Campden & Chorleywood
Food Research Association

Director-General
Prof. C. Dennis
BSc, PhD, FIFST

Chipping Campden
Gloucestershire
GL55 6LD UK
Tel: +44 (0) 1386 840319
Fax: +44 (0) 1386 841306

and
Chorleywood
Hertfordshire
WD3 5SH UK
Tel: +44 (0)1923 284111
Fax: +44 (0)1923 284539

R&D Report No. 3
MAFF Project No. 11186

Risk Factors Associated with Post Process Contamination of Heat Sealed Semi-Rigid Packaging

Part 3: Quantification of Microbiological Hazards Through Heat Seals

February 1995

D. Rose, S.P. Horan, P.M. Withers, B.P.F. Day and D.G. Evans

SUMMARY

Microleakage channels of 15μm and 63μm diameter were made within the polypropylene layers of flat heat seals of 2mm and 5.5mm width by enclosing aluminium wires between the lidding material and the flanges of pots or trays, and then dissolving them away with a solution containing 12% w/v sodium hydroxide and 5% v/v ethyl alcohol.

A new design of apparatus was constructed in which a section of a plastic laminate semi-rigid pot or tray with lid film was sealed. Repetitive biotesting was carried out to evaluate the effects of twelve factors at two levels on bacterial transfer from a challenge suspension of cells in fluid on the "outside" of the container to the "food" side using defined leakage channels. Using a Taguchi exploratory experimental design, 16 tests were carried out involving each factor. Whenever bacteria were transferred through the microleakage channels, the rates of transfer consistently declined in sequential tests.

Significant factors were found to be hole diameter, differential pressure and hole length. The other factors in the study were not found to have a significant effect on microbial transmission. This contrasted with a previously reported stage of the work (Rose *et al* 1994) where other factors, especially pH, were highly significant to the transfer of bacteria through laser drilled "pin holes" in laminated plastic packaging. It was concluded that specific polymers used in these packaging materials affected the results. The implications of this are most important to those companies dealing with ambient shelf stable ready meals in semi-rigid laminate containers, because it reveals that risk factors are more complex than hitherto expected. The results show clearly, however, that the narrowest channel diameter made by 15μm wire across a 5.5mm seal width could result in very high numbers of bacteria being transferred at a hydraulically applied differential pressure of 150 mmHg. As this size of hole is beyond the detection limits of currently available non-destructive integrity test units, even at low production line speeds, any hazards such as "wet handling" which can be avoided will lead to improved confidence in the safety and quality of the products.

CONTENTS

1. INTRODUCTION

The earlier report on MAFF Project No.: N2761, Part 2A, by Rose *et al* (1994) described a multifactorial investigation of parameters at two levels comprising a Taguchi exploratory experimental design. The raw data of the numbers of bacteria (either *Serratia marcescens* or *Micrococcus luteus*) which were recovered as viable counts after passing through a microhole were processed to show the significance of twelve separate factors on the rates of transfer. This gave the size of the difference between two levels of a factor. A ranking between factors was also achieved by setting out Half-Normal scores in Daniel Plots.

These results showed that seven factors were significant in their effects on bacterial transfer with multiplicative values as follows between the two levels:

31 fold more for pH 7.0 cf. pH 4.5
17 fold more for hole length 600μm cf. 107μm
16 fold more for hole diameter 10μm cf. 7μm
14 fold more for pressure differential 150mmHg cf. 0mmHg
11 fold more for challenge level 1×10^8 cf. 1×10^6
5 fold more for D2 solution cf. D2 + 2% starch
4 fold more for exposure time 10 minutes cf. 5 minutes

The remaining five factors were: with or without 1% rinse aid in challenge; *Micrococcus luteus* or *Serratia marcescens*; 18°C or 30°C; with or without 10.3% fat in food; with or without 10.3% fat in challenge. None of these were statistically significant, differences being attributed to random variation.

The objective of this programme was to establish the significance of a similar range of factors by their effects upon the transfer of bacteria through the longer leakage channels of seal faults.

2. MATERIALS AND METHODS

2.1 Preparation of micro-leakage channels

Two samples of commercially available semi-rigid laminated plastic packaging were obtained for the test studies. These were K515 pots of nominal capacity 142ml comprising laminates of polypropylene/polyvinylidene chloride/polypropylene (Polarcup Ltd. (Portadown), 180 Guildford Road, Portadown, Co. Armagh, Northern Ireland, BT63 5LE) and circular trays of capacity 205ml, comprising laminates of polypropylene/ethylene vinyl alcohol/polypropylene (RPC Containers Ltd., Sallow Road, Weldon Industrial Estate, Corby, Northants., NN17 5JX). Lidding film for heat sealing to the two types of containers was Microtort G-101, a general purpose material (Lawson Mardon Flexible, Midsomer Norton, Bath, BA3 4AA). It incorporates nylon, modified polypropylene and a copolymer barrier layer in a film gauge of 107μm. Aluminium wires of diameter 15μm and 63μm (Goodfellow Metals Ltd., Cambridge Science Park, Cambridge, CB4 4DJ) were diametrically located across the seal areas by adhesive tape and heat sealed between the lidding and container flanges at 187°C for 4.3 seconds.

A hole was cut in the base of each container which was then immersed in a solution containing sodium hydroxide (12% w/v) and ethyl alcohol (5% v/v) solution at 30°C, ensuring that both the inside and the outside of the pack were in full contact with the caustic solution; in this way the wire dissolved from both ends.

The channel was visually checked using a low power binocular microscope for residual aluminium, and an electrolytic check was carried out to see if current flowed between the inside and the outside of the container thereby indicating a free leakage channel.

The process of dissolving the aluminium wires took up to one month at 30°C. When the channels were free of aluminium wire, sections of the container rims were cut to fit into test cells. Pot segments were cut 22mm across the radii from the outer edge of the flange into the lidding film and the cuts were angled to coincide with points 35mm down the pot. Trays were similarly cut across the lidding film 25mm from the outer edge of the flange and 35mm and down the side of the tray.

2.2 Model container leakage system

In order to bio-test leakage channels across heat seals a new model container leakage system was developed. The design incorporated a section of container ensuring a liquid barrier between a food simulant on the inside of the packaging and a cooling water simulant on the outside of the packaging. The design also had to permit electrolytic hole detection tests to ensure correct location of the microchannel within the apparatus, and ensure the absence of any material within the microchannel that might block the passage of microorganisms.

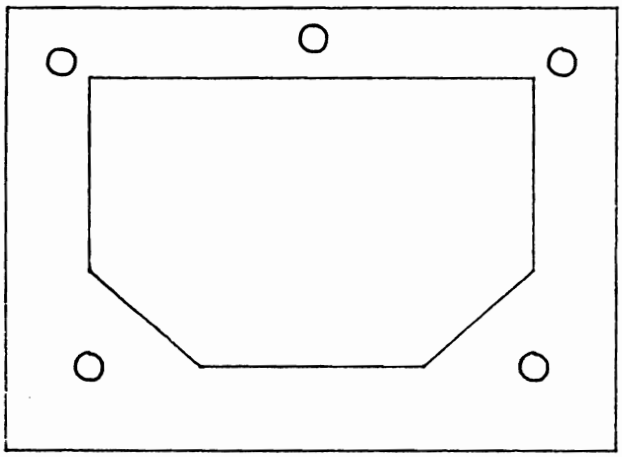
A chamber was built from perspex sheets of thickness 6.5mm. Four layers formed the base of the test cell, the upper three of which had the centre area cut out to form a cavity. The layers were sealed together with silicone of a type free from preservative chemicals. The components of the base layer can be seen in Figure 1 and the dimensions in Figure 2.

The top section of the cell consisted of two sheets of perspex of 6.5mm thickness, the inner layer having an area cut out to match the dimensions of the top plate of the base of the cell (Figure 3). Five inlet/outlet tubes were inserted through the top plate; the tubes were of 2mm (outer diameter) stainless steel. Three were glued centrally within the plate, so that two provided outlets flush with the inner surface of the perspex and the third, to be used as an inlet tube, was inserted to extend into the bottom cavity of the cell. The remaining two tubes were located 15mm from the edge so that one was flush with the inner surface of the perspex and the other, the inlet tube, extended into the base of the cell. The layout and dimensions can be seen in Figure 4.

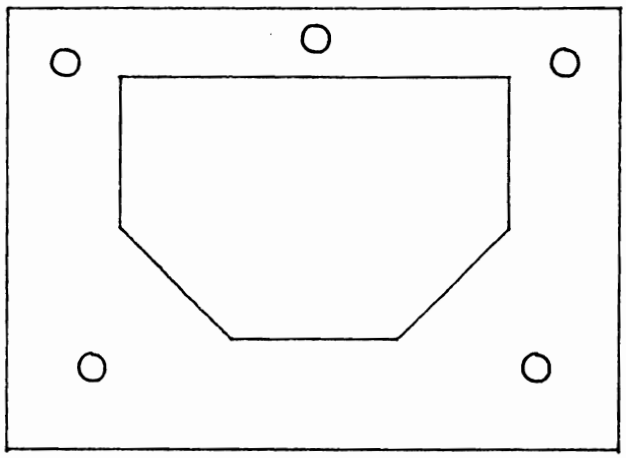
Beneath the underside of the top plate a section of the test container was sealed in place to include the three central pipes. The section of test packaging was sealed within the cell using silicone rubber compound, Part No. 555-588 (R.S. Components Ltd., P.O. Box 99, Corby, Northants) along its cut edges. This was situated so that the preformed discontinuity on the seal was placed centrally within the cell. The packaging sections gave internal volumes of 9.4ml for the pot and 9.0ml for the tray. A silicone rubber gasket of appropriate dimensions (Figure 5) was placed between the top and the base of the cell to seal the two halves and the whole cell was clamped together with 6BA studs (Figure 6). The volume of the chamber containing the packaging section was 55ml.

Attached to the cell was a pressure diaphragm assembly consisting of three layers of perspex 6.5mm thick. The two outer layers had small holes drilled in them into which stainless steel tubes of 2mm outer diameter were glued (Figure 4).

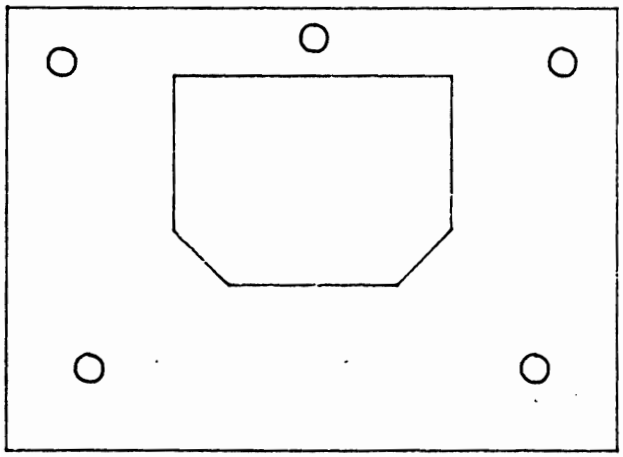
Figure 1. Detail of Lower Chamber Components



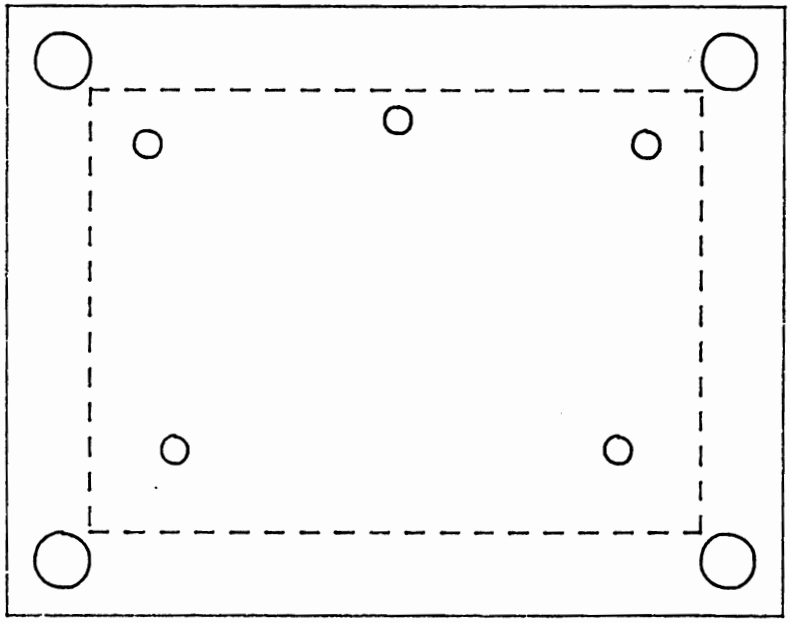
Layer Three



Layer Four



Layer Five



Base Layer

Material: 6.5mm Perspex

Figure 2. Diagram Showing The Lower Chamber Assembly Including All Dimensions

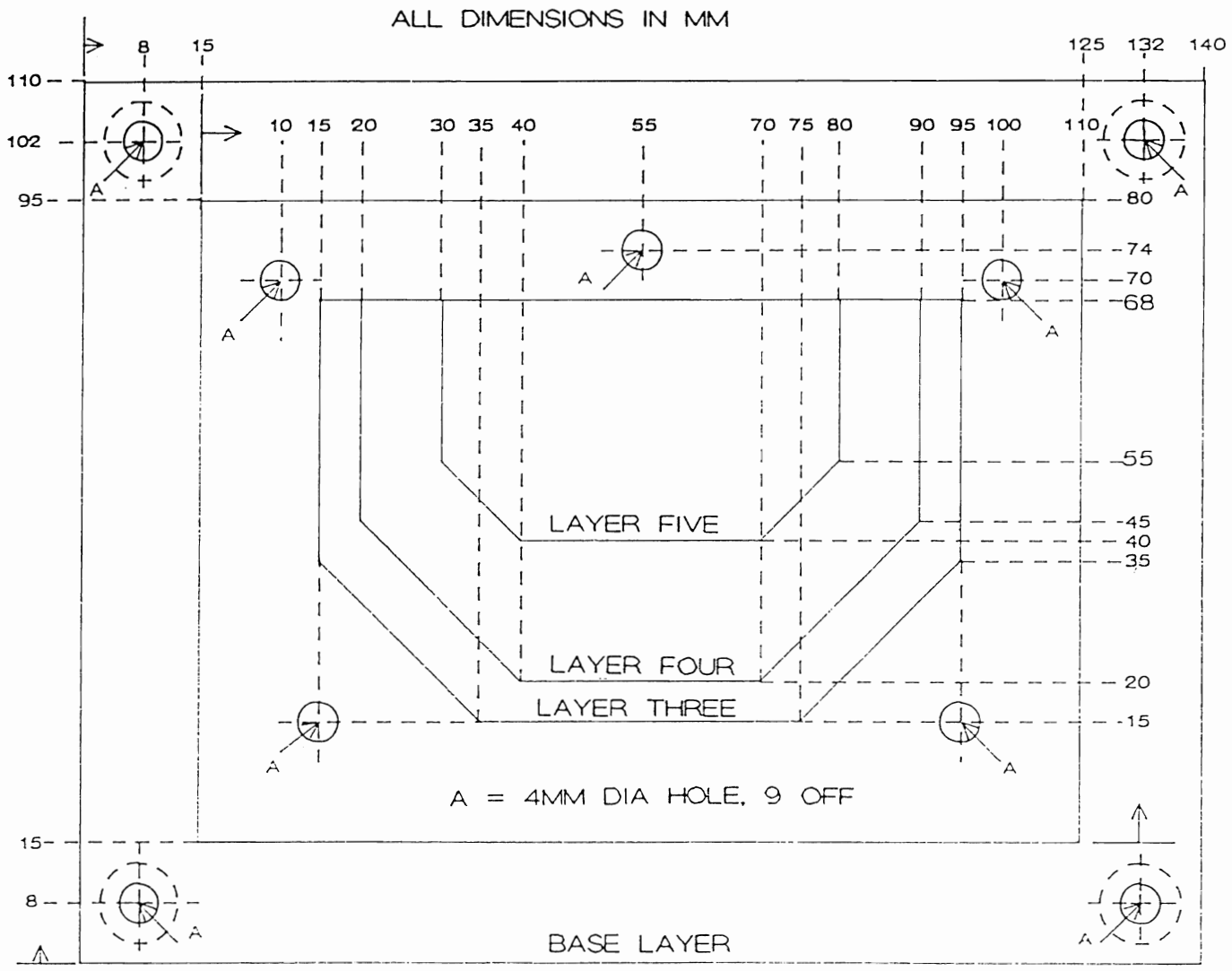
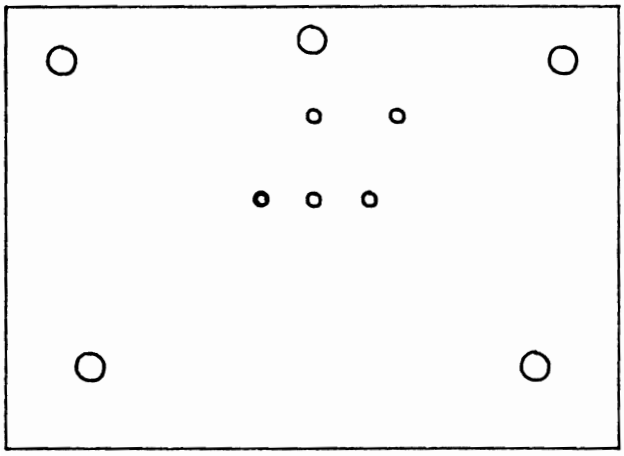
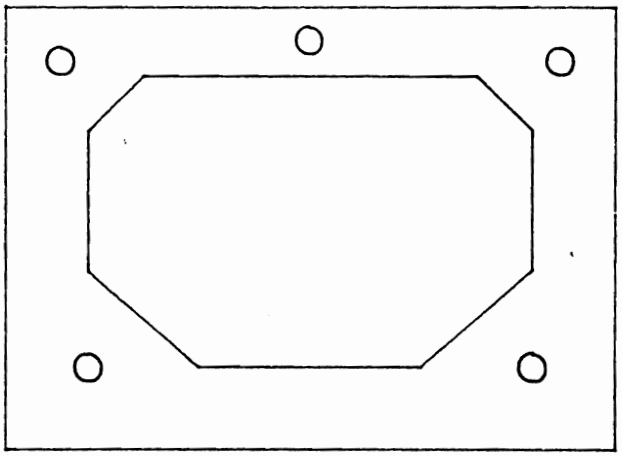


Figure 3. Detail of Top Plate Components

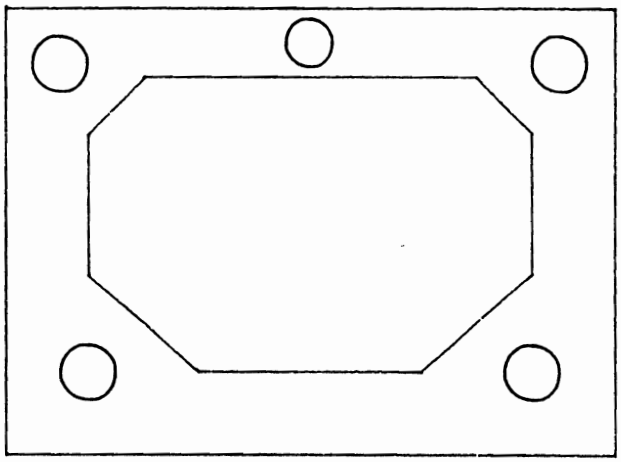


First Layer



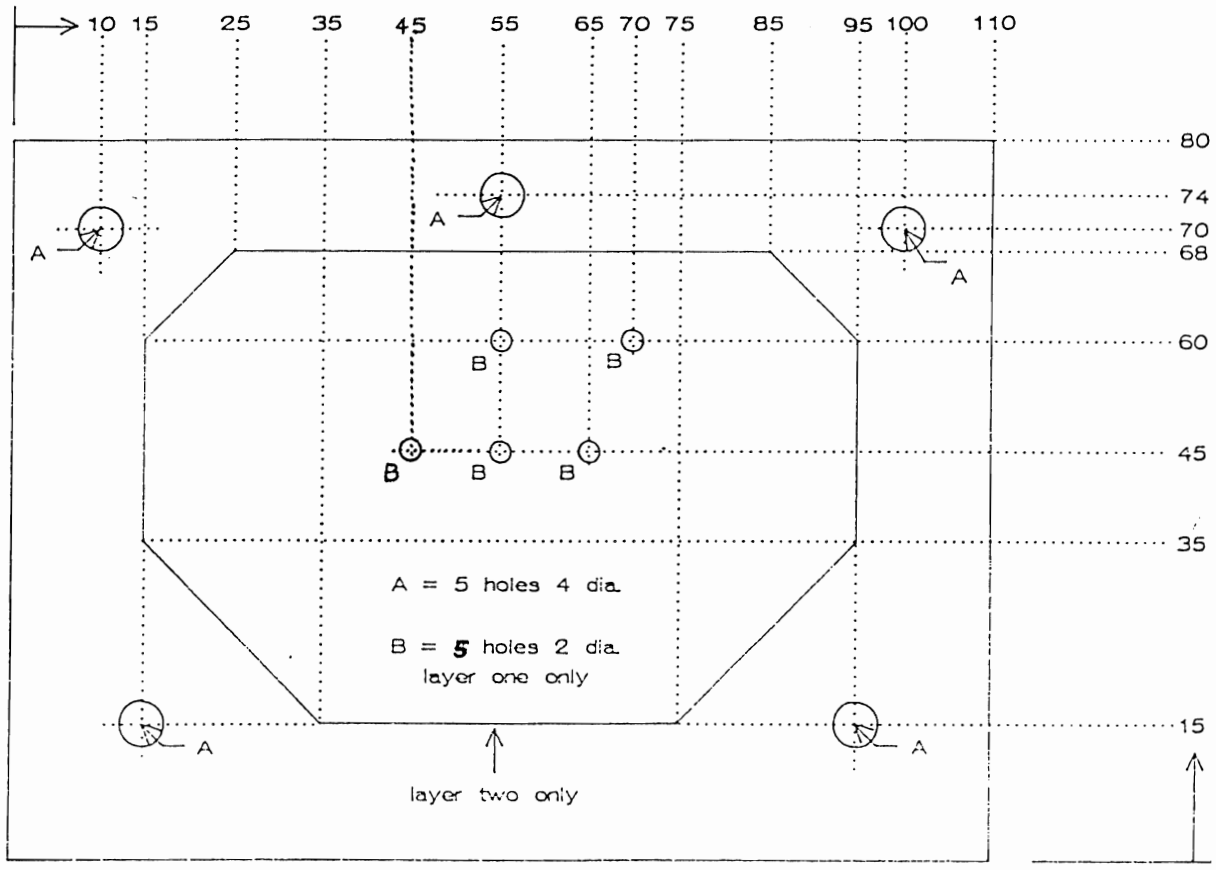
Second Layer

Material: 6.5mm Perspex u.o.s.



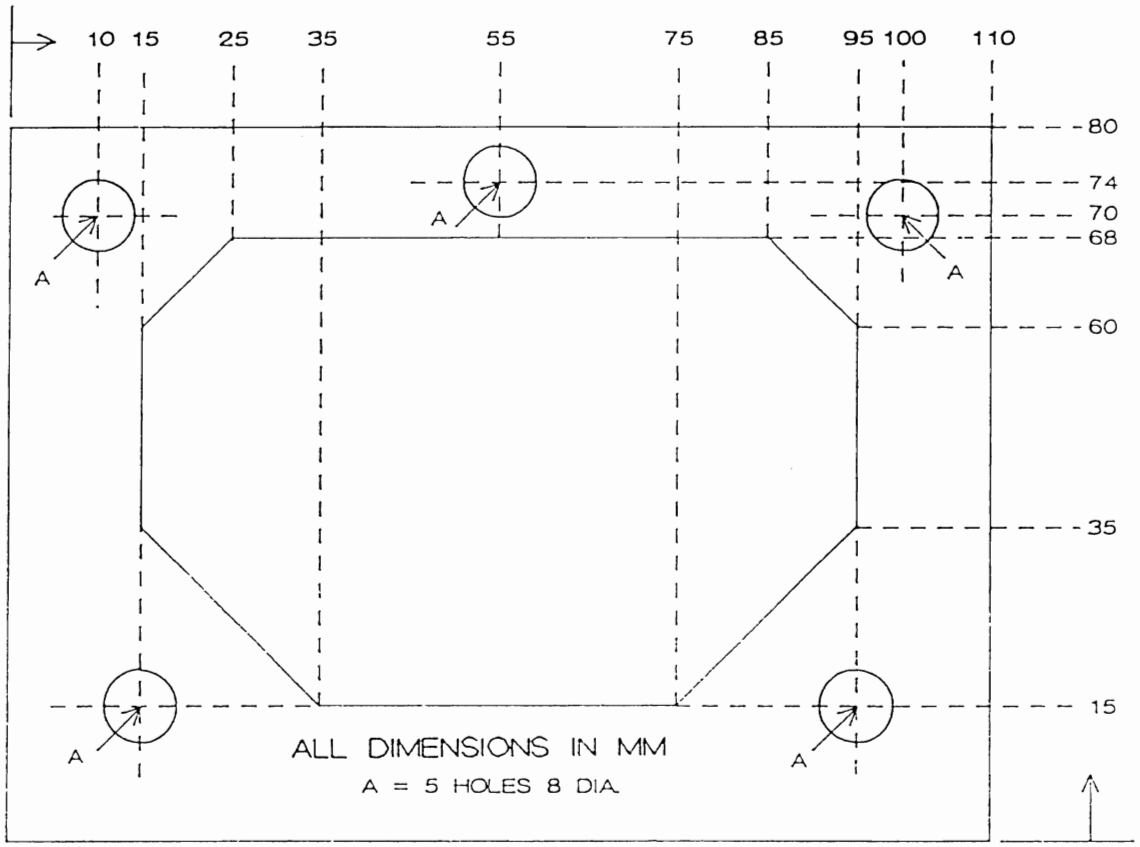
Silicone Rubber Gasket

Figure 4. Diagram Showing Top Plate Construction Including All Dimensions



Top Plate Layout. All Dims. in mm.

Figure 5. Diagram Showing The Silicon Rubber Gasket Construction Including All Dimensions



SILICONE RUBBER GASKET 3MM THICK

Figure 6. General Assembly of Cell

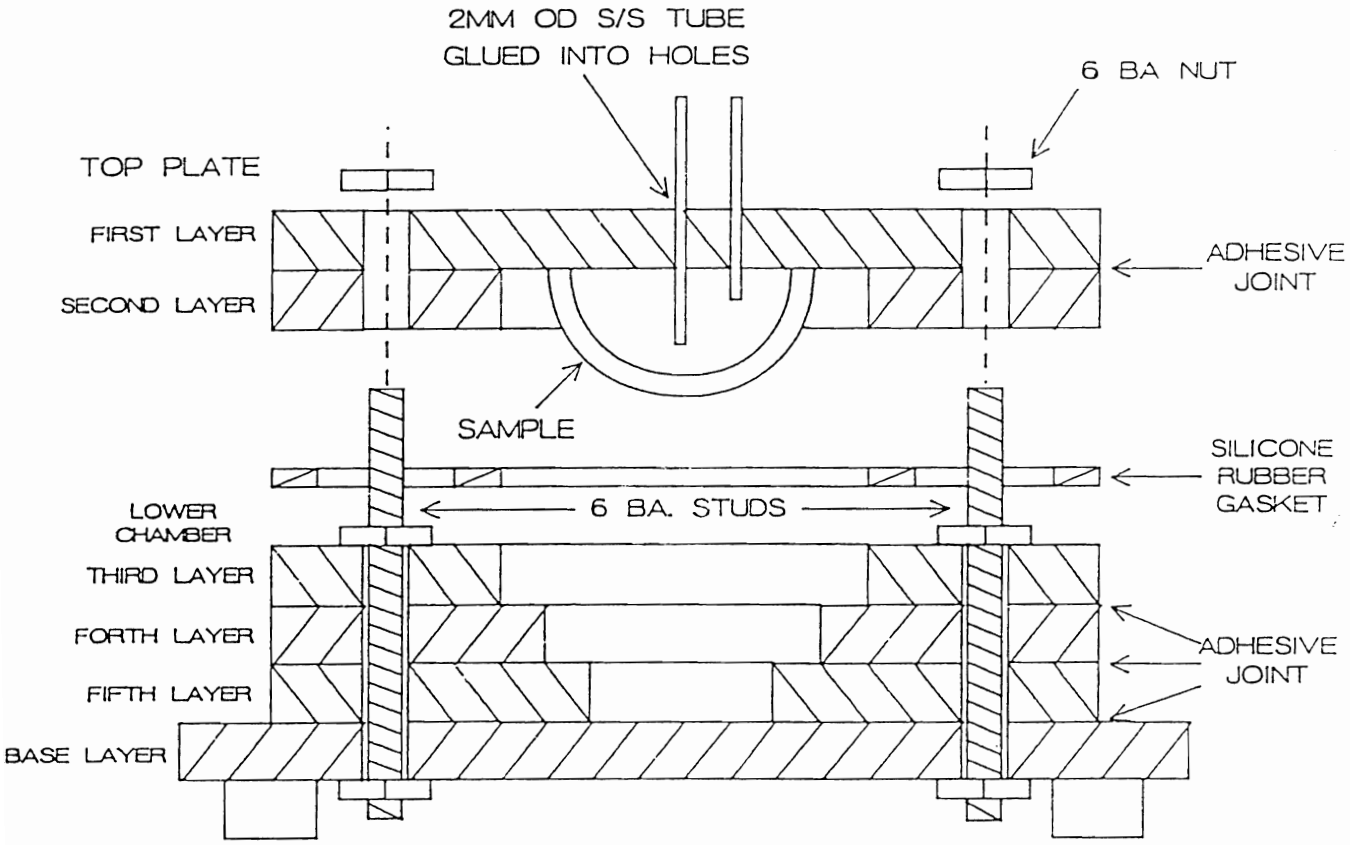
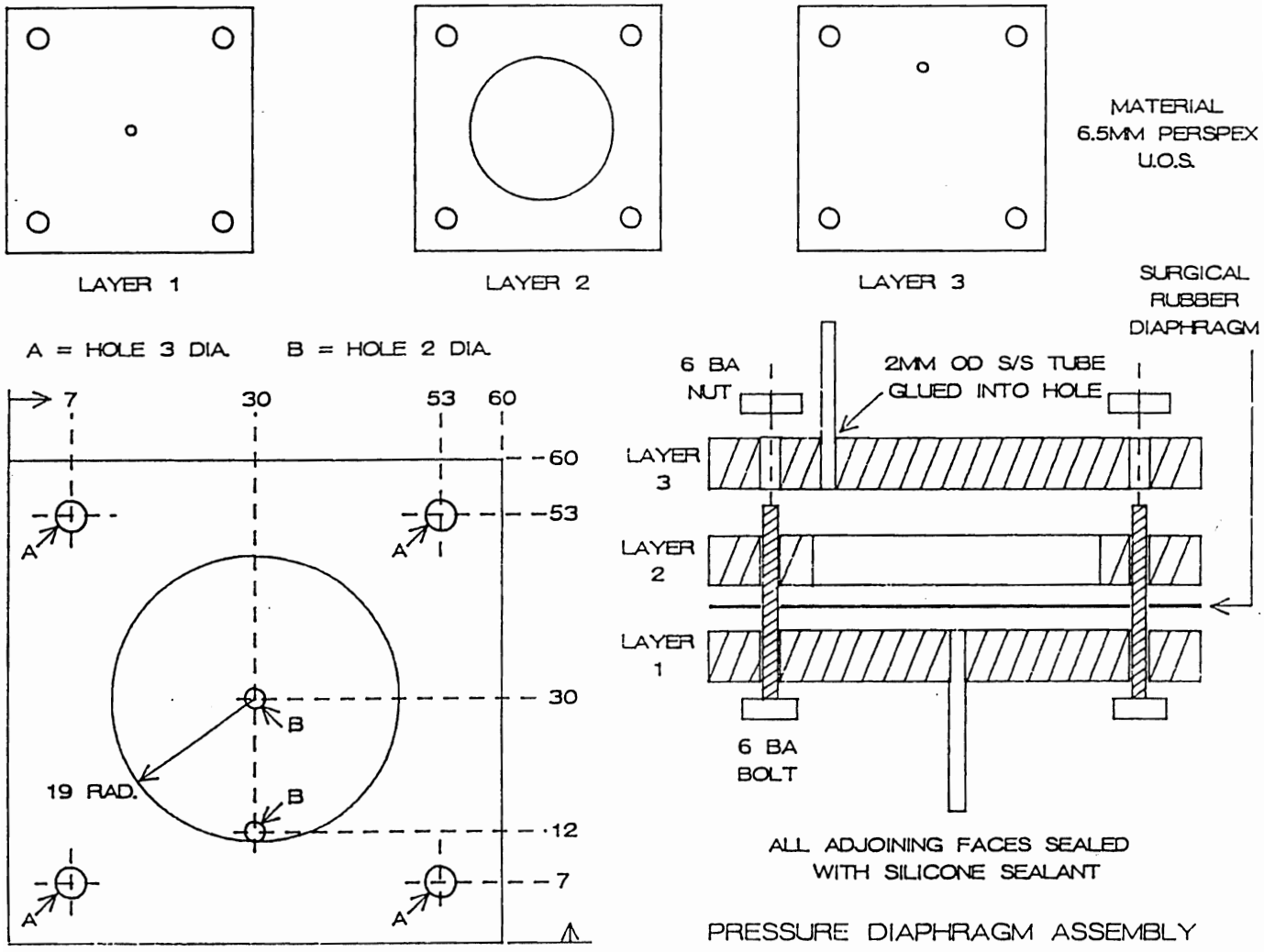


Figure 7. Pressure Diaphragm Assembly



The inner layer had a hole of 19mm radius cut out. A surgical rubber diaphragm was inserted between layers 1 and 2 and all adjoining faces were sealed with silicone sealant, the whole assembly being held together by four 6BA nuts and bolts (Figure 7).

2.3 Electrolytic detection of micro leakage channels

The use of the electrolytic methods for detection of microholes was considered to be critical for the validation of test micro-leakage channels within the test cell. Comprising a power supply unit and an ammeter as reported by Rose, *et al* (1994), the connections were made on the long inlet tube within the package cavity and the long inlet tube within the cavity containing the bacterial challenge. Positive readings showed that the test channel was centrally placed, fully submerged within the challenge media and free from air bubbles and debris which may have blocked the channel. The result of each test was also used to check that the seal between the challenge and food cavities in the chamber was secure, and that there were no spurious leaks in the system. This could be done by comparing the conductivity data from at least six standard microholes both before and after their assembly into the test apparatus.

2.4 Test procedure

Silicone rubber tubes of internal diameter 1mm were attached to the inlet and outlet tubes of the test cell. A silicone rubber tube was also attached to the third tube of the package cavity and this was connected to the pressure diaphragm.

50ml syringes were attached to two 0.4μm disposable sterile filters (Gelman Sciences, 600 South Wagner Road, Ann Arbour, Michigan 48106) and then connected to the inlet lines. The filters were intended to prevent any particles from entering two cavities in the chamber, which were flooded with hydrogen peroxide (35% v/v), including the silicone rubber exit tubes and the pressure diaphragm assembly.

The apparatus was held in this condition for 30 minutes at ambient temperature for decontamination after which it was rinsed with a sterile solution of diluent medium D2 (containing bacteriological peptone (L37 Oxoid, Basingstoke, Hants.), (0.05% w/v) and $KH_2 PO_4$ (Analar) (0.0043% w/v) to reduce residual hydrogen peroxide). Final continuous flow rinsing was done with a filter sterilized solution of bovine catalase (Sigma Chemical Co., Poole, Dorset). Perid test strips (Boehringer-Mannheim GmbH) were used to verify the absence of hydrogen peroxide prior to continuation with all tests.

Sterile D2 solution was used to rinse out the remaining enzyme, after which the filters were disposed of and two 50ml sterile disposable syringes were aseptically attached to the new filters on the flexible inlet tubes of the cell. During the trials, these filters, which had been used to maintain the asepsis of the apparatus had to be removed because different product media often had increased viscosities and contained fat which precluded passage of the media through the filter. At this stage an electrolytic spot test was carried out to establish that a bridge existed between the two cavities within the test chamber.

Trials comprised different combinations of media and conditions of challenge. When a pressure differential was required, a vacuum was applied. The vacuum line was connected to the pressure diaphragm which in turn was connected to a syringe for drawing the vacuum, the measurement of which was made on a manometer. When the cell was required to be operated at 30°C, it was immersed in its entirety in a water bath set at the required temperature. Prior to addition of the inoculum, the collection fluid was filled into the packaging chamber and discharged into a sterile Petri dish; this acted as a negative control to check sterility of the cell.

On addition of the combinations of challenge and collection fluid, the test cell was left for a specified exposure time, upon completion of which 30ml of collection fluid was discharged from the package cavity into a sterile universal tube. This sample was serially diluted (taking 1ml samples) and plated in nutrient agar. The plates were then incubated at 30°C for 24 hours (*Serratia marcescens*) or 72 hours (*Micrococcus luteus*).

After each test, 3ml of inoculum was injected into the challenge cavity of the chamber to ensure complete flooding of the cell and the displaced liquid was collected in a sterile waste bottle.

2.5 Test cultures

Serratia marcescens (Campden Culture Collection No. CRA 130) is a motile bacterium of width 1.0μm and length 1.5μm. It was originally acquired as NCTC 10211 and produces characteristic red pigmented colonies when grown on nutrient agar.

Micrococcus luteus (Campden Culture Collection No. CRA 3503) is a coccus of 1.5μm diameter. It was originally isolated from an environmental sample and it produces yellow pigmented colonies when grown on nutrient agar.

2.6 Preparation of bacterial challenge

Slope cultures of the test bacteria were grown on nutrient agar (10ml in a Universal bottle) for 3 days at 30°C then held for a further 5 days at 3°C before suspension in D2; this was shown to prevent clumping of *Serratia marcescens* cells. In certain trials the microorganisms were resuspended in a fatty emulsion and/or a rinse aid solution in D2.

The culture suspensions of fat in combination with D2 were prepared in 100ml of liquid. The total viable count of this was typically in the order of 1×10^8 cfu/ml for *Serratia marcescens* and 1×10^7 cfu/ml for *Micrococcus luteus.* In order to attain a 1×10^8 cfu/ml challenge for *Micrococcus* sp, slope cultures were suspended in D2 and used to inoculate medical flats of nutrient agar. After 8 days these were resuspended in 100ml of D2 and fat solution. In order to achieve 1×10^6 cfu/ml, slopes were resuspended in 10ml and appropriate serial dilutions were carried out in 9ml of fat emulsion.

A rinse aid, Drewsperse 739 (Drew Ameroid, Unit C4, Broadoak Business Centre, Ashburton Road West, Trafford Park, Manchester, M17 1RW), was added to D2 at 1% v/v. 100ml of this solution was then used to suspend culture slopes which also produced 1×10^8 cfu/ml. Serial dilutions were carried out in 9ml of rinse aid solution to achieve 1×10^6 cfu/ml.

In some of the trials a combination of both fat and rinse aid was needed. In this situation, 1ml of rinse aid was added to 99ml of fat emulsion and this was then used to suspend a slope culture.

2.7 Test fluids

In each case the base solution for collection fluid was D2.

The test required a viscous solution to be used within the cell to replicate a thickened sauce. For this simulation a 2% w/v solution of Colflo 67 (National Starch and Chemicals Ltd., 6730 Neustadt a.d., Germany) made up with D2 was used. Colflo is a modified starch widely used in the food industry. This aqueous solution was heated to above 70°C until the starch had gelatinised and dispensed into 100ml bottles and sterilised.

As a simulated fatty food, a 10.3% w/v fat emulsion was made up with Wesson Green Pure Vegetable Oil (Kraft General Foods, Cheltenham, GL50 3AE) and homogenised at a pressure of 1.02×10^7 N/m^2 (102 bar) prior to dispensing into 100ml bottles and sterilisation.

For a higher acidity food simulant, food grade anhydrous citric acid (Jungbunzlauer AG, Austria) was added to D2 with constant monitoring with a Beckman 71 pH meter until a value of pH 4.5 was achieved. The acidified D2 was dispensed into 100ml bottles and sterilised.

Where a combination of each variable was required the additives were combined in the D2 solution prior to dispensing into 100ml bottles and sterilisation. The solutions in each case were sterilised in steam at 121.1°C for 15 minutes.

3. EXPLORATORY EXPERIMENTAL DESIGN

There were twelve risk factors associated with post process wet handling which were considered necessary to include in the experimental design. These factors had been previously investigated with laser drilled pinholes through laminate materials and the rationale for their selection was discussed by Rose *et al* (1994), but in this study there were changes with respect to packaging types, hole diameters and hole lengths.

The differences between the flange widths of the pot and the tray in conjunction with their heat sealing tools resulted in seals which respectively measured 2mm and 5.5mm across. The hole-making method consequently gave holes of similar length with cross sectional profiles determined by the wire gauges of 15μm and 63μm. This gave an area difference factor of 17.6. It is important to note that for the pot and the tray the sealing polymer was polypropylene, similar to the lid film.

Following initial trials to establish the procedure for carrying out the tests, it was necessary to minimise the number of trials to be performed whilst maximising the data returned by statistical interpretation of the results. A Taguchi L16 experimental design was implemented to investigate the simulated effects of the different factors on microbial transmission.

3.1 Sixteen trials

Each trial represented a particular combination of the factors. If all the combinations had been carried out with just two levels for each factor, 2^{12} (4096) trials would have been required.

The Taguchi experimental design was based upon sixteen individual trials. Such a design is useful, because it offers a great reduction in the number of trials required. It allowed the estimation of twelve factors and also three interactions between the factors. Each factor had two levels (Table 1). It was assumed that all other interactions were nonexistent. If other interactions did exist it would mean that the estimates of the twelve factors and three interactions included in the design would be biased. In the absence of any real interactions, the three included in the design may be regarded as representing purely random variation.

Of course, the large reduction in the number of trials may lead to false conclusions. Firstly, additivity of effects is implied by the exclusion from the design of all first and higher order interactions. Because not all combinations are carried out, final results could be biased if there are strong interactions between the factors. A second drawback is the relatively small size of the experiment; there are only eight replicates for each of the two levels of each factor.

3.2 Risk factors

The factors included and the levels used are given in Table 1.

TABLE 1 : FACTORS AND LEVELS

Factor	Description	Level 1	Level 2	Column in Table 2
A	Acidity of Food	pH 4.5	pH 7.0	c1
B	Hole Length	2mm (pot)	5.5mm (tray)	c2
C	Microorganism	*S. marcescens*	*M. luteus*	c4
D	Pressure	0mmHg	150mmHg	c8
E	Hole Diameter	15 micrometre	63 micrometre	c7
F	Rinse Aid	1%	0%	c11
G	Viscosity	Diluent D2	D2 + 2% starch	c13
H	Fat/Challenge	10.3%	0%	c14
I	Temperature	30°C	18°C	c15
M	Challenge Level	1×10^8 cfu/ml	1×10^6 cfu/ml	c9
N	Exposure Time	5 minutes	10 minutes	c10
O	Fat/Food	10.3%	0%	c12

The design plan is given in Table 2 for the Taguchi L16 exploratory design (Grove and Davis, 1992). All conditions were adhered to exactly so no further variations were introduced except that 5 'repeat' measurements were taken in each trial.

TABLE 2: DESIGN PATTERN FOR TAGUCHI L16 WITH 12 FACTORS

Code 1 = level 1 of factor: Code 2 = level 2 of factor

Column	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Trial No.	A	B	X_1	C	X_2	X_3	E	D	M	N	F	O	G	H	I
1	1	1	2	1	2	2	1	1	2	2	1	2	1	1	2
2	2	1	1	1	1	2	2	1	1	2	2	2	2	1	1
3	1	2	1	1	2	1	2	1	2	1	2	2	1	2	1
4	2	2	2	1	1	1	1	1	1	1	1	2	2	2	2
5	1	1	2	2	1	1	2	1	2	2	1	1	2	2	1
6	2	1	1	2	2	1	1	1	1	2	2	1	1	2	2
7	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2
8	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1
9	1	1	2	1	2	2	1	2	1	1	2	1	2	2	1
10	2	1	1	1	1	2	2	2	2	1	1	1	1	2	2
11	1	2	1	1	2	1	2	2	1	2	1	1	2	1	2
12	2	2	2	1	1	1	1	2	2	2	2	1	1	1	1
13	1	1	2	2	1	1	2	2	1	1	2	2	1	1	2
14	2	1	1	2	2	1	1	2	2	1	1	2	2	1	1
15	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1
16	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Mean 1s	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Mean 2s	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Difference = effect of the factor:	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

X_1 = Interaction of A and B
X_2 = Interaction of A and C
X_3 = Interaction of B and C
X = Calculated results

Reference for design: Grove D.M. and Davis T.P. (1992).

3.3 Sampling regime

The test cell was set up as described in Section 2.4.

During tests the appropriate cavities of the cell were flooded with the challenge suspension of bacteria and the food simulant. This was timed for the specific conditions of each trial and then 30ml of sample was discharged into a sterile universal bottle. Immediately after discharge the timer was set again and the cycle repeated four times, so that five repeat measurements were made for each trial. The 30ml samples were shaken to mix, and of this 1ml was plated out in nutrient agar and 1ml was pipetted into 9ml of D2 to give a 10^{-1} dilution. This was then plated out and the procedure repeated to give 10^{-2} and 10^{-3} dilutions. All results were based upon counts per 1ml sample. Any tests that resulted in zero counts were repeated twice more for confirmation.

4. RESULTS

TABLE 3 : RESULTS FROM TRIALS

Trial	Order of Measurement (All counts are colony forming units per ml of sample)				
	1	2	3	4	5
1	0	0	0	0	0
2	248	105	59	58	36
3	1310	46	6	2	0
4	0	0	0	0	0
5	133	12	2	0	0
6	0	0	1	0	1
7	0	0	0	0	0
8	72,000	1,060	365	186	144
9	0	0	0	0	0
10	3040	235	455	135	94
11	1,292,000	114,000	23,300	12,350	11,650
12	12,650	1,525	396	171	90
13	760,000	500,000	517,000	125,000	100,500
14	7	12	5	4	2
15	52	25	14	23	19
16	174,500	132,000	68,500	49,500	24,900

4.1 Statistical analysis

Results of the 16 trials are shown in Table 3. It should be noted that all counts of zero were adjusted to a nominal value of 0.2 to allow logarithms to be taken.

Examination of Table 3 shows that the repeat counts decline markedly from the first to the fifth repeat. When converted to logarithms, the means for the five repeats, averaged over all 16 trials, were 2.27, 1.76, 1.53, 1.27 and 1.16 respectively. The pattern may be seen clearly in Figure 8 where data from Trials 13, 14, 15 and 16 have been shown as examples.

FIGURE 8

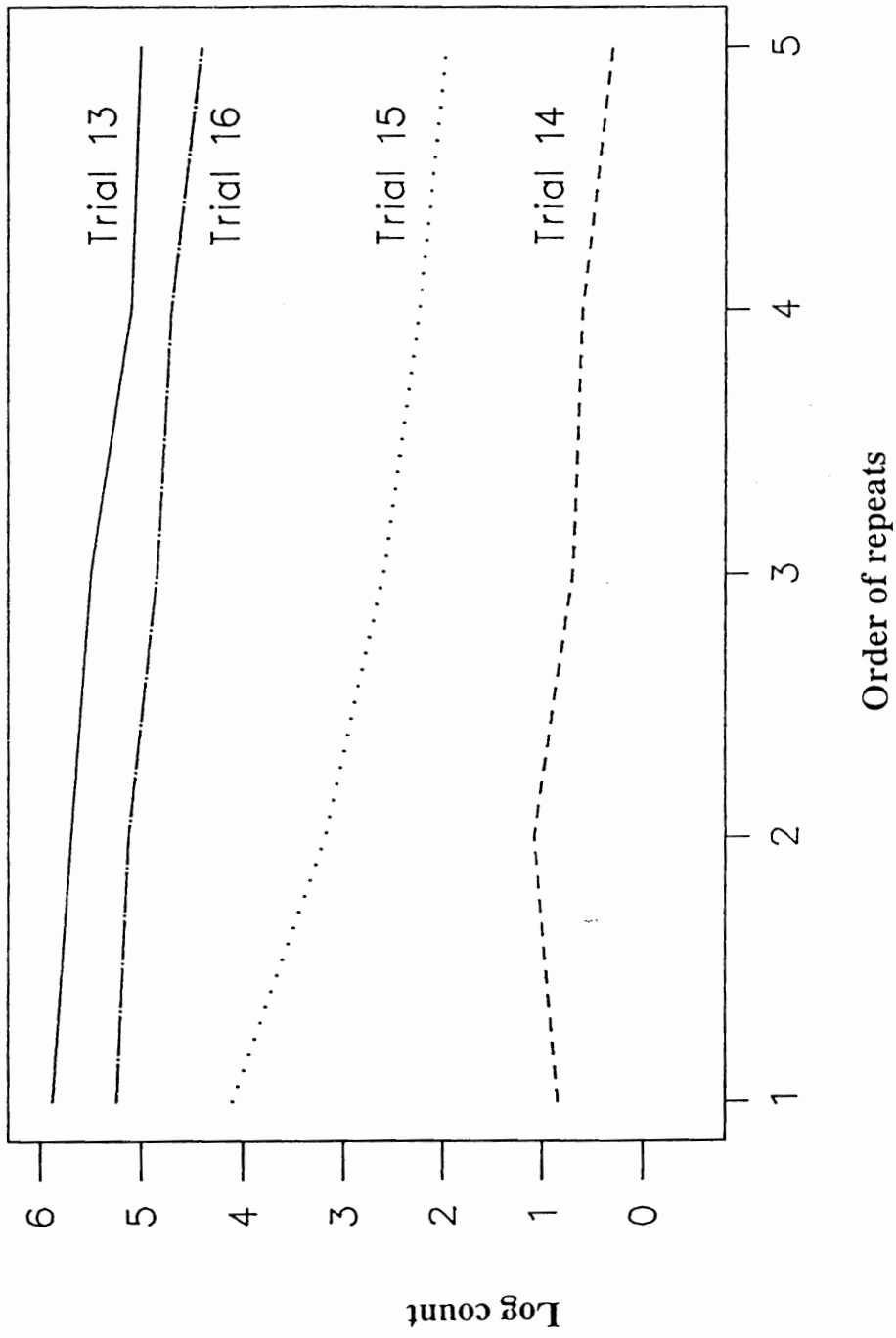


Figure 8: **Five repeats of log count for 4 trials**

In view of the trend in repeat counts shown in Figure 8, it was decided that in case the pattern of decline was unstable it was advisable to use only the first of the counts in the Taguchi analysis.

This analysis involved summing and averaging the log counts in different ways (see Table 2) in order to estimate the effects of the 12 factors and the 3 dummy factors (or possible interactions). Data so derived was used to determine the significant factors (Table 4).

TABLE 4 : MEAN COUNT FOR EACH SIGNIFICANT FACTOR

Factor	Description	Level	Mean Log	Mean Count (cfu/ml)
E	Hole diameter	15 micrometer 63 micrometer	0.396 4.15	2.5 14,200
D	Pressure differential	0mmHg 150mmHg	1.21 3.34	16.3 2160
B	Hole length	2mm 5.5mm	1.58 2.97	37.9 930

The means for the factors that were not significant and which were considered to be due to random variation were also calculated. These results are given in Table 5.

TABLE 5 : MEAN COUNTS OF LESS SIGNIFICANT FACTORS

Factor	Description	Level	Mean Log Count	Mean Count (cfu/ml)
G	Viscosity	D2 D2 + 2% starch	2.72 1.83	525 67
C	Microorganism	S. marcescens M. luteus	1.84 2.71	69 509
M	Challenge level	1×10^8 cfu/ml 1×10^6 cfu/ml	2.66 1.89	453 78
H	Fat in challenge	10.3% 0%	2.55 1.97	356 93
N	Exposure time	5 minutes 10 minutes	2.01 2.54	103 344
O	Fat in food	10.3% 0%	2.02 2.52	106 333
F	Rinse aid	1% 0%	2.52 2.03	328 108
A	Acidity	pH 4.5 pH 7.0	2.40 2.14	254 139
I	Temperature	30°C 18°C	2.31 2.24	203 174

The effects are displayed in a Daniel plot (Figure 9) in which they are plotted in order of 'half-normal score', which is related to the ranking of the effect. The Daniel plot allows effects to be sorted into those which appeared to be real from those which are presumed to have arisen purely from random variation. When the effects are plotted from smallest to largest on a graph, these random points will fall on a straight line, whereas real effects will fall substantially above this line.

The factors are coded on the Daniel plot and in Tables 1 and 2 as A to I, M, N and O, whilst the dummy factors (which can be interpreted alternatively as unseen interactions) are coded X_1, X_2 and X_3.

The Daniel plot of the log counts indicates that factors E, D, and B are significantly above the line of the smaller effects. All of these other effects appear to be non-significant. Note that a formal analysis of variance test is not appropriate with the Taguchi design, due to the absence of replication.

Mean log counts for the two 'levels' of each factor are shown in Tables 4 and 5. Results reconverted from log counts back to counts are also shown.

FIGURE 9

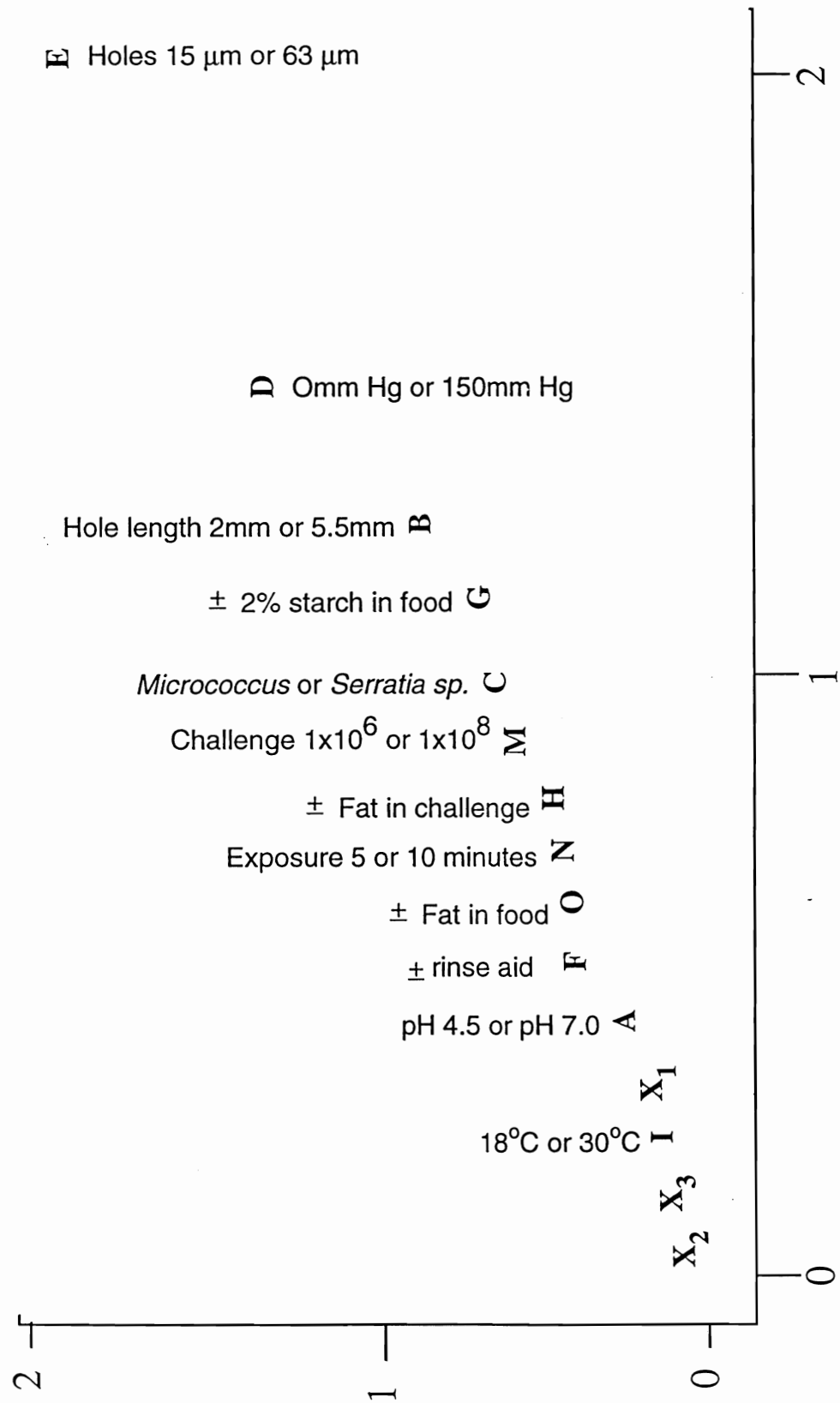


Figure 9 : **Daniel plot of the first measurement for effects on log counts showing significant factors (E,D,B), non significant factors (G, C, M, H, N, O, F, A, I) and spare effects (X_1, X_2, X_3)**

"Effect" = mean log count of factor at one of its levels - the overall mean log count.

"Half-Normal Score" = an integral of Normal Distribution relating to the ranking of the effect

5. DISCUSSION

The decline in microbial transfer on repeat tests in a trial (Figure 8) was considered unlikely to have been caused by overall reductions in populations, since in the extreme case of Trial 16 just 1.8% of challenge cells were transferred out of the system. Because the contents of the chambers in a test cell were not stirred, diffusion could be a factor, but this does not exclude other possibilities such as lodgement of cells within the leakage channels.

The statistical analysis ranked the factors according to the order of importance of their effects on microorganism transmission (Tables 4 and 5). The results and their causes may be explained in the following ways.

5.1 Hole diameter

This factor had the most significant effect on the transmission of microorganisms through leakage channels. As expected the larger hole size allowed a greater number of microorganisms to pass through. The mean penetration count for holes of diameter 63μm was 14,200 cfu/ml compared with 2.5 cfu/ml through the 15μm diameter hole. Therefore increasing the hole area 17.7 times caused an increase in the mean count by a factor of 5,680.

5.2 Differential pressure

Differential pressure had a significant effect on the movement of microorganisms through microleakage channels; a partial vacuum gave a mean count of 2160 cfu/ml compared with the count of 16.3 cfu/ml when no pressure differential was used. This effect substantiates previous work on laser drilled pinholes where an increase in partial vacuum inside the food cavity of the chamber increased the count.

5.3. Hole length

The length of the leakage channel unexpectedly affected microbial transmission. The channel length across the heat seal areas of a pot was 2.75 fold less than the channel of a tray. Previous work on pinholes (Rose *et al* 1994) showed that a thicker laminate allowed greater microbial transmission than a film laminate despite its shorter channel.

Although the channels used in this case were up to 5 times longer than previously studied, a similar result was found in that the longer channel had a higher mean count than the shorter pot channel. This increased transmission is thought to be due to the flexibility of the pot. In the presence of a vacuum the pot flexes and so decreases the actual pressure within the pot. In contrast, the tray is more rigid; therefore, in the presence of a vacuum the pressure difference within the tray is much greater than within the pot, the suction is greater and therefore the microbial transmission is higher.

5.4 Less significant factors

It should be noted that non-significance does not necessarily imply non-existence of an effect; sometimes additional trials or replication will cause a 'non-significant' effect to become 'significant'.

Although not as significant as hole size and differential pressure the following points may have some effect on the movement of microorganisms through micro channels.

5.4.1 Viscosity

An unthickened food simulant had a mean count of 525 cfu/ml compared to the thickened food which had a mean count of 67 cfu/ml. Despite the 8-fold difference this was not deemed to be significant in this set of trials.

5.4.2 Microorganisms

The type of microorganism may have some effect on the microbial transmission since a greater count was found with *Micrococcus luteus* than with *Serratia marcescens.* The mean count for *S. marcescens* penetration was 69 cfu/ml as opposed to 509 cfu/ml for *M. luteus*, a 7-fold difference, although this is not significant in statistical terms.

5.4.3 Challenge level

A challenge level of 1×10^8 produced a higher mean count of transferred bacteria than the challenge level of 1×10^6 (453 cfu/ml cf. 78 cfu/ml), but was found not to be of significance in its effect on microbial transfer. This contrasted with the result from the previous work (Rose *et al*, 1994) when it was found to have a significant effect on microbial transmission, albeit that increasing the challenge level 100-fold produced only a 10-fold transmission increase.

5.4.4 Presence of fat in challenge

The presence of fat in the challenge had no significant effect on microbial transmission. A 10.3% fat solution combined with the challenge organism gave a mean count of 356 cfu/ml; when no fat was present in the challenge a mean count of 93 cfu/ml was achieved. The increased count with the presence of fat could be due to the settling out of the fat at the surface of the challenge solution, thus changing the surface characteristics of the packaging and the method of microbial transmission through the leakage channel, although as this was not a significant factor this may be due to random variation only.

5.4.5 Exposure time

The length of exposure time was considered to be of low importance, which contrasted with Rose *et al* (1994) who found that with pin holes the exposure period was a significant factor. Increasing the exposure time did increase the count however, 10 minutes giving 344 cfu/ml whereas 5 minutes gave 103 cfu/ml.

5.4.6 Fat in food

The presence of fat in the food simulant had no significant effect on the movement of microorganisms through leakage channels. It is of interest to note, however, the similarity of numbers when fat was incorporated in the challenge.

5.4.7 Rinse aid

The presence of rinse aid was found not to have a significant effect on microbial transmission; 33 cfu/ml were transferred in the presence of the product compared with 108 cfu/ml without.

5.4.8 Acidity

The factor considered to be of the most importance by Rose *et al* (1994) was the pH of the product, where increasing the acidity to pH 4.5 caused the mean count to fall 30-fold.

In this work, however, pH had little or no effect on the passage of microorganisms. It was hypothesised by Rose *et al* (1994) that the decrease in count with an acidified product was due to interactive forces between the plastic packaging, pH and the organisms. This same phenomenon was not seen in this stage of the work and this is thought to be due to the exposure to homogeneous packaging material. The last set of trials exposed a range of contact polymers because microholes were drilled through laminates; this work looked at microchannels between the polypropylene heat seal of the films and the containers only. An additional reassurance from this concerns any potential effects that pH "shock" may have had on the bio-test bacteria and the potential bias this may have placed on the result. It reinforces the results of the separate tests which were done on pH sensitivity, which was found to be non-existent.

5.4.9 Temperature

Temperature differences had no significant effect on microbial transmission. Results from trials at 30°C gave a mean count of 203 cfu/ml compared with a mean count of 174 cfu/ml from trials at 18°C.

5.5 Statistical analysis

From the twelve factors identified as possible risk factors in the transmission of microorganisms, three proved to be highly significant. They were the size of the hole, the pressure differential within the pack and hole length. The remaining 9 factors were thought not to be significant and the differences between the high and low levels were attributed to random variation.

6. CONCLUSION

A prototype test chamber was made within which a section of a lidded semi-rigid plastic pot or tray was sealed. This provided two cavities that defined the internal (9ml) and external environments of a semi-rigid plastic container. Means were devised by which 15μm and 63μm leakage channels were made in heat seals equivalent respectively in length to the seals traversed, which were 2mm and 5.5mm. The apparatus was successfully used to repetitively biotest the known discontinuities with appropriate physical and microbiological control to validate the performance of the system and consequently the experimental data.

A Taguchi experimental exploratory design was used similar to that in a proceeding stage of the project (Rose *et al* 1994), but since too many of the test combinations did not result in transfer of bacteria, analysis of variance was not possible.

The Daniel plot for the effects on log counts (Figure 9) shows sharp differentiation and it can be seen that three effects were significant: hole diameter, differential pressure and hole length. Those which were found to be less significant included starch in food, type of bacterium in the challenge, concentration of bacteria in the challenge, fat in challenge, exposure time, fat in "food", temperature (18°C or 30°C), "food" pH (4.5 or 7.0), and the presence or absence of rinse aid. The plots of these all fall along a lower reasonably straight line together with the three spare effects coded as X_1, X_2 and X_3. The latter three plots grouped together well and are attributable to random variation.

It is concluded that not all sets of conditions will result in microbial transfer, as found for example in trials 4 and 7 (Tables 1 and 2) where hole lengths were longest, hole diameters were the most narrow and no pressure differential was applied. By contrast, trials 12 and 15 involved the longest holes with the narrowest diameter but transferred many bacteria (Table 3) under conditions of applied differential pressure. When these considerations are applied to the practice of post-process wet handling of containers, the risks of infection will be high, especially when the presence of bacterial contamination from cooling water, surfaces or skin coincides with pulsed pressure differentials caused by handling. Furthermore, discontinuities smaller than 15μm could still transmit bacteria, whereas no on-line screening machinery can yet be applied economically to factory conditions for detection of holes smaller than 30μm.

Our results showed no effect connected with pH value as we had found in the earlier reported stage of this work (Rose *et al* 1994). On one hand this gives additional support to those findings since this also negates the potential criticism that this effect was spurious and caused by death of bacteria due to shock exposure to acidic conditions. Of extreme importance, however, it does lend weight to the contention that the relationship between package material (in this case a range of plastics), the pH of the food and the biotest organism can control microbial transfer through micro-leakage channels. The implications of this are enormous for a wide range of food packaging technologies where there is a need to hygienically protect foods from infection or reinfection. No reports are known where materials are selected for use because of their inherent ability to resist microbial transfer through channel leaks or pinholes. It would be attractive to food processors to know that wherever a risk to integrity and safety is concerned this may be an option for second-line protection, providing that the mechanisms are understood, and the correct combinations of product/package are chosen.

7. REFERENCES

Grove, D.M. and Davis, T.P. (1992) Engineering, Quality and Experimental Design. Longman Scientific and Technical UK.

Rose, D., Horan, S.P. and Evans, D.G. (1994). Risk factors associated with post process contamination of heat sealed semi-rigid packaging Part 2A : Investigation of microbial ingress through laser drilled microholes in packaging. Technical Memorandum 708, Campden Food and Drink Research Association.

8. ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support from the Ministry of Agriculture, Fisheries and Food for the course of this research project.