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A proposed standard procedure for taint tests with agricultural chemicals

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Summary. This paper has been prepared under the auspices of the Advisory Committee on Taint of the Ministry of Agriculture, Fisheries and Food and is intended to meet the need for a standard procedure for taint tests with agricultural chemicals on processed foodstuffs of plant origin. The paper is divided into two main parts. The first section deals with trial design and site, chemical application, crops, sampling procedure, packing of raw materials, transport and storage of samples, processing and number of tests required to indicate whether a chemical substance may be accepted as being free from tainting characteristics. The second section deals with various aspects of the tasting tests, such as method employed, suitability and numbers of tasters, place and time of tasting and preparation of samples for tasting. The proposed method of tasting is the triangular test and details of calculating the results are given, together with appendices presenting the necessary tables and a worked example.

I. Introduction

The Advisory Committee on Taint of the Ministry of Agriculture, Fisheries and Food is an official committee set up to advise on all aspects of taint on foodstuffs of plant origin. The Committee pays particular attention to taint by agricultural chemicals. The increasing demand by both processors of food products and agricultural chemical manufacturers for information on the taint potential of new agricultural chemicals, and the diversity of methods for assessing taint, has led to a need for a standard procedure. It was thus decided early in 1967 to set up an *ad hoc* committee for the task of proposing a standard procedure for: (1) trials from which raw materials are obtained for taint tests, and (2) tasting tests.

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Although this paper deals exclusively with fruit and vegetable products processed by simulated commercial methods for long term preservation, it can be used as a guide for the detection of taints in fresh fruits and vegetables.

There are many factors which can influence the degree of taint or even decide whether an agricultural chemical will, or will not, in practice cause a taint in food crops. They include the nature and cultivar of the crop, climatic conditions, soil type, method of application, formulation of the active ingredients, interval between application and harvest, and method of processing. However, it is impractical to investigate the effects of all the different combinations and permutations of these factors in relation to each new active ingredient. Consequently, only those factors which have been shown to be important are examined and suggestions for the total number of tests required are detailed in a later section of this paper.

II. Field trials, chemical application and processing

Field trial design and site

An experienced Agricultural Statistician's advice should always be sought *before* starting a trial.

Test crops should be grown under as wide a range of soil and climatic conditions as possible, preferably over a number of years, in order to arrive at a valid assessment. These requirements are not necessarily so important with glasshouse crops and there is no need to test crops under conditions where they are not normally grown. Records of the main climatic factors should be kept. Trials should be statistically designed on a realistic scale: in very small trial sites or plots crops may not behave in the same manner as those grown commercially.

Test crops should be grown on a trial site of known history, making it possible to have complete records of all previous crops and treatments. In this way any anomalous results can be investigated for the effects of residues from earlier treatments.

Test crops should be grown and treated using sound experimental procedures. To do this it is usually necessary to be aware of such factors as the behaviour of a particular crop under local soil and climatic conditions. Cross-contamination and drift from test materials *must* be prevented.

Trial designs should ensure adequate separation between unit plots (both between control and treated, and between adjacent treated plots). Adequate allowance should be made for such factors as topographical and soil variations, the effects on end rows of exposure to wind or frost and so on – all in relation to the specific crop being used for testing.

It should be noted that the tasting method recommended in Section III (p. 189) requires equal quantities of control and treated material, i.e. the amount of control crop must be at least equal to the total amount of treated crop. This must be considered in the design of the trial.

Records should be made of all chemical and fertilizer applications in trials, in case interaction between two chemicals should have an effect which would not have occurred if either had been used alone.

Where possible, it is desirable to select field conditions which are of average suitability for the crop in question in the area concerned. Treatment and cultivation programmes must be closely supervised by skilled and competent workers. Crops left unsupervised for long periods on commercial holdings are unsuitable for taint tests.

A uniform system of cultivation, picking, transport and storage, etc., is essential for any one trial.

Chemical application

Formulations and active ingredients. Different types of formulations of the same chemical such as emulsifiable concentrates, wettable powders and granules, may vary in tainting potential, either directly arising out of the properties of the formulants or impurities or, indirectly, from the effect of the formulation on the distribution and behaviour of the active ingredient.

It may not be practical to test all formulations of one active ingredient by the method given in the section on number of tests. Therefore, the main formulation that will be most widely used should be fully tested as defined on pages 188 and 189. Other formulations may be checked for all processing methods for the crops already fully tested, or being fully tested, using the main formulation.

Dose rate. Treatment should be made at double the expected maximum rate of application recommended for the crop concerned. With liquid sprays this should be done by doubling the maximum concentration. This will allow for overlapping and, consequently, double dosing under commercial conditions. Exceptionally this may not be desirable because of the possibility of serious phytotoxicity.

Method of application. The method used should be consistent with well managed field trials or commercial practice and the amount of carrier used to apply the active ingredient should be optimum for crop retention. It should not be assumed that application at high and low volumes will give the same results, and if both are likely to be used commercially, the one which leaves the heaviest deposit of chemical should be tested. The amount of wetting agent may be important.

Time of application. Times of application recommended by the manufacturers should be followed and the last application should conform with the minimum safety interval before harvest. If the latter is not known, the last time consistent with crop requirements and acceptable residues at harvest should be used.

Where alternative times of application are recommended for one formulation of an active ingredient, such as a herbicide used pre-emergence or post-emergence, the effect

of the latest time of application should be fully tested (see pp. 188 and 189). The earlier times of application may be checked for all processing methods for the crops already fully tested, or being fully tested, using the later time of application.

With seed dressings, unless otherwise recommended for reasons of phytotoxicity, the treated seed should be stored at least 3 months before drilling.

Crops tested

The test chemical should be applied to the chief crops on which it will be used. As the chemical is developed for use on additional crops, a rapid assessment may be possible from the consideration of previous results and additional checks.

Details of suitable varieties for processing are given in Appendix I (p. 193), and the tonnage of processed crops in Appendix II (p. 195).

Sampling procedure

Samples should be representative of the whole crop. A reliable objective random sampling procedure should eliminate subjective effects on the part of the sampler, prevent cross-contamination between samples from different unit plots, and reduce to a minimum the effects of variations inherent in growing crops.

Although the methods rely basically on random procedures it may be necessary to use a stratified, rather than a simple, random pattern of sampling; the stratification being on the basis of, for instance, row, compass orientation or aspect (e.g. fruit trees), height of produce on the plant in relation to maturity (e.g. peas, which mature, from the bottom upwards), prevailing wind or slope of ground. There may also be variations due to, for example, uneven distribution of chemicals both within and over the plant and over the crop as a whole.

The order in which plots are sampled is often important in minimizing the effects of 'time' over the period in which the samples are taken. For instance a sudden change in light intensity may radically alter the sugar composition of a vegetable such as spinach or tomatoes. Samples should always be taken in a manner which ensures that the largest variables or errors operate *between* blocks in a trial and not between unit plots *within* each block; consequently, sampling should be completed one block at a time. In practice it may be desirable to deal first with the control plot(s) within a block to eliminate, as far as possible, contamination risks. But *on no account* should trials be sampled or harvested treatment by treatment and, in general, samples should not be collected when they are wet with dew or rain.

It may be desirable in some instances to collect much larger samples than are needed, or can conveniently be stored, for the tasting tests. A sub-sampling procedure can then be used to reduce the bulk of material to be subsequently handled and stored. The procedure should be carefully chosen as, for example, apples, when transported in deep containers, tend to settle into horizontal strata according to size—a 'vertical' sub-sampling procedure would then be appropriate.

For the tasting tests recommended in Section III (p. 189), the requirement of equal amounts of control and treated material for each individual tasting test will give rise to a proportionately large bulk of control material when several treatments, or levels of a treatment, are included in a trial. This should be obtained by taking the required number of control samples in the standard manner rather than by obtaining a large, atypical sample which has to be handled and stored in different sized containers from those used for the treated sample(s).

Each crop, cultivar and site may require different sampling procedures, and advice may be needed before the precise form of procedure can be decided in order that the samples are not atypical of commercial produce.

Hands, containers, tools, machinery, etc., must always be thoroughly cleaned before sampling or otherwise handling control material, and also between taking each sample from the treated plots. For example, treatments applied as a dust may easily be transferred in dry weather from one plot to another. Adequate cleaning facilities should, therefore, be provided.

All samples from a trial must be handled in an identical manner and their containers should at all times be shaded from direct sunlight.

The literature on sampling for pesticide residue analysis may be a guide to the procedures required for taste test sampling.

Packing of raw materials

The packing method should give adequate physical protection. If necessary, easily damaged fruits or vegetables such as tomatoes, should be individually packed.

Containers should be free from contamination, i.e. thoroughly cleaned to remove the risk of chemical, physical and bacteriological contamination, particularly if the test material is to be stored in an unprocessed form. The packing material should not contaminate the samples either physically or chemically.

The formation of harmful micro-climates should be avoided, e.g. non-ventilated polythene bags and some types of plastic containers can lead to sweating of the samples. Samples in containers with high thermal insulation properties can reach excessive temperatures. In general, packing in shallow layers is preferable to bulk packing, both for physical protection and regulation of temperature.

Transport of samples

Time in transit should be kept to a minimum. The method of transport should be one in which the samples are under the personal supervision of a responsible person, and which is free from the risk of external contamination, extremes of heat, etc. It is strongly recommended that public carriers or normal freight handling facilities should **not** be used.

Storage of raw materials

All raw materials for taint tests should be processed as soon as possible after harvesting. This is particularly important for highly perishable materials such as vined peas, strawberries, etc. Some materials such as potatoes, carrots and apples may need to be stored for varying periods before processing.

Where storage of raw materials is necessary prior to processing, the conditions should be in accordance with the best commercial practice. Advice on storage of raw material may be obtained from such organizations as the Ditton Laboratory, Larkfield, Kent or the Food Research Institute, Norwich.

In some cases it is commercial practice to store raw material in a frozen condition (− 18°C) before manufacture into jam or canned products. Blackcurrants are often frozen to assist strigging. Where the practice is a commercially based one, frozen storage is suitable for material for taint tests.

Processing

Raw material for taint tests should be treated in a manner comparable with recommended commercial practice. For example strawberries should be washed before canning (but not normally for jam making) but, in contrast, raspberries are processed unwashed. Similarly potatoes and carrots should be peeled in a manner which simulates commercial conditions as closely as possible.

Jam making procedures, canning, quick freezing and dehydration should be carried out in the standard manner prescribed by the research station for which the tests are being conducted. The foods concerned must conform to any legal standards applicable.

Number of tests

The difficulties of examining the effects of all known factors on tainting by any new active ingredient have been mentioned in the introduction. The following is the suggested minimum number of tests required to determine whether the treatment is likely to cause a taint.

Relative importance of processing method	No. of tests for each processing method		
	1st year	2nd year	3rd year
Primary processing methods	3	2	2
Secondary processing methods (check tests)	1	1	—

Formulations of a new agricultural chemical should thus be tested seven times in 3 years on an important processing crop the produce of which would be processed in the most important manner (or manners) for that fruit or vegetable (e.g. jam and canning

for strawberries, quick freezing for Brussels sprouts). Secondary tests may also be conducted simultaneously on the same crop in other processed forms.

Canned fruits and vegetables and jams are more likely to be tainted than these products processed in other ways. Therefore, priority should be given to canning and jam making with small scale secondary testing for other processing methods or similar crops. If the produce is not suitable for canning or jam making, freezing becomes a method of processing of primary importance. In the case of peas which are canned in the immature (green) and mature state, immature peas would have priority.

Freedom from tainting characteristics can be assumed tentatively if all results are negative (not significant at $P = 0{\cdot}05$) in the 1st year. Negative results over the full test period would indicate freedom from tainting characteristics for that particular fruit or vegetable.

If some results are positive or some doubt exists, further testing will become essential.

Storage of processed material

All frozen materials for taint tests must be stored at temperatures not exceeding − 18°C for not longer than nine months in an atmosphere free from external contamination.

Other processed materials may be stored at room temperature or temperatures approaching freezing (1–2°C). It is considered that low temperatures may reduce the risk of the loss of taints which may have developed during processing and which could disappear under normal storage conditions.

Length of time of storage under any of the above mentioned conditions will probably vary since the build up of material for taint tests during the growing season may be more rapid than the completion of the taint tests. No taint test should be conducted on material within 7 days of processing.

III. Tasting tests

Method of tasting

The basic method of tasting should be as simple as possible but should also be as accurate as the conditions of the test allow. For this reason, the triangular test is suggested as the standard method for simple taint test work.

In the triangular test, the taster is presented with three samples, two of which are the same (either control or treated materials) and one which is different (treated or control, respectively). The samples should be randomized on each occasion. The taster is asked to pick out the odd sample of the three, distinguishing it by flavour only. At any tasting session more than one triangular test may be carried out to increase the rate of obtaining results. In some tests the taster is also asked to describe the difference in flavour between the single sample and the two similar ones,

and to note a taint if one is present. A further adaption of the triangular test is the sequential triangular test (see Appendix III, Table 2, p. 199) where tastings continue until either the difference or similarity of the treated and control samples becomes significant at $P = 0{\cdot}05$. Where significant differences occur the triangular test may then be followed by a sequential paired comparison test to determine which sample is preferred.

Suitability of tasters

Because the types of flavours or possible taints arising from the use of new chemicals are not known, the selection of a panel on the basis of their sensitivity to a taint is not possible. The panel should, therefore, be composed of those persons who, from experience, have shown their ability to discriminate consistently between flavours of the product under test. A taster whose sense of taste is impaired temporarily should be excluded from the simple triangular test.

Number of tasters

The number of persons required for tasting tests, and the number of times they are required to taste each set of samples will vary according to the type of test. The number of tasters required for simple triangular tests, which are dealing with a wide range of products and flavours, will be about fifteen although numbers may vary. In any case, the number of tasters should not be less than ten and preferably more and each taster should taste the samples on two occasions.

Place of tasting

Taste tests should be conducted in a place from which all outside influence can be excluded. The best conditions are usually those of individual tasting booths where each taster may examine the samples without distraction. The booths should be of a neutral colour throughout, and contain the bare necessities for the test to take place without interruption. Spoons, recording forms, writing materials and palate cleansers, where required, should be provided.

Illumination should only be used when necessary. Suitable coloured lighting must be used to mask variations in the colour of the samples.

Time of tasting

The time of day at which tasting takes place will depend on the work of those conducting tests and the number of tests to be done. Most workers believe that more reliable data will result from morning tests than afternoon ones. It is not uncommon, however, to conduct tests in both the morning and afternoon. Tests should be held at such times that the tasters are neither replete nor hungry. If mid-morning and mid-afternoon breaks for coffee or tea are permitted, tasting should take place before rather than immediately after these times.

Preparation of samples

The preparation of the treated samples for tasting must be identical to that for the controls. Samples may be presented in the form in which they are processed or macerated to a purée. If puréed samples are used the total contents (solid matter plus syrup or brine) of the container should be macerated to a purée but not so thoroughly that fruit seeds are disintegrated. Maceration should be used to blend the sample, small pieces of vegetable being preferable to a smooth overmacerated glutinous paste.

It is recognized that the palate is more sensitive to flavour difference in warmed samples (55°C) than ones at room temperature. Nevertheless, some authorities feel that samples should be tasted at the temperature at which they are normally consumed.

Quick frozen vegetables should be cooked in a minimum, but standard, amount of water and salt until they are tender and palatable. A proportion of the cooking liquor should be used if they are to be macerated and the samples should be tasted warm.

Quick frozen fruits should be brought to room temperature or warmed by immersion of the containers in hot water, macerated (where applicable) and tasted at room temperature or after warming. Sugar should be added to those samples which were not quick frozen in dry sugar or syrup.

Canned fruits and vegetables should be macerated (where applicable) and tasted at room temperature or after warming in a container in hot water.

Jams should be tasted either at room temperature or after warming. Maceration is unnecessary but the jam should be stirred or mashed to ensure that the sample is reasonably homogeneous.

Fruit or vegetable juices should be mixed thoroughly by shaking or stirring. They should be tasted either at room temperature or after warming.

Calculation of results

The calculation of results from the simple triangular test method is most easily achieved by the use of Table 1 and is given in Appendix III (p. 197). The total number of tastings and the number of correct replies is noted. To the latter is added one-third of the no difference replies. If the number of the correct answers plus one-third of the no difference replies is the same as or exceeds the values shown in columns 2 or 6 for the total number of tastings shown in columns 1 and 5 then the difference in flavour is significant at $P = 0{\cdot}05$. The appropriate columns should be used to ascertain whether the difference is significant at the $P = 0{\cdot}01$ and $P = 0{\cdot}001$ levels.

Tables for the evaluation of the results for the sequential triangular test and sequential paired comparison test are also given in Appendix III (p. 197).

Validity of the tasting test results

It is not always appreciated that individuals differ markedly in odour and taste sensitivity to specific substances and that also any particular person's sensitivity may vary on a day-to-day basis, for many reasons.

It must be assumed that this also applies in relation to the taints caused by agricultural chemicals, and that taints will occur from time to time which only a small proportion of the population can detect and find objectionable at a particular concentration of the offending substance. The two sequential schemes of tasting suggested here provide a means of deciding with 95% degree of certainty whether the population is capable of detecting the taint on 50% of occasions or alternatively on 25% of occasions. It is, of course, possible to increase the sensitivity of the test to allow for lower levels of taste recognition within the population, but the increase in tasting required to do so is so large that it is impracticable.

It should thus be appreciated that the suggested scheme of tasting will only detect with any degree of certainty those taints which a reasonable proportion of the population are capable of recognizing and the taints which only small proportions (below 25%) of the population can recognize may escape detection. Nevertheless, scrutiny of replicate results for consistent correct identification of taint by particular individuals may give an indication of the presence of a 'minority' taint, even though the gross results do not reach significance at the accepted level of probability.

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Appendix I

Suggested cultivars suitable for taint tests on processed fruits

Fruit	*Canned*	*Quick frozen*	*Jams*
Apples	Bramley Seedling	—	Bramley Seedling
	Newton Wonder	—	—
Blackcurrants	Baldwin	Baldwin	Baldwin
Cherries	Napoleon Bigarreau	—	—
	Kentish Bigarreau	—	—
Damsons	Shropshire Prune	—	—
Gooseberries	Careless	—	Careless
Greengages	Cambridgeshire Gage	—	—
Pears	William Bon Chrétien (Bartlett)	—	—
Plums	Victoria	—	Victoria
	Pershore (Yellow Egg)	—	Pershore (Yellow Egg)
Raspberries	Malling Jewel	Malling Jewel	Malling Jewel
Redcurrants	—	—	Red Lake
Rhubarb	Prince Albert	—	—
	Timperley Early	—	—
Strawberries	C. Favourite	C. Favourite	C. Favourite
	—	C. Vigour	—

Suggested cultivars suitable for taint tests on processed vegetables

Vegetables	*Canned*	*Quick frozen*	*Dehydration*
Beetroot	Detroit Dark Red	–	–
Broad beans	Triple White	Triple White	–
	Staygreen	Staygreen	–
Brussels sprouts	–	Sanda	–
	–	Jade Cross	
Carrots	New Model Red Cored (Clucas)	New Model Red Cored (Clucas)	New Model Red Cored (Clucas)
	Chantenay Supreme (Elsoms)	–	–
French beans	Processor	Tendercrop	Tendercrop
	Harvester	Processor	–
Green peas	Kelvedon Wonder	Kelvedon Wonder	Kelvedon Wonder
	Dark Skin Perfection	Dark Skin Perfection	Dark Skin Perfection
Potatoes	Arran Pilot	Majestic	Majestic
Processed peas	Maro	–	–
	Pauli	–	–
	Rondo	–	–
Runner beans	Emergo	Emergo	–
Spinach	Elsoms 23	Elsoms 23	–

APPENDIX II

Production of canned vegetables (excluding potatoes) in the United Kingdom

Vegetables	1966 production (tons net can contents)
Peas, processed	191,500
Peas, green	89,300
Carrots	48,500
Broad beans	9,600
Beans, runner and French	7,600
Beetroot	3,100
Celery, spinach, turnips, swedes, parsnips	Not available

N.B. A large proportion of the processed pea pack is derived from peas imported dried from the U.S.A. and the Netherlands.

Production of canned fruits in the United Kingdom

Fruit	1966 production (tons net can contents)
Strawberries	13,600
Rhubarb	11,300
Apples	10,300
Plums, damsons and greengages	9,900
Other berries and currants	7,800
Gooseberries	5,600
Cherries	600

Production of quick frozen vegetables (excluding potatoes) in the United Kingdom

Vegetables	1966 production (tons net contents)
Peas, green	61,000
Beans, runner and French	17,200
Broad beans	9,900
Brussels sprouts	5,000
Spinach, carrots	Not available

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Production of quick frozen fruit in the United Kingdom

The total production of quick frozen fruit in the United Kingdom in 1966 was 1,300 tons. The last year when this total was broken down into individual fruits was 1959 when the order of importance was: Strawberries, apples, raspberries, blackcurrants, gooseberries, blackberries, rhubarb, plums, damsons and greengages

Quantities of potatoes used for processing in the United Kingdom

	1966 production (tons of raw material)
Crisps	320,000
Dehydrated potatoes	60,000
Frozen potatoes	50,000
Canned potato products (excluding new potatoes)	40,000
Other crispy products	15,000
Canned new potatoes	10,000–15,000

Production of dehydrated vegetables

Peas Beans, French	Figures not available

Production of jams in the United Kingdom

Fruits	1966 production (tons)
Raspberry	23,500
Strawberry	22,400
Blackcurrant	15,700
Red plum	7,800
Blackberry	5,200
Damson and greengage	3,900

N.B. The ton used in these tables is the long ton, i.e. 2240 lb.

Appendix III

Tables for the calculation of triangular test results

Table 1. Triangular tests

No. of judges or judgments	Minimum correct judgments to establish significant differentiation			No. of judges or judgments	Minimum correct judgments to establish significant differentiation		
	$P = 0{\cdot}05$	$P = 0{\cdot}01$	$P = 0{\cdot}001$		$P = 0{\cdot}05$	$P = 0{\cdot}01$	$P = 0{\cdot}001$
5	4	5	—	56	25	28	31
6	5	6	6	57	26	28	31
7	5	6	7	58	26	29	31
8	6	7	8	59	27	29	32
9	6	7	8	60	27	30	32
10	7	8	9	61	27	30	33
11	7	8	10	62	28	30	33
12	8	9	10	63	28	31	34
13	8	9	11	64	29	31	34
14	9	10	11	65	29	32	34
15	9	10	12	66	29	32	35
16	9	11	12	67	30	32	35
17	10	11	13	68	30	33	36
18	10	12	13	69	30	33	36
19	11	12	14	70	31	34	37
20	11	13	14	71	31	34	37
21	12	13	15	72	32	34	37
22	12	14	15	73	32	35	38
23	12	14	16	74	32	35	38
24	13	14	16	75	33	36	39
25	13	15	17	76	33	36	39
26	14	15	17	77	33	36	39
27	14	16	18	78	34	37	40
28	14	16	18	79	34	37	40
29	15	17	19	80	35	38	41
30	15	17	19	81	35	38	41
31	16	17	20	82	35	38	42
32	16	18	20	83	36	39	42
33	16	18	21	84	36	39	42
34	17	19	21	85	36	39	43
35	17	19	21	86	37	40	43
36	18	20	22	87	37	40	44
37	18	20	22	88	38	41	44
38	18	20	23	89	38	41	44
39	19	21	23	90	38	41	45
40	19	21	24	91	39	42	45

TABLE 1 (*continued*)

No. of judges or judgments	Minimum correct judgments to establish significant differentiation			No. of judges or judgments	Minimum correct judgments to establish significant differentiation		
	P = 0·05	P = 0·01	P = 0·001		P = 0·05	P = 0·01	P = 0·001
41	20	22	24	92	39	42	46
42	20	22	25	93	40	43	46
43	20	23	25	94	40	43	46
44	21	23	25	95	40	43	47
45	21	23	26	96	41	44	47
46	22	24	26	97	41	44	48
47	22	24	27	98	41	45	48
48	22	25	27	99	42	45	49
49	23	25	28	100	42	45	49
50	23	25	28	200	79	83	88
51	24	26	28	300	114	120	126
52	24	26	29	400	150	156	164
53	24	27	29	500	185	192	200
54	25	27	30	1,000	359	369	381
55	25	27	30	2,000	702	717	733

GOODALL & J. M. COLQUOUHN (1967) B.F.M.I.R.A. Scientific and Technical Surveys No. 49.
N.B. Include one-third 'no difference' results with correct selections.

TABLE 2. Sequential triangular tests

Total No. of tests	Sample 'difference' to be distinguished			
	50%*		25%*	
	D	S	D	S
5	5	0	—	—
6	6	0	—	—
7	6	1	—	—
8	7	1	—	—
9	7	2	—	—
10	8	2	—	—
11	8	3	—	—
12	9	3	10	0
13	9	4	10	1
14	10	4	11	1
15	10	5	11	1
16	11	5	11	2
17	11	6	12	2
18	12	6	12	3
19	12	7	13	3
20	13	7	13	4
21	13	8	13	4
22	14	8	14	4
23	14	9	14	5
24	15	9	15	5
25	15	10	15	6
26	16	10	16	6
27	16	11	16	6
28	17	11	16	7
29	17	12	17	7
30	18	12	17	8
31	18	13	18	8
	- - - -	- - - -		
32	18	14	18	9
33	18	15	18	9
34	18	16	19	9
35	18	17	19	10

Total No. of tests	Sample 'difference' to be distinguished	
	25%*	
	D	S
40	21	12
45	23	14
50	25	16
55	28	18
60	30	20
65	32	22
70	34	24
75	36	26
80	38	28
85	40	30
90	42	33
95	44	35
100	46	37
105	48	39
110	50	41
115	52	43
120	54	45
125	56	47
130	59	49
	- - - -	- - - -
135	59	53
140	59	58

E. H. STEINER (1964) B.F.M.I.R.A. Tech. Circ. No. 274.
E. H. STEINER (1966) *J. Fd Technol.* **1**, 41.

* Tasters are capable of genuinely distinguishing samples on the indicated percentage of occasions.

Number of correct selections, N, required for a decision in a sequential triangular test (probability level = 0·05). If $N \geq D$ samples are different. If $N \leq S$ samples are

similar. If D > N > S continue testing. Include one-third 'no difference' results with correct selections.

TABLE 3. Sequential paired comparison test

Total No. of tests	Sample 'difference' to be distinguished 50%* D	50%* S	25%* D	25%* S	Total No. of tests	Sample 'difference' to be distinguished 25%* D	25%* S
10	10	—	—	—	80	53	—
12	11	—	—	—	90	58	—
14	13	—	—	—	100	64	50
16	14	—	—	—	110	69	56
18	15	—	—	—	120	75	61
20	16	—	19	—	130	81	67
22	18	11	20	—	140	86	72
24	19	12	21	—	150	92	78
26	20	13	22	—	160	98	84
28	21	14	23	—	170	103	89
30	23	16	24	—	180	109	95
32	24	17	26	—	190	114	100
34	25	18	27	—	200	120	106
36	27	20	28	—	210	126	112
38	28	21	29	—	220	131	117
40	29	22	30	—	230	137	123
42	30	23	31	—	240	143	129
44	32	25	32	—	250	148	134
46	33	26	33	—	260	154	140
48	34	27	35	—	270	159	145
50	35	28	36	—	280	165	151
52	37	30	37	—			
54	38	31	38	—	290	169	158
56	39	32	39	—	300	169	163
58	40	33	40	—			
60	42	35	41	—			
62	43	36	42	—			
64	44	38	44	—			
66	45	40	45	—			
68	45	42	46	—			
70	45	44	47	—			

E. H. STEINER (1964) B.F.M.I.R.A. Tech. Circ. No. 274.
E. H. STEINER (1966) *J. Fd Technol.* **1,** 41.

* Tasters are capable of genuinely distinguishing samples on the indicated percentage of occasions.

Number of preferences (or higher ratings), N, required for decision in a sequential two-sided paired comparison test (probability level 0·05). If $N \geqslant D$, where N refers to the sample with the greater number of preferences, the sample is preferred. If $N \leqslant S$ samples are similar. If $D > N > S$ continue testing. Include one-half 'no difference' results with preferences.

Similarity cannot be established until at least twenty-two tests are made in the procedure to distinguish a 50% 'difference', or 100 tests in the more selective procedure.

APPENDIX IV

Calculation of results—a worked example of a simple triangular test

Material: Samples of strawberries were canned from a trial designed to test the efficacy of a fungicide.
Treated and control fruit were processed in an identical fashion and the cans carefully labelled.

Method: Equal quantities of treated and control canned strawberries were heated to about 55°C and macerated in separate containers. Samples were placed in tasting booths and each of twelve tasters was asked to complete a simple questionnaire on two separate tasting occasions.
The order of samples was:
First tasting: A *B* C (treated sample italic).
Second tasting: *A* *B* C (treated sample italic).

The questionnaire was as follows:

Triangular test

Two samples are similar, one is different.

Question 1. Which is the odd sample?..........

Question 2. If you are unable to detect any difference mark ND..........

Question 3. If you think there is a difference, how does the odd sample differ from the other two?

....

Question 4. Could this difference be described as a taint or off-flavour?

....

NAME.......... DATE..........

The results are tabulated below for ease of calculation.

Taster	First tasting question				Second tasting question			
	1	2	3	4	1	2	3	4
1	–	ND	–	–	–	ND	–	–
2	B	–	Slightly sweeter	No	C	–	Slightly sweeter	No
3	–	ND	–	–	–	ND	–	–
4	A	–	Improved flavour	No	–	ND	–	–
5	–	ND	–	–	–	ND	–	–
6	B	–	Weaker flavour	No	A	–	Sweeter	No
7	–	ND	–	–	–	ND	–	–
8	–	ND	–	–	C	–	Flavour difference	No
9	–	ND	–	–	B	–	Sweeter	No
10	B	–	Sweeter	No	A	–	Sharper	No
11	A	–	Sweeter	No	–	ND	–	–
12	A	–	Better flavour	No	–	ND	–	–
	Answers: three, correct. six, no difference.				two, correct. seven, no difference.			

Only one-third of the no difference answers should be added to the correct answers, thus the results are:

First tasting, five out of twelve.

Second tasting, four out of twelve.

Making a total of nine correct answers from a total of twenty-four tastings.

Thirteen correct answers are necessary to establish a significant difference ($P = 0{\cdot}05$) between the flavours of treated and untreated material when twenty-four tastings have been made. Thus the tasting tests failed to demonstrate any significant effect in the fruit due to the fungicide applied in the field prior to canning.

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'Thaw-rigor' and 'cold-shortening' in rabbit muscle

R. A. LAWRIE

WHEN unrestrained muscles are frozen pre-rigor they shorten markedly on relatively slow thawing. This is the phenomenon of 'thaw-rigor' (Chambers & Hale, 1932). If thawing is virtually instantaneous, however, shortening is quickly followed by relaxation to pre-thaw length (Bendall, 1960). Bendall concluded that when pre-rigor-frozen muscle is thawed there is initially an extensive release of calcium ions by the sarcotubular system. These ions stimulate myofibrillar ATPase to an abnormal degree, thus causing marked contraction. When such thawing is sufficiently fast, however, the released calcium ions are recaptured by the sarcotubular system and myofibrillar ATPase is thereby depressed before much ATP has been hydrolysed. Retention of the high pre-rigor ATP level causes relaxation in these circumstances.

In 1963 Locker & Hagyard reported that when the muscles of beef animals are unrestrained during post-mortem glycolysis they shorten considerably more if held at 0–2°C than at 15°C. Cassens & Newbold (1967) attributed this phenomenon also – 'cold-shortening' – to release of calcium ions from the sarcotubular system, this being a reflection of its response to the low temperature. They suggested that release of calcium ions in these circumstances is less marked than when caused by the disorganization of 'thaw-rigor'. If the mechanisms in the two phenomena are basically the same, however, and differ only in degree, is it not immediately clear why, although both *psoas* and *semitendinosus* muscles of the rabbit exhibit 'thaw-rigor', only the latter resembles beef musculature in undergoing 'cold-shortening' (Locker & Hagyard, 1963; Bendall, 1966). It is possible, however, to account for the discrepancy on the basis of the biochemical and physiological differentiation of the muscles concerned (Lawrie, 1968); and the present communication reports evidence in support of this view.

In the experiments which first demonstrated the presence of a relaxing factor in muscle (the Marsh–Bendall factor: Marsh, 1952) the myofibrils of homogenates of fresh muscle were found to swell on the addition of ATP from which they hydrolysed inorganic phosphate (P_i) at a relatively slow rate. When the soluble and suspended sarcoplasmic components were removed from the homogenate by centrifuging and washing, the addition of ATP to the myofibrils now caused them to hydrolyse it swiftly – and to synaerese strongly. Subsequent investigations have shown that the factor represented fragments of the sarcotubular system; and that they acted by removing calcium ions, whereby stimulation of myofibrillar ATPase was inhibited. Indeed,

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Marsh had found in 1952 that the addition of calcium ions to unwashed homogenates of muscle eliminated the protective action of the suspended factor. At the same time (Lawrie, 1952) it was shown that the mechanism which suppressed myofibrillar ATPase was more effectively developed in so-called white muscle (in which mechanisms for *anaerobic* energy production were marked) than in so-called red muscle (in which mechanisms for *aerobic* energy production were marked). In these experiments the ATPase activities had been measured at room temperature. From the above considerations it seemed that it would be illuminating to compare the ATPase activity of homogenates of rabbit *psoas* (a 'white' muscle) and *semitendinosus* (a 'red' muscle) at 15° and 0°C.

Psoas and *semitendinosus* muscles were immediately excised from rabbits killed after relaxation by myanesin (500 mg/kg, intraperitoneally). Samples (~ 30 mm long × ~ 5 mm wide) for measuring 'cold-shortening' were suspended in moist air at 0° and 15°C. Similar strips for measuring shortening in 'thaw-rigor' were frozen in liquid nitrogen and thawed at 20°C. The ATPase activity of muscle homogenates was determined by a method previously described (Lawrie, 1953) before and after removing suspended fragments of the sarcotubular system. These are referred to as 'unwashed' and 'washed' samples respectively (cf. Marsh, 1952). Results are shown in Table 1.

TABLE 1. ATPase activity and shortening in rabbit *Psoas* and *Semitendinosus* muscles

Muscle	Homogenates: ATPase activity ($\mu g/P_i/min/g$ muscle)				Strips (~ 30 mm × ~ 5 mm)	
	15°C		0°C		Cold shortening at 0°C	Shortening in thaw-rigor
	Unwashed	Washed	Unwashed	Washed	(as % initial length)	
Psoas	81 (5)	193 (5)	164 (5)	52 (5)	3 (4)	73 (8)
Semitendinosus	80 (3)	103 (3)	116 (3)	39 (3)	24 (4)	39 (4)

Figures in parentheses refer to numbers of experiments on which values are based.

At 15°C the ATPase activities of the unwashed homogenates of *psoas* and *semitendinosus* are the same; but on removing the 'factor' particles, the ATPase activity increases in the *psoas* to a considerably greater extent than in *semitendinosus*, this confirming that the sarcotubular system is more active in the 'white' muscle. The data on ATPase at 0°C, however, show that in the unwashed samples the rates are 100% greater in *psoas*, and 50% greater in *semitendinosus* than at 15°C. This is presumably a further reflection of the different factor activity in the two muscles. For if, as Cassens & Newbold (1967) believe, the sarcotubular system discharges calcium ions on exposure to 0°C, the stimulation of myofibrillar ATPase by this ion would be expected to be more marked

in a 'white' muscle (where the enzyme is more active). In support of this view, the data on the ATPase of washed homogenates at 0°C from which factor particles (and the calcium ions discharged from them at this temperature) had been removed, indicate that the myofibrils of both muscles hydrolysed ATP at a relatively low rate. (The addition, to the washed homogenates of *psoas* at 0°C, of calcium ions to a final concentration of 4 mM increased the ATPase rate three-fold.)

The fact that the ATPase activity of unwashed *psoas* homogenates at 0°C, is greater than that found in those of *semitendinosus* would lead one to anticipate a greater degree of 'cold-shortening' at this temperature in the former muscle. As the table indicates, and in confirmation of the results of Locker & Hagyard (1963) and of Bendall (1966), the converse is true. 'Cold-shortening', however, is a phenomenon exhibited by the *intact* muscle. It may be presumed that the degree of organization of the sarcotubular system in the latter is much higher than in homogenates; and further, that the greater development of the system in the *psoas* more readily permits recapture of calcium ions released at 0°C. In this way, notwithstanding the more active myofibrillar ATPase in the *psoas* even at 0°C, the intact muscle successfully suppresses ATP breakdown and it fails to shorten appreciably. When the organization of the intact muscle is disrupted, as in pre-rigor freezing and thawing, the sarcotubular system would be less able to capture calcium ions before they stimulated myofibrillar ATPase (unless thawing were instantaneous, as referred to above). In these circumstances no inhibition of myofibrillar ATPase would be possible in either muscle; and the intrinsically greater activity of this enzyme in *psoas* would be reflected in a greater degree of shortening in thaw-rigor. The data in Table 1 support this view.

The failure of rabbit *psoas* to show appreciable 'cold-shortening', on the one hand, and its marked ability to shorten in 'thaw-rigor', on the other, appears therefore to conform to the behaviour anticipated of a so-called 'white' muscle; and does not invalidate the proposition that the basic mechanisms of both phenomena are similar.

The technical assistance of Mr M. Lenton is gratefully acknowledged.

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The correlation of the extract-release volume of stored beef with other spoilage values

D. PEARSON

Summary. Various relationships between the extract-release volume (ERV) and the protein and fat contents of fresh beef are studied in relation to theoretical and other considerations. Inter-relationships between the pH, total volatile nitrogen and the extract-release volume of stored beef samples confirm that the drop in the filtrate volume as spoilage advances is due to changes in the protein. The correlations between the ERV and the pH values take into account the changes which occur in both the 'reverse' pre-rigor and post-rigor stages.

Introduction

The water-holding capacity (WHC) of freshly slaughtered meat is high, but it drops markedly within a few hours and then increases slowly during further storage. Hamm (1956) attributed two-thirds of the post-mortem hydration drop to the breakdown of the ATP and one-third to the fall in pH, so that there is probably a connection between the hydration decrease and rigor development. Most of the methods described for determining the WHC measure the 'free water' released by pressing or heating the meat under specified conditions. Grau & Hamm (1953) calculated the area of water diffused from the meat under a standardized pressure. Wierbicki & Deatherage (1958) gave further consideration to the standardization of the procedure and recommended the use of an 8-ton hydraulic jack. The results of the changes in WHC which occur during chilling and conditioning agreed closely with those reported by Hamm.

Jay (1964) developed a method for determining the extract-release volume (ERV) which is related to the water-holding capacity and appears to have considerable possibilities for assessing the degree of spoilage of beef. The procedure is based on the measurement of the volume of aqueous filtrate released from a slurry of meat in a fixed time. The ERV decreases as spoilage progresses and no filtrate at all is obtained with putrid meat. Jay showed that there was a good correlation between ERV and bacterial numbers, and, in view of its simplicity, rapidity in performance and apparently consistent decrease during spoilage the ERV has proved useful for routine control assessments of meat quality.

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Experimental

Water was determined by drying the comminuted beef samples at 100°C.

Fat was determined by extraction from the dried sample with light petroleum.

Nitrogen was determined on 1·4–1·8 g of sample by the macro-Kjeldahl method. Protein was calculated as $N \times 6{\cdot}25$.

Total volatile nitrogen (*TVN*) was estimated by a modification of the macro-distillation technique of Lucke and Geidel (Pearson, 1962).

Extract-release volume was obtained by a modification of Jay's method employing 15 g meat + 60 ml buffer solution (Pearson, 1967).

Results and discussion

By considering the ERV figures for fresh beef, it is possible to assess how the fat affects the meat's water-holding capacity. The data quoted in Table 1 includes the ERV figure calculated to 100 g meat for the fresh samples of varying fat content. The ratios of this ERV value to protein and fat (all calculated as percentages) are also included.

TABLE 1. Calculated data related to ERV of fresh beef samples

% fat	% water	% protein*	ERV (ml)	% ERV†	% ERV / % protein	% ERV / % fat	100 ERV / % protein	ERV / % fat
5·2	70·2	23·8	22·0	147	6·18	28·2	88·8	4·23
21·3	56·5	21·2	23·2	155	7·31	7·2	104·5	1·089
30·4	51·0	18·6	27·5	183	9·84	6·0	140·3	0·905
30·6	40·8	27·6	28·0	187	6·77	6·1	97·9	0·915
42·7	42·1	14·2	28·2	188	13·23	4·4	185·4	0·660
49·3	33·7	16·0	32·0	213	13·31	4·3	188·2	0·649
57·4	29·4	12·2	31·2	208	17·06	3·6	236·5	0·543
74·5	15·1	9·4	35·7	238	25·30	3·2	343·2	0·479

*Protein (by difference) = 99·0 − (% water + % fat).
†ERV calculated to 100 g meat.

Some observations on ERV values as applied to fresh samples of beef

The increased ERV with rising fat content appears to be largely due to the immiscibility of fat with the buffer solution. Alternatively the fat can be considered as a diluent of the protein, the amount of which obviously has some influence on the ERV. The type of curve found in Fig. 1 which relates % ERV/% fat with increasing fat content, however, suggests that the effect of the fat is something more than merely as a diluent of the protein.

The graph of % ERV/protein *vs.* % fat (Fig. 2) displays the effect of fat better than % ERV/% fat *vs.* % fat. From Fig. 2 it is apparent that what is virtually ERV (ml)/g

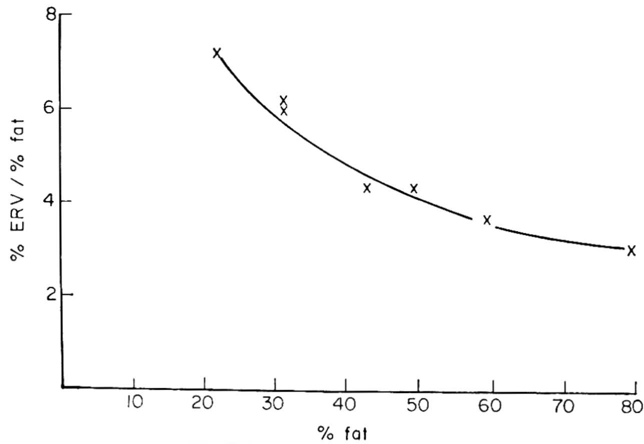

FIG. 1. Relationship between % ERV/% fat and % fat of fresh samples of beef.

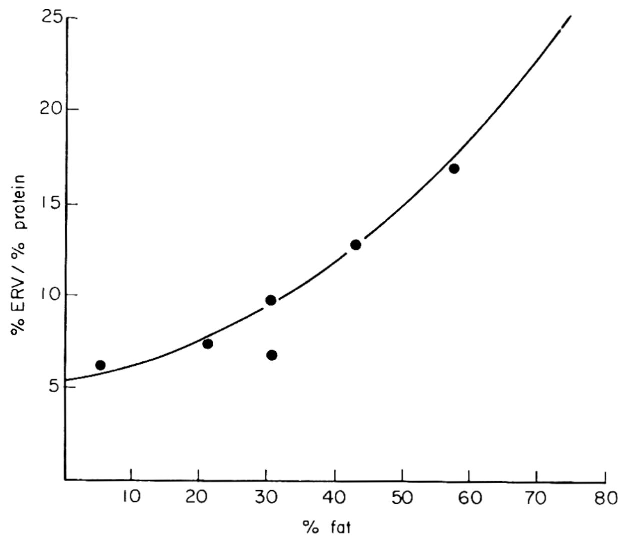

FIG. 2. Relationship between % ERV/% protein and % fat of fresh samples of beef.

protein increases as more fat is present due to: (a) physical interference by fat on the uptake of water, and (b) increase in lipoprotein which has fewer water binding centres than intact meat protein.

The correlation coefficients of the various relationships for fresh samples are as follows:

	Correlation coefficient
% protein/ERV (ml)	−0·93
% protein/ERV (%)	−0·93
% fat/(% ERV/% protein)	+0·94
% fat/(% ERV/% fat)	−0·74

Comments on the application of the ERV method to stored minced beef

The ERV of minced beef stored at 5°C was included in various storage experiments. In all instances there was a steady decrease in the ERV as the meats deteriorated. The main exceptions to this were the results at the beginning of the storage period in experiments in which the meat used was taken from a carcase at the slaughterhouse and the examination was commenced within a few hours of slaughter. In these instances the ERV increased during the first few days of the storage period and then decreased in a manner similar to that found in meat purchased from retailers.

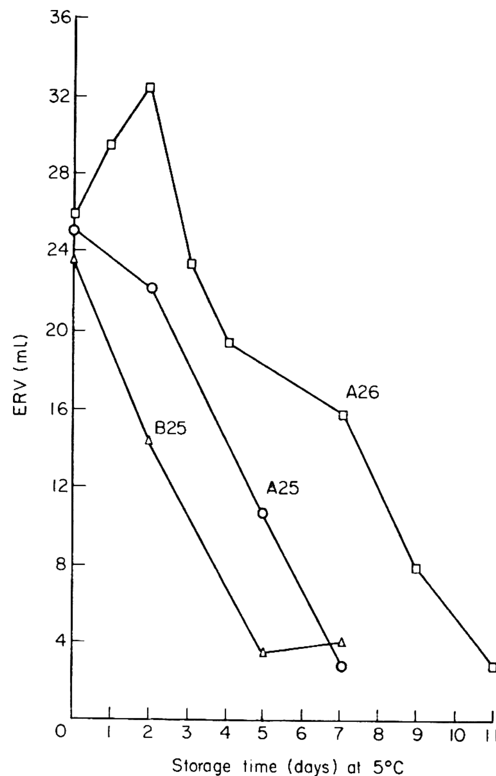

Fig. 3. Changes in ERV of beef during storage at 5°C. ○, A.25; △, B.25; □, A.26.

The changes in the ERV in Experiments 26 (A.26, slaughterhouse meat) and 25 (A.25 and B.25, butcher's beef) are represented graphically in Fig. 3. The ERV figures for the fresh samples examined (excluding those obtained from the slaughterhouse) showed a considerable variation, e.g. 14·1–25·0 ml (mean 20·2). The corresponding ERV figures for samples adjudged organoleptically to be 'just spoiling' ranged from 6 to 14 ml (mean 10·6). It would appear from these results, therefore, that the ends of the two ranges are more or less coincidental.

Inter-relationship between pH and TVN and ERV

The relationship between the pH value and the TVN in Experiment No. 26 (slaughterhouse meat) is shown graphically in Fig. 4. By excluding the pre-rigor

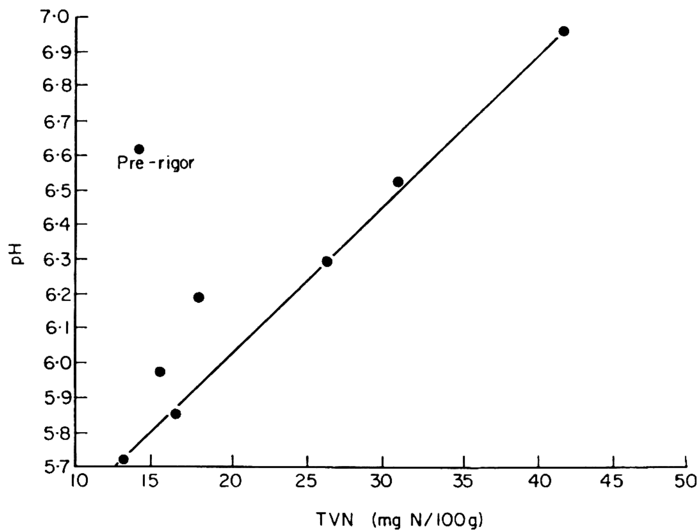

FIG. 4. Relationship between pH and TVN in beef taken from slaughterhouse and stored at 5°C.

sample, the regression equation for the straight line relationship is:

$$pH = 0{\cdot}043\ TVN + 5{\cdot}16.$$

As the TVN dropped from 14·0 to 12·4 mg N/100 g during the first 2 days of storage, it would appear that ammonia was lost at first by simple volatilization. Similarly the pH value fell in this period, i.e. until the rigor process had been completed. After the 2nd day, however, lactic acid production was presumably complete so that the pH value began to rise and this increased alkalinity is also reflected in the rising TVN figure as the meat deteriorated.

From Fig. 5 it is apparent that the ERV decreases as the pH of the meat (prior to adding buffer solution for the test) increases. In order to compare the effects during storage on the extract-release volume, the reciprocal of the ERV was calculated as it tends (unlike other values) to decrease during storage (Fig. 3). Figs. 6 and 7, therefore,

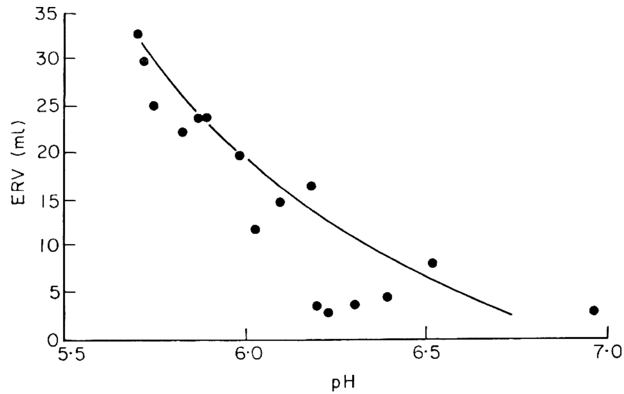

Fig. 5. Relationship between ERV and pH of beef stored at 5°C.

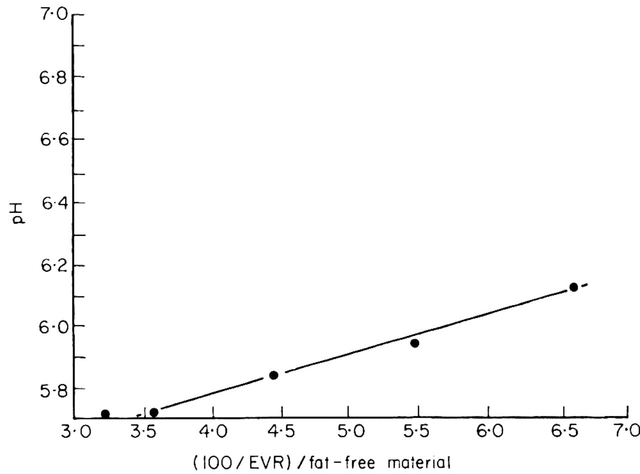

Fig. 6

relate 100/ERV in the fat-free material with the pH value for the storage of the slaughterhouse meat in Experiment No. 26. This figure, therefore, increases concomitantly with the pH value and the TVN (Figs. 6 and 8). This further confirms that the drop in the volume of ERV filtrate is due to changes in the protein as spoilage advances.

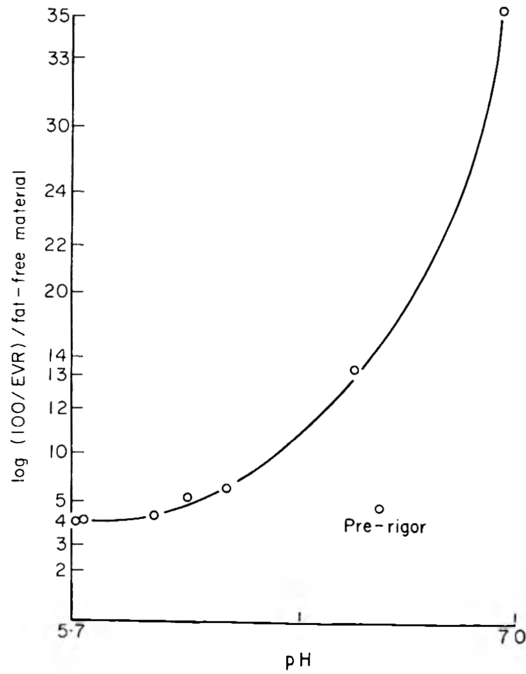

Fig. 7

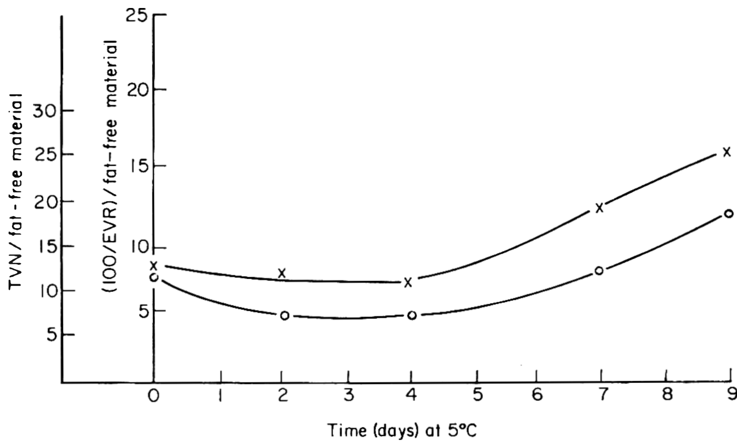

Fig. 8. ×, TVN/fat-free material; O, (100/EVR)/fat free material.

Also, apart from the first pH reading taken soon after slaughter, these relationships take into account the rising ERV and falling pH in the pre-rigor stage (see Figs. 6 and 7).

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J. Fd Technol. (1968) **3**, 215–221.

The connective tissues of fish

I. The influence of biological condition in cod on gaping in frozen-thawed muscle

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Summary. 'Gaping' is a phenomenon in which slits or holes appear between the muscle blocks, so that in a bad case the fillet falls to pieces and cannot be processed or sold. The gaping in whole fish frozen *in* rigor mortis, thawed and filleted was shown to be related to the biological condition of the fish, strong healthy fish gaping a great deal, while spent or starving fish did not gape at all. It is postulated that when fish are frozen *in* rigor mortis the ice growing in the connective tissue weakens it, so that it may break under the rigor mortis contraction. The contraction of spent or starving fish appears to be too weak to break the connective tissue. No gaping was shown by similar fish frozen *before* rigor mortis. The practical importance of the findings is discussed.

Introduction

The main structures of a fish fillet are the contractile cells which run in a roughly anterior–posterior direction, and sheets of connective tissue which cut across them (Fig. 1).

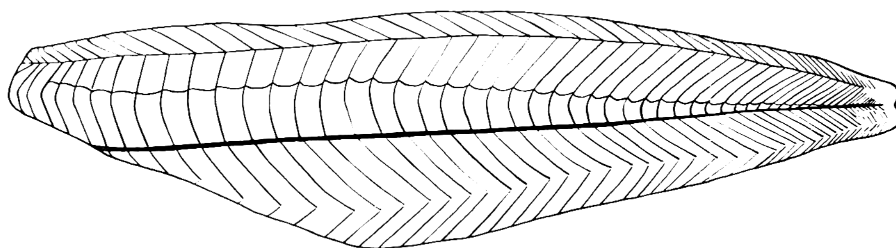

FIG. 1. Diagrammatic representation of the musculature of cod, showing the surface removed from the backbone. The pattern of lines represents the sheets of connective tissue (myocommata).

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The connective tissue sheets ('myocommata') do not run vertically from the cut surface to the skin surface, but curve in a complex manner inside the thickness of the fillet; Nursall (1956) has described the structure in relation to the way in which the fish swim.

Usually all the tissues hold together, so that the cut surface of the fillet, as shown in Fig. 1, is smooth and continuous, but on occasion the connective structures break down. When this occurs the muscle blocks (myotomes) become separated from the myocommata, and holes or slits appear in the fillet. The effect is known in industry as 'gaping', and on occasion it is so bad that the fillets cannot be sold, although their taste and texture after cooking may have been satisfactory. While gaping is well known, its causes and mechanisms are not, and there is no general agreement about when it occurs, except that some fishing grounds almost invariably seem to yield gaping fish. The problem is therefore of considerable economic importance.

Scientific investigation of gaping is virtually non-existent, apart from Jones' report (1964) that whole cod entering rigor mortis at a temperature of 17°C or above gaped after filleting, the gaping becoming progressively worse as the temperature was raised. It was suggested that the connective structures may have been weakened by the higher temperature, and also that the contraction of rigor mortis was more violent at the higher temperature, thus disrupting the myocommata (Jones *et al.*, 1965). In a subsequent paper Jones (1965) reported that the weakness of the connective tissue appeared to vary from one stock of cod to another.

The only existing chemical studies on fish connective tissue concern skin, swim bladder, scales or bones [Takahashi & Yokoyama, 1954a, b; Solomons, 1955; Takahashi, unpublished (quoted by Gustavson, 1956); Doty & Nishihara, 1957; Chun & Doty, 1957; McBride, MacLeod & Idler, 1960; Piez & Gross, 1960; Young & Lorimer, 1961; Bai & Kalyani, 1961; Geiger, Roberts & Tomlinson, 1962]. Studies on myocommata, separated from muscle tissue, are limited to a few measurements of their solubility in sodium chloride solution (Ironside & Love, 1958).

In the present work, therefore, it has been necessary to start from the beginning and try to find out what holds the fish together, also the limits of the conditions under which gaping can occur. The series will comprise histological, physical and chemical studies on the myocommata. The work has been made easier by the fact that biological 'condition' can now be satisfactorily measured on an objective scale (Love, 1960, 1962a).

The present paper reports a relationship between 'condition' and gaping in cod from different grounds frozen *in* rigor mortis and then thawed.

Material and methods

The fish used were cod (*Gadus morhua* L.) caught with a trawl net by the research vessel of Torry Research Station, 'Sir William Hardy'. In the 'one-ground' experiments carried out by one of us (I.R.) the fish were packed into melting ice immediately after

landing, still with guts in, for various periods of time, then gutted, washed with sea-water and frozen in ½-cwt blocks in a vertical plate freezer at −30°C (2–3 hr). The blocks were stored at −30°C for 4–8 weeks before thawing and examining. They were thawed by leaving in air at room temperature, about 18°C, for 19 hr. During this time they were covered with a single sheet of paper to reduce moisture loss. They were then filleted and examined for gaping.

In the big experiment in which fish from many grounds were taken, the fish were gutted immediately after catching and then stowed in melting ice. After a day, one fillet was removed for the determination of muscle water-content and pH. The remainder of the fish, complete except for the removal of guts and one fillet, was then wrapped in aluminium foil, and placed on the floor of a room at −30°C, where it froze in about 3 hr. The work was arranged so that the samples started to freeze exactly 24 hr after landing. The fish had all been laid straight in the ice, so that when they became stiff (rigor mortis) they had no curvature in their bodies. One fillet could, therefore, be easily removed without changing the curvature of the remainder. When wrapping in foil, carrying down to the cold room of the ship and laying on the floor, great care was taken not to bend the fish, which was still in rigor. These fish were also stored at −30°C until needed, then thawed overnight, filleted and examined for gaping.

The water content of the flesh, a sensitive measure of biological 'condition' (Love, 1960) was determined in the following way, since samples could not be weighed in a moving ship. Two to 3 g of muscle from the anterior end of the fillet, dissected free from connective tissue, was pushed into a polyethylene tube so as almost to fill it. A polyethylene stopper was then inserted, the air space being made as small as possible. After this the tube was frozen at − 30°C until required. On only one occasion out of nearly 300 was the stopper pushed out by the freezing. Back at the research station, several tubes were thawed out at a time in a desiccator at 4°C overnight. After bringing to room temperature in the desiccator (3 hr) they were weighed full, most of the tissue was rapidly transferred to a weighed and dried basin, and the polyethylene stopper was replaced to prevent loss of the remaining film of tissue fluid. The tube was then weighed again and the tissue weight obtained by difference. The sample was dried at 100°C for 1 week, the loss in weight representing the tissue water.

For pH determination, a lump of whole tissue (myotomes plus myocommata) was closely wrapped in aluminium foil and stored at −30°C until required. After thawing 10 g of the sample was homogenized with 20 ml of distilled water and the pH measured with a Beckman instrument.

For the histological pictures of connective tissue in the frozen state, the method of Koonz & Ramsbottom (1939) was used, vacuum-drying being done at −29°C, while the different speeds of freezing were achieved essentially by the method of Love (1957).

Results

Four short trips were made on the 'Sir William Hardy' and cod taken from fishing

grounds as follows:

April 1966: Hebrides (west Scotland).
June 1966: Iceland.
November 1966: Hebrides.
April 1967: Iceland.

The experimental procedure was the same in each case. When the fish from the first trip were thawed, filleted and examined, there was found to be little difference in gaping between those frozen immediately after death and those frozen after being packed in melting ice for 20 hr. This was in line with general experience at this Research Station that cod frozen within 3 days of catching under the best conditions, i.e. chilling as rapidly as possible and not subjecting to mechanical damage, made an acceptable frozen product.

However, those from the other three trips showed a marked increase in gaping with time after death before freezing, even as little as 6 hr. Between 6 and 36 hr after death, the longest period for which the fish were kept in ice, there was a further, though less marked, increase in gaping. This divergence from the first trip was not altogether unexpected, since it is well known in a qualitative way that gaping is subject to seasonal variation and also varies with the fishing ground.

The gaping after 6 hr was not caused by mechanical damage during rigor, nor by a temperature rise of the type described by Jones (1964).

In addition to keeping in melting ice before freezing, other fish from the same batches as those kept chilled were kept for up to 18 hr at 18°C, without mechanical disturbance, before being frozen. On thawing out and filleting, these were found to show much more gaping than in the 'iced' series, some fillets virtually falling to pieces and being completely unacceptable. The observations underline the extreme thermal lability of cod connective tissue.

Seasonal variation in gaping has always been assumed to be linked to the condition of the fish, a soft post-spawned one being more likely to gape than a firm, well-fed one in good condition.

The big trip in June 1966 was designed to relate gaping to condition.

Cod were taken from Faroe Bank, Faroe Plateau, S.E. Iceland, W. Greenland (two grounds), St Kilda (an island in the Atlantic west of Scotland) and the west coast of Scotland. On studying the results it was found that all of the 282 cod taken showed the same relationship with condition, regardless of the fishing ground. They have, therefore, been grouped together for the purpose of this report. Gaping was assessed on an agreed subjective scale, '0' being no gaping at all, '1' a single hole appearing and so on, '6' being the point where the fillet was virtually dropping to pieces. Condition was assessed from the water content of the muscle and, secondarily, by the pH.

The results (Fig. 2) show a remarkable linearity considering the comparative crudity of the technique used for assessing gaping, and that the fish were handled during freezing, thawing and filleting, all of which would tend to tear the connective tissue.

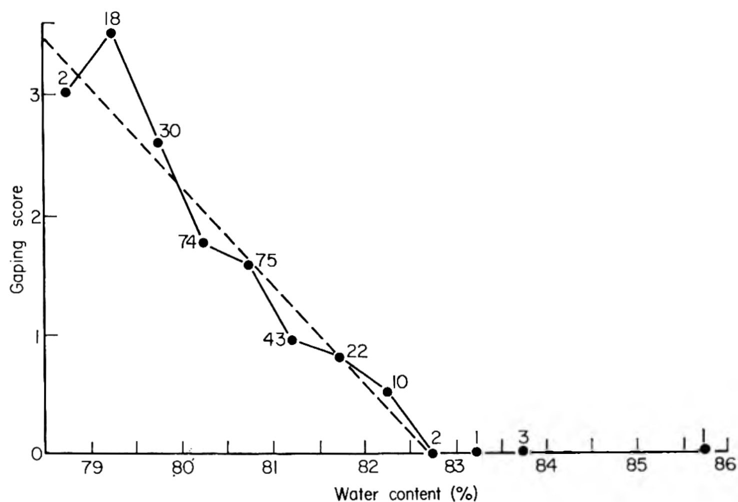

FIG. 2. Relationship between the amount of gaping, as measured on a subjective scale, and the biological condition of the fish, as measured by the water content. Values have been averaged for each range of water content, and figures beside each point show the number of fish. The regression line (broken line) relates to all the individual values.

The regression line relates to individual values, so takes into account the different numbers of fish falling within each range of water content. The relationship between gaping and pH was similar. The correlation coefficient between water content and pH was 0·444.

Discussion

The most noteworthy finding to emerge is that it is the healthy firm fish, not the weak soft ones, which gape most when they are frozen 24 hr after death (*in* rigor mortis). This probably explains why almost no gaping was seen in the fish caught in April 1966 off Scotland and frozen 20 hr after death — Scottish cod spawn at the beginning of March and are in very poor condition in April. The Icelandic cod caught in April 1967 gaped more than these when frozen *in* rigor mortis, but they are presumed to have been in better condition than the comparable Scottish cod because they spawn later, around the end of April. The important point appears to be that all of these fish were frozen *in* rigor mortis, when the opposing muscles were pulling against each other.

If freezing caused ice to form inside the connective tissue, it would weaken it and render it liable to be ruptured by the muscular contraction. However, Gersh (1965) had already noted that ice crystals had never been seen in collagen fibres or cartilage matrix, and Kaess (1966) found that small rapidly-frozen beef samples showed no ice crystals in the connective tissue bordering the cells. It was therefore necessary to observe the situation in cod muscle.

Many sections of cod frozen at various speeds were examined, and it can now be stated that, when cod muscle freezes, some ice invariably forms in the myocommata at all rates of freezing apart perhaps from extremely slow.

Plate 1(a) shows in-rigor cod muscle in the frozen state, frozen at the rate of a vertical plate freezer, about 2 hr. Colourless areas denote the ice, which appears to be extracellular. The connective tissue, slightly darker than the muscle tissue, runs from lower left to upper right, as indicated at the edges of the picture. Large ice crystals can be seen within the connective tissue region, and it is obvious that after thawing such tissue would have been much weakened, as it is split almost completely down the middle. Plate 1(b) shows cod muscle frozen in about 3 min, each muscle cell now being filled with a large number of minute ice crystals. In this picture the band of connective tissue is easier to discern, and ice crystals pervade it in large numbers, being slightly smaller than those in the muscle cells. The findings of Fig. 2 most probably result from different degrees of strengths of pull by the contracting muscle. Fish in good condition 'kick' furiously when landed on the deck and move their bodies vigorously. Those in poor condition often die without struggling, and any muscular movement is weak and slow. It is therefore not unreasonable to suppose that the strength of the rigor mortis contraction is great in healthy fish, and that when the connective tissue has weakened through ice crystal growth the contraction is sometimes sufficient to tear it apart, and vice versa in fish of poor condition.

This phenomenon could not take place in loose fillets which are free to shrink—it applies only to whole fish. However, the freezing of fillets before or in rigor is itself fraught with difficulties, and can give rise to an undesirably tough texture (Love, 1962b).

The gaping produced in this way can be avoided by freezing all the fish *before* the onset of rigor mortis. If this is not possible, the largest fish should be frozen last, since they are frequently of poorer condition than the smaller fish (Love, 1960).

Thus gaping in fish is now known to be brought about in several ways: handling (bending, bruising, etc.) when in rigor mortis, temperature rise while lying on the deck and good physiological condition coupled with freezing as whole fish after the onset of rigor mortis.

It should, however, be emphasized that this is not the whole story. As pointed out earlier in the present report, gaping continues to increase somewhat as time in ice after death increases before freezing. Also, fish from certain grounds are found to gape in the fresh state very soon after catching. It is hoped to deal with these aspects in future reports.

Acknowledgment

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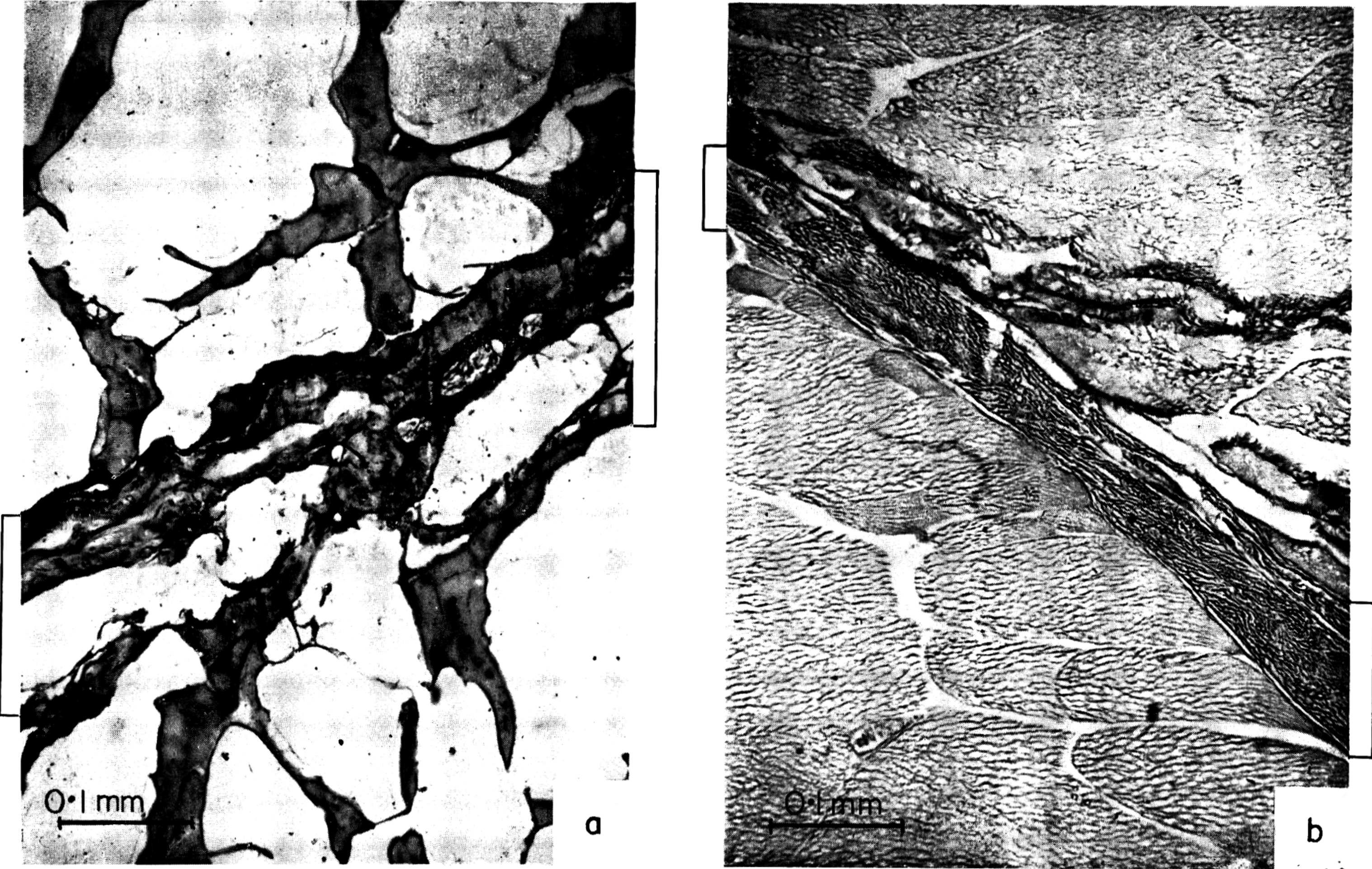

PLATE 1. Transverse sections of cod muscle as it appears in the frozen state after freezing in about: (a) 2 hr, and (b) 3 min. The connective tissue is that part enclosed by brackets at the edge of the picture. The figure shows that ice does form in cod connective tissue.

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The survival of pathogenic bacteria in, and the microbial spoilage of, salads, containing meat, fish and vegetables

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Summary. An investigation was carried out on the fate at 9° and 20°C of: (i) two types of non-spore-bearing bacteria, causing food-borne disease, viz. *Staphylococcus aureus* and *Salmonella* spp., and two spore-bearing enteropathogens (*Clostridium perfringens* and *Bacillus cereus*); and (ii) Lactobacillaceae and yeasts in one type of meat and vegetable salad (pH = 4·2, acetic acid content 0·5%) and in one type of shrimp salad (pH = 5·3, acetic acid content 0·3%), preserved at two levels of benzoic acid plus sorbic acid. The pathogens were inoculated at initial densities of 10^4–10^9/g; for lactic acid bacteria and yeast reliance was placed on naturally occurring organisms.

The non-spore-bearing pathogens died out under all conditions, albeit much faster at 20°C. Spore-bearing cells of the Bacillaceae tested remained mostly dormant under the conditions of the tests. Lactobacillaceae developed fast, also at 9°C and eventually caused souring of the products. Yeasts appeared to play a role at 3°C only. The main antibacterial effect of the salads was demonstrated to be due to its vinegar content and pH.

Introduction

Salads manufactured from meat, poultry, egg, fish, vegetables and salad cream or mayonnaise are, in principle, perishable products. Dependent on their pH-value and the addition, or not, of chemical preservatives, they form a more or less suitable medium for certain bacteria, yeasts and moulds (Wethington & Fabian, 1950; Gram, 1957; Lerche, 1961; Roemmele & v.d. Wall, 1961; Lorenzen & Sieh, 1962; Coretti, 1963; Beck & Schneider, 1964; Wurziger, 1965; Tuynenburg Muys, van Gils & de Vogel, 1966; Rasmussen & Strong, 1967). Outbreaks of food poisoning due to the consumption of bacteriologically unsound products of this type have been described (Ellemann, 1956; Bøvre, 1958; Meyer & Oxhoi, 1964); also typical spoilage of such products by Lactobaccillaceae had been reported (Baumgart, 1965; Sinell & Baumgart, 1965).

The increasing use of fully prepared salads in The Netherlands prompted a study

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of their microbiological condition. Special attention was paid to the fate of some food-borne pathogenic organisms, when such products were stored at temperatures well above the range of refrigeration.

Materials and Methods

Commodities

The salads used in the present studies are described in Table 1. They were all taken from freshly manufactured commercial batches of materials. The type of preservation used had been found necessary in preliminary investigations, particularly for the inhibition of rapid fermentation of the products by yeasts. The levels of preservatives used were below those allowed in certain semi-preserved fisheries products by Section 3.2 of the Fish Decree, Netherlands Food Law.

TABLE 1. Properties of salads used in the present study

Code used in subsequent tables	Main compositional characteristics			Preservation aimed at (% of mixture benzoic acid: sorbic acid 1 : 2)	Chemical analysis				
	Salad dressing (type and %)	Type of other ingredients	Acidity (pH)		Water (%)	Acetic acid (%)	NaCl (%)	Benzoic acid (%)	Sorbic acid (%)
H 0·30	Salad cream (containing 50% oil), 40%	Meat, vegetables and paprika	4·2	0·30	66·3	0·5	1·7	0·12	0·18
H 0·15				0·15	67·3	0·5	1·7	0·06	0·08
G 0·30	Mayonnaise (containing 80% oil), 55%	Shrimps	5·3	0·30	42·0	0·3	1·3	0·28	0·14
G 0·15				0·15	43·2	0·3	1·3	0·24*	0·08

* Interference with attempted formulation due to heterogeneous distribution of benzoic acid in the consignments of preserved, frozen shrimps used as an ingredient.

Bacteriological examination of these raw materials (*vide infra*) revealed that they contained far too low numbers of organisms of Public Health significance to be suitable for the planned studies. Hence they had to be inoculated with pure cultures of pertinent bacteria.

Inoculation

Strains used. Organisms were chosen, that represent the most frequent health risks in North-Western Europe. The strains used are presented in Table 2.

TABLE 2. Origin and properties of bacterial strains used for inoculation of salads

Species	Code No.	Isolation made from:
Staphylococcus aureus	662260 phage t. 42 D	Meat croquette
	662289 phage t. 47 D/75/77	Ice cream
Salmonella typhimurium	S 66-AL	Fresh minced meat
Salmonella panama	S 71-AL	(Dr P. A. M. Guinée, Utrecht)
Clostridium perfringens	C 19-LO	Patients suffering from *Cl. perfringens* food poisoning
	C 43-LO	Dr B. C. Hobbs, London)
Bacillus cereus	B 01-AC	Vanilla pudding, incriminated in food poisoning (Professor S. Hauge, Oslo)
	B 29-AC	Grilled rice dish, incriminated in food poisoning (Dr M. van Schothorst, Utrecht)

Mode of inoculation. Fresh cultures of the aerobic strains under study were prepared by streaking on to the surface of infusion agar layers contained in large Roux flasks incubating overnight at 35°C and emulsifying the cell crop in about 5 ml of sterile peptone saline (Straka & Stokes, 1957). The *Cl. perfringens* cultures were obtained by culturing in 300-ml flasks of Tarozzi's liver liver chalk broth, covered with a 2-cm seal of sterile beef tallow, for 40 hr at 37°C.

Prior to mixing with the salad samples, the bacterial cultures, as well as the salads, were cooled to *ca.* 5°C. This was done with the purpose of making the bacteria as little sensitive as possible to immediate death due to the hostile environment of the acid salads (Sherman & Cameron, 1934), and thereby obtaining the highest possible initial counts.

Inoculation was carried out at a rate of 1% v/v. Salads and inoculum were carefully mixed in sterile bottles, using a spatula.

Storage

Inoculated samples were stored in ground glass stoppered bottles at two different temperatures.

One series was incubated at 20° ± 2°C in a conventional air incubator; this range represents conditions of storage in ordinary shops in The Netherlands.

A second series was stored in a refrigerated cabinet at 9° ± 1°C.

A non-inoculated series of samples was kept in an electric refrigerator at 3° ± 1°C.

Microbiological examination

Preparation of dilutions. Samples of *ca.* 10 g were taken after thorough re-homogenization of the inoculated and stored materials.

They were, first of all, homogenized with 100 ml of sterile peptone saline (Straka & Stokes, 1957). After sedimentation for 30 sec, the supernatants were adjusted to pH = 7·0 ± 0·2 by the addition of a few millilitres of sterile, 5% K_3PO_4 solution (Mossel & v.d. Meulen, 1960).

Further dilutions were prepared with peptone saline until the viable counts of the organisms to be enumerated were of the order of 20–300/ml.

Salmonella. Examination for Enterobacteriaceae of the commercially manufactured salads used in this investigation revealed that they contained consistently very much less than 10/g. Hence the natural Enterobacteriaceae-flora of the commodities could be neglected in relation to the artificial inoculation which was at the order of magnitude of 10^4–10^9/g. Therefore, the fate of the *Salmonellea* in the salads could be studied by a simple enumeration of all Enterobacteriaceae.

Viable counts of these bacteria were assessed by making poured plates of suitable dilutions in violet red bile glucose (VRBG) agar (Mossel, Mengerink & Scholts, 1962a). After solidification the plates were covered with a second layer of *ca.* 20 ml of VRBG-agar to secure partial anaerobiosis, and incubated for 20–24 hr at 37°C.

Upon completion of this incubation the numbers of violet colonies surrounded by a violet precipitate, were counted.

Staph. aureus. The salads showed very low initial counts of *Staph. aureus.* In addition almost no Micrococci and only a few other types of bacteria that form black colonies on Baird-Parker's agar (Baird-Parker, 1962; de Waart *et. al.* 1968) were detected. Hence, counts on the latter agar could be relied on in studying the behaviour upon storage of *Staph. aureus* inoculated into salads.

Quantities of 0·1 ml of suitable dilutions were brought in the centre of plates containing freshly prepared Baird-Parker agar. After carefully spreading the inoculum over the entire surface of the plates, these were incubated for 29 ± 1 hr at 37°C, whereupon the black colonies, surrounded by a halo were counted.

Cl. perfringens. The salads used in these experiments were first of all examined for their natural contamination with *Cl. perfringens.*

For this purpose 1-ml quantities of dilutions, made with peptone saline of pH = 7·0 containing 0·1% cystein–HCl (Mossel *et al.,* 1965), were mixed with *ca.* 10 ml of freshly prepared tryptone iron polymyxin neomycin agar (Marshall, Steenbergen & McClung, 1965), using air-impermeable plastic pouches (Bladel & Greenberg, 1965; de Waart & Smit, 1967). These were sealed and incubated at 46°C for 24 hr. Any black colonies isolated in this way were subcultured on to Willis & Hobbs' (1959) lactose

egg yolk neomycin agar at 46°C and if necessary further examined for identity with *Cl. perfringens*.

No *Cl. perfringens* organisms were isolated from any of the products examined. In fact, even the numbers of all types of sulphite reducing clostridia (Mossel, 1959; Narajan, 1967) never exceeded $0{\cdot}2 \times 10^3$/g and could hence be neglected against the initial counts of the order 10^4–10^6/g achieved by artificial inoculation with *Cl. perfringens*.

These organisms were, therefore—very much simpler—determined by making poured pouch counts in sulphite–iron–polymyxin agar at 32°C (Mossel, 1959).

In these survival studies it was essential to assess also the degree of sporulation of the cultures used for inoculation. This was done by an enumeration, in addition to the total specific count, of the numbers of colonies obtained after heating suitable dilutions of the inoculum for 1 min at 80°C, which had been found earlier to give a fair impression of the numbers of spores, the less thermoresistant ones included (Mossel, 1967).

Bac. cereus. The salads appeared to contain appreciably lower numbers of *Bac. cereus* than 10^2/g, and virtually no other mannitol negative, egg yolk positive bacteria. Hence it was justified to study the fate of inoculated *Bac. cereus* by the method, currently used for the enumeration of these organisms in foods.

For this purpose, 0·1-ml quantities of suitable dilutions were spread on to plates containing freshly prepared mannitol egg yolk phenol red agar and incubated for *ca.* 30 hr at 32°C. At this point the numbers of colonies surrounded by a dense white halo and developed against a distinctly purple background were determined (Mossel, Koopman & Jongerius, 1967).

The numbers of spores present in the inoculum were estimated in the same way as described for *Cl. perfringens*.

Lactobacillaceae and total count. It has been demonstrated that the spoilage association of the type of vinegar products studied, consists mainly of Laetobacillaceae such as *L. buchneri, L. plantarum, L. brevis* and *Pediococcus cerevisiae* (Baumgart, 1965). Therefore, the predominating microflora of the salads during storage was studied by making poured plate counts in Rogosa, Mitchell & Wiseman's (1951) acetate agar of pH = 5·4. Plates were layered with about 20 ml of sterile Rogosa-agar and incubated for 5 days at 32°C.

A number of colonies, equal to the square root of the total and taken strictly at random, was examined for morphology (Gram stain) and catalase reaction. In case of doubt, the mode of attack on a few key sugars, such as maltose, lactose, saccharose, xylose and raffinose, was studied.

In a few instances, total bacterial counts were also carried out. Poured plates of tryptone dextrose yeast extract peptonized milk agar, incubated at 32°C were used

for this purpose. This medium allows the quantitative development of all Lactobacillaceae, fastidious types included (Mossel, 1964).

Yeasts. Viable yeasts were enumerated by making poured plates in oxytetracycline glucose yeast extract agar, incubated for 5 days at 30°C (Mossel, Visser & Mengerink, 1962b).

Chemical examination

The fresh materials used in the experiments were examined for NaCl content, pH, total acidity and benzoic and sorbic acid. During storage an attempt was made to examine the samples periodically for microbial metabolites, particularly acetic and lactic acid. Because it was detected in tentative tests that part of the pre-existent and additionally formed acetic acid was lost due to volatilization, only the level of lactic acid was followed in the final experiments.

Preparation of samples. All samples used for chemical examination were homogenized in a Braun household blendor of 100 ml capacity, at high speed.

Sodium chloride. Homogenized samples of *ca.* 15 g, were weighed into a beaker and transferred with *ca.* 150 ml distilled water into a measuring flask of 250 ml. After the addition of potassium ferrocyanide and zinc sulphate solutions, the volume was made up to 250 ml. To 25 ml of corrected aliquots of these solutions 5 ml nitric acid (1 + 1) and 50 mg of nitro-prusside sodium were added and the chloride ion present titrated with 0·1 N-Hg $(NO_3)_2$ until a definite turbidity was observed (Meyer, 1962).

Total acidity. Homogenized samples of *ca.* 15-g size were weighed into Erlenmeyers, diluted to *ca.* 200 ml and thoroughly hand shaken. The suspension was then titrated with 0·1 N solution of NaOH, using phenolphthaleine as the indicator (*Official Methods of Analysis of the Association of Official Analytical Chemists*, 10th edn).

Results were calculated as acetic acid and expressed as % total acid.

Lactic acid. An approximate determination of the lactic acid content of stored salads was carried out as follows. Samples of 50 g were mixed with *ca.* 50 g sand and dried *in vacuo* (oil pump) at 70°C. The residue was taken up in distilled water and titrated with 0·1 N-NaOH, using phenolphthaleine as the indicator. It was verified that no acetic acid would remain in the residue, when this procedure was followed.

In some instances a more specific method was used. Twenty-gram samples were very intensively mixed, in a mortar, with 20 g of sand. This mixture was dried *in vacuo* at 70°C and thereupon extracted for 2 hr with pure ethanol. The extract was concentrated to *ca.* 15 ml, transferred to a 25-ml measuring flask and brought to volume with ethanol. Quantities of 5, 10 and 20 μl of this solution and the usual standards

were spotted on Whatman No. 1 filter paper and developed with a solvent containing ethyl ether, 13 volumes; glacial acetic acid, 3 volumes and water, 1 volume. The spots were detected by spraying with a solution of 40 mg/100 ml of bromocresol green in 95% ethanol (Denison & Phares, 1952). The amounts of lactic acid in the spots were estimated visually.

Preservatives. Benzoic and sorbic acid were determined by submitting 1-g quantities of the homogenized products to steam distillation in the presence of 2 ml 1 N-sulphuric acid. Subsequent quantities of 100 ml distillate were acidified with 1 ml of 1 N-sulphuric acid and examined in a Zeiss PMQII spectrophotometer at wavelength 230 nm (benzoic acid) and 263 nm (sorbic acid), respectively.

Results and discussion

Storage at 20°C

Microbiological data. The results obtained in the tests at 20°C are presented in Tables 3 and 4.

Staph. aureus. These bacteria obviously died off quite rapidly, i.e. at a rate of > 6 decimal reductions (*D*) per 7 days, in the meat and vegetable salad of pH 4·2 (code H). This decay was independent of the mode of preservation followed, or the strain used. In the shrimp salad of pH = 5·3 (code G) spontaneous destruction was much slower, approximately 1 *D*/7 days. Anew, no influence of the concentration of preservatives was observed.

Salmonellae. These showed a similar behaviour upon storage in the salads. The effect of the decreased pH of the meat salad (H) was obvious again and, once more, differences in susceptibility of strains were not observed.

Cl. perfringens. The fate of *Cl. perfringens* in the salads was somewhat irreproducible. Particularly in the second test with strain C 19-LO the impression was obtained that the spores survived sojourn in the salads, independent of the latter's pH or degree of preservation. The less clear picture emerging from the other experiments may be due to the well known capricious sporulation behaviour of *Cl. perfringens* and to the essential shortcomings of the mode of enumeration of these spores. However this may be, it is obvious that the salads, even those of pH *ca.* 5·3 do not allow any proliferation of these enteropathogenic organisms.

Bac. cereus. These spore-forming bacteria showed a behaviour in salads quite similar to that of *Cl. perfringens* in that the spores maintained themselves independent of the intrinsic antimicrobial properties of the environment. A quite different survival rate

TABLE 3. Survival of *Staph. aureus* phage t. 42D, *Salmonella panama*, *Cl. perfringens*, strain C 43-LO and *B. cereus* strain B 01-AC and development of Lactobacillaceae and yeasts in various types of salads at 20°C

	Days of storage			
	0	13	21	35
Staph. aureus phage t. 42 D				
H 0·30	$0·5 \times 10^{10}$*	$< 0·1 \times 10^{3}$	$< 0·1 \times 10^{3}$	
H 0·15	$0·4 \times 10^{10}$	$< 0·1 \times 10^{3}$	$< 0·1 \times 10^{3}$	
G 0·30	$0·7 \times 10^{10}$	$0·2 \times 10^{5}$	$< 0·1 \times 10^{3}$	
G 0·15	$0·6 \times 10^{10}$	$0·8 \times 10^{4}$	$< 0·1 \times 10^{3}$	
Salmonella panama				
H 0·30	$0·2 \times 10^{7}$†	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
H 0·15	$0·2 \times 10^{7}$	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
G 0·30	$0·6 \times 10^{9}$	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
G 0·15	$0·9 \times 10^{9}$	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
Cl. perfringens strain C 43-LO				
H 0·30	$0·7 \times 10^{6}$‡	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
H 0·15	$0·6 \times 10^{6}$	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
G 0·30	$0·2 \times 10^{6}$	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
G 0·15	$0·2 \times 10^{6}$	$< 0·1 \times 10^{2}$	$< 0·1 \times 10^{2}$	
B. cereus strain B 01-AC				
H 0·30	$0·3 \times 10^{4}$§	$0·2 \times 10^{4}$	$0·2 \times 10^{4}$	$0·1 \times 10^{4}$
H 0·15	$0·4 \times 10^{4}$	$0·3 \times 10^{4}$	$0·2 \times 10^{4}$	$0·2 \times 10^{4}$
G 0·30	$0·2 \times 10^{5}$	$0·3 \times 10^{4}$	$0·1 \times 10^{4}$	$0·1 \times 10^{4}$
G 0·15	$0·1 \times 10^{6}$	$0·4 \times 10^{4}$	$0·2 \times 10^{4}$	$0·9 \times 10^{3}$
Lactobacillaceae				
H 0·30	$0·2–0·4 \times 10^{6}$	$0·3–0·5 \times 10^{9}$	$0·4–0·6 \times 10^{9}$	
H 0·15	$0·3–0·6 \times 10^{7}$	$0·4–0·5 \times 10^{9}$	$0·7–1·0 \times 10^{9}$	
G 0·30	$0·6–1·0 \times 10^{3}$	$0·3–1·0 \times 10^{8}$	$0·1–0·4 \times 10^{9}$	
G 0·15	$0·6–2·0 \times 10^{3}$	$0·1–0·7 \times 10^{9}$	$0·9–5·0 \times 10^{8}$	
Yeasts				
H 0·30	$< 0·1 \times 10^{3}$	$0·2–0·6 \times 10^{2}$	$< 0·1–0·2 \times 10^{2}$	
H 0·15	$< 0·1–0·6 \times 10^{3}$	$0·4–0·9 \times 10^{2}$	$< 0·1–0·1 \times 10^{2}$	
G 0·30	$< 0·1–1·0 \times 10^{3}$	$< 0·1–0·5 \times 10^{2}$	$< 0·1–0·1 \times 10^{2}$	
G 0·15	$0·3–0·6 \times 10^{3}$	$0·3–4·0 \times 10^{4}$	$0·6–6·0 \times 10^{2}$	

* Viable count of inoculum $0·1 \times 10^{13}$/ml, hence initial count in salads $0·1 \times 10^{11}$/g.

† Viable count of inoculum $0·2 \times 10^{12}$/ml, hence initial count in salads $0·2 \times 10^{10}$/g.

‡ Viable count of inoculum $0·2 \times 10^{9}$/ml, hence initial count in salads $0·2 \times 10^{7}$/g. Spore counts $0·2 \times 10^{5}$/ml and $0·2 \times 10^{3}$/g, respectively.

§ Viable count of inoculum $0·1 \times 10^{10}$/ml, hence initial count in salads $0·1 \times 10^{8}$/g. Spore counts $0·2 \times 10^{9}$/ml and $0·2 \times 10^{7}$/g, respectively.

of the strains B 01-AC and B 29-AC upon inoculation in the salads was observed. The former suffered a 3 *D* loss in spore count upon mixing with the salads of pH *ca.* 4·2 and a 1–2 *D* loss when homogenized with the salads of pH *ca.* 5·3, correlating with

the degree of preservation. Strain B 29-AC did not show such a behaviour. This difference may be due to the presence of spores, or pre-spores, of relatively low acid resistance (Hauschild, Hilsheimer & Thatcher, 1967) in the spore population of strain B 01-AC.

Lactobacillaceae. The course of the counts in viable Lactobacillaceae in the various substrates was quite striking. Virtually independent of the pH, the composition, the degree of preservation and the accompanying microflora, final counts converged to a level of approximately 10^9/g after storage under conditions which may be considered representative of what may happen in commercial practice. This confirms earlier observations, made in Germany (Baumgart, 1965; Sinell & Baumgart, 1965). It is also quite intelligible from the point of view of ecology: Lactobacillaceae are known to be resistant to sorbic acid (Emard & Vaughan, 1952; Costilow, Ferguson & Ray, 1955), while, confirming earlier findings (von Schelhorn, 1951; Rehm, 1961; Rehm, Wittmann & Stahl, 1961; Lubieniecki-von Schelhorn, 1967), we found the antimicrobial activity of the other preservative used, i.e. benzoic acid, insufficient to inhibit the isolated Lactobacillaceae, even in a concentration of 0·1% at pH = 3·7.

To obtain an impression of the types of Lactobacillaceae, predominant among the spoilage association of the salads, 140 representative colonies, taken at random from Rogosa agar plates obtained in the twelve tests, were examined for morphology and catalase activity. All appeared to be catalase negative; fifteen (11%) were cocci, the other rod shaped bacteria.

Yeasts. In the meat salads of low pH (code H), no proliferation of yeasts occurred at all, but rather a dying off, at a rate up to over 2 *D*/7 days. In the shrimp salad of pH = 5·3 (code G) some initial proliferation of yeasts leading to a count of *ca.* 10^4g was observed, e.g. in the second series of tests summarized in Table 4.

However, this was followed by a subsequent decrease in numbers in the next 7-day period; this becomes particularly clear from the data for the G 0·15 samples in Tables 3 and 4. Yeasts were, therefore, found much more sensitive to the preservatives used — as could be anticipated from our introductory experiments described in the section on Commodities (p. 224).

Chemical data. The course of the lactic acid content of the salads during storage for up to 3 weeks at 20°C has been summarized in Table 5.

It is clear, that a significant formation of lactic acid always occurs. However, the increase in lactic acid content in the meat salads is, on the average, twice as high as that observed in the shrimp salad. This correlates very well with the reducing sugar content of the two salads which was estimated at 1·1% for the meat salad and 0·6% for the shrimp salad.

TABLE 4. Survival of *Staph. aureus* phage t. 47/D/75/77, *Salmonella typhimurium*, *Cl. perfringens* strain C 19-LO and *B. cereus* strain B 29-AC and development of Lactobacillaceae and yeasts in various types of salads at 20°C

	Days of storage						
	First test				Second test		
	0	13	21	35	0	7	14
Staph. aureus phage t. 47/D/75/77							
H 0·30	0.5×10^{9}*	$<0.1 \times 10^{3}$	$<0.1 \times 10^{3}$		0.9×10^{9}†	0.2×10^{3}	$<0.1 \times 10^{3}$
H 0·15	0.5×10^{9}	$<0.1 \times 10^{3}$	$<0.1 \times 10^{3}$		0.1×10^{9}	$<0.1 \times 10^{3}$	$<0.1 \times 10^{3}$
G 0·30	0.7×10^{9}	0.5×10^{4}	$<0.1 \times 10^{3}$		0.1×10^{10}	0.1×10^{9}	0.7×10^{4}
G 0·15	0.1×10^{10}	0.3×10^{5}	0.1×10^{3}		0.2×10^{10}	0.7×10^{8}	0.9×10^{4}
Salmonella typhimurium							
H 0·30	0.5×10^{10}‡	$<0.1 \times 10^{2}$	$<0.1 \times 10^{2}$		0.5×10^{8}§	$<0.1 \times 10^{2}$	
H 0·15	0.2×10^{10}	$<0.1 \times 10^{2}$	$<0.1 \times 10^{2}$		0.4×10^{8}	$<0.1 \times 10^{2}$	
G 0·30	0.3×10^{10}	$<0.1 \times 10^{2}$	$<0.1 \times 10^{2}$		0.3×10^{9}	0.3×10^{8}	$<0.1 \times 10^{2}$
G 0·15	0.5×10^{10}	$<0.1 \times 10^{2}$	$<0.1 \times 10^{2}$		0.3×10^{9}	0.1×10^{8}	$<0.1 \times 10^{2}$
Cl. perfringens strain C 19-LO							
H 0·30	0.1×10^{5}¶	0.1×10^{3}	0.2×10^{3}		0.5×10^{4}‖	0.5×10^{4}	0.5×10^{4}
H 0·15	0.4×10^{5}	0.1×10^{3}	0.8×10^{2}		0.5×10^{4}	0.4×10^{4}	0.3×10^{4}
G 0·30	0.5×10^{5}	0.2×10^{2}	0.3×10^{2}		0.8×10^{5}	0.6×10^{4}	0.6×10^{4}
G 0·15	0.6×10^{5}	0.2×10^{2}	0.3×10^{2}		0.1×10^{6}	0.5×10^{4}	0.4×10^{4}
B. cereus strain B 29-AC							
H 0·30	0.1×10^{7}**	0.1×10^{7}	0.5×10^{6}	0.3×10^{6}	0.1×10^{8}††	0.4×10^{7}	
H 0·15	0.1×10^{7}	0.4×10^{6}	0.3×10^{6}	0.9×10^{5}	0.3×10^{8}	0.5×10^{7}	

G 0·30	0·1 × 10^7	0·6 × 10^7	0·1 × 10^7	0·2 × 10^6	0·1 × 10^8	0·6 × 10^7
G 0·15	0·8 × 10^6	0·2 × 10^7	0·5 × 10^6	0·6 × 10^6	0·3 × 10^8	0·6 × 10^7
Lactobacillaceae						
H 0·30	0·2–2·0 × 10^6	0·3–0·4 × 10^9	0·2–0·5 × 10^9		0·4–0·9 × 10^5	0·6–4·0 × 10^8
H 0·15	0·3–0·4 × 10^7	0·4–0·5 × 10^9	0·6–1·0 × 10^9		0·5–0·9 × 10^7	0·6–0·8 × 10^9
G 0·30	0·2–7·0 × 10^3	0·5–1·0 × 10^8	0·1–0·7 × 10^9		0·2–6·0 × 10^4	0·3–0·9 × 10^9
G 0·15	0·2–1·0 × 10^4	0·5–2·0 × 10^8	0·1–3·0 × 10^9		0·7–3·0 × 10^4	0·6–0·9 × 10^9
Yeasts						
H 0·30	< 0·1 × 10^3	0·1–1·0 × 10^2	< 0·1 × 10^2		0·1–0·5 × 10^3	< 0·1 × 10^3
H 0·15	< 0·1–0·4 × 10^3	0·1–3·0 × 10^2	< 0·1–0·1 × 10^2		0·5–0·8 × 10^5	< 0·1–2·0 × 10^3
G 0·30	0·4–0·9 × 10^3	0·1–0·4 × 10^2	< 0·1–0·2 × 10^2		0·4–1·0 × 10^3	0·1–0·3 × 10^5
G 0·15	0·2–1·0 × 10^3	0·2–1·0 × 10^4	0·1–3·0 × 10^4		0·5–2·0 × 10^3	0·3–2·0 × 10^4

* Viable count of inoculum 0·7 × 10^{12}/ml, hence initial count in salads 0·7 × 10^{10}/g.

† Viable count of inoculum 0·1 × 10^{12}/ml, hence initial count in salads 0·1 × 10^{10}/g.

‡ Viable count of inoculum 0·2 × 10^{12}/ml, hence initial count in salads 0·2 × 10^{10}/g.

§ Viable count of inoculum 0·1 × 10^{11}/ml, hence initial count in salads 0·1 × 10^9/g.

¶ Viable count of inoculum 0·4 × 10^8/ml, hence initial count in salads 0·4 × 10^6/g. Spore counts 0·1 × 10^6/ml and 0·1 × 10^4/g, respectively.

‖ Viable count of inoculum 0·2 × 10^7/ml, hence initial count in salads 0·2 × 10^5/g. Spore counts 0·6 × 10^7/ml and 0·6 × 10^5/g, respectively, probably higher than total counts due to heat activation.

** Viable count of inoculum 0·2 × 10^{10}/ml, hence initial count in salads 0·2 × 10^8/g. Spore counts 0·1 × 10^7/ml and 0·1 × 10^5/g, respectively.

†† Viable count of inoculum 0·1 × 10^{10}/ml, hence initial count in salads 0·1 × 10^8/g. Spore counts 0·6 × 10^9/ml and 0·6 × 10^7/g.

TABLE 5. Formation of lactic acid in salads, after storage for 2 weeks at 20°C

Type of salad	Lactic acid (%)			
	Initial determined by:		Final determined by:	
	Titration	Chromatography	Titration	Chromatography
H 0·30	0·5–0·3	0·1	1·5	2·5–1·3 av. 1·9
H 0·15	0·5–0·3	0·1	1·6–1·0 av. 1·4	2·4–0·7 av. 1·6
G 0·30	0·3–0·1	< 0·1	0·7	0·8–0·7
G 0·15	0·3–0·1	< 0·1	0·7	0·8–0·6

Storage at 9°C

Microbiological results. The microbiological data obtained in the course of the storage tests at 9°C have been summarized in Table 6.

Staph. aureus. The strain tested died off much slower at 9°C than they did at 20°C in the same environments. In the meat salad of pH = 4·2 the decay rate was > 6 $D/13$ days at 20°C and 3–6 $D/13$ days at 9°C, the latter figure being much influenced by the degree of preservation of the salad by vinegar. In the shrimp salad of pH = 5·3 the corresponding figures were approximately 5 D *versus* 1 D.

These results are quite intelligible in view of: (i) the well-known increasing spontaneous decay with increasing temperature (Mossel & de Bruin, 1960; Siegmund, 1960); and (ii) the observed faster development of Lactobacillaceae and hence acidification at the higher temperature.

Salmonella typhimurium. The inhibitory effect of decreased temperature on the destruction was observed again, particularly, for analytical reasons, in the shrimp salad. Whereas at 20°C a decay date of over 8 $D/13$ days was observed, this figure was of the order of only 1–2 $D/13$ days at 9°C.

Cl. perfringens. Spores of this organism remained dormant in both salads for about 28 days at 9°C. Thereupon a reduction in numbers of cells was observed amounting to slightly over 2 $D/28$ days.

Bac. cereus. Contrary to the behaviour of the clostridial spores the numbers of these aerobic spores remained almost constant for up to 56 days at 9°C.

Lactobacillaceae. The growth of the lactic acid bacteria was somewhat impaired at 9°C. This becomes particularly clear from the counts of these bacteria in the H 0·30

Table 6. Survival of *Staph. aureus* phage t. 47/D/75/77, *Salmonella typhimurium Cl. perfringens*, strain C 19–LO and *Bac. cereus*, strain B19-AC and development of Lactobacillaceae and yeasts in various types of salads at 9°C

		Days of storage			
		0	13	21	56
Staph. aureus phage t. 47/D/75/77					
H 0·30	$0{\cdot}9 \times 10^{9}$*	$0{\cdot}3 \times 10^{3}$	$<0{\cdot}1 \times 10^{3}$		
H 0·15	$0{\cdot}1 \times 10^{9}$	$0{\cdot}9 \times 10^{5}$	$<0{\cdot}1 \times 10^{3}$		
G 0·30	$0{\cdot}1 \times 10^{10}$	$0{\cdot}2 \times 10^{9}$	$0{\cdot}8 \times 10^{8}$	$0{\cdot}3 \times 10^{6}$	$<0{\cdot}1 \times 10^{3}$
G 0·15	$0{\cdot}2 \times 10^{10}$	$0{\cdot}4 \times 10^{9}$	$0{\cdot}3 \times 10^{8}$	$0{\cdot}6 \times 10^{5}$	$0{\cdot}1 \times 10^{3}$
Salmonella typhimurium					
H 0·30	$0{\cdot}5 \times 10^{8}$†	$<0{\cdot}1 \times 10^{2}$	$<0{\cdot}1 \times 10^{2}$		
H 0·15	$0{\cdot}4 \times 10^{8}$	$<0{\cdot}1 \times 10^{2}$	$<0{\cdot}1 \times 10^{2}$		
G 0·30	$0{\cdot}3 \times 10^{9}$	$0{\cdot}1 \times 10^{9}$	$0{\cdot}3 \times 10^{8}$	$0{\cdot}4 \times 10^{6}$	$<0{\cdot}1 \times 10^{2}$
G 0·15	$0{\cdot}3 \times 10^{9}$	$0{\cdot}4 \times 10^{7}$	$0{\cdot}2 \times 10^{7}$	$0{\cdot}1 \times 10^{4}$	$<0{\cdot}1 \times 10^{2}$
Cl. perfringens C 19-LO					
H 0·30	$0{\cdot}5 \times 10^{4}$‡	$0{\cdot}5 \times 10^{4}$	$0{\cdot}5 \times 10^{4}$	$0{\cdot}4 \times 10^{4}$	$<0{\cdot}1 \times 10^{2}$
H 0·15	$0{\cdot}5 \times 10^{4}$	$0{\cdot}5 \times 10^{4}$	$0{\cdot}5 \times 10^{4}$	$0{\cdot}3 \times 10^{4}$	$<0{\cdot}1 \times 10^{2}$
G 0·30	$0{\cdot}8 \times 10^{5}$	$0{\cdot}6 \times 10^{4}$	$0{\cdot}6 \times 10^{4}$	$0{\cdot}5 \times 10^{4}$	$<0{\cdot}1 \times 10^{2}$
G 0·15	$0{\cdot}1 \times 10^{6}$	$0{\cdot}6 \times 10^{4}$	$0{\cdot}6 \times 10^{4}$	$0{\cdot}3 \times 10^{4}$	$<0{\cdot}1 \times 10^{2}$
B. cereus B 29-AC					
H 0·30	$0{\cdot}1 \times 10^{8}$§	$0{\cdot}5 \times 10^{7}$	$0{\cdot}7 \times 10^{7}$	$0{\cdot}5 \times 10^{7}$	$0{\cdot}6 \times 10^{7}$
H 0·15	$0{\cdot}3 \times 10^{8}$	$0{\cdot}6 \times 10^{7}$	$0{\cdot}6 \times 10^{7}$	$0{\cdot}4 \times 10^{7}$	$0{\cdot}3 \times 10^{7}$
G 0·30	$0{\cdot}1 \times 10^{8}$	$0{\cdot}5 \times 10^{7}$	$0{\cdot}5 \times 10^{7}$	$0{\cdot}6 \times 10^{7}$	$0{\cdot}3 \times 10^{6}$
G 0·15	$0{\cdot}3 \times 10^{8}$	$0{\cdot}7 \times 10^{7}$	$0{\cdot}5 \times 10^{7}$	$0{\cdot}5 \times 10^{7}$	$0{\cdot}2 \times 10^{7}$
Lactobacillaceae					
H 0·30	$0{\cdot}4\text{–}0{\cdot}9 \times 10^{5}$	$0{\cdot}1\text{–}0{\cdot}6 \times 10^{6}$	$<0{\cdot}1\text{–}0{\cdot}2 \times 10^{7}$	$0{\cdot}1\text{–}1{\cdot}0 \times 10^{7}$	$0{\cdot}1\text{–}0{\cdot}3 \times 10^{9}$
H 0·15	$0{\cdot}5\text{–}0{\cdot}9 \times 10^{7}$	$0{\cdot}9\text{–}2{\cdot}9 \times 10^{8}$	$0{\cdot}6\text{–}2{\cdot}0 \times 10^{8}$	$0{\cdot}1\text{–}0{\cdot}5 \times 10^{9}$	$0{\cdot}5\text{–}2{\cdot}0 \times 10^{8}$
G 0·30	$0{\cdot}2\text{–}6{\cdot}0 \times 10^{4}$	$0{\cdot}4\text{–}1{\cdot}0 \times 10^{8}$	$0{\cdot}1\text{–}1{\cdot}0 \times 10^{8}$	$0{\cdot}2\text{–}1{\cdot}0 \times 10^{9}$	$0{\cdot}1\text{–}0{\cdot}5 \times 10^{9}$
G 0·15	$0{\cdot}7\text{–}3{\cdot}0 \times 10^{4}$	$0{\cdot}1\text{–}0{\cdot}3 \times 10^{9}$	$0{\cdot}1\text{–}0{\cdot}3 \times 10^{9}$	$0{\cdot}2\text{–}0{\cdot}5 \times 10^{9}$	$0{\cdot}1\text{–}0{\cdot}3 \times 10^{9}$
Yeast					
H 0·30	$0{\cdot}1\text{–}0{\cdot}5 \times 10^{3}$	$<0{\cdot}1\text{–}0{\cdot}3 \times 10^{2}$	$<0{\cdot}1\text{–}0{\cdot}3 \times 10^{2}$	$<0{\cdot}1\text{–}0{\cdot}3 \times 10^{2}$	
H 0·15	$0{\cdot}5\text{–}0{\cdot}8 \times 10^{5}$	$0{\cdot}1\text{–}6{\cdot}0 \times 10^{3}$	$0{\cdot}1\text{–}7{\cdot}0 \times 10^{3}$	$0{\cdot}2\text{–}1{\cdot}0 \times 10^{4}$	
G 0·30	$0{\cdot}4\text{–}1{\cdot}0 \times 10^{3}$	$0{\cdot}1\text{–}9{\cdot}0 \times 10^{3}$	$0{\cdot}8\text{–}3{\cdot}0 \times 10^{4}$	$0{\cdot}1\text{–}3{\cdot}0 \times 10^{7}$	
G 0·15	$0{\cdot}5\text{–}2{\cdot}0 \times 10^{3}$	$0{\cdot}2\text{–}4{\cdot}0 \times 10^{4}$	$0{\cdot}2\text{–}5{\cdot}0 \times 10^{4}$	$0{\cdot}9\text{–}1{\cdot}0 \times 10^{7}$	

* Viable count of inoculum $0{\cdot}1 \times 10^{12}$/ml, hence initial count in salads $0{\cdot}1 \times 10^{10}$/g.

† Viable count of inoculum $0{\cdot}1 \times 10^{11}$/ml, hence initial count in salads $0{\cdot}1 \times 10^{9}$/g.

‡ Viable count of inoculum $0{\cdot}2 \times 10^{7}$/ml, hence initial count in salads $0{\cdot}2 \times 10^{5}$/g. Spore counts $0{\cdot}6 \times 10^{7}$/ml and $0{\cdot}6 \times 10^{5}$/g, respectively, probably due to heat activation.

§ Viable count of inoculum $0{\cdot}1 \times 10^{10}$/ml, hence initial count in salads $0{\cdot}1 \times 10^{8}$/g. Spore counts $0{\cdot}6 \times 10^{9}$/ml and $0{\cdot}6 \times 10^{7}$/g, respectively.

salad. Whereas after 13 days at 20°C the enumeration of Lactobacillaceae amounted to *ca.* $0{\cdot}4 \times 10^9$/g, it reached only *ca.* $0{\cdot}2 \times 10^6$/g after the same period of storage at 9°C.

Yeasts. Again, as at 20°C, no proliferation of yeasts, but rather a slow decrease, initially of *ca.* 1 *D*/13 days, was observed in the most strongly preserved meat salad. In the meat salad with only 0·15% total preservative, occasionally some growth was observed after 28–56 days.

Definite growth did occur in the shrimp salads, leading to counts of the order 10^6–10^7/g. The maximal value of these viable counts was apparently not reached after 56 days.

Chemical data. The concentration of lactic acid in all salads was determined after storage for 56 days at 9°C. The titrimetric procedure was used in this instance.

The percentages found are summarized in Table 7. Anew, the lactic acid formation was higher in the meat than in the shrimp salad, although the difference was less pronounced than at 20°C.

TABLE 7. Lactic acid formation in salads, stored for 56 days at 9°C

Type of salad	Lactic acid (%) determined by titration	
	Fresh	After storage
H 0·30	0·1	1·4–1·1
H 0·15	0·1	1·2
G 0·30	< 0·1	1·0–0·9
G 0·15	< 0·1	1·1–0·9

The velocity of acid formation in general was very much reduced at 9°C: *ca.* 1% in 56 days, in contrast to the order of 0·5–1·5% in 14 days at 20°C.

Storage at 3°C

Uninoculated samples of the four types of salads were stored at 3°C and examined after 8, 21, 35, 63 and 85 days for viable Lactobacillaceae. These bacteria appeared to remain fully dormant during the entire period of investigation.

However, slow growth of yeasts was observed in all samples, i.e. at a rate of *ca.* 2 log cycles in 63 days and about 3 in 85 days. The latter data are in agreement with the known psychrotrophic behaviour of quite some yeast species and the generally lowered sensitivity to antimicrobial agents at reduced temperatures.

Conclusions

From the results discussed in the previous section the following conclusions can be drawn.

Amongst the pathogenic organisms tested for survival in the two types of salad, the non-spore-forming types, i.e. *Staph. aureus* and *Salmonella* definitely so not proliferate in any of the salads kept at 20°C, but rather die off at a rate determined by the intrinsic antimicrobial properties of the salads. The spore-bearing enteropathogenic species investigated, viz. *Cl. perfringens* and *Bac. cereus* maintained themselves in a state of dormancy. However, the Lactobacillaceae, initially present in numbers of an order varying from 10^3 to 10^6/g, developed rapidly to numbers of the order 10^9/g, thereby forming about 1 to 1·5% of lactic acid and rendering the medium more inhibitory to pathogenic bacteria.

At 9°C, the storage temperature mostly used in The Netherlands, the non-spore-bearing pathogenic organisms died off much more slowly. Again the amount of vinegar used and the initial pH determined the rate of destruction of these bacteria. *Cl. perfringens* was destroyed slowly, *Bac. cereus* remained dormant for up to 2 months.

The wholesomeness of meat and fish salads of the type examined therefore depends entirely on their initial contamination with enteropathogenic organisms. The commercial consignments used in this investigation contained consistently less than ten Enterobacteriaceae of all types, and less than 10^2 *Staph. aureus, Cl. perfringens* and *Bac. cereus*/1 g. In this condition they are obviously completely innocuous; and in view of the fate of these organisms during storage just referred to, they will remain entirely wholesome under any condition of storage to which they might be exposed. In other words, salads of the type examined, which are prepared under satisfactory conditions of sanitation will be fully acceptable from the point of view of Public Health.

However, the keeping quality of such salads is limited. They will tend to souring well within a week, when stored at 20°C and within 2 weeks when refrigerated at 9°C. When kept at 3°C they will tend to fermentation by yeasts well after 2 months. Both types of spoilage can obviously be retarded by securing low initial counts of the spoilage agents concerned. This, according to our experience, can be achieved by carefully controlled heat processing of raw materials, such as meat, egg, fish and vegetables and excellent sanitary conditions of manufacture.

In this connection it has to be kept in mind that the salads used in the present investigation were preserved in accordance with the preservation authorized for certain semi-preserved foods in The Netherlands Food Law, Fish Decree. Where no preservatives are allowed, the keeping quality of such salads is likely to be more restricted (Beck & Schneider, 1964; Baumgart, 1965; Sinell & Baumgart, 1965) and yeast spoilage may even be quite imminent.

However, the fate of the pathogenic bacteria, according to our results, does not seem to be very much influenced by the presence of preservatives, but rather depends predominantly on pH and vinegar concentration.

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Spoilage of canned milk products by flavobacteria

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Summary. It is recognized that, arising from their clump-forming characteristics, the flavobacteria are resistant to the disinfectants commonly used in canneries; these organisms are, therefore, frequently found as contaminants in cooling water and on wet post-process can handling equipment. Hitherto, there has been no suggestion of flavobacteria being involved in the spoilage of canned foods, and laboratory studies confirmed their inability to develop in canned vegetables.

It has now been demonstrated that there is a spoilage hazard from the flavobacteria for at least some varieties of milk-based canned products. A somewhat prolonged delay occurs between infection and the manifestation of spoilage, and contaminated packs do not become blown.

Introduction

The yellow-orange chromogenic flavobacteria are widely distributed in nature and their presence in water supplies has been known for many years (Levine, Heller & Bender, 1942; Allen & Brooks, 1949; Everton & Dutson, 1964). These organisms have been recovered consistently from chlorinated waters and the mechanism of their resistance to the disinfectant probably resides in an ability to form clumps and aggregates, the innermost cells being protected from the bactericide.

The occasional leakage of bacteria into cans having double seams of commercial quality is a well-known phenomenon, the bacteria gaining access during water cooling or the subsequent handling of wet cans (Scott, 1937; Merrill, 1938; Bashford, Gillespy & Tomlinson, 1960; Cockburn, 1960; Everton & Herbert, 1962). However, it is considered that flavobacteria surviving in chlorinated water are unlikely to gain access to normal cans directly via the cooling water because of their probable aggregation in relatively large clump forms in this environment.

Investigational work carried out some years ago, both by the Fruit and Vegetable Preservation Research Association and in the authors' laboratory, indicated that flavobacteria were unable to cause spoilage of vegetables owing to their inability to grow and multiply in the canned product. Therefore, little interest has been shown

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in this particular group of non-pathogenic bacteria in canned foods, and they have been generally regarded as non-spoilage types (Gillespy & Thorpe, 1961; Lerke, Adams & Faber, 1965).

However, during a recent investigation of an outbreak of spoilage in creamed rice pudding involving blown cans, it was observed that amongst a large number of apparently sound cans held at room temperature for a period of several months, some showed a marked 'thinning' or decrease in consistency of the product which could be readily detected in unopened cans by the increased mobility of the contents. A small collection of micro-organisms having characteristics common to flavobacteria was recovered from some of these 'thinned' cans. At least one of these cultures, together with another isolated from post-process can lines in the particular cannery, reproduced the condition of 'thinning' on reinoculation into rice pudding in laboratory 'test tube' experiments. This observation was thought to be significant because, over a number of years, complaints from the trade had been reported. These complaints concerning canned creamed rice pudding were somewhat ambiguous since the description varied from 'off' flavour to marked souring; they were also spasmodic in occurrence and there was variation in the numbers of allegedly spoiled cans. There was a general assumption that spoilage was associated with the outgrowth of facultative thermophiles or some other heat-resistant organisms that had survived the thermal process. This suggestion, however, could not be substantiated by a number of studies carried out at the plant both by the resident technical staff and ourselves.

Against this background, an investigation was initiated into the spoilage potential of flavobacteria in milk-based canned products.

Experimental methods

(a) *Collection of chlorine resistant flavobacterium cultures*

Some 172 yellow-orange pigmented cultures were collected during plate count studies of chlorinated can cooling water and of post-process can handling equipment. From this collection, 132 flavobacteria cultures were isolated, identification of the genus *Flavobacterium*, being based on the description given in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957).

(b) *Milk products employed*

Portions of creamed rice, semolina and tapioca puddings were transferred aseptically from processed cans to sterile 1-oz screw-capped bottles, the original proportion of cereal to milk being retained as nearly as possible. The bottles containing milk pudding were heavily inoculated with sixty-two selected strains of flavobacteria, sealed and incubated along with uninoculated controls.

Following this '*in vitro*' work, 8- and 16-oz cans of creamed rice pudding, 16-oz

cans of creamed tapioca and 16-oz cans of semolina puddings were inoculated with selected cultures of flavobacteria.

(c) *Viscosity measurements*

Consistency of rice puddings was compared, using a Bostwick type consistometer (Rutgus, 1958). Briefly, this apparatus is a simple trough of rectangular section having a hinged gate near one end, behind which a standard volume of sample is retained. Following instant release of the gate, flow distance of the test material is recorded over 30 sec with the trough inclined at an angle of seven degrees. By comparing the flow distances of inoculated and non-inoculated control material, a decrease in consistency could be measured. Where the flow distance in 30 sec exceeded the length of the consistometer (17 cm), the time to flow this distance (17 cm) was recorded. However, the initial high consistency of both semolina and tapioca puddings used in the 1-oz bottle experiments made flow rate determinations impracticable and visual examination alone was employed with these two products.

Results

It was thought that 'thinning' of milk-based products was probably associated with hydrolysis of caseinogen, fat and/or starch, and the 132 flavobacteria collected were accordingly divided into six groups on the basis of these hydrolytic reactions.

Sixty-two flavobacteria cultures, representing these six groups, were inoculated into creamed rice pudding in 1-oz screw-capped bottles and incubated at 25°C for 4 weeks. Twenty of these cultures slowly produced a marked decrease in consistency of this pack (see Table 1). It was observed also that these twenty spoilage strains fell into five of the six arbitrary hydrolytic groups; because of these findings, this attempted classification was subsequently abandoned.

Eleven of the twenty cultures which produced 'thinning' in *in vitro* experiments were selected for inoculation into cans of creamed milk puddings. An inoculum level of 50–400 organisms/can was chosen (closer agreement in numbers was unobtainable because of the clumping characteristics of the organisms). Uninoculated control cans and cans inoculated with cultures giving negative results in the *in vitro* tests were also set up. All cans were incubated at 25°C for 3 months.

After incubation the cans were sampled bacteriologically and the contents examined for evidence of 'thinning', alteration in pH value or change in colour. Extensive growth of the eleven flavobacteria recurred in the inoculated can contents and five of the cultures reduced the consistency of one or more of the milk puddings (see Table 2). No significant alteration in pH of the canned milk puddings was recorded. Sour or cheesy odours, and yellowing and separation of the contents were often but not always associated with reduced viscosity. No gas evolution was observed in any of the test cans, no growth occurred in the uninoculated cans, and no spoilage developed in the cans inoculated with the non-spoilage flavobacteria strains.

Table 1. Viscosity measurements produced by flavobacteria cultures causing spoilage on inoculation into 1-oz bottles of rice pudding

Culture No.	Mean flow distance in 30 sec	
	Test	Control (cm)
12	17 cm in 8 sec	3·6
14	17 cm in 4 sec	3·6
36	12·3 cm	4·4
37	13·0 cm	5·3
37*	17 cm in 23 sec	3·6
39	8·5 cm	3·6
41	12·6 cm	3·6
43	12·6 cm	3·6
51	17·0 cm	5·3
65	11·8 cm	2·7
70	15·0 cm	4·2
75	10·3 cm	2·7
111	11·4 cm	2·7
125	8·0 cm	2·2
130	17 cm in 8 sec	2·2
134	7·2 cm	2·2
135	17 cm in 11 sec	4·4
144	8·8 cm	2·2
149	5·4 cm	2·2
151	14·5 cm	5·3
158	17 cm in 2 sec	2·2

* Repeat inoculation using a second variety of rice pudding.

Seventeen of the twenty cultures which produced a reduction in viscosity of milk-based products in 1-oz bottles hydrolysed both caseinogen and tributyrin. Of the three remaining cultures, one hydrolysed caseinogen but not tributyrin, one hydrolysed tributyrin but not caseinogen and the third failed to hydrolyse either of these two compounds. Further can inoculation work indicated that these last three organisms were unlikely to cause 'thinning' of canned milk-based products.

The flavobacteria cultures were subjected to the techniques of numerical taxonomy by computation of dissimilarities, as described by Floodgate (1962), over a range of twenty-eight characteristics. This proved to be a more positive method of classification than the initial concept based solely on hydrolytic action. By this new approach six distinct pleista were formed, ninety-six of the 132 cultures falling into these, the remaining thirty-six being intermediate between two or more pleista.

TABLE 2. Flavobacteria cultures causing spoilage on inoculation into cans of milk products

Culture No.	Product	Pleiston
37	Creamed rice pudding	B2
51		B5
70		B5
85		B2
138		A
37	Creamed tapioca pudding	B2
51		B5
70		B5
138		A
37	Creamed macaroni pudding	B2
51		B5
37	Creamed semolina pudding	B2
51		B5
70		B5

Canned milk-based soups

Chance discussions of these observations with two soup canners revealed features in common with unresolved complaints associated with milk or cream based soups. As a result, one of the canners provided samples of cream soup for inoculation studies, while the other obtained cultures of appropriate flavobacteria strains from us to conduct his own checks.

Both investigations established that flavobacteria known to cause thinning in milk puddings could also produce a similar spoilage in cream-style soups.

Discussion

The work reported in this paper has shown that members of the genus *Flavobacterium*, contrary to previous reports in the literature, are capable of spoilage of canned foods particularly milk-based products such as creamed puddings and cream or milk-based soups. However, the characteristic spoilage effect viz. 'thinning' of the contents often subsequently accompanied by odour change, becomes apparent only after prolonged storage such as 6–12 weeks at room temperature. Flavobacteria are frequently prevalent in canning factories since these organisms are, in general, tolerant to the concentrations of disinfectants commonly used. Spoilage strains of this genus have been isolated from eight canneries, selected at random for investigation. Protection from chlorine disinfection in can cooling water is believed to reside in the ability of these organisms to

form clumps. It is unlikely that the clumps could pass through the seams of commercially acceptable cans; however, they may be carried on the can and deposited onto handling equipment where the clumps would not only be disintegrated by the mechanical action of passing cans, but could then quite readily continue to multiply as single cells capable of ingress through the can seam.

The prolonged delay between infection and manifestation of spoilage is a feature of these organisms, which are often slow growing types. This characteristic, allied to the well-known difficulties in isolating them and the fact that they do not necessarily produce acid from glucose in the presence of available organic nitrogen (Weeks, 1955) – e.g. as in Tryptone Dextrose Broth, a recovery medium widely employed in canned food bacteriology – could explain why these organisms have been overlooked in the past as potential spoilage types.

The ability of flavobacteria to develop in milk-based soups is now accepted by two of the larger soup producers in the United Kingdom as a potential flat-can type spoilage hazard. The characteristic defect brought about by flavobacteria in milk puddings was recognized by these canners as resembling spoilage encountered in the past, but not previously identified.

In attempting to discover the particular properties of the organisms capable of producing spoilage in milk-based products, the 132 cultures making up the flavobacteria collection studied were divided into six groups on the basis of their hydrolysis of caseinogen, tributyrin and starch. However, the potential spoilage cultures were found to fall into five of these six groups and this arbitrary division was, therefore, abandoned. Numerical analysis of the results of computation of dissimilarity values by the method of Floodgate (1962) revealed six distinct pleista, designated A–F, in the flavobacteria collection. By this taxonomic analysis the majority of the spoilage flavobacteria were shown to exhibit closely related characteristics; most of them being placed into subdivisions of pleiston B. Although there is some evidence to show that caseinogen hydrolysis may be a prerequisite of spoilage by flavobacteria it cannot be assumed that the mechanism of thinning of canned milk-based products is necessarily associated with the breakdown of caseinogen. However, it can be said that caseinogen hydrolysis is a characteristic common to the majority of the spoilage flavobacteria studied in this investigation and this feature could, therefore, be one of the means of identifying potential spoilage organisms of this genus.

It is of interest to note that during the course of these studies, ten of the yellow pigmented cultures isolated were shown to be enterobacteria and from limited examination were tentatively placed in the *Hafnia* and *Cloaca* groups, (Enterobacteria Sub-Committee Report, 1958). These organisms, while closely resembling flavobacteria in colonial appearance and pigmentation, are gas producing and therefore likely to cause blowing on leakage into canned foods. It is emphasized that there is a danger, therefore, in regarding all yellow pigmented colonies arising from water sample or swab cultures as belonging to the flavobacteria group since the yellow pigmented

enterobacteria are likely to cause spoilage in a wide range of food products in which flavobacteria do not appear to be a problem.

Whilst cannery infection by flavobacteria alone may not lead to spoilage in canned foods other than milk-based products, it may serve as a useful indication of poor or ineffective sanitation measures.

Note: A report on the detailed taxonomy of the organisms studied during the course of this investigation has been accepted for publication elsewhere (Bean & Everton, 1968).

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In-Can shelf life of tomato paste as affected by tomato variety and maturity

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Summary. An increase in de-tinning activity, and hence a decrease in shelf life, has occurred in South African tomato paste during recent years.

Corrosion rate is shown to be related to the variety of tomato used in the manufacture of paste and to its maturity.

Of the changes in composition associated with these factors, total water-insoluble solids appear to be particularly associated with variations in corrosion rate.

Introduction

The shelf life of canned 28–30% tomato paste is especially important to South Africa's fish canning industry, which uses most of the tomato paste canned in South Africa. A minimum useful shelf life of 18 months is required to provide adequately for seasonal fluctuations in tomato crops and fish canning activities.

The corrosivity of tomato paste has been associated with hydroxymethylfurfural, dehydroascorbic acid, diketogulonic acid and demethylated pectins. The concentrations of these compounds were found to be primarily dependent on the amount of heat applied as governed by factory practices (Luh, Leonard & Marsh, 1958; Hernandez & Feaster, 1960; Hernandez, 1961; Luh *et al.*, 1964).

The incidence of hydrogen swelling and rapid corrosion of South African tomato paste has been reviewed by Knock (1965). It was reported that until 1960, tomato paste producers in South Africa obtained a shelf life of 24–30 months in plain cans made from hot-dipped tinplate, 1·25 pot yield. In 1961 the first isolated instance of detinning occurred and in the following year perhaps 20% of the Transvaal product became hydrogen swells within 6 months. It was also indicated that during these years efforts had been made to increase consistency by hot-breaking the tomatoes, to intensify colour by replacing round varieties such as French Marvel, Marglobe and Pearson by the Roma, and to reduce the Howard Mould count by picking at a less mature stage.

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An indication that maturity might be important arose from the observation that paste prepared from tomatoes grown in fields adjacent to a cannery gave rise to rapid detinning, while paste produced from these tomatoes at a factory some distance away, and processed 48 hr after picking, did not cause rapid detinning. A further detailed study of the detinning problem in one factory showed that the percentage swells developing in each day's production 6 months after the commencement of the packing season bore a good relationship to the proportion of tomatoes received each day by road on the day of picking and by rail 48 hr after picking (Knock, 1965).

Accordingly, the effects of variety and maturity on de-tinning rate were selected for study.

Experimental

Material

To provide comparable material and to examine the effect of delays between picking and processing known to occur in industry about 30 tons of Roma (pear) tomatoes were picked at the yellow-orange maturity stage from a single field. Four random 7-ton lots were drawn and processed after holding on the factory floor at 80°F for 24, 48, 72 and 96 hr, respectively. A similar procedure was adopted for the S. Pierre (round) variety except that the 96-hr lot was replaced with a 7-ton lot of vine-ripened tomatoes, processed on the day of picking. This was done because round varieties are not normally held for 96 hr after picking but are sometimes processed at a fully mature stage off the vine.

In both varieties maturity advanced through the orange-red (24 hr) and red (48 hr) stages to the canning-ripe stage after 72 hr. The Roma fruit was soft-ripe after 96 hr while the vine-ripened S. Pierre fruit was soft-ripe and riper than those held for 72 hr.

Preparation of pastes

For the Roma tomatoes a hot-break (185°F in less than 30 sec after crushing) with a 0·025-in. screen in the final finisher was used, while for S. Pierre a cold break (145°F in less than 45 sec after crushing) and a 0·027-in. screen in the final finisher was selected to represent earlier practices. The resultant juices were vacuum concentrated to 28–30% total soluble solids, pasteurized and filled at 180–190°F, closed and water cooled to 130°F before air cooling to room temperature.

These pastes were filled into both plain and fully lacquered 308 × 308 cans made from hot-dipped plate. The plain cans were made from a single delivery of tinplate with a mean actual tin-coating weight of 18·3 oz/b.b.

The contents of plain cans were analysed for tin and iron at monthly intervals during storage at 71°F for 15 months and twice thereafter at bi-monthly intervals. Paste stored frozen in fully lacquered cans was used for determining paste composition.

Polarization resistance

A polarization cell with calomel reference electrode, platinum as working electrode and hot-dipped tinplate as test electrode was used to measure polarization resistance according to the method reviewed by Phelps (1962).

Tin and Iron

A *ca*. 5-g sample of tomato paste was wet-ashed using 10 ml conc. HNO_3 plus 10 ml of a 5 : 1 : 1 mixture of conc. HNO_3, conc. $HClO_4$ and conc. H_2SO_4. One millilitre conc. HCl was added to the final H_2SO_4 solution before dilution to [illegible] ml. Aliquots of this solution were used for tin and iron determination. For tin determinations 10 ml conc. HCl was added to a 10-ml aliquot before dilution to 25 ml. A Cathode Ray Polarograph was used. The *o*-phenanthroline method (Vogel, 1953) was used for iron determinations.

Acidity and non-volatile organic acids

Titratable acidity was determined according to the A.O.A.C. (1965) procedure using a pH meter, while pH was measured on an expanded scale instrument.

Citric acid and malic acid were isolated on a Duolite A–A resin column and further separated on Whatman No. 3 MM paper. The acids were finally determined by titration.

Tomato solids

Total solids were determined using the vacuum oven method of the A.O.A.C. (1965).

To determine total water insolubles 5- to 10-g samples of paste were simmered for a few minutes in hot water before filtering through Whatman No. 12 filter paper. The insoluble matter and paper were washed five times with hot water before drying at 100°C at a pressure of less than 100 mmHg in a slow stream of dried air.

Nitrogenous fractions

Nitrates were determined by the method described by Kamm, McKeown & Smith (1965).

The Kjeldahl method was used on both the 10% trichloroacetic acid precipitates and centrifugates. The digests were diluted to 100 ml from which 10-ml aliquots were taken to determine nitrogen using a Markhams still. The results were used to express protein nitrogen, non-protein nitrogen and total nitrogen.

Consistency

Tomato paste consistency was determined using the F.I.R.I. Blob Test (F.I.R.I., 1958) as amended by F.I.R.I. (1961). The results were expressed as %, TSS at 58 mm blob diameter.

Mould count

Mould counts were conducted according to procedures by A.O.A.C. (1965).

Results and discussion

Table 1 presents the regression equations of the amount of tin corroded during storage by tomato pastes made from Roma and S. Pierre tomatoes at each of four different stages of maturity. Curvilinear regression presented the best fit during the first months while linear regression was fitted to the data for the remainder of the 19 months' storage period. Tomato paste made from the vine-ripened S. Pierre tomatoes was the exception: linear regression could be fitted throughout the storage period.

TABLE 1. Equations for the regression of corroded tin on storage time for eight tomato pastes

Paste type		Storage period (months)	Equations
Variety	Maturity		
Roma	Orange—red	1–10	$C = 7{\cdot}383 + 76{\cdot}490t - 5{\cdot}064t^2$
		6–19	$C = 247{\cdot}055 + 4{\cdot}960t$
	Red	1–12	$C = 74{\cdot}874 + 38{\cdot}317t - 1{\cdot}476t^2$
		10–19	$C = 257{\cdot}127 + 5{\cdot}748t$
	Canning-ripe	1–10	$C = -12{\cdot}133 + 68{\cdot}018t - 3{\cdot}394t^2$
		7–19	$C = 275{\cdot}812 + 5{\cdot}512t$
	Soft-Ripe	1–10	$C = 21{\cdot}934 + 106{\cdot}967t - 6{\cdot}500t^2$
		8–19	$C = 408{\cdot}860 + 5{\cdot}675t$
Saint Pierre	Orange—red	1–10	$C = 145{\cdot}650 + 56{\cdot}681t - 3{\cdot}140t^2$
		10–19	$C = 353{\cdot}139 + 5{\cdot}513t$
	Red	1–10	$C = 127{\cdot}601 + 49{\cdot}503t - 3{\cdot}030t^2$
		6–19	$C = 272{\cdot}856 + 6{\cdot}417t$
	Canning-ripe	1–7	$C = 83{\cdot}428 + 52{\cdot}928t - 4{\cdot}643t^2$
		4–19	$C = 177{\cdot}990 + 10{\cdot}668t$
	Vine-ripened	1–19	$C = 97{\cdot}302 + 8{\cdot}416t$

C = Corroded tin (oz $\times 10^{-6}$/oz paste). t = Storage time (months).

Corrosion rates calculated from the regression curves for various intervals during storage are presented in Fig. 1. The observed decrease in corrosion rate (Fig. 1) indicates a reduction in the rate of direct attack on tin by de-polarizers or oxidants.

The paste from vine-ripened S. Pierre tomatoes was the exception. With corrosion rates tending to constant values it can be deduced that some of the corrosion compounds are used up in the process of tin corrosion. The diminishing differences in the corrosion rates indicate that initially the various pastes contained these compounds in varying concentrations.

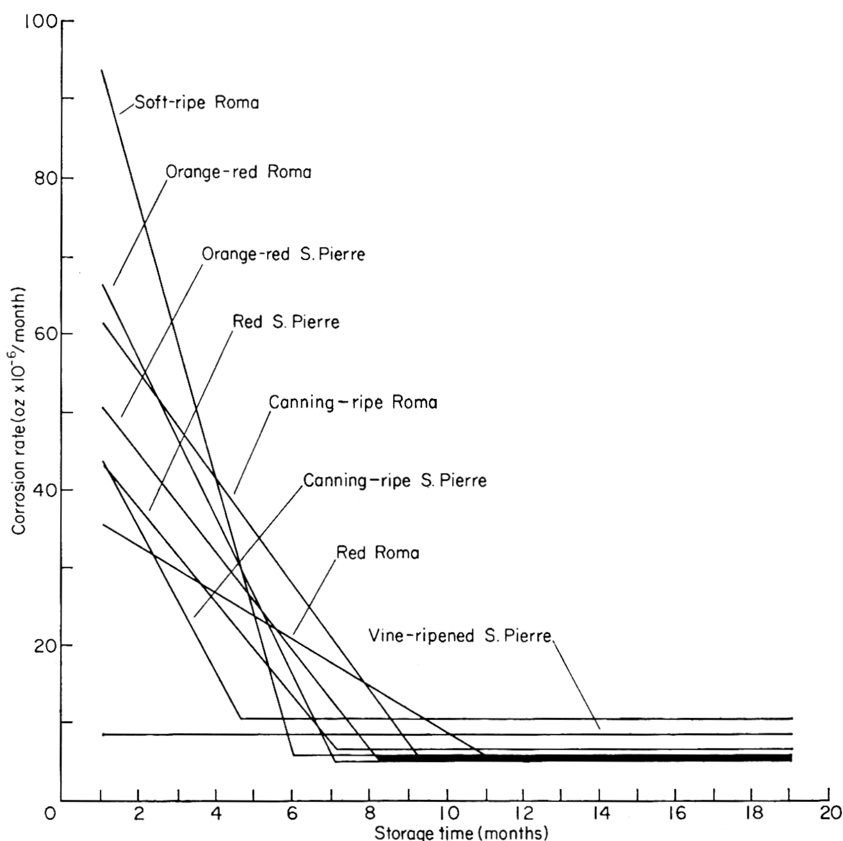

FIG. 1. The tin corrosion rates/oz paste for eight tomato pastes in 308 × 308 plain 1·25 H.D. cans during storage at 71°F.

To test the relationship between the corrosion rate and the possible accelerators, simple correlation coefficients were calculated and evaluated. To allow for the initial interference of residual headspace oxygen (Shiga *et al.*, 1964–65) and to utilize the period of rapid corrosion, corrosion rates at the 3-month storage interval were chosen.

Constituents not related to corrosion rate

Pectinic acid, pectic acid, protopectin, total pectin, galacturonic acid, ascorbic acid, dehydroascorbic acid, diketogulonic acid, pyrrolidonecarboxylic acid (PCA), total

tannins and hydroxymethylfurfural (HMF) were examined by customary methods and were found to be unrelated to corrosion rate.

Good paste quality and sound processing conditions were confirmed by the presence of less than 0·5 ppm HMF and less than 6·63 mEq/100 g PCA in all pastes. With galacturonic acid not exceeding 0·40 g AGA/100 g and with dehydroascorbic acid and diketogulonic acid concentrations below 3·0 and 5·0 expressed as g ascorbic acid/100 g a good shelf life might have been expected (Rice & Pederson, 1954; Villarreal, Luh & Leonard, 1960; Hernandez, 1961; Luh *et al.*, 1964; Davies, 1966).

Acidity and non-volatile inorganic acids

Table 2 indicates the effect of titratable acidity and non-volatile organic acids on the corrosion rate. The corrosion rates of Roma pastes increased with increasing pH and

TABLE 2. Effect of acidity and non volatile organic acids on corrosion rate

Paste type			Titratable	Malic	Citric	
Variety	Maturity	pH	acidity (mEq/100 g)	acid (mEq/100 g)	acid (mEq/100 g)	Corrosion rate*
Roma	Orange-red	4·34	28·7	1·90	28·3	46
	Red	4·24	32·1	2·63	30·4	29
	Canning-ripe	4·32	27·5	1·60	27·8	48
	Soft-ripe	4·39	25·4	2·05	26·1	68
Variety mean		4·32	28·4	2·05	28·2	48
Saint Pierre	Orange-red	3·98	37·8	8·10	39·5	38
	Red	4·01	35·8	8·64	37·1	31
	Canning-ripe	4·06	32·8	7·02	34·3	25
	Vine-ripened	4·18	25·0	5·88	26·5	9
Variety mean		4·06	32·9	7·41	34·3	26
Student's *t*-values for difference between variety means		4·814†	1·432	8·268†	2·081	2·096

		Roma	Saint Pierre
Correlation coefficients ($n = 4$)	pH	0·963‡	− 0·995†
	Titratable acidity	− 0·974‡	0·996†
	Malic acid	− 0·517	0·901
	Citric acid	− 0·989†	0·998†

* Expressed as oz $\times$ 10^{-6} tin/month at 3 months.
† Significant at 1% level.
‡ Significant at 5% level.

were slower at higher titratable acidity and citric acid concentrations. The opposite was found with S. Pierre pastes. These relationships are confirmed by significantly large correlation coefficients (Table 2).

In both varieties titratable acidity and citric acid concentrations decreased during ripening with corresponding increases in pH. These results are in agreement with those discussed by Luh *et al.* (1960), Winsor, Davies & Massey (1962a) and Davies (1966).

Malic acid tended to correlate with corrosion rate in S. Pierre pastes, but not in Roma pastes. Analogous to this, malic acid decreased during ripening of S. Pierre fruit while no definite trend was observed in Roma pastes. Similar anomalies in the corrosion of tinplate by acid foods were reviewed by Brittain (1951) and are not unexpected in corrosion studies.

Roma pastes had a significantly higher mean pH while S. Pierre pastes were significantly higher in malic acid.

Nitrogenous fractions

Table 3 presents the relationships between the different nitrogenous fractions of the final paste, tomato maturity and corrosion rate.

Nitrate in foods is a known de-polarizer in the mechanism of tin corrosion (Culpepper & Moon, 1928; Strodtz & Henry, 1954; Horio, Iwamoto & Oda, 1964–65a, b). The corrosion rate of pastes from both varieties correlated with nitrate content (Table 3). High nitrate levels markedly increased the attack on tin.

The nitrate concentration was lowest in Roma paste made from fruit held for 48 hr. The concentrations in Roma pastes from orange-red and canning-ripe tomatoes were comparable, while that of the paste from soft-ripe fruit was relatively high. In S. Pierre pastes, however, nitrate decreased with advancing maturity of the fruit. It is of interest to note that the paste from vine-ripened S. Pierre fruit contained no nitrate. Although it is outside the scope of this paper to evaluate possible physiological reasons for the change in nitrate content during maturation, mention should be made of the relationship between nitrate concentration and citric acid. The correllation coefficients obtained were $-0{\cdot}900$ and $0{\cdot}973 > P_{0{\cdot}05(2)}$ for Roma and S. Pierre pastes, respectively.

Although the mean nitrate content of Roma pastes was higher, the difference between the means for the pastes from the two varieties was statistically insignificant.

To test the general effect of nitrate on the corrosion rate, the data relating to the tomato varieties were pooled. The correlation coefficient was found to be $0{\cdot}898 > P_{0{\cdot}01(6)}$. The regression equation for the relationship in all pastes was as follows:

$$\text{Corrosion rate} = 11{\cdot}646 + 0{\cdot}869\ (\text{Nitrate}).$$

Corrosion rate of S. Pierre pastes tended to correlate with protein nitrogen. Although it is known that protein additions in model systems can increase the attack on tin (Goss,

TABLE 3. Effect of nitrogenous fractions and nitrate on corrosion rate

Paste type: Variety	Paste type: Maturity	Protein nitrogen (g/100 g)	Non-protein nitrogen (g/100 g)	Total nitrogen (g/100 g)	Nitrate (ppm)	Corrosion rate*
Roma	Orange-red	0·171	0·573	0·743	26	46
	Red	0·160	0·601	0·761	18	29
	Canning-ripe	0·157	0·573	0·730	30	48
	Soft-ripe	0·156	0·627	0·783	65	68
Variety mean		0·161	0·593	0·754	35	48
Saint Pierre	Orange-red	0·126	0·383	0·510	37	38
	Red	0·128	0·380	0·508	27	31
	Canning-ripe	0·121	0·399	0·520	28	25
	Vine-ripened	0·112	0·469	0·581	0	9
Variety mean		0·122	0·408	0·529	23	26
Student's *t*-values for difference between variety means		7·860†	7·690†	10·830†	0·900	2·096

		Roma	Saint Pierre
Correlation coefficients ($n = 4$)	Protein nitrogen	− 0·330	0·922
	Non-protein nitrogen	0·455	− 0·949‡
	Total nitrogen	0·488	− 0·941
	Nitrate	0·945‡	0·968‡

* Expressed as oz $\times$ 10^{-6} tin/month at 3 months.
† Significant at 1% level.
‡ Significant at 5% level.

1917) this apparent effect should be viewed with caution because a lack of correlation was observed with Roma pastes.

Protein nitrogen decreased in the pastes along with ripening of the tomatoes. This was true for pastes from both varieties. Roma pastes had a significantly larger mean protein nitrogen content than S. Pierre pastes.

Non-protein nitrogen and total nitrogen were higher in pastes made from riper fruit, which agrees with the trend reported by Winsor, Davies & Massey (1962b). The increases were progressive. Both of these nitrogenous fractions were significantly higher in pastes from the Roma variety. No definite relation between these fractions and corrosion rate was found.

Tomato paste solids

Table 4 presents the total dissolved solids, total water insoluble solids, total solids and consistency of the various tomato pastes as affected by tomato maturity. The relationships of these properties to corrosion rate are indicated by correlation coefficients.

Total water insoluble solids decreased slightly and then increased in the pastes along with advancing maturity of the Roma tomatoes. A similar pattern was observed in

TABLE 4. Effect of tomato paste solids and consistency on corrosion rate

Paste type: Variety	Paste type: Maturity	Total dissolved solids (g/100 g)	Total water insoluble solids (g/100 g)	Total solids (g/100 g)	Consistency*	Corrosion rate†
Roma	Orange-red	26·05	4·19	30·24	12·3	46
	Red	26·22	3·80	30·02	12·1	29
	Canning-ripe	24·87	4·05	28·92	11·3	48
	Soft-ripe	25·04	4·42	29·46	10·1	68
Variety mean		25·55	4·12	29·66	11·45	48
Saint Pierre	Orange-red	27·09	3·65	30·74	13·3	38
	Red	26·64	3·36	30·00	14·0	31
	Canning-ripe	26·33	3·42	30·75	14·1	25
	Vine-ripened	25·66	2·68	28·34	16·6	9
Variety mean		26·43	3·28	29·96	14·5	26
Student's t-values for differerence between variety means		1·972	3·418‡	0·468	3·474‡	2·096

		Roma	Saint Pierre
Correlation coefficients ($n = 4$)	Total dissolved solids	− 0·726	0·994‡
	Total water insoluble solids	0·955‡	0·962‡
	Total solids	− 0·423	0·863
	Consistency	− 0·858	− 0·974‡

* Expressed as % TSS at 58 mm blob diameter.

† Expressed as oz × 10^{-6} tin/month at 3 months.

‡ Significant at 5% level.

S. Pierre pastes made from fruit ripened off the vine. The vine-ripened S. Pierre tomatoes yielded a paste with a very low total water insoluble solids content, while paste from soft-ripe Roma tomatoes had the highest total water insoluble solids content.

Water insoluble solids correlated with the corrosion rate of pastes from both varieties (Table 4). Pastes with high water insoluble solids had higher corrosion rates. The general relationship between water insoluble solids and corrosion rate of all the pastes was tested by pooling the data of both varieties. The correlation coefficient for the pooled data was $0{\cdot}933 > P_{0{\cdot}01(6)}$.

The influence of water insoluble solids on the direct attack of tin was further investigated by measurement of the polarization resistance and corrosion current of a citrate buffer at pH 4·35 containing varying amounts of washed tomato paste. Table 5 presents the results. These results indicate that the corrosivity of the solution towards tin was substantially increased with increasing amounts of washed tomato paste.

TABLE 5. Effect of washed tomato paste on corrosion of tin in citrate buffer

Washed paste*†	Polarization resistance (KΩ/cm²)	Corrosion current‡ (μA/cm²)
0	77·4	0·12
10	26·0	0·15
20	17·0	0·34
37	11·0	0·43

* Percentage tomato paste pre-washed with distilled water followed by citrate buffer at pH 4·35.

† Paste from orange-red S. Pierre tomatoes was used.

‡ Direct attack on tin based on Tafel slope extrapolated from cathodic curve to rest potential.

It has been shown by Bigelow (1916) that tin is absorbed from solution by the insoluble portion of food products. The role of insolubles in the attack on the baseplate steel of tinplate has been reviewed by Brittain (1951). Insolubles are able to absorb tin, thereby reducing the protection to steel of dissolved tin. The role of water insolubles appears to be analogous when tin is directly attacked by de-polarizers such as nitrate. Dissolved tin is removed from solution and its protective effect on the corrosion of tin is lost (Uhling, 1963). The polarization studies with washed tomato paste added to a citrate buffer seems to confirm this view.

The importance of water insoluble solids is further substantiated by the observation that at comparable nitrate concentrations Roma paste from canning-ripe tomatoes corroded tin at almost twice the rate, at the 3-month interval, of its S. Pierre counter-

part. The only constituent investigated which differed and correlated with corrosion rate was the water insoluble solids. The faster corrosion of tin by nitrate at lower pH values reported by Strodtz & Henry (1954) was apparently negligible in comparison.

Because the tomato pastes were concentrated to a predetermined refractometer reading of 28–30° Brix, total soluble solids and total solids were reasonably constant in all pastes. A casual correlation between total soluble solids and corrosion rate was found in the S. Pierre pastes but no correlation between total solids and corrosion rate was observed.

Consistency

These values are reported in Table 4. The mean consistency of Roma pastes was found to be significantly higher than the mean for S. Pierre pastes. When the data of both varieties were pooled consistency correlated with the quantitative amounts of total water insoluble solids ($r = -0{\cdot}968 > P_{0{\cdot}01(6)}$) and total pectin ($r = -0{\cdot}824 > P_{0{\cdot}05(6)}$). The total pectin concentrations as g AGA/100 g, with advancing maturity were 1·40, 1·21, 1·08 and 1·24 for Roma and 0·97, 0·97, 0·98 and 0·60 for S. Pierre pastes.

These results extend the quantitative role of water insoluble solids reported by Hand *et al.* (1955), Robinson *et al.* (1956) and York *et al.* (1967) and support the effect of pectic substances reviewed by Doesburg (1965).

In general, it would appear that consistency of Roma pastes increased while that of S. Pierre pastes decreased as fruit of these varieties ripened.

Consistency was found to be related to corrosion rate (Table 4). In paste from both varieties thicker pastes had a higher corrosion rate. When the data of both varieties were pooled a correlation coefficient of $-0{\cdot}921 > P_{0{\cdot}05(6)}$ was obtained.

The evaluation of the role of consistency in corrosion was difficult because consistency correlated with water insoluble solids ($r = 0{\cdot}968 > P_{0{\cdot}01(6)}$) and nitrate ($r = -0{\cdot}739 > P_{0{\cdot}05(6)}$) when data of both varieties were pooled. It seems, however, possible to infer that the experimental tomato pastes with high consistencies were more corrosive because such pastes contained larger amounts of water insoluble solids and nitrate.

The combined effect of water insoluble solids and nitrate on corrosion rate

With the data of both varieties pooled, the rate at which tomato pastes corrode tin was dependent on the amount of water insoluble solids and nitrate present. The correlation coefficients between corrosion rate and water insoluble solids and between corrosion rate and nitrate were $0{\cdot}933 > P_{0{\cdot}01(6)}$ and $0{\cdot}898 > P_{0{\cdot}01(6)}$, respectively. To test the joint effect of both independent variables the multiple correlation coefficient was calculated and found to be $0{\cdot}975 > P_{0{\cdot}01(5)}$, an improvement on the previously found simple correlation coefficients.

The F-values summarized in Table 6 indicate that water insoluble solids as well as nitrate contributed significantly to the variation in corrosion rate. The coefficient of determination R^2, indicated that 95·06% of the variation in corrosion rate can be accounted for by water insoluble solids and nitrate content.

The relationship between corrosion rate, water insoluble solids, and nitrate is expressed by the following multiple regression equation:

Corrosion rate = − 45·245 + 18·771 (water insoluble solids) + 0·437 (nitrate).

Because water insoluble solids and nitrate correlated ($r = 0{\cdot}764 > P_{0{\cdot}05(6)}$) in the pooled data, the relative importance of each of these two independent variables needs examining. This can be done by keeping one of the two independent variables constant

TABLE 6. Analysis of variance for multiple regression of corrosion rate on water insoluble solids and nitrate (fitting total water insoluble first)

Source of variation	Degrees of freedom	Mean square	F
Due to b_0	1	10,804·50	
Due to b_1 1 b_0	1	1,895·69	87·549†
Due to b_2 1 b_0, b_1	1	187·54	8·661*
Residual	5	21·65	
Total	8		

* Significant at 5% level.
† Significant at 1% level.

and testing the effect of the other on corrosion rate. To avoid model systems and their pitfalls, the relative effects of the two independent variables were examined by selecting suitable pastes from the eight pastes under investigation. Two groups in which one of the two respective variables was reasonably constant were formed. For approximately constant nitrate values pastes from orange-red and canning-ripe Roma were grouped with pastes from orange-red, red and canning-ripe S. Pierre. A group with reasonably constant water insolubles was formed by pooling the data of pastes from orange-red, red and canning-ripe S. Pierre and red Roma tomatoes. With nitrate values reasonably constant, correlation was found between water insoluble solids and corrosion rate ($r = 0{\cdot}931 > P_{0{\cdot}05(3)}$) but lack of correlation between nitrate and water insolubles. When water insolubles were held reasonably constant no correlation between any of the three variables existed.

From these statistical results it can be inferred that the water insoluble solids of tomato paste has the greater contribution towards accounting for the variation in corrosion rate. It also suggests that the correlation between water insoluble solids and nitrate observed earlier is perhaps casual.

Conclusions

The data presented indicate that depending on maturity and variety, 1 oz of tomato paste processed using sound factory procedures, undamaged by heat and stored at a relatively low temperature of 71°F could corrode tin at rates from as little as 9×10^{-6} oz/month to as much as 68×10^{-6}oz/month, 3 months after canning. If canned in 603 × 700 and 611 × 912 plain 1·25 H.D. cans, the useful shelf life of these pastes could vary from the 24 months obtained in earlier years to as little as 3 months.*

The effects of maturity and variety can be attributed to related differences in water insoluble solids and nitrate. These two constituents accounted for 95·06% of the variation in corrosion rate at the 3-month storage interval.

It seems, therefore, justifiable to conclude that the sudden outbreak of early hydrogen swelling was a result of industry substituting the round varieties for the Roma tomato of the same colour grading in order to meet demands for paste of improved colour and thicker consistency.

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* By calculating the quantity of tin on the inside of a can and substitution into the appropriate regression equation in Table 1, shelf life estimates could be made for various can types.

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The production of brined cauliflower for the pickle industry

K. G. ANDERSON

Summary. The production of brined cauliflower for the pickle industry is reviewed, consideration being given to the raw material, preparation and brining method. Some pitfalls and causes of softening and discoloration are discussed and the necessity of adequate agitation to prevent stratification during the early part of brining is emphasized.

The possible use of sulphur dioxide in brined cauliflower is briefly reviewed.

Introduction

This paper attempts to provide a guide to the principles involved in the production of brined cauliflower for the pickle industry.

For a considerable time most of the cauliflower used by British pickle manufacturers has come from Holland, the trade having originated in the Rynsburg area and subsequently developed in North Holland where a product of superior quality could be obtained. The remainder was produced in England where renewed interest in brining of cauliflower has occurred in recent years, perhaps because of the entry of other countries into the market, some supplies having recently come notably from Italy and Canada. Trials are also being carried out in Spain and Eire with a view to subsequent commercial exploitation.

Both the true cauliflower and to a lesser extent broccoli (winter cauliflower) are used, but the name broccoli is virtually unknown in the pickle industry and the two types may here be regarded as synonymous. Cauliflower is harvested during summer and autumn until the hard frosts come, while broccoli is more hardy, withstands frost, and is hence available in spring and early summer. The cultivation of cauliflower is well covered by a Ministry of Agriculture, Fisheries and Food Bulletin (1964). This publication covers a wide field including choice of variety for time of harvesting, packaging methods, pests and diseases.

The manufacture of a satisfactory brined product is not difficult, and is well within the capacity of those prepared to take a little trouble in the early stages.

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Raw materials

The pickle manufacturer requires a head of brined cauliflower which is hard in texture, white in colour, and is, to use a trade expression, 'good and tight'. The terms 'tight' and 'loose' are used to describe what is really the relative proportion of stalk to curd. Ideally, the stalks should be as short as possible, this producing a head which is hard to break apart into smaller segments. In a 'loose' head, excessive stalk growth has occurred, and the smaller segments do not hold together well.

To achieve these qualities in the brined product, the same characteristics must be present in the cut flowers. Harvesting at the right time is essential, and such factors as frost, rain and exposure to sunlight, which can cause curd discoloration, must be avoided. It is, for example, common practice to break a leaf over an unharvested plant to protect the curd from the light and perhaps frost. Leaves must be left on the plant to afford protection from bruising during transport to the brinery.

Trimming and grading

Leaves and surplus stalk are removed from the head, as much stalk as possible being removed without causing the heads to fall apart. Heads may be graded either at this stage or after the initial brining process. Alternatively they may be roughly graded at trimming and graded more selectively during subsequent processing.

The grades may be described in various ways, such as 'best', 'No. 1', 'No. 2', etc., but there are, in fact, only three common grades. These are the normal good quality head usually known as 'No. 1' or 'best', a slightly poorer grade with heads of a looser texture is known as 'No. 2' or 'seconds' and a very good grade with heads which are particularly firm, white and uniform, and which is usually called 'extra selected'.

There is some demand for specially cut or diced cauliflower and even for stalk and stump. This provides an outlet for heads damaged only in part, e.g. by a fork or partially discolored or bruised. Before embarking on manufacture of this type of product, however, it is most necessary to consult the potential customer since pickle manufacturers often specify a particular cut or dice.

If the supply at harvesting is too great, careful storage of the heads will be necessary, since cauliflower remains in good condition only for a relatively short time. Storage was investigated by Smith (1952) at the Ditton Laboratory, who showed the necessity for refrigeration. Generally speaking, however, the sooner cauliflower is brined after harvesting the better, this being especially important in warm weather.

Brining

The initial treatment is known as 'shrinking' after which, in the brining proper, full fermentation occurs. In the shrinking process cauliflower are put into brine for a short period, during which, although volume change is negligible, the cauliflower becomes more pliant so that 50–90% more can be packed in the same volume. Thus more

vegetable per container can be packed. It is at this stage that the necessary weights are recorded.

Shrinkage is normally carried out in vats or in casks, the vegetables being held in the shrinking brine for at least 24 hr, and not more than 48 hr, after which they are packed into the final containers, covered with brine, and allowed to ferment naturally.

The suitability of brines for cauliflower has been studied by Morpeth (1952), and although there appears to be a considerable latitude it is generally recommended that a 50° salinometer brine (i.e. 50% saturation) is satisfactory for both shrinking and storage.

Morpeth (1951) has also shown that stratification occurs unless the brine is agitated during the process, and Dakin (1961) has more recently emphasized this in his study of freshening, a reversal of the brining procedure in which salt is removed before processing. A considerable degree of agitation is vital during the early stages of brining to prevent a gradient in the brine; this produces a weak brine at the top of the vessel, while at the bottom it is almost as strong as the original brine. The barrels or drums are usually agitated by rolling them at least once a day for the first 7–10 days; if other containers are used it is necessary to employ some form of mechanical agitation.

Gaseous fermentation proceeds for 6–8 weeks, depending on ambient temperature; during this time it is necessary to vent the container and also to top up with brine at regular intervals. After the fermentation process is completed the containers are finally topped up, sealed, and are ready for despatch.

Softening

Although casks of cauliflower which have become much softened are rare, casks which have softened to a lesser extent are, unfortunately, more common. Softening has been investigated by Dakin & Milton (1964) and has been found to be due to the enzyme polygalacturonase (PG). PG destroys pectin in plant tissues, pectin being virtually the cement holding individual plant cells together. It was established that the PG originated from mould growing in empty barrels which had not been thoroughly cleaned. The PG can be absorbed by the wood to be later released slowly into the contents. Barrels should, therefore, not be allowed to remain dirty or empty and after several soakings in water should be thoroughly cleaned. When this is not possible they should be well steamed before use. Other causes of softening are excessive dwell times without agitation in the shrinking tank, and the use of diseased raw material.

Discoloration

Discoloration is one of the most frequent causes of complaint, and several investigations into this have been reported (Morpeth, 1952; Strachen, 1960; Chandler, 1964). Pink discoloration is caused by the use of acidified brine; some apparent discoloration results, of course, from the use of cauliflower with an initially unsatisfactory colour.

Discoloration most usually encountered by the writer is, however, the darkening attributable to iron contamination, and every possible precaution should be taken to prevent this.

Some mention should be made of choice of containers. Chestnut casks are particularly unsuitable (B.F.M.I.R.A., 1954) as the wood contains tannins and tannic acid which will combine with the iron naturally present in the vegetables to give a dark blue or black colour. If chestnut casks must be used, they should be coated internally with wax to give some protection or alternatively the whole contents may be enclosed in a large sealed polythene bag. In this latter case it will also be necessary to put brine around the bag to prevent the wood from drying out. Needless to say any casks which have previously contained some strongly coloured product are quite unsuitable.

Plastic drums with wide screw-on caps are now beginning to replace the traditional wooden casks for brined vegetables. These are very suitable, having the advantages of ease of cleaning, freedom from leakage, and not requiring the services of a skilled cooper.

Sulphur dioxide

The effects of sulphur dioxide on pickles and sauces have been considered by Dakin (1963); there is no doubt that this preservative can often be beneficially employed. The Preservatives in Food Regulations 1962 now permit the use of sulphur dioxide up to 100 parts per million (ppm) in pickles and sauces, and also allow its use in ingredients to a higher level provided that the necessary declaration is made to the importer or buyers.

Sulphur dioxide is of value in preserving white colour of brined cauliflower, but before it is used the requirements of the user must be ascertained and care must be taken to conform with the Regulations.

The time of addition of sulphur dioxide is important and it is the opinion of the writer that it should be added only after fermentation is complete, since earlier addition may retard fermentation undesirably. It may also mask defects such as iron contamination which may come to light during the pickle manufacturer's processing.

Experiments carried out by the writer on sulphiting of English grown cauliflower and observations on sulphited imported cauliflower and the removal of sulphur dioxide during processing, have shown that for this company's requirements optimum results are obtained from the addition of 50–100 ppm sulphur dioxide after fermentation. This quantity seems to achieve the best results without excessively masking defects and yet is not so large as to render its removal a serious problem during later processing. It has been shown that fermentation can be inhibited by quantities in excess of 50 ppm when sulphur dioxide is incorporated before full fermentation is complete.

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The phenolic compounds of blackcurrant juice and their protective effect on ascorbic acid

I. The characterization and estimation of phenolic compounds in blackcurrant juice

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Summary. The anthocyanins, flavonols and other phenolic compounds present in blackcurrant juice have been characterized and estimated using column, paper and thin-layer chromatography, and absorption spectrophotometry. New minor phenolic compounds isolated and identified include 5-methyl quercetin, quercitrin, dactylifric acid and *p*-coumaric acid. The presence of previously reported phenolic compounds in blackcurrant juice has been confirmed. A quantitative assessment of anthocyanin and other phenolic substances has been made.

Introduction

The anthocyanins of blackcurrants were first investigated by Robinson & Robinson (1931) who suggested the presence of a cyanidin-3-bioside and, possibly, delphinidin. Subsequently, the occurrence of cyanidin and delphinidin and their glycosides was confirmed (Chandler & Harper, 1958; Mehlitz & Matzick, 1958; Reichel & Reichwald, 1960) and the glycosides conclusively identified as cyanidin-3-rhamnoglucoside, cyanidin-3-glucoside, delphinidin-3-rhamnoglucoside and delphinidin-3-glucoside (Chandler & Harper, 1962).

Pollard (1942) noted that blackcurrants were rich in flavonoids. These pigments, and other polyphenolic substances, were examined by Kajanne & Sten (1958), Ayres, Charley & Swindells (1961) and Samarodova-Bianki (1965), all of whom identified kaempferol, quercetin and myricetin. Williams, Ice & Wender (1952) had previously isolated iso-quercitrin and quercetin.

Only a few divergent figures have been reported for the concentration of flavonoid substances in blackcurrants and their expressed juices. These values may be expected to vary with the variety of fruit, time of season and country of origin. A commercial black-

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currant juice concentrate was selected for this investigation of the qualitative and quantitative flavonoid composition of blackcurrants.

Experimental and results

Isolation and identification of anthocyanins

Blackcurrant juice (1 litre), reconstituted from a 5 : 1 concentrate, was saturated with salt and exhaustively extracted with 400-ml volumes of n-butanol. The combined extracts were reduced to 15 ml by evaporation under vacuum and dissolved in 40 ml anhydrous methanol. The methanolic solution was added to 120 ml anhydrous diethyl ether to precipitate the anthocyanins. The precipitate was dissolved in methanol and re-precipitated with ether a further three times. The final precipitate was dried over P_2O_5 at 37°C and stored in a sealed glass ampoule.

The precipitated anthocyanins were dissolved in the minimum volume of the upper phase of a n-butanol–2 N-HCl (1 : 1 v/v) solvent mixture and subjected to preparative column chromatography (Chandler & Harper, 1958). Six coloured fractions were collected and concentrated under vacuum. The fractions were taken up in 10 ml methanol and aliquots applied as a thin streak to plates coated with Kieselgel G for preparative thin-layer chromatography (PTLC) (Morton, 1967). The plates were developed in ethyl acetate–ethyl methyl ketone–formic acid–water (6 : 3 : 1 : 1 by volume) and the anthocyanin bands were eluted with acidified methanol (CH_3OH : 0·5 N-HCl, 4 : 1 v/v). The separated anthocyanins were concentrated under a stream of nitrogen.

Identification of the purified pigments was achieved by paper chromatography (Harborne, 1958) using known compounds as reference materials, acid and alkali hydrolysis (Chandler & Harper, 1962), visible, ultraviolet and infrared absorption spectrophotometry (Hsia, Luh & Chichester, 1965) and by alkaline fusion. In the latter case, the phenolic compounds were identified using thin layer chromatography (TLC) on Kieselgel G plates developed in toluene–ethyl formate–formic acid (5 : 3 : 1 by volume), examining the dried plates under ultraviolet light and spraying with the following reagents:

(a) Bartons reagent (Barton, Evans & Gardner, 1952).
(b) Vanillin reagent (Rohan & Connell, 1964).
(c) Toluene *p*-sulphonic acid (Roux, 1957).
(d) Sodium borohydride (Horowitz, 1957).
(e) Sodium carbonate.
(f) Bis-diazotized benzidene (Pictet & Brandenberger, 1960).
(g) Aluminium chloride (Gage & Wender, 1950).
(h) Sodium acetate (Jurd, 1962).
(i) Höpfner reagent (Walker, 1962).

The sugars, liberated by acid hydrolysis, were examined by TLC on sulphite impregnated Kieselgel G plates (Adachi, 1965).

Quantitative determination of anthocyanins

Cyanidin-3-glucoside and delphinidin-3-glucoside isolated from blackcurrant juice by column chromatography and purified by PTLC were dissolved in acidified methanol and diluted so as to give solutions spanning the range 2–10 μg/ml. The change in optical density at 525 mμ, before and after the addition of hydrogen peroxide, was determined (Swain & Hillis, 1959). A standard curve was prepared also for the total unfractionated anthocyanins in the n-butanol extract of blackcurrant juice. Using these curves the concentration of the two glycosides of cyanidin and of delphinidin was determined after quantitative chromatographic separation and elution; the concentration of the two aglycones was assessed by difference from the total anthocyanin content and the determined glycosides, the values are given in Table 1.

TABLE 1. The distribution and identification of anthocyanins in blackcurrant juice

Fraction	Colour in visible light	Identification	Concentration of anthocyanins (mg/100ml)
		Total anthocyanins	145·0
A	Red	Cyanidin } by difference	17·0
B	Purple	Delphinidin } by difference	
C	Red	Cyanidin-3-glucoside	12·0
D	Red	Cyanidin-3-rhamnoglucoside	33·0
E	Purple	Delphinidin-3-rhamnoglucoside	21·3
F	Purple	Delphinidin-3-glucoside	61·8

Isolation and identification of phenolic compounds exclusive of anthocyanins

Blackcurrant juice (1·5 litres) was saturated with salt and extracted with 4·5 litres ether. The residue was then extracted with 4·5 litres ethyl acetate. The ether extract was concentrated to dryness under vacuum and dissolved in boiling 6% acetic acid, the residue was designated Fraction 1 and the aqueous solution Fraction 2. The ethyl acetate extract was concentrated under vacuum and treated with chloroform to give a residue, Fraction 3, and a chloroform solution, Fraction 4.

The fractions were investigated by ultraviolet and visible absorption spectrophotometry, paper chromatography (Seikel, 1964) and TLC using the following plates and solvent system:

(i) Polyamide plates with the solvent system water–ethanol–ethyl methyl ketone–acetylacetone (13 : 3 : 3 : 1 by volume) (Egger, 1961).

(ii) Kieselgel G plates with the solvent system toluene–ethyl formate–formic acid (5 : 4 : 1 by volume).

(iii) Kieselgel G plates with the solvent system ethyl acetate–ethyl methyl ketone–formic acid–water (5 : 3 : 1 : 1 by volume) (Stahl & Schorn, 1965).

The spray reagents listed above were an aid to classification.

Fraction 1, a greenish yellow residue, when subjected to TLC (ii) resolved three spots Nos. 1, 2 and 3 which were found to be the flavonols kaempferol, quercetin and myricetin, respectively.

Fraction 2, subjected to TLC (i), also resolved three spots Nos. 4, 5 and 6. Spot 4 was shown to correspond to rutin. Subsequent elution of spot 5 and re-chromatographing according to TLC (iii) separated two spots which were identified as quercitrin and iso-quercitrin. Spot 6, when eluted, gave no spectral change with aluminium chloride but a bathochromic shift with sodium acetate was recorded. This information suggested that the material had a blocked 5–OH group and a free 7–OH group and was probably a flavonol methyl ether. The 5-methyl ethers of quercetin, kaempferol and myricetin were prepared (Simpson & Beton, 1954) and spot 6 was found to have the same properties as the 5-methyl ether of quercetin.

Fraction 3 was subjected to TLC (iii). Two poorly defined zones near the baseline, spots 7 and 7a, were treated with the diagnostic sprays and their ultraviolet absorption spectra investigated. These spots, and baseline material, were not fully characterized but it is suggested that they contained polymerized phenols.

Fraction 4 was characterized by a large number of pale blue fluorescent spots, numbered 8–19, when chromatographed by TLC (ii) and examined under ultraviolet light. The majority of these spots were identified by comparison with authentic compounds and by the use of spray reagents. The findings are given in Table 2. Spot 13 was present in sufficient quantity to elute and to establish by absorption spectrophotometry and paper chromatography as *p*-coumaric acid. Spots 17 and 17a were examined also in detail; their spray response and ultraviolet absorption spectra were similar to chlorogenic acid but differed in chromatographic behaviour. Maier & Metzler (1965) reported a new class of substances, namely, caffeoyl-shikimic acid esters which are similar to the chlorogenic acid group. Samples of *cis*- and *trans*-dactylifric acid were obtained and all treatments showed them to be the same as spots 17 and 17a, respectively. Spots 18 and 19 had the same chemical and physical properties as *cis*- and *trans*-chlorogenic acid.

Quantitative determination of phenolic compounds exclusive of anthocyanins

Blackcurrant juice (600 ml) was extracted with three 200-ml volumes of ethyl acetate and the extract was washed with saturated salt solution. The extract was evaporated to dryness under vacuum, dried over P_2O_5 at 37°C and weighed. A known amount was applied to a Kieselgel G PTLC plate and the phenolic substances separated by multiple development in solvent (ii). The phenolic substances were located under ultraviolet light, eluted with ethanol, bulked according to their classification and made up to 10 ml with water. Each group was reacted with Folin–Denis reagent and the optical density measured at 725 mμ (Swain & Hillis, 1959). The results are summarized in Table 3.

TABLE 2. Thin layer chromatographic separation of ethyl acetate extract (Fraction 4) of blackcurrant juice

Spot No.	Identification	Colour developed				
		Aluminium chloride	Ammonia	Bis-diazotized benzidene	Hopfner	Vanillin
8	Genistic acid	Y	B	Br	G O	None
9	Syringic acid	B Gr	B	Br	G O	None
10	Flavone?	Y Gr	Pale Y	None	None	P
11	*cis*-Caffeic acid	Pu	B	R	O	Y
11a	Caffeic acid?	Pu	B	R	O	Y
12	Protocatechuic acid	Pale B	Pale B	G	G O	None
13	*p*-Coumaric acid	Pale P	B	O	Br	Y
14	Cinnamic acid	Gr Y	B Gr	None	None	Y
15a, b, c, d	Cinnamic acid esters?	Pale B	Faint Gr	Br	Y	Y
16	Flavonol glycosides	Y Gr	Y	Not tested	Not tested	Not tested
17, 17a	Dactylifric acids	B	Gr	Br	Y	Y
18	*cis*-Chlorogenic acid	Pale B	Gr	Faint Br	Y	Y
19	*trans*-Chlorogenic acid	Pale B	Gr	Y Br	Y	Y

B, Blue; Br, brown; G, grey; Gr, green; O, orange; P, pink; Pu, purple; R, red; Y, yellow.

TABLE 3. Approximate distribution of phenolic compounds in blackcurrant juice, exclusive of anthocyanins

Fraction class of ethyl acetate extract	mg/100 ml
Benzoic acid derivatives	10
Cinnamic acid derivatives	15
Flavonol glycosides	15
Flavonol aglycones	
(a) myricetin	3
(b) quercetin	3
(c) kaempferol	3
Condensed polyphenolics and other material	20

Discussion

The identified anthocyanins from the commercial blackcurrant juice were the same as those found by Chandler & Harper (1962). Timberlake (1960) reported a value of 720 mg/100 ml for the anthocyanin content of blackcurrant juice but the present finding of 145 mg/100 ml was closer to the 76 mg/100 ml recorded by Gleisberg & Aumann (1958). The anthocyanin glycosides were found to account for 88% by weight of the total anthocyanin content; cyanidin-3-rhamnoglucoside was the predominant cyanidin glycoside whereas delphinidin-3-glucoside was the major delphinidin glycoside.

Flavonol aglycones occurred mainly in Fraction 1 and flavonol glycosides in Fraction 2 of the ether extract of blackcurrant juice. With TLC on polyamide plates the flavonol glycosides separated according to their glycosidation pattern. The presence of the aglycones kaempferol, quercetin and myricetin and the glycosides rutin and iso-quercetin was confirmed. Although Williams *et al.* (1952) isolated iso-quercitrin they did not report finding quercitrin which was identified in the present investigation.

The presence of cinnamic acid derivatives, in particular the dactylifric acids not previously observed in blackcurrant juice, was of interest in view of the recent attention to these substances as possible antioxidants (Pratt, 1965; Thewlis, 1967). Also, *p*-coumaric acid had not been reported in blackcurrants previously.

The 5-methyl ether of quercetin was identified but, although methylated flavonols are widely distributed in plant tissue, the corresponding derivatives of myricetin and kaempferol were not found. Similarly, no identification of glycosides of kaempferol and myricetin was made.

The quantitative estimation of phenolic compounds, exclusive of anthocyanins, should be taken as an approximate guide. Kajanne & Sten (1958) reported a value of 44 mg flavonols/100 g for blackcurrant skins which contain a higher proportion than pulp or juice (Bacharach & Coates, 1944), and Gleisberg & Aumann (1958) gave an average figure of 4·5 mg/100 ml juice. In the present study, the phenolic substances in blackcurrant juice were found to be approximately 69 mg/100 ml of which 24 mg were flavonols.

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The phenolic compounds of blackcurrant juice and their protective effect on ascorbic acid

II. The stability of ascorbic acid in model systems containing some of the phenolic compounds associated with blackcurrant juice

K. MARY CLEGG AND A. D. MORTON*

Summary. Model systems of ascorbic acid in citrate buffer have been used to investigate the antioxidant effect of flavonols, phenolic acids, anthocyanins and extracts of blackcurrant juice for ascorbic acid. In the presence and absence of added copper salts the flavonol aglycones, and quercetin in particular, gave positive protection to the ascorbic acid, but the anthocyanins showed no antioxidant properties except in the presence of copper. The stability of ascorbic acid in blackcurrant juice has been discussed.

Introduction

The stability of ascorbic acid in fruit and vegetable products is known to vary and several naturally occurring flavonoid substances have been reported to be protective (Davidek, 1960; Samarodova-Bianki, 1965).

The oxidation of ascorbic acid is catalysed by metallic ions, especially Cu^{++}, and any protective effect is generally attributed to the metal chelating properties of the factor than to antioxidant characteristics. The ascorbic acid content of blackcurrants is particularly stable (Hooper & Ayres, 1950) and this fruit contains a wide range of anthocyanins, flavonols and other phenolic substances (Morton, 1968). Flavonols have strong antioxidant properties for lipid materials (Crawford, Sinhuber & Aft, 1961; Letan, 1966) and the present study has been undertaken to investigate any protective effect by these naturally occurring pigments for the oxidation of ascorbic acid in an aqueous medium. Model systems, with and without the addition of copper salts, have been used to test the stability of ascorbic acid in the presence of extracts of blackcurrant juice, and of pure compounds which have been shown to occur in this product.

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Experimental

Determination of ascorbic acid

The method of Evered (1960) involving titration with *N*-bromosuccinimide, which is selective for the oxidation of ascorbic acid, was chosen. This procedure overcomes interference from pigments in blackcurrant juice which limits the use of 2 : 6-dichlorophenolindophenol.

Composition of the basic model system

Citric acid and malic acid (approximately 17 : 3 w/w) represent the two main organic acids in blackcurrants (Whiting, 1958). A corresponding 1·5% citrate–malate (w/v) buffer solution, adjusted to pH 2·9, was compared with a similarly neutralized 1·5% citric acid solution for ascorbic acid stability. No difference was detected and the simpler 1·5% citric acid solution, adjusted to pH 2·9 with 10 N-NaOH, was adopted for the basis of the model system.

Design of experiment

Copper contamination was reduced to a minimum by soaking the glassware in concentrated nitric acid for a few hours, followed by repeated rinsing with de-ionized water. From the determined copper contamination of the citric acid, the model system was calculated to have a negligible copper content of the order of 2×10^{-5} ppm. Morin was the only additive where repeated recrystallization from hot 10% acetic acid was necessary to remove trace copper contamination.

In a typical experiment the stability of ascorbic acid in citrate buffer was investigated in four model systems:

(1) 1·5% citrate buffer, pH 2·9, + 0·225% ascorbic acid.
(2) 1·5% citrate buffer, pH 2·9, + 0·225% ascorbic acid + 1 ppm Cu^{++}.
(3) As (1) + phenolic additive.
(4) As (2) + phenolic additive.

The phenolic additives were used in varying concentrations depending on the availability of the material. The compounds investigated and their concentrations are listed in Table 1. To aid their dissolution in the model systems the phenolic compounds were first dissolved in the minimum quantity of ethanol, the same small quantity of ethanol was added to the controls.

The model systems were contained in phials of 33 ml capacity closed with plastic cap drilled with two 1-mm diameter holes to provide aerobic conditions (Clegg & Morton, 1965). The phials were incubated at 37°C and one sample from each of the four treatments was withdrawn at intervals for the determination of ascorbic acid. The incubation period lasted 10–17 days; the addition of a preservative, such as thymol, was unnecessary. The development of browning in the model systems during incubation was measured by the increased absorption at 400 mμ (Clegg & Morton, 1965).

TABLE 1. Protection factors for phenolic compounds and phenolic rich extracts of blackcurrant juice in model systems

	Concentration	Protection factor in absence of Cu^{++}	Protection factor in presence of Cu^{++}
Phenolic compounds	M × 10^{-5}		
Kaempferol	8·8	1·32	1·16
Quercetin	8·0	1·60	1·52
Morin	8·0	1·24	1·28
Robinetin	8·0	1·36	1·16
Dihydroquercetin	8·0	1·38	1·18
Quercitrin	8·2	1·28	1·14
Rutin	8·2	1·10	1·02
Robinin	8·0	1·00	1·00
Chlorogenic acid	14·0	1·20	1·08
p-Coumaric acid	14·0	1·20	1·08
Cyanidin-3-rh.gl.†	50·0	0·56	1·10
Cyanidin-3-gl.*	25·0	0·56	1·10
Delphinidin-3-rh.gl.	31·0	0·74	1·10
Delphinidin-3-gl.	119·0	0·77	1·09
Quercetin + Kaempferol	4·0 + 4·0	1·64	1·32
Quercetin + Morin	4·0 + 4·0	1·44	1·32
Quercetin + Cyanidin-3-rh.gl. + Delphinidin-3-gl.	4·0 + 21·0 + 21·0	1·26	1·22
Solvent extracts of blackcurrant juice	mg/100 ml		
Ethyl acetate	500	1·11	1·17
Diethyl ether	500	1·28	1·24
Chloroform	500	1·26	1·28

*gl. = Glucoside.
† rh.gl. = Rhamnoglucoside.

The extent and nature of the decomposition products of the additive after incubation for 96 and 240 hr were investigated by extraction with ethyl acetate, ether and n-amyl alcohol for flavonols, phenolic acids and anthocyanins, respectively. The extracts were subjected to identification techniques outlined in the previous paper (Morton, 1968). The build up of carbonyl compounds in the model systems was followed by thin layer chromatographic separation of the 2 : 4-dinitrophenylhydrazine derivatives.

Calculation of results

Fig. 1 demonstrates the linear response obtained when the log % retention of ascorbic acid was plotted against time for the four model systems.

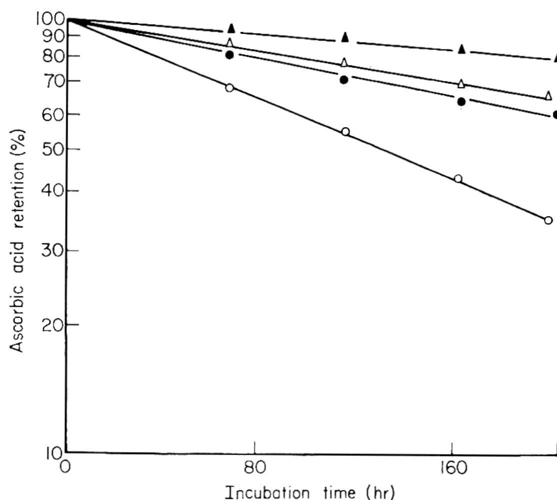

Fig. 1. The retention of ascorbic acid in citrate buffer, pH 2·9, in the presence of Cu^{++} and quercetin, incubated at 37°C. ●, Control; ○, 1 ppm Cu^{++}; ▲, $8{\cdot}0 \times 10^{-5}$ M-quercetin; △, 1 ppm Cu^{++} + $8{\cdot}0 \times 10^{-5}$ M-quercetin.

A method for assessing the protection of the phenolic additives, with and without additional Cu^{++}, was derived as follows:

$$\text{Protection factor} = \frac{\%\ \text{ascorbic acid in system containing additive}}{\%\ \text{ascorbic acid in corresponding control system}}.$$

All measurements were taken at the time when the control system retained 50% ascorbic acid. This calculation utilized the control systems of each experiment and thereby ensured standardization.

From experimental results it was calculated that, at the 95% level of chance, there was no significant difference on the addition of the phenolic substance when the protection factors were in the range 0·97–1·07.

A satisfactory reproducibility of the method was shown by examining the control systems with and without Cu^{++}. In all experiments at the time when the copper-containing control retained 50% ascrobic acid the copper-free model system retained 67–72% ascorbic acid.

Results

The incubated solutions containing chlorogenic acid, quercitrin, rutin, dihydroquercetin and quercetin were solvent extracted and the extracts were chromatographed. Decomposition was noted with only the first two additives. Approximately 25%

chlorogenic acid was degraded to caffeic acid and quinic acid, and approximately 50% quercitrin was hydrolysed to yield quercetin, after 10 days incubation at 37°C.

Anthocyanins as additives to the model systems showed most degradation. After 100–136 hr incubation no characteristic pigmentation remained and a red-brown precipitate developed on further incubation. The precipitate was partially soluble in n-amyl alcohol and chromatographic treatment demonstrated the presence of phloroglucinol and other fluorescent material.

Analysis of the dinitrophenylhydrazine derivates of the carbonyl compounds which developed during incubation in all of the systems showed the same pattern as found in model systems simulating lemon juice (Clegg & Morton, 1965). Similarly, the degree of browning, irrespective of the presence of Cu^{++} and/or pure flavonols or their glycosides, was directly proportional to the oxidation of ascorbic acid and provided a useful confirmation for the ascorbic acid determinations. However, this relationship no longer held on the addition of pure anthocyanins, the phenolic substances separated by cellulose column chromatography from a n-butanol extract of blackcurrant juice, or an ether or ethyl acetate extract of blackcurrant juice. The anthocyanins were degraded to brown pigments and the model systems containing chlorogenic acid gave more browning than would be expected from the loss of ascorbic acid, due to the liberation of caffeic acid. This phenolic acid was shown to contribute to the development of a brown colour in model systems.

The protection factors of pure samples of five flavonol aglycones and three flavonol glycosides, some of which are known to occur in blackcurrants, were compared (Table 1). All the aglycones demonstrated a protective effect for ascorbic acid, with and without the addition of Cu^{++}, quercetin being the most powerful. Quercitrin was the only flavonol glycoside which gave a positive protection factor, rutin and robinin were without effect.

The protection factors of the phenolic acids, such as chlorogenic acid and *p*-coumaric acid, were not as high as for the flavonols. The solvent extracts of blackcurrant juice were rich in phenolic acids, and, on addition to model systems, gave a degree of protection for ascorbic acid in the absence of copper similar to that of inidvidual phenolic acids (Table 1).

The effect of anthocyanins was complex. In the absence of copper, anthocyanin glycosides accelerated the oxidation of ascorbic acid whereas a protective effect was detectable in the presence of copper. The pigments degraded slightly faster in the copper-containing systems.

A mixture of quercetin and kaempferol, at the same concentrations as found in blackcurrant juice, gave a slightly higher protection factor than for either flavonol singly. The solvent extracts of blackcurrant juice, and a combination of quercetin and anthocyanin glycosides, were the additives which showed approximately equal protection in the presence and absence of additional Cu^{++} (Table 1). The majority of flavonols and phenolic acids on their own were relatively less effective in the presence

of copper. In the systems containing quercetin and anthocyanin glycosides not only was the ascorbic acid protected but the degradation of the anthocyanins was slowed down compared with anthocyanins as the sole additive.

Discussion

Citric acid is known to form a stable complex with trace copper (Parry & Du Bois, 1952). Therefore, the stability of ascorbic acid in the citrate buffer should be at a maximum due to the unavailability of the catalytic copper ions. Despite this chelation, the oxidation of ascorbic acid proceeded in citrate buffer as demonstrated in this investigation and by other workers (Joslyn & Miller, 1949; Timberlake, 1960). Furthermore, the flavonols have been shown to retard the oxidation of ascorbic acid in the presence and absence of additional copper. Because of the low concentration of flavonols involved it is unlikely that the dissociation constants of the copper–citrate complex allowed the flavonols to extract copper preferentially from the large excess of citrate. The increased stability of ascorbic acid in the presence of flavonols cannot be explained by the substitution of one copper-chelating agent for another. It is noteworthy that dihydroquercetin, which has no metal complexing ability (Letan, 1966), was as efficient as the other aglycones (except quercetin) in protecting ascorbic acid.

The chromone ring of kaempferol, quercetin and morin is identical and the molecules differ only in their hydroxylation pattern in the phenol ring. The *ortho*-hydroxylation of quercetin was the most efficient for the protection of ascorbic acid and a similar finding has been reported in the use of flavonoids for antioxidants in fats (Mehta & Seshadri, 1958). A combination of quercetin and kaempferol, two flavonol aglycones known to occur in blackcurrants, further increased the stability of ascorbic acid in the absence of copper. Unfortunately, a sample of myricetin, the third aglycone identified in blackcurrant juice, was not available. However, robinetin has the same trihydroxylation of the phenol ring as myricetin and differs only in the absence of a hydroxyl group in the C – 5 position of the chromone ring. In view of the good protection factor obtained for robinetin, myricetin may be expected to make a similar contribution to the stability of ascorbic acid in blackcurrant juice. Quercetin was the only flavonol glycoside found to hydrolyse and liberate quercetin under the experimental conditions and this would account for its higher protection factor compared with the other glycosides.

The natural stability of ascorbic acid in lemon juice and blackcurrant juice was compared with similar ascorbic acid–citric acid model systems; commercial lemon juice was fortified with ascorbic acid to a level (0·225%) equivalent to that of blackcurrant juice, and citric acid was added to the blackcurrant juice to equate the pH to that of lemon juice (pH 2·5). The lemon juice and blackcurrant juice both had copper contents of approximately 1 ppm but copper was not added to the comparable model systems. The results given in Table 2 show that, with and without additional Cu^{++}, the blackcurrant juice contained protective agents for ascorbic acid whereas in the natural

TABLE 2. The stabilization of ascorbic acid in lemon juice with various additives

	Concentration		Protection factor in absence of Cu^{++}	Protection factor in presence of Cu^{++}
	mg/100 ml	$M \times 10^{-5}$		
Blackcurrant juice*	–	–	1·24	1·56
Lemon juice*	–	–	0·88	1·16
+ Quercetin†	–	8·00	1·16	1·18
+ Quercetin }† + Tween 80 }	– / 300	8·00 / –	1·10	1·18
+ Ether extract of blackcurrant juice†	500	–	1·14	1·10

* Compared with equivalent model system.
† Compared with untreated lemon juice.

lemon juice the ascorbic acid was oxidized faster than in the comparable model system. These findings provide further evidence of the high stability of ascorbic acid in blackcurrant juice. The possibility of increasing the stability of the ascorbic acid in lemon juice by the addition of quercetin or a solvent extract of blackcurrant juice, whose protective effects have been demonstrated, was investigated and confirmed. Quercetin has the disadvantage of being only slightly soluble in water and in an aqueous solution of 2·5 mg/100 ml, has a tendency to separate. By use of an emulsifying agent, such as Tween 80, the quercetin was held in solution but the protection factor was not augmented by the increased solubility. Thus, for practical purposes no advantage would be gained by seeking a palatable, odour-free, emulsifying agent. From this investigation it would appear that phenolic rich extracts of blackcurrant juice, or pure flavonols, may be added advantageously to less stable natural sources of ascorbic acid. Davidek (1960) made a similar suggestion on the basis that rosehip and blackcurrant flavonoids stabilized ascorbic acid in acetate buffers.

Another aspect of the differing stability of the two fruit juices concerned their flavour. In an organoleptic triangular test with a panel of nineteen untrained tasters, all the members identified the lemon juice which had been incubated for 14 days compared with the non-incubated juice (dye was added to both samples to mask the colour differences). In a similar trial with blackcurrant juice the tasters were unable to differentiate between the incubated and control products. The slower oxidation of ascorbic acid to carbonyl compounds in the blackcurrant juice, because of its high content of phenolic compounds with antioxidant properties, could contribute to this flavour factor.

It is interesting that the stability of blackcurrant juice must be attributed to the

phenolic substances which are present at less than half the concentration of the characteristic anthocyanin pigments (Morton, 1968).

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Factors affecting the heat resistance of *Bacillus stearothermophilus* spores

I. The effect of recovery conditions on colony count of unheated and heated spores

A. M. COOK* AND R. J. GILBERT†

Summary. Maximum colony counts of unheated and heated spores of *Bacillus stearothermophilus* were obtained after incubation at 50–65°C and 45–50°C, respectively. The composition of the plating medium was found to have a marked effect upon recovery of unheated and heated spores. The effects of diluent and incubation time on recovery have also been investigated.

Introduction

Bacillus stearothermophilus is of particular importance to the food processor because the spores of this organism often exhibit a resistance in excess of that upon which the conventional thermal processes for canned foods are based and the vegetative form, which is capable of growth at 65°C, is often responsible for flat sour spoilage of low-acid canned foods. Also, the extreme heat resistance of these spores has found important application in the bacteriological control of heat sterilization processes (Brewer & McLaughlin, 1961; Kelsey, 1961).

The purpose of this and subsequent papers is an attempt to relate the effects of several environmental conditions before, during and after the heating process on the apparent heat resistance of *B. stearothermophilus* spores, and was initiated for its possible theoretical and practical applications in food microbiology.

Materials and methods

Organism and preparation of spore suspension

The organism used was *Bacillus stearothermophilus* (NCIB 8919). Spores were obtained using the medium and method of Cook & Brown (1964), except that the sporulation

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conditions were 7 days at 60°C. Vegetative cells were removed by washing ten times and separating by differential centrifugation using a refrigerated centrifuge. The aqueous suspension ($>$ 99% phase bright spores) was stored at 4°C. No heat shock was used during the preparation of the suspension.

Media and plate counting methods

A spread plate count method was used throughout. Dilutions were prepared in sterile distilled water unless specified and 0·5 ml quantities were plated. Five replicate plates were used at two dilution levels for each count. At low levels of survival the heated spore suspension was washed directly onto the medium without prior dilution.

Viable counts were made on Antibiotic Assay Medium A (AAM) (British Pharmacopoeia, 1963), pH 7·3 with 0·1% (w/v) of added starch unless specified. This medium is referred to as AAMS. Other media examined were Dextrose Tryptone Agar (DTA) (Oxoid Ltd, London, S.E.1), and Shapton Medium (SM) (Oxoid Ltd, London, S.E.1). The use of the following supplements to enhance recovery of unheated and heated spores was examined; soluble starch (Analar grade, B.D.H. Ltd, Poole) 0·1% (w/v), charcoal (Bacteriological quality, Oxoid Ltd, London, S.E.1) 0·2% (w/v), and furfuraldehyde (General Purpose Reagent grade, B.D.H. Ltd, Poole) 1×10^{-4}% (v/v). The plates were incubated for 3 days at 50°C unless specified.

Determination of heat resistance

Six drop (*c.* 0·1 ml) samples of aqueous spore suspension from a calibrated dropping pipette (Cook & Yousef, 1953) were distributed into 5 ml powder ampoules (BSS 795, 1961, type C) and were sealed under air. Ampoules were heated at 115°C by total immersion in a constant temperature oil bath (Gilbert, 1966). Ampoules were removed at appropriate time intervals, immediately cooled in water to $<$ 25°C and the contents quantitatively washed out, diluted and plated. Heating times were calculated from the time the ampoules were immersed in the heating bath.

Statistical analyses

Survival curves of log percentage survivors or log colony count/ml against time were constructed, using the mean count from one or two unheated ampoules as 100% or the initial colony count. After the initial heat activation effect, the rate of inactivation was exponential over the survivor range studied since correlation coefficients, obtained by the method of least squares, were always greater than tabulated values at $P = 0{\cdot}05$ for the appropriate degrees of freedom. Pairs of calculated regression lines were compared by a modified *t*-test (Bailey, 1959). Blocks of regression lines were tested for parallelism by an analysis of variance and when parallelism was established a further analysis showed whether a common regression line could be plotted.

The use of decimal reduction (D) values is a convenient method of expressing heat resistance when survivor curves are linear. However, D values do not take into con-

sideration any initial heat activation effects on survivor curves, and care must therefore be exercised in their correct interpretation. In the present studies this has been overcome by quoting two inactivation times—a calculated D value for the exponential section of the curve, and the calculated time to give a 10^4 reduction of the initial (unheated) count (i.e. 99·99% kill).

Results

The effect of incubation temperature

Suitable dilutions from samples of unheated and heated spores were plated out and replicate plates incubated at different temperatures within the range 32–65°C (Table 1). Maximum colony counts from unheated spores were obtained after incubation at 50–65°C. In contrast, maximum colony counts from spores surviving heat treatment were obtained after incubation at 45–50°C. Consequently all plates were routinely incubated at 50°C since slight fluctuations in temperature would have little effect on colony counts.

TABLE 1. The effect of incubation temperature upon colony counts of unheated and heated spores of *Bacillus stearothermophilus*

Time of heating at 115°C (min)	Mean count of five plates: incubation temperature				
	32°C	45°C	50°C	55°C	65°C
0	0	58·8	71·6	74·2	71·8
10	0	86·8	100·8	101·4	98·0
30	0	37·2	42·4	40·0	29·8
60	0	24·2	27·0	23·0	12·0
70	0	12·4	12·2	9·8	6·2

The effect of incubation time

Suitable dilutions from samples of unheated and heated spores were plated out and colony counts made after incubation at 50°C for various intervals of time (Table 2). There appeared to be no evidence for delayed germination of spores, maximum colony formation occurring after 1–3 days incubation. Consequently all plates were routinely incubated for 3 days.

The effect of recovery medium

Viable counts of spores were made before and after heating at 115°C, using different media with added supplements. The results from Experiment 1 (Table 3, Fig. 1) show that colony counts for unheated and heated spores were higher on AAMS, at pH 6·6

TABLE 2. The effect of incubation time upon colony counts of unheated and heated spores of *Bacillus stearothermophilus*

Time of heating at 115°C (min)	Total count of five plates: incubation time		
	1 day	3 days	7 days
0	305	344	344
10	410	414	413
60	107	108	108

or 7·3, than on DTA with or without added supplements. Colony counts for unheated and heated spores were higher on DTA, at pH 7·3 with added starch, than at pH 7·3 with added furfuraldehyde, or at pH 6·9 with or without added charcoal. The results from Experiment 2 (Table 3) show that highest counts were always obtained on AAMS at pH 7·3. Consequently this medium was used for all viable counts on *B. stearothermophilus* spores unless specified.

TABLE 3. The effect of composition and pH of plating medium on unheated *Bacillus stearothermophilus* spores and on their recovery after heating at 115°C

Experiment	Counting medium			Initial count of unheated spores/ml	D (min)	Time to inactivate 10^4 spores (min)
	Medium*	Supplement	pH			
1	AAMS	Starch	7·3	$3·60 \times 10^6$	24·4	122·0
	AAMS	Starch	6·6	$3·27 \times 10^6$	20·5	108·7
	DTA	Starch	7·3	$2·72 \times 10^6$	22·3	108·2
	DTA	Furfuraldehyde	7·3	$1·40 \times 10^6$	17·4	82·1
	DTA	Charcoal	6·9	$1·36 \times 10^6$	17·8	78·0
	DTA	–	6·9	$6·00 \times 10^5$	8·5	43·5
2	AAMS	Starch	7·3	$4·43 \times 10^6$	22·9	120·0
	AAMS	Starch	6·6	$3·89 \times 10^6$	18·9	100·1
	AAM	Charcoal	7·3	$3·82 \times 10^6$	22·8	113·4

* For media, see text.

There were also significant differences in the heat activation responses using the various plating media. Fig. 1 illustrates that there were four to five-fold increases in viable count when AAMS was used compared to one to two-fold increases when DTA, with or without added supplements, was used.

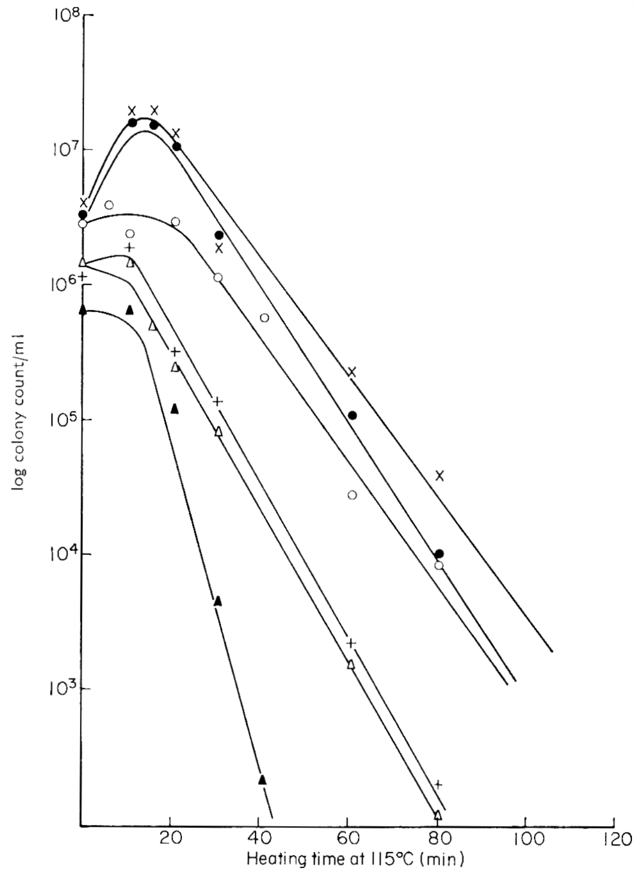

FIG. 1. The effect of composition of the recovery medium on colony counts of unheated and heated spores of *Bacillus stearothermophilus*. ×, AAMS, pH 7·3; ● AAMS, pH 6·6; ○, DTA + 0·1% (w/v) starch, pH 7·3; +, DTA + 0·2% (w/v) charcoal, pH 6·9; △, DTA + 1·0 × 10^{-4}% (w/v) furfuraldehyde, pH 7·3; ▲, DTA, pH 6·9.

Shapton & Hindes (1963) have recommended a new medium for the enumeration of organisms of the *B. stearothermophilus* group and this medium is now commercially available. Suitable dilutions from samples of unheated and heated spores of *B. stearothermophilus* were plated out on AAMS and SM with and without added starch (Table 4). Colony counts were consistently higher on AAMS than on SM. The addition of 0·1% (w/v) starch to SM gave increased colony counts especially after a severe heat treatment. For unheated and heated spores there was no significant difference between colony counts made on AAMS containing 0·1 or 0·5% (w/v) of starch.

The effect of diluent

B. stearothermophilus spores suspended in water were heated at 115°C for different

TABLE 4. The effect of composition of the plating medium upon colony counts of unheated and heated spores of *Bacillus stearothermophilus*

Time of heating at 115°C (min)	Mean count of five plates: counting medium*			
	SM, pH 7·4		AAMS, pH 7·3	
	No starch	0·1% (w/v) starch	0·1% (w/v) starch	0·5% (w/v) starch
0	84·6	107·2	127·8	123·4
10	31·0	35·0	40·2	40·0
60	9·8	53·0	99·6	96·4

* For media, see text.

periods of time, after which various diluents were used for making dilutions and subsequent platings onto the recovery medium. The results are summarized in Table 5.

TABLE 5. The effect of diluent on the recovery of *Bacillus stearothermophilus* spores surviving heat treatment at 115°C

Diluent	D (min)	Time to inactivate 10^4 spores (min)
Water	21·2	110·6
Water + 0·1% (w/v) peptone	20·4	107·9
Water + 1×10^{-4}% (v/v) furfuraldehyde	20·5	103·7
Water + 0·85% (w/v) sodium chloride	16·4	89·1
Ringer's solution (quarter-strength)	18·7	96·4

A test for parallelism indicated that there was no significant difference between the slopes of the exponential sections of the five survivor curves which may therefore be regarded as parallel [calculated variance ratio F^4_{25} = 2·40; tabulated F^4_{25} (Documenta Geigy, 1962) = 2·76 (P = 0·05)]. However, when that curve using sodium chloride solution as diluent was omitted, a closer relationship was obtained [calculated variance ratio F^3_{20} = 1·66, tabulated F^3_{20} = 8·66 (P = 0·05)]. It was felt that the weighting effect of the other four survivor curves was sufficient to render the curve using sodium chloride solution as diluent not significantly different when compared by an analysis of variance of all five curves. This was confirmed since a modified *t*-test between the regression coefficients of survivor curves using water and sodium chloride solution as diluent, showed a significant difference [calculated *t* value = 2·88; tabulated *t* value = 2·29 (7·3 d.f. at P = 0·05)].

Of the various diluents studied water gave the highest recovery of heat-treated spores.

Discussion

Many workers have shown that the growth requirements of bacterial spores surviving heat treatment are more exacting than those of unheated spores: present studies have endorsed this. Little work has been done on the optimal incubation temperature for the recovery of heat-treated spores but such information as is available suggests that a level below the optimal for unheated spores is the most suitable. Williams & Reed (1942) have reported greater recovery of *Clostridium botulinum* spores in the range 24–31°C than at 37°C and Edwards, Busta & Speck (1965) greater recovery of *Bacillus subtilis* spores at 32°C than at 45°C which was the reverse of the results with unheated or slightly heated spores. Gordon & Smith (1949) have shown that of eighty-seven unheated strains of *B. stearothermophilus*, eighty-one grew at 45°C, eighty-seven grew at 50–65°C and forty-five grew at 70°C. The optimal incubation temperature for *B. stearothermophilus* spores surviving heat treatment has not been previously reported.

Results for unheated or heat-activated (10 min at 115°C) *B. stearothermophilus* spores show that maximum colony counts were obtained after incubation at 50–65°C (Table 1). However, for spores surviving a severe heat treatment (60–70 min at 115°C) recovery was greatest after incubation at 45–50°C. The greater count of heated spores at the lower temperatures of incubation may possibly be due to a form of temperature dependent mutant in which specific nutritional requirements appear at the higher temperature but not at the lower ones. In addition, any sensitivity to inhibitors to spore germination, which increases upon heating spores (Olsen & Scott, 1950), may be greater at the higher incubation temperatures.

DTA with or without added supplements is widely used as a suitable medium for detection of 'flat-sour' thermophiles (Hersom & Hulland, 1963) and as plating medium for heat activation and heat resistance studies on *B. stearothermophilus* spores (Molyneux, 1952; Shull & Ernst, 1962; Finley, 1964). The present studies have clearly indicated that AAMS at pH 7·3 is superior to DTA at pH 6·9 or 7·3 with or without added supplements, for colony counts of unheated or heated spores of *B. stearothermophilus* (Fig. 1). Viable counts on these spores after maximum heat activation also varied considerably with the various plating media. Thus, after heating at 115°C, maximum increases in colony counts (from counts on unheated spores) were about five-fold on AAMS at pH 6·6 or 7·3, compared to less than two-fold on DTA at pH 6·9 or 7·3 with or without added supplements. The present results are at variance with the heat activation and heat resistance data for *B. stearothermophilus* spores, where DTA, with or without added starch, has been used as plating medium: the results of Molyneux (1952), Brachfeld (1955), Titus (1957), Shull & Ernst (1962), Finley & Fields (1962), Fields (1963, 1964) and Finley (1964) are in this category. It is conceded that all of these workers used strains of *B. stearothermophilus* different from that used in the present

studies and most used a different commercial brand of DTA (Difco as against Oxoid). Nevertheless the superiority of AAMS compared to DTA has been noted by other workers in this laboratory (Cook & Brown, 1964; Briggs, 1966).

The composition of DTA has been modified by Shapton & Hindes (1963) to give a more reliable medium for the enumeration of organisms of the *B. stearothermophilus* group. Present results show that SM at pH 7·4 was still inferior to AAMS especially for counting spores surviving a severe heat treatment: the addition of 0·1% (w/v) starch to the medium increased counts of both unheated and heated spores.

In view of the findings of Brachfeld (1955) and Brown (1962) of the inhibitory action of bromo-cresol purple on the development of heated spores of *B. stearothermophilus*, it is suggested that both DTA and SM would be improved if bromo-cresol purple was omitted. Also, the uncoloured plates of AAMS were more easily counted and the smaller colonies were seen more readily. However, it is conceded that the acid indicator does have its advantages when used in quality control work in food processing plants for the evaluation of flat-sours.

Fig. 1 shows that colony counts of unheated and heated spores made on DTA can be considerably increased by pH adjustment from 6·9 to 7·3 and inclusion of 0·1% (w/v) starch. The addition of 1×10^{-4}% (v/v) furfuraldehyde (Mefferd & Campbell, 1951) or 0·2% (w/v) charcoal to DTA also increased counts. The importance of starch or charcoal in the medium for the recovery of heated spores has been stressed by Molyneux (1952), Schmidt (1955) and Brown (1962). It is probable that these substances increase the germination of heated spores by virtue of their capacity for adsorption of substances present in the medium which inhibit spore germination. The pH of the recovery medium is also important, for example after a severe heat treatment recovery of *B. stearothermophilus* spores was greatest on media of pH 7·0 (Yokoya & York, 1965) and 7·3 (Cook & Brown, 1965).

There is no general conclusion about the advantages and disadvantages of including nutrients in diluents for damaged and undamaged vegetative cells and spores, although it is important that the diluent should not cause death or reproduction during the dilution process (Jayne-Williams, 1963). The present results show that water was the best diluent for the recovery of spores after a severe heat treatment. The use of quarter-strength Ringer's solution or water containing 0·1% (w/v) peptone or 1×10^{-4}% (v/v) furfuraldehyde (Mefferd & Campbell, 1951) had little effect on the recovery of spores. When water containing 0·85% (w/v) sodium chloride was used as diluent there was a small but significant fall in the calculated D value as compared to water.

In view of the work of Roberts & Ingram (1966) and Roberts, Gilbert & Ingram (1966) that with increasing severity of heat treatment spores become increasingly sensitive to sodium chloride in the plating medium, it would seem possible that the fall in D value was due to a failure of some spores to germinate and grow in the presence of this diluent added to the plating medium.

There appears to be no comparable information in the literature on the effects of diluent on the recovery of spores of other *Bacillus* species surviving heat treatment.

Acknowledgment

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Factors affecting the heat resistance of *Bacillus stearothermophilus* spores

II. The effect of sporulating conditions and nature of the heating medium

A. M. COOK* AND R. J. GILBERT†

Summary. With increase in sporulation temperature (50–55–60°C) there was a significant increase in the heat resistance at 115°C of *Bacillus stearothermophilus* spores produced at these temperatures. The heat resistance of spores was not related to the manganese sulphate concentration in the sporulation medium except at high concentrations (1000 ppm). The heat resistance of spores in McIlvaine's citric acid–phosphate or Sörensen's phosphate buffers was greatest at pH 7·0: survivor curves showed an exponential death rate with no heat activation response. Spores were significantly more resistant when heated in water as compared with the various buffers.

Introduction

The effect of recovery conditions on colony counts of unheated and heated spores of *Bacillus stearothermophilus* has been described (Cook & Gilbert, 1968a). This paper reports the effects of sporulation temperature, manganese sulphate concentration in the sporulation medium and the nature of buffer composition and pH of the heating medium on the heat resistance of *B. stearothermophilus* spores.

Materials and methods

The organism used was *Bacillus stearothermophilus* (NCIB 8919). Spores were obtained using the medium and method of Cook & Brown (1964) as modified by Cook & Gilbert (1968a) at sporulation temperatures of 50°, 55° and 60°C and in sporulation media at 55°C containing different concentrations (0, 1, 5, 10, 20, 50, 100 and 1000 ppm) of manganese sulphate $4H_2O$ (Analar grade, B.D.H. Ltd, Poole).

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The viability of each of the spore suspensions was measured by comparing the mean viable count of unheated spores with the mean total spore count from five haemacytometer slides (depth 0·1 mm with improved Neubauer ruling). Details of spore suspensions are given in Table 1.

The method of determining heat resistance, the plate counting technique and statistical analyses used have been described (Cook & Gilbert, 1968a). The recovery medium was Antibiotic Assay Medium, pH 7·3 with 0·1% (w/v) of added starch.

McIlvaine's citric acid–phosphate buffer and Sörensen's phosphate buffer (M/15 and M/40) were prepared at various pH values according to formulae given in tables (Documenta Geigy, 1962).

TABLE 1. Details of spore suspensions of *Bacillus stearothermophilus*

Suspension	Sporulation temperature (°C)	Manganese sulphate concentration in sporulation medium (ppm)	Total count (spores/ml)	Unheated spores capable of forming colonies (%)	Heat-activated* spores capable of forming colonies (%)
A	50	1	$6·36 \times 10^7$	18·2	25·8
B	55	1	$3·12 \times 10^7$	12·4	31·1
C	60	1	$6·36 \times 10^8$	7·0	37·6
G	55–60	1	$7·39 \times 10^7$	9·9	32·1
H	55	0	$1·10 \times 10^8$	8·0	9·8
I	55	1	$1·33 \times 10^8$	8·6	21·8
J	55	5	$1·17 \times 10^8$	7·0	14·1
K	55	10	$1·25 \times 10^8$	5·1	13·9
L	55	20	$8·24 \times 10^7$	4·2	9·1
M	55	50	$6·71 \times 10^7$	4·0	6·8
N	55	100	$1·37 \times 10^8$	3·3	6·9
O	55	1000	$6·59 \times 10^7$	0·02	0·03

* After maximum heat activation (5–10 min at 115°C).

Results

The effect of sporulation temperature

Viable counts of spores were made before and after heating in water at 115°C using spore suspensions produced at 50°, 55° and 60°C. The results are summarized in Table 2.

The effect of sporulation temperature on the heat resistance of spores was examined by comparing the exponential sections of curves of log percentage survivors against time by means of modified '*t*'-tests as described previously (Cook & Gilbert, 1968a).

TABLE 2. The effect of sporulation temperature on the heat resistance at 115°C of *Bacillus stearothermophilus* spores

Suspension	Sporulation temperature (°C)	D*(min)	Time to inactivate 10^4 spores(min)
A	50	12·2	74·1
B	55	16·2	88·9
C	60	24·4	122·0

* Decimal reduction time.

Suspensions A and B: calculated '*t*' = 2·41; tabulated '*t*'-value = 2·36 (P = 0·05).
Suspensions A and C: calculated '*t*' = 4·88; tabulated '*t*'-value = 2·37 (P = 0·05).
Suspensions B and C: calculated '*t*' = 4·13; tabulated '*t*'-value = 2·34 (P = 0·05).
The regression coefficient for the survivor curve of suspension A (50°C) was significantly different from that of suspension B (55°C) and both in turn significantly different from that of suspension C (60°C).

The effect of manganese sulphate concentration in the sporulation medium

Viable counts of spores were made before and after heating in water at 115°C using spore suspensions produced in sporulation media (other medium ingredients contained < 1 ppm Mn^{++}) containing different concentrations of manganese sulphate. The results are summarized in Table 3.

TABLE 3. The effect of manganese sulphate concentration in the sporulation medium on the heat resistance at 115°C of *Bacillus stearothermophilus* spores

Suspension	Manganese sulphate concentration in sporulation medium (ppm)	D(min)	Time to inactivate 10^4 spores (min)
H	0	16·6	90·3
I	1	16·7	91·4
J	5	16·6	93·8
K	10	17·4	95·0
L	20	17·9	94·9
M	50	16·6	83·2
N	100	17·4	81·9
O	1000	12·7	61·6

With the exception of suspension O there was very little difference between the calculated D values and times to inactivate 10^4 spores for suspensions H–N. A common regression line calculation showed that the five regression lines (suspensions H–L) could be represented by a common line [calculated variance ratio $F^8_{20} = 1{\cdot}08$: tabulated $F^8_{20} = 2{\cdot}45$ ($P = 0{\cdot}05$)]. Modified 't'-tests showed that the regression coefficient for the survivor curve of suspension O was significantly different at $P = 0{\cdot}05$ from the regression coefficients of suspensions H–N.

The effect of buffer composition and pH of the heating medium

Heat resistance experiments were made at 115°C using spores suspended in McIlvaine's citric acid–phosphate buffer at pH values 5·0, 6·0, 7·0 and 8·0 in and Sörensen's phosphate buffer (M/15) at pH values 6·0 and 7·0 and (M/40) at pH 7·0. The results are summarized in Table 4 and Fig. 1.

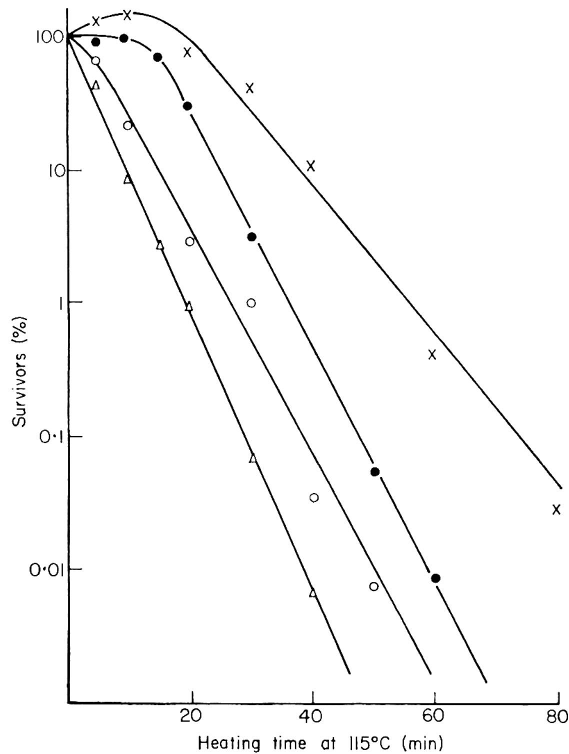

FIG. 1. The effect of using various buffer solutions as the heating medium, on the heat resistance at 115°C of spores of *Bacillus stearothermophilus* (suspn G). ×, Water; ●, Sörenson's buffer (M/40), pH 7·0; ○, Sörensen's buffer (M/15), pH 7·0; △, McIlvaine's buffer, pH 7·0.

Modified 't'-tests showed that the slopes of the two regression lines representing McIlvaine's buffer at pH 6·0 and 7·0 were not significantly different [calculated

TABLE 4. The effect of buffer composition and pH of the heating medium on the heat resistance at 115°C of *Bacillus stearothermophilus* spores (suspension G)

Heating medium	pH	D(min)	Time to inactivate 10^4 spores (min)
Water		16·5	87·8
McIlvaine's buffer	5·0	4·2	20·0
	6·0	7·4	28·3
	7·0	9·2	37·7
	8·0	7·7	27·9
Sörensen's buffer (M/15)	6·0	10·8	45·8
	7·0	11·3	48·2
Sörensen's buffer (M/40)	7·0	11·2	58·6

't' = 1·23; tabulated 't' = 3·18 (P = 0·05)] and also that the slopes of the two regression lines representing Sörensen's buffer (M/15) at pH 6·0 and 7·0 were not significantly different [calculated 't' = 0·46; tabulated 't' = 2·32 (P = 0·05)]. A further test showed that the slopes of the two regression lines representing water and Sörensen's buffer (M/40) at pH 7·0 were very significantly different [calculated 't' = 8·24; tabulated 't' = 2·76 (P = 0·05)].

Discussion

Various incubation temperatures have been used to produce spore crops of *B. stearothermophilus* for heat resistance studies: 50°C, Anand (1961); 52°C, Finley (1964); 53°C, Curran & Pallansch (1963); 55°C, Yokoya & York (1965); 56°C, Brown (1962); 60°C, Thompson & Thames (1967); 62–64°C, Brewer & McLaughlin (1961); and 65°C, Molyneux (1952). The effect of such temperatures on the heat resistance and viability of the spores produced has not been fully investigated although Williams & Robertson (1954) using ten strains of *B. stearothermophilus* reported that in general there was an increase in the heat resistance of the spores with increase in the sporulation temperature (37–45–55°C) for five 'facultative' strains and (55–60°C) for five 'obligate' strains.

Table 2 shows that with increase in sporulation temperature (50–55–60°C) there was a significant increase in the heat resistance of spores produced. These results are important as they show how even small differences (5°C) in sporulation temperature may have a significant effect on the heat resistance of spores produced. In view of a report that the optimum temperature of growth for some strains of *B. stearothermophilus* is 63–65°C (Brewer & McLaughlin, 1961) it was possible in the present studies that a growth and sporulation temperature of 60°C was nearer the optimum than either 50° or 55°C. In this respect it is interesting to note that total counts of spores produced at

60°C were higher than those for spores produced at 50° or 55°C. Higher sporulation temperatures were not used because of the serious overdrying of the solid media.

With increase in sporulation temperature (50–55–60°C) the percentage viability of unheated spores decreased (18·2–12·4–7·0%): however, after maximum heat activation the percentage viability of spores increased (25·8–31·1–37·6%). The phenomenon of heat activation is lost on storage of spores at 4°C (Cook & Gilbert, 1968b). The results indicate that the dormancy found in a spore suspension is directly related to the temperature of sporulation. This may be because one or more of the enzyme systems involved in spore formation has a temperature optimum of 60°C or over.

Although various amounts of Mn^{++}: 3 ppm, Berlin, Curran & Pallansch (1963); 5 ppm, Curran & Pallansch (1963); 10 ppm, Finley (1964); 35 ppm, Wang, & Scharer Humphrey (1964) have been included in the sporulation media to promote the formation of spores of *B. stearothermophilus*, the effect of such ions on the heat resistance and viability of these spores has not been fully investigated. Present results show that the heat resistance of spores was not related to the manganese sulphate concentration except at high concentrations (1000 ppm ≡ 250 ppm Mn^{++}) when there was a significant fall. The results are in contrast to those of Amaha & Ordal (1957) who reported a large increase in heat resistance of *Bacillus coagulans* var. *thermoacidurans* spores with increase in Mn^{++} concentration (0·33–17 ppm) in the sporulation medium.

The percentage viability of *B. stearothermophilus* spores either unheated or after maximum heat activation was greatest when the sporulation medium contained 1 ppm of manganese sulphate. In the absence of, or in the presence of > 1 ppm manganese sulphate there was a decrease in viability for unheated or heat-activated spores. These results are in agreement with those of Richardson (1965) who found that the viability of *Bacillus subtilis* spores decreased with increase in the Mn^{++} concentration in the sporulation medium.

Molyneux (1952), Thorpe (1960) and Tramer (1964) have used McIlvaine's citric acid–phosphate buffer at pH 6·0 as heating medium for heat resistance studies on *B. stearothermophilus* spores, but no comparisons were made with the same buffer at pH 7·0 or with water: heat activation and shoulder effects were not encountered. Present results show that although the slopes of the survivor curves for spores heated in McIlvaine's buffer at pH 6·0 and 7·0 were not significantly different, they were both considerably different in shape and slope from that for spores heated in water. There were no heat activation or shoulder effects for spores heated in McIlvaine's buffer at pH 5·0–8·0 and survivor curves were exponential.

Several workers have used other phosphate buffers as heating media for *B. stearothermophilus* spores, at concentrations similar to those used in the present study: M/10, Brown (1962); M/15, Harper Curran & Pallansch (1964); M/20, Anand (1961) and Marshall, Murrell & Scott (1963); and M/40, Yokoya & York (1965). Present results show that although the slopes of the survivor curves for spores heated in Sörensen's phosphate buffer (pH 7·0 at M/15 and M/40) were not significantly different, they

were both significantly different from that for spores heated in water. There were no heat activation or shoulder effects for spores heated in Sörensen's buffer M/15 at pH 6·0 or 7·0. However, in M/40 buffer at pH 7·0 there was a definite shoulder on the survivor curve.

Brachfeld (1955) studying the effect of phosphate on the heat activation response of *B. stearothermophilus* spores reported that M/20 buffer significantly decreased average plate counts, whereas M/80, M/200, M/800 and M/2000 phosphate buffers had no effect. Williams & Hennessee (1956) reported a stimulation of germination and outgrowth when *B. stearothermophilus* spores were heated in M/120 phosphate buffer. However, under the same conditions Finley & Fields (1962) reported a definite inhibitory effect. Williams & Hennessee (1956) also reported that with decreasing concentrations of phosphate in the heating medium over the range M/15 to M/120, resistance of spores to lethal heat (120°C) increased. Most of the observed differences, however, were attributed to a 'carry-over' of phosphate into the plating medium rather than to combined phosphate-heating effects. Phosphate 'carry-over' effects have also been stressed by Cook & Brown (1964).

The present results illustrate that although the use of buffers is desirable for accurate pH control of heating media, significant variations in heat activation and overall heat resistance of spores may occur when different buffers are used.

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Methods of thermal process evaluation

N. D. COWELL

In his recent paper, Jones (1968) compared specimen thermal processes evaluated on the alternative assumptions of a constant z-value and a constant activation energy (E). In making such comparisons it is important to distinguish between effects due to the assumed mathematical model and those due to the bacteriological data employed.

Experimental data on the thermal destruction of bacteria are both subject to error and are available over a limited temperature range. The same data can often be interpreted equally as well on the basis of a constant z-value as by assuming that E is constant. Where this is true, one may expect reasonable agreement between the two methods of thermal process calculation over the temperature range covered by the original data. Outside this temperature range the Arrhenius equation, by its very nature will give increasingly conservative estimates of the efficiency of a heat process compared with the constant z assumption.

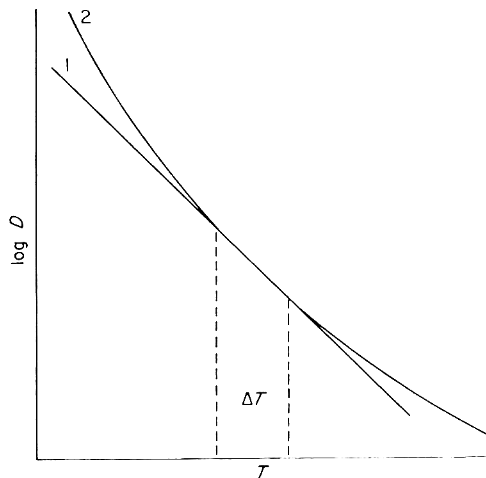

Fig. 1. Diagrammatic plot of the logarithm of the decimal reduction time (log D) as a function of the absolute temperature, T. Curves of type (1) result from assuming a constant z-value and curves of type (2) from assuming that E is constant. Over a limited temperature range (ΔT) either curve may be used to represent experimental thermo-bacteriological data.

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This may be easily demonstrated by reference to Fig. 1. To assume that z is constant is equivalent to assuming that log D is a linear function of T, while to assume that E' is constant is to assume that log D is a linear function of $1/T$ (Gillespy, 1947). The latter curve is part of a hyperbola and is concave upwards as shown. Both curves will adequately describe the same data over a limited range of temperature, the former being approximately tangential to the latter in that range. Outside this range the curvature of the hyperbola will result in the Arrhenius equation predicting relatively longer decimal reduction times.

The above situation is demonstrated in the second line of Table 2 of the paper by Jones (1968). This is based entirely on the decimal reduction time data of Stumbo, Murphy & Cochran (1950) (over the temperature range 104·4–126·6°C) for spores of *Cl. botulinum* strain 62A suspended in puréed peas. Gillespy (1948) had previously demonstrated the same effects from a consideration of the data of Esty & Meyer (1922) on the maximum heat resistance of spores of *Cl. botulinum* suspended in phosphate solution.

Unfortunately Jones (1968) confuses this picture in the first and last lines of his Table 2 by attempting comparisons in which the z-value is calculated from one set of experiments and the activation energy from another. Such a procedure cannot provide an unambiguous comparison of the methods of calculation since differing bacteriological constants are being included in the comparison. For instance, it is known that the heat resistance of *Cl. botulinum* spores is considerably different in phosphate solution and pea purée (Townsend, Esty & Baselt, 1938), yet in the first line of his Table 2 (*Cl. botulinum* (a)) the activation energy (E) is derived from work using suspensions in pea purée and the z-value from data on phosphate solution suspensions. The z-values from these two sources differ so much that calculations on this basis alone would not yield comparable results.

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Reply to N. D. Cowell: 'Methods of thermal process evaluation'

M. C. JONES

Mr Cowell's criticisms are valid. His Fig. 1 demonstrates clearly, but qualitatively, the effect that equation (8) of my paper expressed quantitatively.

My object was to demonstrate a problem facing the designer of sterilizing equipment when he wishes to specify the time and temperature needed to destroy a harmful organism. The two calculation methods that I described will predict different times at a specified temperature, whatever bacteriological constants, consistent or inconsistent, he uses. This is perhaps commonplace to experts in the field (I am not one) but, in my limited experience, not recognized by some engaged in the design of food sterilizing equipment, where the tendency is to extrapolate results to higher and higher temperatures in the expectation of quality improvements.

I accept, of course, that it is invalid to compare predictions based on different sets of data. However, $z = 10°C$ is the 'general purpose' value used for *Cl. botulinum* whilst $E = 82{,}100$ cal/mole was the only value of E for *Cl. botulinum* that Deindoerfer and Humphrey provide in their method. In comparing unlike I put myself in the position of the designer who uses the information at his disposal. Since, as Dr Cowell's Fig. 1 shows, the 'E method' is conservative when applied to consistent data, it is unfortunate that the 'z method' is the more widely used, at least in my experience, and as reflected by the availability of z-value data.

May I repeat my suggestion that we need more experimental evidence, particularly at high temperatures? It is surely unsatisfactory that we cannot decide whether log D varies linearly or reciprocally with T.

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J. Fd Technol. (1968) **3**, 307–310.

Book Reviews

A Symposium on Drugs and Sensory Functions. Ed. by A. Herxheimer
London: Churchill, 1968. Pp. 338 + xiii. 75s.

Of the five sections of this symposium only the one on smell and taste will be of *direct* interest to the food scientist. This carries six papers with substantial discussion, among them papers of an introductory nature by R. W. Moncrieff and R. Harper, which provide excellent summaries of the chemical, physiological and psychological aspects of taste and smell.

The remainder of the book provides much interesting information about the action of hallucinatory, tranquillizing and antidepressant drugs in common clinical use.

E. C. Bate-Smith

Odour Description and Odour Classifications. By R. Harper, E. C. Bate-Smith and D. G. Land
London: Churchill, 1968. Pp. 191 + viii. 38s.

Scientists labour under peculiar disability when they attempt to describe aesthetic sensations. A physicist may be able to describe the nature of sound and can set down the amplitude and frequency of a musical note. Yet he can no more describe with any useful precision of meaning what Yehudi Menuhin draws out of his violin that he can produce it by electronic oscillations. Musicians, however, do have at their disposal a system of musical notation and this perfumers and food scientists concerned with smell do not possess for odours. Once again it is borne in upon us that physics, dealing as it does with the few concepts of heat, light and sound, electricity and magnetism, is a simple subject compared with chemistry with more than a hundred elements quite apart from the extravagant combinations and permutations of the compounds of carbon. And smell is, we must suppose, a chemical sensation. To link its chemistry and physics with the further biological complexity of physiology and the psychological intricacies of memory and meaning, likes and dislikes is a daunting task.

Progress is undoubtedly being made in the understanding of odours. Robert Wright in British Columbia in his impressive study using insects as biological reagents has deduced that, provided the human nose possessed only twenty primary receptors, the number of categories of odour which could be detected would be 1,048,576, which may well be about the number that there are. And he is beginning to talk about 'breaking the olfactory code' by matching the molecular oscillations of odorous compounds to the pattern of frequency components in the response shown by the olfactory bulbs.

But even if he is correct, this only brings us as far as the physicist who knows the spectrographic analysis of Erythrosine BS without being able to describe precisely what its colour is.

Harper, Bate-Smith and Land have now gathered together everything of substance that any one who has had anything to say about describing smell has said. But to bring order out of the complexity of the literature is the problem. The measurement of odours has been put on a firm basis. The existence of differences in smell detected by the use of some kind of 'triangle test' can be subjected to the rigours of statistics. Threshold values, just noticeable differences and the assessment of the number of 'olfacties' contributed by this or that component of a blend of compounds can be measured with known precision. By such means a great deal can be learned about the odorous activities of homologous series of compounds of various types. But when we come to the description of a smell, even when a precise and carefully thought-out method such as that of Schultz is used or the 'semantic differential' technique of Osgood, the matter becomes more difficult. The very fact that by using the appropriate computer programmes, figures calculated from 'semantic differential' tests of the smell of beer, bacon or perfume can be submitted to quite sophisticated calculations of 'factor analysis' and the values obtained 'rotated' by even more subtle mathematical techniques may lead the enthusiast to forget that the purpose of these seemingly exact operations is to express the smell of coffee or corned beef or whatever else may be under examination in terms of units of 'aromaticness', 'etherialness', 'empyreumaticness' (burnt, to you) or 'pleasantness'. The panels which provide the figures which the computer processes are made up of people whose judgement and ability may vary—the authors even refer to one subject (admittedly a special case) who could not distinguish the smell of coffee from that of faeces. The most explicit revelation of where Harper, Bate-Smith and Land stand in all this is when they write 'Odorous substances do not, of themselves, smell; they are smelt . . .'.

This is a difficult book to read. It covers a wide area of scholarship and in appending a bibliography of over 200 references. which yet makes no claim to be exhaustive, further justifies its subtitle, 'a multidisciplinary examination'. But while giving much, the authors demand something in return. Readers are sometimes expected to know a good deal about the published sources which come up for discussion. Some understanding of such topics as the nature of memory or the philosophy of science would help as well. They are sometimes expected to hold in mind a topic discussed in one part of the work until it is referred to again later. 'Nature and Nature's laws', wrote Alexander Pope, 'lay hid in night,/God said, "Let Newton be!", and all was light'. The problems of odour classification have not all been solved yet. But before Newton could conceive his grand simplifying generalizations, he needed Kepler's calculations to start with. Harper, Bate-Smith and Land have supplied us with a basis upon which the Newton of smell for whom we are seeking can build.

MAGNUS PYKE

Olfaction and Taste, Vol. 2. Ed. by T. HAYASHI
Oxford: Pergamon Press, 1967. Pp. 835. £10.

This book is the record of the fifty-three papers given at the Second International Symposium on Olfaction and Taste held in Tokyo in 1965. It followed the highly successful initial meeting held in Stockholm in 1962, and by comparison with the original book, reflects progress being made in this field. As these meetings are held in conjunction with International Physiological Congresses, it is not surprising that the interest is largely neurophysiological. The emphasis is still largely on taste, although the papers on olfaction do reflect the considerable increase in interest in this sense.

Papers on taste range from the psychophysical study by Stone on characterization of a number of amino acids in terms of the four basic taste modalities, through animal behaviour, which includes a paper on a conditioning method for studying flavour discrimination in dog foods to the purely electrophysiological. The latter include an excellent review, by Beidler, of ionic influences on taste receptor responses in the light of present knowledge of membrane structure and a very interesting correlation between signals recorded from the chorda tympani nerve and the perceived intensities of sugar and citric acid, but not with salt solutions in man. The signals following sugar or saccharin, but not salt, acid or bitter stimuli, were also clearly abolished after treatment with *Gymnema* extracts, confirming the perceptual observations. Other studies demonstrated the effect of nucleotides and MSG in increasing the activity of salt fibres and depressing bitter fibres.

Olfaction is similarly covered by a wide range of different approaches. Amoore gives extensive data on 107 substances in support of his 'Stereo Chemical Theory' and Hughes and Hendrix have given a new lead on the 'Olfactory Code' with their study of frequency analysis of olfactory bulb signals leading to the 'Frequency Component Hypothesis'. Wright proffers further evidence for his 'Vibrational Theory' in a joint paper with Demerdache which ends up with a discussion between the two authors in which there seems to be considerable disagreement as to the validity of the deductions and conclusions. Dravnieks gives a useful analysis of the properties of different odorant molecules in relation to threshold values and considers the nature of possible interactions with the receptor surface. A number of papers deal with differences between signals from different stimuli in an effort to determine methods of transfer of information, and a number are purely electrophysiological. Further papers deal with olfaction in birds, fish and insects.

These illustrations of content may be taken as a reflexion of the current state of knowledge of taste and olfaction. This has been a period of consolidation and classification of ideas rather than of spectacular advance. The book brings together many different approaches and no doubt reflects a very stimulating meeting, although unfortunately it contains no record of the discussion following papers. It contains much valuable reference material and is essential reading for anyone involved in this field.

D. G. LAND

Book Received

European Food Processing Industry. By S. A. MANN
Noyes Development Corp., U.S.A., 1968. Pp. 200. $35.

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The British Journal of Nutrition

Vol. 22, *No.* 3, *September* 1968. *Partial contents*

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Severe undernutrition in growing and adult animals. 18. R. A. MCCANCE, P. D. A. OWENS and C. H. TONGE

The fate of water in the rumen. 1 and 2. A. C. I. WARNER and B. D. STACY

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gram(s)	g	second(s)	sec
kilogram(s)	kg	cubic millimetre(s)	mm^3
milligram(s) (10^{-3} g)	mg	millimetre(s)	mm
		centimetre(s)	cm
microgram(s) (10^{-6} g)	μg	litre(s)	l
		millilitre(s)	ml
nanogram(s) (10^{-9} g)	ng	pound(s)	lb
		gallon(s)	gal
hour(s)	hr	milliequivalent	mEq
minute(s)	min	R_F values	R_F

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