane filtration, foam flotation, electrophoresis, ion-exchange, affinity chromatography, etc) and any detection method (microscopy, flow-cytometry, CCD/luminescence, etc). Without worrying about exactly which techniques we might use for the separation stages, how long does it take us if we try to extract target cells in a single stage, or two or more less ambitious stages?

Single-Stage Process: Consider a single-stage concentration process as the operation of passing a *capture element* (in reality, the final volume) through a sample N times greater in volume, until it has passed through the whole sample and captured the target microbes from it. If it takes t seconds for the capture element to pass through its own volume in the sample (*specific sweep time*) and this remains constant during the capture pass, the time T required for the capture element to reach the end of its pass is:

$$T = t(N - 1) \qquad\qquad\qquad 1$$

Two-Stage Process: Now concentrate the sample in two stages, each of which is less efficient (yields a smaller increase in analyte concentration) than the above. Instead of just one, use n capture elements for the first stage and, after concentrating the sample into these, concentrate these n elements into one in the manner above. The total time T for concentration is:

$$T = (T_1 + T_2) = t(n + \frac{N}{n} - 2) \qquad\qquad 2$$

and is minimum when $n = N^{1/2}$, i.e., when the overall concentration factor is achieved in two stages of approximately equal effectiveness.

Three or More-Stage Process: A three-stage process is a two-stage process with a stage added at the front-end. If t is the same for all three processes the total time needed is:

$$T = t(2n + \frac{N}{n^2} - 3) \qquad\qquad\qquad 3$$

and is minimum when $n = N^{1/3}$, i.e., three stages of approximately equal effectiveness yield the minimum concentration time. Similarly, p multiplexed processes execute in a minimum time when $n = N^{1/p}$.

Relative Separation Times: To give perspective to this imagine an extreme case of concentrating pathogens from 1,000 ml of suspension into one high-power microscope field (10^{-9} ml), ignoring practicalities such as the need to prepare the product of one stage for introduction to the next (e.g., eluting from a column). To derive a plausible value for t one might consider membrane filtration, where a 1 ml sample might be reduced to 1.10^{-4} ml when it is captured as a layer approximately 1 µm thick in 10s, giving t = 1/1000s for this process. Other processes (e.g., antibody-coated beads) have different specific sweep times, but we can ignore it in illuminating the relative efficiency of multiplexed procedures. By using t = .001s for all stages in concentrating the microbes from a 1,000 ml sample into a 10^{-9} ml microscope field, we find that for single-, two- and three-stage processes, total concentration times would be 1.10^9s (32 years), 2.10^3s (33 min), and 30s, respectively.

Even allowing for different specific sweep times of different processes, the effect of multiplexing separation stages, shown by this simple treatment, *is so dramatic* that one should keep it in mind. The possibilities are endless. One might, for example, combine a large column as the first stage (plus washing and elution), second stage centrifugation, a third stage in the flow-cytometer, and so on. Or, a process might be repeated on a smaller scale. As sample volume comes down through the stages, adsorption to an antibody-coated filament or a pin-point area on a microscope slide, might be considered. *The important thing is not to expect too much of any one*

stage or the overall process slows down. I emphasize that this is not a solution, just a philosophical guide to process development.

3.2 Membrane filtration separations
This prime separation method is capable (not necessarily without difficulty) of separating microbial cells from food tissues and concentrating them for detection. It can lower limits of detection in direct microscopic methods by several orders of magnitude, for example in the Direct Epifluorescent Filter Technique (Pettipher 1989). In a related procedure *E. coli* O157 has been detected directly in juice and meat at 10 cells/g (Tortorello and Stewart 1994).

The utility of filtration depends very strongly on the filterability of food suspensions. Suspensions of unprocessed foods (raw meats, fish, vegetables) generally filter easily. With increasing levels of processing, gums, fillers, etc, food suspensions become less filterable, and dairy products are often a problem. A great deal of work on improving the membrane filterability of food suspensions without killing bacteria was carried out during development of the hydrophobic grid membrane filter (Sharpe et al 1979, Peterkin et al 1982, Sharpe and Peterkin 1988). For microscopy, where the ability of cells to multiply is not important, digestion with trypsin and Triton X-100 surfactant efficiently removes unwanted debris (Pettipher 1989, Rodrigues and Kroll 1985, Yamaguchi et al 1994).

3.3 Centrifugation
Despite being cumbersome, centrifugation will probably continue to have a major role in microbial separation. Centrifugation at 2,000 g for 10 s prior to estimating biomass by ATP measurement removed virtually all meat particles without loss of bacterial count (Stannard and Wood 1983). Density gradient centrifugation can remove food debris without loss of bacterial count; a 15 min method using colloidal silica clarified food suspensions (Basel et al 1983), and the automated density gradient apparatus of the Bactoscan instrument (see below) also allow concentration of food-related microbes (Linhardt 1987). Sedimentation Field-Flow Fractionation separates pure bacterial cultures and is probably applicable to foods; cells are injected into an open, unpacked channel, first sedimented by a low (5-10 RCF) centrifugal field, then fractionated by a parabolic fluid-velocity field as diluent passes through the chamber (Sharma et al 1993).

3.4 Biphasic partitioning
The tendency of bacteria, viruses, and other bodies to partition themselves between the phases of aqueous *biphasic* systems (e.g., of polysaccharide and gelatin mixtures), permits some degree of separation (Betts 1993). Not only can *Salmonella* and *Escherichia coli* be separated, but also rough/smooth mutants *of Salmonella typhimurium* (Stendahl et al 1973a,b

3.5 Dielectrophoresis
Conducting particles, suspended in liquid in a non-uniform electric field between a plate and a pin electrode, migrate to or from the pin electrode. The direction of movement depends on the relative conductivities of particle and liquid. Unlike *electrophoresis*, which needs a DC field, dielectrophoresis occurs in AC or DC fields. Electrode assemblies are barely larger than the cells they are used with, and may be fabricated on silicon semiconductor chips. Complex electrode arrays can also rotate cells. Particles modify the applied fields, allowing electronic analysis of the situation (Huang et al 1992). The small size of dielectrophoresis units is unlikely to allow their use for processing primary suspensions, but the ability to use electronic signal processing and control suggests that they are potentially useful for final stages of microbial separation.

3.6 Immunomagnetic separations
Microbes can be made ferro- or paramagnetic by adsorbing particles of magnetic iron oxides on their surfaces, treating them with Erbium (Er^{3+}), or precipitating ferromagnetic ions on their surfaces. However, more popular methods involve immobilizing them on paramagnetic polystyrene beads (2.8-4.5 μm, Dynabeads, Dynal, UK) or primed, silanized magnetic iron oxide particles (BioMag, Metachem Diagnostics Ltd., UK) by means of lectins or, particularly, antibodies. The attraction of immunomagnetic methods lies in the speed and simplicity with which the target species may be separated by means of a powerful magnet. Techniques can be as simple as collecting a pellet of magnetic cells, washing them by resuspending, and recollecting, or more sophisticated as in thin-layer magnetophoresis (Payne et al 1992, Safarik et al 1995). Separated cells are detected by plating them on normal growth media, electrical impedance methods, or PCR where immunamagnetic separation can remove inhibitory materials (Fluit et al 1993, Olsvik et al 1991, Widjojoatmodjo et al 1991), ELISA (Krusell and Skovgaard 1993), or other methods.

Immunomagnetic methods can detect dead or severely damaged microbes that are undetectable by standard culture techniques (Mansfield and Forsythe 1993).

Separation works best with high concentrations of immunomagnetic particles (10^6 -10^7 particles/ ml for salmonellae and *E. coli* O157:H7 (Skjerve et al 1990, Vermunt et al 1992, Framatico et al 1992). Incubations of 10-60 min are required, and attachment increases with time; however, non-specific attachment and heavy contamination with non-target microbes reduces effectiveness on unduly long incubation. Limitations are the small volume treatable because of the short range of magnetic fields, and a tendency for less-than-quantitative attachment even with great excesses of particle to target cell. A range of techniques and kits based on immunomagnetic separation (either directly from the initial suspension or after a short enrichment) provides earlier detection (Lund et al 1988, Skjerve et al 1990, Luk and Lindberg 1991, Skjerve and Olsvik 1991, Tomoyasu 1992, Chapman and Siddons 1996). Commercial magnetic particles primed with lectins or secondary antibodies facilitate the methodology.

3.7 Standing wave ultrasound
Reflection of sound waves in a tube to yield standing waves causes suspended particles to concentrate at nodes, which can then be moved by varying the frequency (Whitworth et al 1991, Miles et al 1995). It is tempting to think of detaching cells from a test surface, then moving them to a place of collection by the same ultrasonic forces. Particle size, concentration, energy levels, and other factors affect efficiency and there has not yet been a practical application of the technique in food microbiology.

3.8. Ion-exchange and affinity chromatography
This topic is covered in publications elsewhere and is not discussed here.

4. PREPARATION OF SAMPLE SUSPENSIONS
The mechanism by which the sampling technique detaches microbes from foods is complex and poorly understood. A puzzling feature is that during rinsing, stomaching, blending, etc, the concentration of suspended microbes quickly reaches a plateau; however (where it can be done), repeating the process with fresh diluent liberates more microbes, often at a barely reduced level. If the sampling process is repeated many times the sum of the counts is much higher than a single processing suggests (Price 1938, Lillard 1988), and my own (unpublished) observations.

Some authors assume that microbial release follows a second-order reaction, as though microbes in suspension inhibit detachment of others; however, it is difficult to believe that Mass Action has a significant effect. Shaking, swabbing, stomaching or blending all disrupt tissues and perhaps microbes quickly reattach to newly exposed surfaces, or to the sampling device (e.g., swab fibres or stomacher bag). At any rate, one cannot assume that the yield of microbes from a sampling technique (even blending) represents more than a small fraction of their actual level. This consideration is important to rapid pathogen detection methods, since they are compared against traditional pre-enrichment (where single viable cells grow out into a broth and are detected, regardless of their state in the sample). *For samples of low cell concentrations, a sampling technique that does not suspend every pathogen cell will result in poor performance by a rapid detection method.*

Several microbe suspending methods are described below. For "older" methods based on microbial growth it was most important that the technique yielded a maximum level of suspended cells. Faster, more direct detection methods, also demand suspensions that contain a minimum of suspended debris to interfere with the analysis.

4.1. Swabs. Though yielding minimal debris, microbe removal is poorly reproducible and less than needed for quantitative sampling. In the most detailed study, a "wet and dry" swab method gave counts of 1-24% for beef carcass, 27-52% for mutton, 13-67% for pork, and 25-89% for pork belly, compared with counts from excised, blended surfaces (Ingram and Roberts 1976).

4.2. Improved swabs. The poor performance of swabs may be due to newly exposed surfaces or the swab itself trapping liberated microbes, a result of the high concentration of microbes built up at contact surfaces and an inability to distribute them uniformly through the diluent in the swab. The *Rotorinser* holds a large diluent volume (10 ml for 50 cm^2 area) in a sponge which scrubs the test surface, compressing and rotating to pump liquid around and bring suspended microbes into equilibrium with diluent. The Rotorinser removed more microbes from pork carcass than did excision followed by stomaching (98% at 60 s operation for pork, compared with 86% for a Stomacher) (Sharpe et al, 1996).

4.3. Spray methods. Sprays yield low debris levels. A spray method yielded bacteria counts as high as by blender from meats(Clark 1963); other workers claimed various success with similar techniques (Reuter et al 1979).

4.4. Ultrasound and vortex stirring. Insonation gives good microbial removal from hard surfaces. For food samples in glass tubes in an ultrasonic tank, microbe removal compared well with blending and yi(elded suspensions with very low debris content (Sharpe and Kilsby 1970). Insonation was less effective for prawns, which protected surfaces from the energy source and very poor with comminuted meat. Sonication conditions must be a compromise between effectiveness and lethality from cavitation. Vortex stirring suspended microbes very effectively. Small sample size is a problem for both techniques, and both have lapsed into obscurity.

4.5. Blenders. Although appearing in many standard methods and long assumed to give "quantitative" suspensions of microbes from foods, the need to clean and sterilise after use, overheating, and high levels of suspended debris are severe problems.

4.6. Stomachers. These problems led to development of the Stomacher™ [1] which processes samples in disposable plastic bags. Two paddles reciprocating at 300 rpm crush the sample and drive diluent from side to side in the bag. It causes less tissue disruption than a bladed blender (Sharpe and Jackson 1972). Early suspicion about this led to many performance evaluations. In eight studies the Stomacher yielded about the same count as a bladed blender, in six there was no difference, and in about twenty the count was slightly lower. The Stomacher replaced the bladed blender in many laboratories, and today at least five "clones" of the original Stomacher are marketed. They are referred to generically as *paddle-type* processors.

4.7. Pulsifier™. A recent advancement in sample processing, both for traditional microbiological analyses, but particularly for newer detection techniques that are facilitated by "cleaner" starting points, is the Pulsifier [2] (Microgen Bioproducts Ltd., Camberley, Surrey, UK). The Pulsifier also accepts samples in disposable plastic bags. The microbe-suspending energy is applied to the sample bag by a *Beater Ring*, vibrating at 2,900-3,500 rpm. The action is a combination of shock waves and intense agitation. Because it does not crush samples the Pulsifier greatly reduces tissue disruption, even compared to paddle-type processors. An incidental benefit is that hard objects such as rice, bones, and gravel cause less bag-damage. A removable, transparent door allows the action to be viewed and gives excellent accessibility for cleaning.

Since microbes usually exist on surfaces or in easily accessible structures *pulsification* efficiently liberates them. In a comparative trial the average ratio of total aerobic counts *Pulsifier:Stomacher* for 96 samples of representative foods was 1.4 (Fung et al 1998). At very low count levels total counts by Pulsifier were approximately twice those obtained by Stomacher, were significantly higher for samples with $<10^5$ CFU/g, and not significantly different for samples with 10^5 - 10^7 CFU/g. As counts at the lower levels were obtained on sample suspensions that were not diluted further, it is probable that the ratios reflect better visibility of colonies, or reduced interference by food components, compared with those from the Stomacher. Counts of coliforms, and *Escherichia coli* in *pulsificates* did not differ significantly from *stomachates* for most food types, were slightly higher for mushrooms, and slightly lower for ground pork(Fung et al, 1997).

Except with pre-comminuted or powdered foods, pulsified suspensions contained less food debris, although microbial levels were not inferior. For example, for celery and carrot *Pulsifier:Stomacher* total count ratios were 1.3 and 2.5, respectively, but pulsificates were clear while stomachates contained enough debris to interfere with pipetting (Fung and Hart 1997). Membrane filterability (which deteriorates quickly if there are appreciable levels of suspended particulates) was considerably better for pulsificates (Sharpe et al 2000). Filtration rate ratios ranged from 1.3x (feta cheese), to 10.7x (broccoli) and 12.2x (beef liver) compared with stomachates. Suspended solids contents ranged from <1% (fresh shrimp) to 102% (oregano and chili powders), and total solids (which include dissolved salts, sugars, etc), ranged from 28% (ocean perch) to 101% (chili powder).

The cleaner suspensions the Pulsifier yields should prove particularly beneficial for techniques in which interference by food components is a problem. For example, improved membrane filterability could improve the limit of detection of DEFT-type tests of pathogens such as the detection of *E. coli* O157:H7 described above(Tortor et al), to levels close to those of regulatory interest. The reduced debris level could also be of benefit in polymerase chain reactions, ATP bioluminescence, flow-cytometry and other analyses.

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[1] STOMACHER™ is a registered trademark of Seward Medical, London, UK

[2] PULSIFIER™ is a registered trademark of Filtaflex Ltd., Almonte, Ontario, Canada.

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