3

Separation and concentration of samples

A.N. Sharpe, Filtaflex Ltd, Canada

3.1 Introduction: the need for separation and concentration

To detect even 100 cells of microbial pathogens per gram of food or raw material within 30 minutes would be very useful, but if we could confirm the presence (or absence), of one viable cell in 25 g of food within 30 minutes we could reject shipments at loading bays, divert raw materials, monitor HACCP Critical Control Points, etc. The reality is that rapid detection of microbial pathogens in foods at limiting regulatory levels is confounded by our inability to detect their minute signals amid the noise presented by food components, including competing flora.

The identification kits and systems that have proliferated recently are mainly immunological, DNA hybridization, or conductance methods needing levels of about 10^5 organisms/ml for reliable detection. Reaching this level from 1 cell/25 g means a concentration gain of about 10^7 or 22 doublings. This gain is similar to overnight enrichment, making new methods unlikely candidates for direct tests. DNA amplification cannot be applied directly to 25 g food samples. However, if we could extract the target into a suitably small sample aliquot and amplify it without noise, a five-minute Polymerase Chain Reaction cycle would achieve the necessary concentration gain in about two hours. Current analyses use time inefficiently because we take for detection only a small aliquot of the microbes we have slowly cultivated during enrichment. Using a larger fraction of enrichment (e.g. by immunomagnetic separations) speeds analyses. However, the volume which can be handled is still small and takes us only part way towards the real challenge of rapid detection at regulatory levels.

The need to amplify microbial signals to detectable levels in relatively large volumes (10–100 μL) thus remains a major barrier to more rapid detection.

However, ease of detection of microbes depends not on *number* but rather their *concentration*, which affects the signal-to-noise ratio. Physical, chemical or immunological methods might achieve massive gains in efficiency, not by increasing microbial numbers but by separating them from the food components and reducing the volume of suspension in which they exist. We could then employ rapid identification procedures that currently are impractical owing to poor signal-to-noise. As an example, microscopes or cytometers can, if there is no interfering noise, identify volumes of 10^{-12} L (i.e. single cells).

The concentration gains ($\geq 10^7$) needed to improve microbial signal-to-noise ratios for direct detection are still out of reach. Even 'DEFT-type' enzyme/surfactant/membrane filtration treatment – still one of the best separation methods and quite successful for spoilage organisms – needs improving by several orders of magnitude before it can be applied to direct pathogen detection at regulatory levels. The effectiveness of separation and concentration techniques may depend on whether target organisms can be brought into a free planktonic state or have to be dealt with whilst still attached to larger particles.

3.2 General approaches to removal, separation and detection

Approaches to directly detecting pathogens in foods include:

- extracting whole cells then identifying them after suitable concentration steps
- using the cell's phenotypy (serological or enzymological properties)
- chemical extraction (e.g. DNA or RNA) to provide consistent analytical starting bases
- detecting targets directly in product by confering on target cells some property (light emission, radioactivity, etc.) detectable against the background.

This discussion is based on an assumption that for the forseeable future it will be necessary to first remove microorganisms from samples into liquid suspension, then concentrate them. Reliable pathogen detection then depends on two fundamental processes:

1. removing them (quantitatively?), from the sample into a primary suspension
2. moving them quantitatively from this primary suspension into the detection device.

There is a pressing need for new methods to generate primary suspensions. With present technology (rinses, stomachers, blenders, etc.), one cannot suspend a high proportion of microorganisms from foods without producing large volumes of suspension (100–250 ml). Yet the powerful separation techniques (IMS, flow cytometers, etc.), cannot handle more than a few millilitres of suspension. Moreover, microbial capture reagents are prohibitively costly for large volumes. The most important (and most difficult) problem to solve or

avoid is that of reducing a primary suspension to a volume of a few millilitres or less. It is the most difficult because

- the primary suspension has a very variable composition, and
- it cannot be attacked with expensive materials because of its volume.

Once a suspension has been reduced to a few millilitres one can consider more 'sophisticated' techniques to concentrate the target further.

3.2.1 How efficiently must pathogens be extracted from foods?

This question affects the credibility of any method not dependent on microbial multiplication. Any detection method based on separation will be measured against the performance of traditional methods, for which single viable cells can be detected given sufficient time and suitable enrichment conditions, even if organisms exist in the food in a tightly bound or protected state. The test of a novel (separation-based) method is not when there are >100 cells/g, but when there is only a single viable cell in the analytical sample. If it does not find that single cell it will prove inferior to the traditional method. Either it must be capable of collecting and detecting every cell of the target organism, or the probability of analytical samples containing this limiting concentration of cells must be low.

For example, if the target occurs in a lot as sporadic colonies of a few million cells, the statistical distribution of local contamination levels will be such that samples tend to be either 'clean' or else heavily contaminated (i.e. only a very small proportion of samples will contain target cells at the limit of detectability of the rapid method). For such samples it will not matter if the novel method only detects samples containing >100 cells/g. However, if the target is distributed uniformly through a lot at a level of 1 cell/g (i.e. all samples are positive but each contains only a few target cells), then a separation-based method might perform poorly. Clearly, much depends on the statistics of the distribution of target cells in a typical lot. For the time being we should aim to develop techniques capable of detecting *all* target organisms in the conventional sample, or (preferably), techniques that provide an even better chance of detecting the target than do traditional methods.

3.2.2 Separation efficiency, speed and multiplexed separations

Conventional detachment methods (blenders, etc.), yield large suspension volumes throughout which the target cells are distributed. To concentrate target cells they must be contacted by whatever means is effecting their separation, so it is vital to make rapid contact ('hits') with all the target cells. Unless one uses a 'capture means' (filter, column, beads, etc.), having a surface area and volume commensurate with the volume being processed, the hit rate is low and the method slow. How, at affordable costs, can we quickly capture target cells from large volumes? One can show that concentration processes will be faster if they are broken down into several smaller steps, each of lesser efficiency.

In a single-stage concentration process a capture element (in reality, the final volume), passes through a sample N times greater in volume, until it has gone through the whole sample to capture the target from it. If it takes t s (specific sweep time) for the capture element to pass through its own volume in the sample (this factor remaining constant during the capture pass), one can show (Sharpe, 1991) that for single-stage, two-stage, three-stage, or p-stage, processes, the minimum times required for separation vary in the ratios:

$$(N-1); N^{1/2}; N^{1/3};\ \text{and}\ N^{1/p}.$$

To give perspective, imagine concentrating pathogens from 1,000 ml of sample suspension into a volume of 1×10^{-9} ml (one high-power field), for microscopy, ignoring practicalities such as the need to prepare the product of one stage for introduction to the next. A value of $t = 0.001$ s can be assumed for membrane filtration (other processes such as sweeping antibody-coated beads through samples will have different specific sweep times). Using this value for all steps used in reaching the required concentration gain we find that, for single-, two- and three-stage processes, total concentration times would be 1×10^{9} s (32 years), 2×10^{3} s (33 min), and 30 s, respectively. Even allowing for different specific sweep times of different processes one can appreciate that if we expect too much of any one stage the overall process is likely to slow down, and that multiplexing separations steps could yield dramatic improvements in speed.

3.3 'Primary' microbial removal methods

3.3.1 Swabs

Of early techniques only the 'Total Object Swab' (Mossel and Buchli, 1964), or 'Danish Swab Method' (Olgaard, 1977) yielded reproducible fractions of surface counts provided by more rigorous methods, and only a few (using cellulose sponges or polyester-bonded cloths), permitted sampling the larger areas needed for environment sampling (Silliker and Gabis, 1975; Quevedo *et al*., 1977; Kirschner and Puleo, 1979). Ingram and Roberts (1976), found a 'wet and dry' swab method (a wet swab followed by rubbing down the area with a dry one), for carcasses and carcass meats gave summed counts from the two swabs ranging from 1–24 per cent (average 10) for fresh beef carcass, 27–52 per cent (average 37) for fresh mutton, 13–67 per cent (average 44) for fresh pork, and 25–89 per cent (average 39) for chilled pork belly, compared with counts from the excised, blended surface. Gill *et al*. (2001) showed that for fresh beef and pork carcasses, swabbing with cotton and wool recovered 30 per cent and gauze 10 per cent of counts compared with excision/stomaching, rising somewhat as carcasses aged. Palumbo *et al*. (1999) found that, for pork carcasses, a three-site swab method yielded the same incidence of *E. coli* as an excision method, while a one-site swab yielded rather less than half the positives. Thus, while swabs yield minimal debris, microbial removal is usually below the required efficiency.

3.3.2 Is there is a 'mass action' effect?

Data by Price (1938), Lillard (1938), and others, and my own (unpublished) observations, indicate that bacteria detach from surfaces until they reach a limiting concentration in suspension. The effect is important to rinsing, swabbing, stomaching, blending, etc. One explanation is that as rinsing, blending or stomaching continues, more and more food particles are released into suspension, so that rapid re-attachment of microbes to previously uncontaminated particles reduces their countability and minimizes further count increases. For example, Price (1938) had subjects scrub their hands, rinse them and repeat the process several times, counting the bacteria in each rinse and, using curves plotted from serial scrubbings, determined total skin bacterial counts. Fifty per cent of the skin's bacterial population was removed after six minutes of scrubbing and the decrease on successive washes followed a first order reaction kinetic; other workers (Pohle and Stuart, 1940; Cade, 1952; Ulrich, 1961; Sheena and Stiles, 1983) confirmed or disagreed with these conclusions. Attenuation of the count per wash/rinse is of a similar order of magnitude to effects found for meat-based products: Leistner and Szentkuti (1970) reported a constant proportion of bacteria removed (17–24%), during each of six rinses of roosters; Carson *et al*. (1987) found rinsing poultry skin up to 30 times with saline only removed 90 per cent of *Salmonella typhimurium*; and Lillard (1988) found a continual strong removal by stomachings for up to 40 rinses of chicken skin, showing that even blending does not give a representative count. We can infer that bacterial removal techniques that are only as good as blending might not yield, say, the number of *Salmonella* positives as techniques where the whole specimen is first incubated in broth, and that novel techniques are needed to pull, say, >90 per cent of the organisms off a carcass.

While not wishing to suggest that bacteria communicate with one another while being removed from foods, one has to wonder whether some sort of 'mass action' effect determines detachment of cells. If 'mass action' actually exists, a means for quickly capturing detached target cells, (reducing their suspension concentration to zero) could improve detachment methods. We normally think of dispersing all microbial cells, then trying to concentrate them, but just as chemical reactions can be driven to one side of the equation by removing the reaction product, if we could apply a 'sink' for the target organism to the sample we could ignore the remaining flora.

If 'mass action' exists, the high concentration of removed microbes built up at a swab/sample interface would inhibit further removal, and inability to distribute removed microbes uniformly through the small quantity of diluent in the swab could explain the inefficient and variable swab performance. To test the idea of treating surfaces with a larger swab diluent volume and distributing and diluting the removed organisms throughout that volume, Sharpe developed the 'Rotorinser'. It held 10 ml of diluent in an open-cell urethane foam cylinder; compressions and rotations of the cylinder against it pumped liquid repeatedly through the foam to bring microbes from the test surface into equilibrium with the diluent. On pork skin and beef carcass surfaces it proved more efficient at

removing microbes than excision followed by stomaching (Sharpe *et al*., 1996), but did not become available commercially.

3.3.3 Paddle-type blenders and the Pulsifier®

By causing less tissue disruption than bladed blenders, paddle-type blenders (originally the Stomacher® by Seward Medical, London, England) are useful in separating and concentrating microbes from foods. One should be concerned as to whether stomaching removes the majority of target cells from the food. The original Stomacher evaluation (Sharpe and Jackson, 1972, 1975) used saline or peptone diluent, but later studies recommended addition of 1 per cent Tween 80 to cope with foods containing fat (Sharpe and Harshman, 1976). In general, paddle-type blenders yield about the same count as bladed blenders and, until recently, this was assumed to yield the closest representation of the actual microbe content.

A recent advancement is the Pulsifier® (Sharpe, 2001), available through Microgen Bioproducts Ltd. (Surrey, UK). Instead of kneading and crushing samples, it applies a combination of shock waves and intense agitation. The Pulsifier yielded counts of total aerobes, coliforms and *E. coli* biotype 1 as high as or higher than a Stomacher. Significantly for separation technology, 'pulsificates' contained much less food debris unless the sample already was highly comminuted. 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 *et al*., 1998) and membrane filtration rates were up to 12 times greater (Sharpe *et al*., 2000).

3.3.4 Sticky tapes

There are many descriptions (even patents) using adhesive tapes for removing microbial cells from surfaces for direct microscopy or for plating and incubating in the manner of an agar contact technique (e.g., Thomas, 1961; Fung *et al*., 2000; Saika *et al*., 2001). An interesting publication (Imam and Gould, 1990) describes adhesion of amylolytic bacteria (*Arthrobacter* spp.) to a starch-based film – killed or inactivated cells did not adhere, suggesting that adhesion required cell viability and that surfaces might be tailored for binding specific microbes. Kyogashima *et al*. (1989) describe glycolipid receptors (N-glycolylsialoparagloboside, etc.) to bind *E. coli* K99, and several kits or patents have been based on similar compounds (Krivan *et al*., 1993; Ginsburg *et al*., 1996). Hydroxyapatite surfaces also specifically bind bacteria (Schilling and Doyle, 1995).

3.3.5 Sprays

Sprays are relatively non-destructive and yield low debris levels, yet will remove bacteria from surfaces. In fact, they are used to reduce carcass bacterial

loads, though a common combination of sanitizing agents with sprays clouds the issue of just how many they actually remove. Anderson *et al*. (1977) found 99.9 per cent reductions in count when samples were washed with three per cent acetic acid at a pressure of 14 kg/cm^2 and Roberts (1980) quoted carcass spray pressures of 228 and 1,137 kPa as removing 50–80 per cent of the bacterial contamination. In view of the inefficiency of unsanitized high-pressure sprays, some older reports seem surprising. Clark (1963) reported higher counts of *Pseudomonas fluorescens* by spraying inoculated chicken skin, lean beef and pork fat with diluent at a pressure of 35–207 kPa over sample surfaces, than either a blender or swabbing; favorable reports were given by Baumgart and Kussmann (1975), Hess and Lott (1970), Leistner (1979) and Reuter *et al*. (1979). Interest in sprays during that era was high enough that a commercial unit – the PASS (Portable Automatic Spray System, Pool Bioanalysis Italiana, Milan, Italy) – could be purchased.

What would be the effect of jets of much higher pressure, such as are used to cut rocks? Pressure pulse immunization guns vaccinate through skin without other mechanical contact. Some foods obviously would not be suited, but skin would probably resist the cutting action of quite strong jets, while attached bacteria might be removed, even from crevices. A device that might be worth trying is the dental hygiene system 'Water-Pik' (Teledyne Water-Pik, Fort Collins, CO, USA); its use to remove bacteria from human skin was described by Staal and Noordzij (1978).

3.3.6 Ultrasound

There are few studies on insonation for removal of microbes from foods. An ultrasonic tank gave good recovery for peas and beans, was less effective for intact meat pieces which clumped together to hide surfaces from the energy source, and worse with comminuted meat; however, it yielded suspensions with very low debris content (Sharpe and Kilsby, 1970). Dispersive (even chemical) effects of insonation occur only at intensity levels high enough to cause cavitation, which is quickly lethal to bacteria – sonication conditions must be a compromise between effectiveness and lethality.

Several workers describe the use of ultrasound to move suspended particles around (e.g. Coakley *et al*., 1989; Grundy *et al*., 1989; Whitworth *et al*., 1991) and particle size, concentration, and other factors affecting efficiency were described by Miles *et al*. (1995). The idea of detaching cells from test surfaces and moving them off to a place of collection by using the same ultrasonic forces is tempting.

3.3.7 Gas bubbles

Gas bubbles expanding from nuclei dislodge materials from surfaces. 'Household Hints'-type books often suggest soaking stubborn deposits in Coca-Cola, and the advertising for some cleaning fluids suggests the same effect

occurs. Perhaps more credible evidence – in electrolytic cleaning of metals – 'gas scrubbing' is very effective in removing scale and other unwanted surface material, and it is more effective when the metal part is the cathode because the volume of hydrogen liberated there is twice that of oxygen from the anode. Could this be applied to foods? The closer organisms are to the nucleation site of a bubble the greater the force they would experience. Catalase-positive organisms might be a good subject for study – they would be right at the spot. Could they be lifted off with dilute H_2O_2? Would it be possible to 'aim' catalase at target organisms by conjugating it to specific antibodies? It might not be necessary to use lethal levels of H_2O_2 – lower levels, followed by sudden evacuation of the vessel to cause outgassing might be sufficient.

3.4 Separation and concentration of cells once they have been removed

Once an extraction method has detached cells from the food into suspension they must be brought into a more handleable state. Centrifugation and membrane filtration are absolute methods, in that all the target can be brought into the ultimate volume or area; other techniques may not collect the target so completely. The most important factor in trapping microbes from suspension to effect a concentration gain is the true state of those microbes. We have little idea when we blend, stomach, vortex stir, rinse, or otherwise suspend microbes, whether suspended microbes exist as single (or clumped) cells, or are still attached to tissue cells. While tissue cells may still be small enough to stay in suspension when we pipette in conventional plating procedures, they are many times larger than microbes. We can expect an enormous difference in the performance of capture systems, depending on whether bacteria are freely floating 1 μm particles or 1 μm particles attached to 20 μm particles. Even if target bacteria were on the outside of such particles the forces needed to capture large composites must be many times greater (many times less probable) than for free bacteria.

3.4.1 Membrane filtration

While the right pore size removes all target cells from a suspension, the success of membrane filters depends strongly on the filterability of food suspensions. Suspensions of unprocessed foods (raw meats, fish, vegetables) filter easily. With increasing levels of processing, addition of gums, fillers, etc., food suspensions become less filterable, and dairy products usually present problems. Filtration problems can be overcome: much work on the filtration of foods (temperature, pressure, flow direction, inclusion of Tween 80, and enzyme treatments, etc.) was carried out during development of the hydrophobic grid membrane filter (ISO-GRID HGMF, Neogen Inc., Lansing, MI, USA) (Sharpe *et al.*, 1979; Peterkin and Sharpe, 1980, 1981; Entis *et al.*, 1982). Around the

same time Pettipher and co-workers settled on trypsin/Triton X-100 digestion for milks and other foods as a means of removing unwanted debris while developing the Direct Epifluorescent Filter Technique (DEFT) (Cousins *et al*., 1979; Pettipher and Rodrigues, 1982; Pettipher, 1989; Rodrigues and Kroll, 1985). A complete discussion of enzyme treatments can be found in Sharpe and Peterkin (1988).

3.4.2 Centrifugation

Despite being inconvenient, centrifugation has a major role in microbial separation. Centrifugation at 2000 g for 10 s prior to estimating biomass by ATP measurement removed virtually all meat particles without decrease in bacterial count (Stannard and Wood, 1983). Density gradient centrifugation removed food debris without loss of bacterial count in 15 min using colloidal silica (Basel *et al*., 1983) and the automated density gradient apparatus of the Bactoscan instrument (Foss Electric, Hillerod, Denmark) allows concentration of food-related microbes (Linhardt, 1987). Sedimentation Field-Flow Fractionation separated pure bacterial cultures – cells were injected into an open, unpacked channel, first sedimented by a low (5–10 RCF) centrifugal field, then fractionated by the parabolic fluid-velocity field as diluent passed through the chamber (Sharma *et al*., 1993). The method is probably applicable to foods.

3.4.3 Immunomagnetic separations (IMS)

Microbes can be made ferromagnetic or paramagnetic by adsorbing submicron particles of magnetic iron oxides on their surfaces, treating them with Er^{3+} ions (Zborowski *et al*., 1993) or precipitating ferromagnetic ions on their surfaces (Ellwood *et al*., 1992). However, the popular methods involve immobilizing them, in suspension, on paramagnetic polystyrene beads (Dynabeads, Dynal, UK) or primed, silanized magnetic iron oxide particles (BioMag, Metachem Diagnostics Ltd, UK) by means of lectins or antibodies. The attraction lies in the speed and simplicity with which target species may be separated by a powerful magnet. Techniques may be as simple as collecting a pellet of magnetic cells, washing them by resuspending and recollecting, or more sophisticated processes such as thin-layer magnetophoresis (Payne *et al*., 1992; Safarik *et al*., 1995). Separated cells may be detected by plating on normal growth media, electrical impedance, or PCR where immunomagnetic separation can remove inhibitory materials (Fluit *et al*., 1993; Olsvik *et al*., 1991; Widjojoatmodjo *et al*., 1991) or ELISA (Krusell and Skovgaard, 1993). IMS can capture microbes that are dead or severely damaged and undetectable by standard culture techniques (Mansfield and Forsythe, 1993).

In general, separation works best with high levels of immunomagnetic particles. For *Salmonellae* and *E. coli* O157:H7 particle concentrations of 10^6–10^7/ml have been used (Skjerve *et al*., 1990; Vermunt *et al*., 1992; Fratamico *et al*., 1992). Incubations of 10–60 min are required, and attachment increases with

time; however, with unduly long incubation non-specific attachment and heavy contamination with non-target microbes reduces the selectivity of immunomagnetic beads; surfactants such as Tween-20 or protamine reduce non-specific attachment. The utility of immunomagnetic beads is limited by 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 bead to target cell. Plate counts from immobilized microbes tend to be lower than the actual number of cells owing to a tendency for multiple attachment of cells; a common rule is that one colony may represent up to six target organisms (Skjerve *et al*., 1990; Skjerve and Olsvik, 1991).

There are now hundreds of papers, techniques and kits related to IMS for separating suspended microbes, either directly from initial suspensions or after short enrichments. Just a small sample of current literature is indicated by descriptions of detection of *Vibrio parahaemolyticus* from shellfish (Hara-Kudo *et al*., 2001); *E. coli* O157:H7 from sprouts (Weagant and Bound, 2001), carcasses (Kang *et al*., 2001) or raw meats or milks (Chapman *et al*., 2001; Coia *et al*., 2001); *Listeria monocytogenes* and *Salmonella* from foods (Hsih and Tsen, 2001; Hudson *et al*., 2001); and *Campylobacter jejuni* from foods (Waller and Ogata, 2000).

3.4.4 Dielectrophoresis

When an electric field is applied between plate and pin electrodes, conducting particles suspended in liquid in the non-uniform field migrate to or from the pin electrode, the direction depending on the relative conductivities of particle and liquid. Unlike electrophoresis, dielectrophoresis occurs in both 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, and since particles modify applied fields one can make some electronic analysis (Huang *et al*., 1992). While the small size of dielectrophoresis units prohibits their use for processing primary suspensions, the ability to combine them with electronic signal processing and control will make them useful for final microbe separations.

3.4.5 Biphasic partitioning

Bacteria, viruses and other bodies partition themselves between the phases of aqueous biphasic systems (e.g. of polysaccharide and gelatin mixtures) permitting some degree of separation (Magnusson and Stendahl, 1985; Mattiason, 1983; Betts, 1993). Not only can *Salmonella* and *E. coli* be separated, but also rough/smooth mutants of *Salmonella typhimurium* (Stendahl *et al*., 1973). Practical applications in food microbiological analysis have not been described.

3.4.6 Ion-exchange columns and sponges

In general, microorganisms can only be separated from other material if they make contact with the separating element. In the 1980s considerable interesting work on separation by ion-exchange bead columns was described by the Leatherhead Food Research Association (Surrey, UK) in their in-house reports, though little seems to have been published in the regular journals. The advantage of bead columns is that target microorganisms are never more distant from a trapping surface than the maximum interbead spacing, optimizing the probability of hits. Disadvantages are that void volumes are relatively small so there is need for continuous flow (short contact times) to treat reasonable suspension volumes; they block easily; and bound microorganisms must be removed by a somewhat voluminous elution mixture.

The 'physical negative' of a bead column is an open-cell sponge. I have not seen descriptions of the use of sponges in microbial separations, yet they have many potential attractions. Some sponge materials (e.g. the polyurethane used in the Filtaflex Ltd 'FiltaTips') have void volumes of about 97 per cent, superb flexibility, and are non-toxic to most species. Sponges suitably treated with binding receptors could permit:

- initial separation of target from debris by the inherent filter effect of the sponge
- treating of much larger suspension volumes per given volume of separating element – filled sponges could be left alone for extended periods, to optimize trapping
- distances of target from trapping surface as close as with beads
- compressibility to a fraction of the initial volume prior to adding eluting agent
- ability to compress the sponge even further to express the liberated microorganisms.

Thus, sponges might permit both separation and concentration in one operation.

3.5 Future trends

If HACCP procedures do manage to control pathogens to low levels in foods, an increasingly important goal of microbial separation should be to improve the efficiency of sampling procedures. Microorganisms generally are not distributed uniformly through lots or over surfaces. If they are distributed relatively uniformly but in low numbers, statistics dictates whether any particular sample does, or does not, contain a detectable target organism. Even when numbers are high, microbes may be present as a few colonies of many cells each. In either case, the microbiologist's success in detecting the target depends considerably on the luck of sampling the right area at the right time. Statistics exerts a dismal effect on our probability of accepting or not accepting lots that contain appreciable proportions of defectives; the effect is well illustrated in Table 3.1.

Table 3.1 Probability of accepting a lot for two-class attribute plans with $c = 0$

Proportion of defectives	Number of samples taken					
	5	10	20	60	200	2000
0.0000	1.00	1.00	1.00	1.00	1.00	1.00
0.0001	1.00	1.00	1.00	1.00	1.00	0.98
0.001	1.00	0.99	0.98	0.94	0.82	0.14
0.002	0.99	0.98	0.98	0.89	0.94	0.09
0.01	0.95	0.90	0.82	0.55	0.40	>0
0.05	0.77	0.60	0.36	0.05	>0	
0.1	0.59	0.35	0.12	>0		
0.5	0.03	>0				

Adapted from data in ICMSF (1986).

It is not possible to guarantee the microbiological status of a lot without taking 100 per cent of that lot for examination.

Similar situations occur locally when levels of contamination are low or fluctuate. For example, an animal's fur may remain contaminated after pathogen excretion stops; a rinsewater that was temporarily contaminated may be free of pathogen by the time it is sampled; or a low level of pathogen may be missed through swabbing the wrong area of a carcass. The traditional way of collecting samples places an upper limit on the reliability of any detection procedure. Given that there is a limit to the size of sample that can be sent off to the microbiology lab, are there ways to improve the probability of the analytical sample containing the target – for example, by increasing the apparent volume of product sampled or the duration of the sampling period?

There may be advantages to changing the way we think about microbiological testing. For example, we may speak of 'screening for a pathogen in 48 h …'. This actually means that the test method detects (or does not) its presence within 48 h of a sample reaching the lab. The irony is that the pathogen may be in the test environment (cattle pen, truck, crate, fruit, lettuce, conveyor, grinder, mixer, floor) hours or days before samples are taken, but analysis only begins when samples reach the lab, so this other lifetime of the pathogen is lost as far as the analysis is concerned. If we could begin the separation process *before* the traditional start time we might shorten the lab time needed to get the answer.

The Microbe Trap®, jointly under development by Pharmacon Research, Inc. (Ottawa, ON, Canada) and Filtaflex Ltd, comprises a self-adhesive flexible substrate coated with binding receptors specific to microbial targets. It can be placed in the test site and left to capture target cells for any length of time before being removed to the laboratory. For example, it can be attached to cattle pens, cows' tails, lettuce plants, apples, linemen's overalls, plant equipment, grocery store chill cabinets, domestic kitchen counters, cutting boards, refrigerators, etc. The Microbe Trap attacks the sampling problem because it has more time, and more patience, to spend on its task than a human inspector (anyone who has

fished for lobster will appreciate the difference in catch between dangling a net over the side of a boat during the time available, compared with leaving a baited trap on the sea floor for several days). But it also attacks the speed problem since, at the lab, non-selectively attached materials can be removed by rinsing, leaving the target in a relatively purified state suitable for rapid detection. The Microbe Trap is the subject of various patent applications.

3.6 References

ANDERSON, M E, R T MARSHALL, W C STRINGER and H D NAUMANN (1977), 'Combined and individual effects of washing and sanitizing on bacterial counts of meat – a model system', *J Food Prot*, **40** 668–70.

BASEL, R M, E R RICHTER and G J BANWART (1983), 'Monitoring microbial numbers in food by density centrifugation', *Appl Environ Microbiol*, **45** 1156–9.

BAUMGART, J and H KUSSMANN (1975), 'Eine Sprühmethode zur Ermittlung des Oberflächenkeimgehaltes bei Schlachttieren', *Fleischwirtschaft,* **55** 113–14.

BETTS, R P (1993), 'The separation and rapid detection of microorganisms', pp 107–19 in *Rapid Methods and Automation in Microbiology and Automation*, eds R C Spencer, E P Wright and S W B Newsom, Athenaeum Press, UK (*Proceedings of the Seventh International Congress on Rapid Methods and Automation in Microbiology and Automation*, London, UK, Sept 12–15.

CADE, A R (1952), 'An in-vivo method for determining the degerming efficiency of soaps containing hexachlorophene', *Amer Soc Testing Materials, Tech Publication* No. 115, 33–9.

CARSON, M O, H S LILLARD and M K HAMDY, (1987), 'Transfer of firmly attached ^{32}P-*Salmonella typhimurium* from raw poultry skin to other surfaces', *J Food Prot,* **50** 327–9.

CHAPMAN, P A, M ELLIN, R ASHTON and W SHAFIQUE (2001), 'Comparison of culture, PCR and immunoassays for detecting *Escherichia coli* O157 following enrichment culture and immunomagnetic separation performed on naturally contaminated raw meat products', *Int J Food Microbiol,* **15** 11–20.

CLARK, D S (1963), 'Method of estimating the bacterial population on surfaces', *Can J Microbiol*, **11** 407–13.

COAKLEY, W T, D W BARDSLEY and M A GRUNDY (1989), 'Cell manipulation in ultrasonic standing wave fields', *J Chem Tech Biotechnol*, 44, 43–62.

COIA, J E, Y JOHNSTON, N J STEERS and M F HANSON (2001), 'A survey of the prevalence of *Escherichia coli* O157 in raw meats, raw cow's milk and raw-milk cheeses in south-east Scotland', *Int J Food Microbiol*, **66** 63–9.

COUSINS, C M, G L PETTIPHER, C H MCKINNON and R MANSELL (1979), 'A rapid method for counting bacteria in raw milk', *Dairy Ind Int*, **44** 27–39.

ELLWOOD, D C, M J HILL and J H P WATSON (1992), 'Pollution control using microorganisms and magnetic separation', in *Microbial Control of Pollution,* eds J C Fry, G M Gadd, R A Herbert, C W Jones and I A Watson-Craik, *Soc Gen Microbiol*, Symposium No. 48, pp. 89–112, Cambridge University Press, Cambridge.

ENTIS, P, M H BRODSKY and A N SHARPE (1982), 'Effect of pre-filtration and enzyme treatment on membrane filtration of foods', *J Food Prot*, **45** 8–11.

FLUIT, A C, R TORENSMA, M J VISSER, ET AL (1993), 'Detection of *Listeria monocytogenes* in

cheese with magnetic immuno-polymerase chain reaction assay', *Appl Environ Microbiol*, **59** 1289–93.

FRATAMICO, P M, F J SCHULTZ and R L BUCHANAN (1992), 'Rapid isolation of *Escherichia coli* O157:H7 from enrichment cultures of foods using an immunomagnetic separation method', *Food Microbiol.*, **9** 105–13.

FUNG, D Y C, A N SHARPE, B C HART and Y LIU (1998), 'The Pulsifier, a new instrument for preparing food suspensions for microbiological analysis', *J Rapid Meth Autom Microbiol*, **6** 43–9.

FUNG, D Y C, L K THOMPSON, B A CROZIER-DODSON and C L KASTNER (2000), ' ''Hands-free'', ''pop-up'' adhesive tape method for microbial sampling of meat surfaces', *J Rapid Meth Autom Microbiol*, **8** 209–17.

GILL, C O, M BADONI and J C MCGINNIS (2001), 'Microbiological sampling of meat cuts and manufacturing beef by excision or swabbing', *J Food Prot*, **64** 325–34.

GINSBURG, V, H C KRIVAN and D D ROBERTS (1996), 'Diagnostic kit and diagnostic method for mycoplasma utilizing carbohydrate receptors', United States Patent 5,529,904.

GRUNDY, M A, W T COAKLEY and D J CLARKE (1989), 'Rapid detection of Hepatitis B virus using a haemagglutination assay in an ultrasonic standing wave field', *J Clin Lab Immunol*, **30** 93–6.

HARA-KUDO, Y, K SUGIYAMA, T NISHINA, A SAITOH, H NAKAGAWA, T ICHIHARA, H KONUMA, J HASEGAWA and S KUMAGAI (2001), 'Detection of TDH-producing *Vibrio parahaemolyticus* O3,K6), from naturally contaminated shellfish using an immunomagnetic separation method and chromogenic agar medium', *Kansenshogaku Zasshi*, **75** 955–60.

HESS, E and G LOTT (1970), 'Kontamination des Fleisches wahrend und nach der Schlachtung', *Fleischwirtschaft*, **50** 47–50.

HSIH, H Y and H Y TSEN (2001), 'Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp in food samples', *J Food Prot*, **64** 1744–50.

HUANG, Y, R HOLZEL, R PETHIG and X B WANG (1992), 'Differences in the AC electrodynamics of viable and non-viable yeast cells determined through combined dielectrophoresis and electrorotation studies', *Phys Med Biol*, **37** 1499–517.

HUDSON, J A, R J LAKE, M G SAVILL, P SCHOLES and R E MCCORMICK (2001), 'Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction', *J Appl Microbiol*, **90** 614–21.

ICMSF (INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS) (1986), *Microorganisms in Foods 2. Sampling for microbiological analysis; principles and specific applications* (2nd). University of Toronto Press, Toronto.

IMAM, S H and J M GOULD (1990), 'Adhesion of an amylolytic *Arthrobacter* spp. to starch-containing plastic films', *Appl Environ Microbiol*, **56** 872–6.

INGRAM, M and T A ROBERTS (1976), 'The microbiology of the red meat carcass and the slaughterhouse', *Roy Soc Health J*, **96** 270–6.

KANG, D H, G A BARKOCY-GALLAGHE, M KOOHMARAIE and G R SIRAGUSA (2001), 'Screening bovine carcass sponge samples for *Escherichia coli* O157 using a short enrichment coupled with immunomagnetic separation and a polymerase chain reaction-based (BAX), detection step', *J Food Prot*, **64** 1610–12.

KIRSCHNER, L E and J R PULEO (1979), 'Wipe-rinse technique for quantitating microbial contamination on large surfaces', *Appl Environ Microbiol,* **38** 466–70.

KRIVAN, H C, V GINSBURG and D D ROBERTS (1993), 'Carbohydrate receptor for bacteria and method for use thereof', United States Patent 5,217,715.

KRUSELL, L and N SKOVGAARD (1993), 'Evaluation of a new semi-automated screening method for the detection of Salmonella in foods within 24 h', *Int J Food Microbiol*, **20** 123–30.

KYOGASHIMA, M, V GINSBURG and H C KRIVAN (1989), '*Escherichia coli* K99 binds to N-glycolylsialoparagloboside and N-glycolyl-GM3 found in piglet small intestine', *Arch Biochem Biophysm,* **270** 391–7.

LEISTNER, L and L SZENTKUTI (1970), 'Zwei Methoden zur bakteriologischen Untersuchung von Schlachtgeflugel', *Fleischwirtschaft*, **50** 81.

LEISTNER, L (1979), 'Oberflachenkkeimgehalt von Schlachttierkorpen', *Arbeitstagung des Arbeitsgeb Lebensmittelhygienischer tierarztlicher Sachverstandiger, Berlin*, **21** 23, 5.

LILLARD, H S (1988), 'Comparison of sampling methods and implications for bacterial decontamination of poultry carcasses by rinsing', *J Food Prot,* **51** 405–8.

LINHARDT, C H (1987), 'Density gradient centrifugation as a means to concentrate food-related microorganisms from liquid samples', *J Appl Bacteriol* **63**(6) pp. XXIII (Conference paper).

MAGNUSSON, K-E and O STENDAHL (1985), 'Partitioning of bacteria, virus, and phage', in *Partitioning in Aqueous Two-Phase Systems*, eds H Walter, D E Brooks and D Fisher, Academic Press, pp 415–52.

MANSFIELD, L P and S J FORSYTHE (1993), 'Immunomagnetic separation as an alternative to enrichment broths for *Salmonella* detection', *Lett Appl Microbiol*, **16** 122–5.

MATTIASON, B (1983), 'Applications of aqueous two-phase systems in biotechnology', *Trends Biotechnol*, **1** 16–20.

MILES, C A, M J MORLEY, W R HUDSON and B M MACKEY (1995), 'Principles of separating micro-organisms from suspensions using ultrasound', *J Appl Bacteriol*, **78** 47–54.

MOSSEL, D A A and K BUCHLI (1964), 'The Total Object Swab (''TOS''), technique', *Lab Pract*, **13** 1184–7.

OLGAARD, K (1977), 'Determination of relative bacterial levels on carcasses and meats – a new quick method', *J Appl Bacteriol*, **42** 321–9.

OLSVIK, O, Y WASTESON, A LUND and E HORNES (1991), 'Pathogenic *Escherichia coli* found in food', *Int J Food Microbiol*, **12** 103–14.

PALUMBO, S A, P KLEIN, J CAPRA, S EBLENA and A J MILLER (1999), 'Comparison of excision and swabbing sampling methods to determine the microbiological quality of swine carcass surfaces', *Food Microbiol*, **16** 459–64.

PAYNE, M J, S CAMPBELL, R A PATCHETT and R G KROLL (1992), 'The use of immobilized lectins in the separation of *Staphylococcus aureus, Escherichia coli, Listeria* and *Salmonella* spp from pure cultures and foods', *J Appl Bacteriol*, **73** pp 41–52.

PETERKIN, P I and A N SHARPE (1980), 'Membrane filtration of dairy products for microbiological analysis', *Appl Environ Microbiol*, **39** 1138–43.

PETERKIN, P I and A N SHARPE (1981), 'Filtering out food debris before microbiological analysis', *Appl Environ Microbiol,* **42** 63–5.

PETTIPHER, G L and U M RODRIGUES (1982), 'Rapid enumeration of microorganisms in foods by the Direct Epifluorescent Filter Technique', *Appl Environ Microbiol*, **44** 809–13.

PETTIPHER, G L (1989), 'The Direct Epifluorescence Filter Technique', In *Rapid Methods in Food Microbiology*, eds M R Adams and C F A Hope, *Progress in Industrial Microbiology*, Vol. 26, Elsevier, pp. 19–56.

POHLE, W D and L S STUART (1940), 'The germicidal action of cleaning agents – a study of a modification of Price's procedure', *J Inf Dis*, **67** 275–81.

PRICE, P B (1938), 'The bacteriology of normal skin; a new quantitative test applied to a study of the bacterial flora and the disinfectant action of mechanical cleaning', *J Inf Dis*, **63** 301–18.

QUEVEDO, F, J A LASTA and J A DINELLI (1977), 'Control microbológico de superficies con esponjas de poliuretano', *Rev Lat-amer Microbiol*, **19** 79–82.

REUTER, G, D SASSE and G SIBOMANA (1979), 'Entwicklung und Prufung eines Abspulgerates zur Erfassung des Oberflachenkeimgehaltes an Schlachttierkorpen', *Arch Lebensmitt*, **30** 126–9.

ROBERTS, T A (1980), 'Contamination of meat: the effects of slaughter practices on the bacteriology of red meat carcass surfaces', *Roy Soc Health J*, **100** 3–9.

RODRIGUES, U M and R G KROLL (1985), 'Increased selectivity, sensitivity and rapidity in the Direct Epifluorescent Filter Technique (DEFT)', *J Appl Bacteriol*, **59** 493–9.

SAFARIK, I, SAFARIKOVÁ, M and FORSYTHE, S J (1995), 'The application of magnetic separations in applied microbiology', *J Appl Bacteriol,* **78** 575–85.

SAIKA, T, S SENDA, Y KAZUSE, A IWAMA, T SUGII, M NASU, K TAMI and N YAMAGUCHI (2001), 'Pressure sensitive adhesive sheet for detection of microorganisms and method for detection of microorganisms', United States Patent 6,203,900.

SCHILLING, K M and R J DOYLE (1995), 'Bacterial adhesion to hydroxyapatite', Chapter 43 in *Methods in Enzymology*, eds, R J Doyle and I Ofek,Academic Press Inc., New York, pp. 536–41.Vol 253, *Adhesion of Microbial Pathogens*.

SHARMA, R V, R T EDWARDS and R BECKETT (1993), 'Physical characterization and quantification of bacteria by sedimentation field-flow fractionation', *Appl Environ Microbiol*, **59** 1864–75.

SHARPE, A N and D C KILSBY (1970), 'Ultrasound and vortex stirring as bacteriological sampling methods for foods', *J Appl Bacteriol*, **33** 351–7.

SHARPE, A N and A K JACKSON (1972), 'Stomaching, a new concept in bacteriological sample preparation', *Appl Microbiol*, **24** 175–8.

SHARPE, A N and A K JACKSON (1975), 'Automation requirements in the microbiological quality control of foods', i*n Automation in Microbiology and Immunology,* eds C-G Heden and T Illeni Wiley pp 116–37.

SHARPE, A N and G C HARSHMAN (1976), 'Recovery of *Clostridium perfringens*, *Staphylococcus aureus* and molds by the Stomacher, effect of fat content, surfactant concentration and blending time', *Can Inst Food Sci Technol J*, **9** 30–4.

SHARPE, A N, P I PETERKIN and I DUDAS (1979), 'Membrane filtration of food suspensions', *Appl Environ Microbiol*, **37** 21–35.

SHARPE, A N and P I PETERKIN (1988), '*Membrane Filter Food Microbiology*', ed. A N Sharpe, *Innovation in Microbiology Research Studies Series*, Research Studies Press, Letchworth, UK.

SHARPE, A N (1991), 'On multiplexed separations in quick detection of micro-organisms in foods', *Food Microbiol*, 8, 167–70.

SHARPE, A N, C I BIN KINGOMBE, P WATNEY, L J PARRINGTON and I DUDAS (1996), 'Efficient non-destructive sampler for carcasses and other surfaces', *J Food Prot*, **59** 757–63.

SHARPE, A N HEARN, E M and KOVACS-NOLAN, J (2000), 'Membrane filtration rates and HGMF coliform and *Escherichia coli* counts in food suspensions by Pulsifier?', *J Food Prot,* **63** 126–30.

SHARPE, A N (2001), 'Microbe suspender having a vibrating beater for agitating the contents of a bag', United States Patent 6,273,600.

SHEENA, A Z and M E STILES (1983), 'Comparison of barrier creams and germicides for hand hygiene', *J Food Prot*, **46** 943–6.

SILLIKER, J H and D A GABIS (1975), 'A cellulose sponge sampling technique for surfaces', *J Milk Food Technol,* **38** 504.

SKJERVE, E, L M RORVIK and O OLSVIK (1990), 'Detection of *Listeria monocytogenes* in foods by immunomagnetic separation', *Appl Environ Microbiol*, **56** 3478–81.

SKJERVE, E and O OLSVIK (1991), 'Immunomagnetic separation of *Salmonella* from foods', *Int J Food Microbiol*, **14** 11–18.

STAAL, E M and A C NOORDZIJ (1978), 'A new method for the quantitative determination of micro-organisms on human skin', *J Soc Cosmet Chem*, **29** 607–15.

STANNARD, C J and J M WOOD (1983), 'The rapid estimation of microbial contamination of raw meat by measurement of adenosine triphosphate (ATP)', *J Appl Bacteriol* **55** 429–38.

STENDAHL, O, K-E MAGNUSSON, C CUNNINGHAM and R EDEBO (1973), 'Characterisation of mutants of *Salmonella typhimurium* by counter current distribution in aqueous two-polymer phase systems', *Infect Immun* **7** 573–7.

THOMAS, M (1961), 'The sticky film method of detecting skin staphylococci', *Monthly Bull Min of Health, London,* **20** 37–40.

ULRICH, J A (1961), 'Technics of skin sampling for microbial contaminants', *Health Lab Sci,* **1** 133–6.

VERMUNT, A E M, A A J M FRANKEN and R R BEUMER (1992), 'Isolation of salmonellas by immunomagnetic separation', *J Appl Bacteriol*, **72** 112–18.

WALLER, D F and S A OGATA (2000), 'Quantitative immunocapture PCR assay for detection of *Campylobacter jejuni* in foods', *Appl Environ Microbiol*, **66** 4115–18.

WEAGANT, S D and A J BOUND (2001), 'Evaluation of techniques for enrichment and isolation of *Escherichia coli* O157:H7), from artificially contaminated sprouts', *Int J Food Microbiol,* **71** 87–92.

WHITWORTH, G, M A GRUNDY and W T COAKLEY (1991), 'Transport and harvesting of suspended particles using modulated ultrasound', *Ultrasonics,* **29** 439–44.

WIDJOJOATMODJO, M N, A C FLUIT, R TORENSMA, B H KELLER and J VERHOEF (1991), 'Evaluation of the magnetic immuno PCR assay for rapid detection of *Salmonella*', *Eur J Clin Microbiol Infect Dis*, **10** 935–8.

ZBOROWSKI, M, Y TADA, P S MALCHESKY and G S HALL (1993), 'Quantitative and qualitative analysis of bacteria in erbium(III), solution by thin-film magnetophoresis', *Appl Environ Microbiol*, **59** pp 1187–93.