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Soybeans as a Food Source

W. J. Wolf and J. C. Cowan

The recent interest in soybeans as a source of food proteins and as the basis of unconventional foods stimulated the revision of this monograph. New developments in processing and technology are outlined and the growing potential of soybeans is discussed.

Selection from Contents Soybean production; Conversion to edible oil products; Food uses of soybean proteins; Physical and chemical properties; Nutritional properties; Food containing soy proteins; Problem areas.

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The assay of conjugable oxidation products applied to lipid deterioration in stored foods

L. J. PARR AND P. A. T. SWOBODA

Summary

A new analytical procedure, determining oxidation products of polyenoic fatty acids, is applied to both fresh and stored foods and fats. The extent of deterioration of the extracted lipid is measured by changes in the ultraviolet spectrum resulting from the chemical reaction steps of the assay—the results are compared with the traditional peroxide value. In the assay not only hydroperoxides of polyenoic fatty acids but also hydroxy and carbonyl compounds derived from them, yield 'conjugable oxidation products' which are measured together and expressed as the 'C.O.P. value'. At the same time an 'oxodiene value' independently determines the unsaturated carbonyl compounds. Another feature of the assay is that is distinguishes between the oxidation products derived from dienoic fatty acids and those of more highly unsaturated polyenoic fatty acids and this is measured by the 'C.O.P. ratio'.

For possible greater convenience in quality control, a quicker procedure is also described in which only the final spectrum derived from the conjugable oxidation products is measured.

Introduction

The extent of lipid oxidation in a foodstuff is often the dominant factor in determining its palatability. The polyenoic fatty acids not only oxidize the fastest but also yield the volatile scission products which, as off-flavours, limit acceptability. We now report the application of a new analytical procedure (Fishwick & Swoboda, 1976) which specifically assays certain oxidation products of polyenoic fatty acids, to monitor the extent of lipid oxidation in stored foods. Chromophoric groups are assayed in the ultraviolet by the change in absorbance resulting from their formation or disappearance due to the chemical reaction steps of the analytical procedure. A simplified version of the method is also described which only measures gross absorbance and is much quicker to perform. For rapid quality control it may, indeed, prove more useful than the full procedure.

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In the assay, hydroperoxides of polyenoic fatty acids as well as hydroxy and carbonyl compounds derived from them, are converted by the two chemical reaction steps, first reduction and then dehydration, into more conjugated chromophores. The assay is, therefore, described as that of conjugable oxidation products (abbreviated to COP assay). It distinguishes between the oxidation of dienoic fatty acids (e.g. linoleate) and that of the more highly polyunsaturated acids (e.g. linolenate, arachidonate, and the five and six double bond polyenoates). The former yield a conjugated triene chromophore whereas the latter form a conjugated tetraene chromaphore (viz. Fig. 2) as a result of the final dehydration step of the analytical procedure. The first step of the analytical procedure, reduction by sodium borohydride, results in the disappearance of the characteristic ultraviolet absorbance of the carbonyl compounds of oxidized polyenoic fatty acids, more correctly described as oxodienes. The decrease in absorbance at 275 nm as a result of the reduction step is defined as the 'oxodiene value'.

Changes in absorbance at 268 and 301 nm during the dehydration step measure the formation of conjugated triene and tetraene chromophores, respectively. The sum of the absorbance increase at these two wavelengths is defined as the conjugable oxidation product value (abbreviated to 'COP value'). The relative proportion of tetraene to triene products is measured by the ratio of the absorbance change at the two wavelengths (termed the 'COP ratio'). Whereas the ratio is dimensionless, all other numerical values resulting from the COP assay are expressed in units of absorbance for a 1% w/v lipid solution measured in a 1-cm cell.

Although the hydroperoxides of polyenoic fatty acids (and indeed the hydroxydienes) have an absorption maximum around 230 nm, measurement at such wavelength is not so definitive as the background spectrum required for correction is uncertain. Measurement at this shorter wavelength, although often used in laboratory investigations of the oxidation of lipids, is not so applicable to oxidized foodstuffs. Measurements at the longer wavelengths used in the COP assay are less susceptible to error and yield more information.

The traditional peroxide value method (British Standard, 1958), the iodmetric assay of peroxides, does not distinguish between types of unsaturated fatty acid undergoing oxidation, neither does it give any information about secondary products which are derived from further reaction of the first formed hydroperoxides. Hydroxydiene and oxodiene groups do not liberate iodine in the assay, whereas hydroperoxides and peroxides do so whether derived from monoenoic or polyunsaturated fatty acids.

In this paper we report the results of the application of these assay methods to lipids from a variety of foodstuffs before and after further storage and to some commercial vegetable oils and food grade lecithins before and after oxidation at elevated temperatures.

Whereas oils and fats can be assayed directly, the lipids of foods have first to be extracted. The soxhlet extraction procedure with petroleum or diethyl ether although a recommended method for triglyceride content (Statutory Instruments, 1968;

A.O.A.C., 1970), does not efficiently extract polar lipids. This selectivity could be even more adversely affected by the polarity of oxidized unsaturated fatty acids. In the present investigation, therefore, it was deemed necessary to use an extraction procedure which had been well characterized for the efficient extraction of polar and oxidized lipids. A suitable modification of the rapid, cold extraction method of Bligh & Dyer (1959) using polar solvents was chosen since it provides a quantitative, representative and unchanged lipid sample. The efficiency of such procedures has been evaluated by Ambrose & Knobl (1966); Atkinson *et al.* (1972), whilst Smith (1969) and Winter (1963) have reviewed rapid methods for estimation of total fat.

Experimental

Materials

The foodstuffs listed in Table 1 were purchased from commercial outlets. The lecithins (from egg and soya-bean) were samples of food grade materials used in the confectionary trade. In some cases the foods were subjected to further storage (under conditions which allowed access of air whilst preventing loss or gain of water) at the storage temperature indicated in Table 1. Vegetable oils and mixtures with lecithin were oxidized at 100°C, the temperature often employed for accelerated stability tests. All chemical reagents and solvents used were of A.R. quality.

Extraction of lipid from the foods

The method of Bligh & Dyer (1959) was modified so that the disintegration, mixing and phasing could be carried out quickly and routinely at room or lower temperature in one vessel, using a high speed top drive homogenizer (M.S.E., Crawley, Sussex). Mixtures of chloroform, methanol and water, including that present in the material, are used to produce, initially, monophasic extracting conditions and, finally, a biphasic system with the lipid in the chloroform phase. The solvent composition, by volume, which initially is 1 : 2 : 0·8 chloroform, methanol and water becomes 2 : 2 : 1·8 in the final biphasic system.

The procedure for a given foodstuff of known approximate water content was as follows.

Sufficient weight was taken (into a 500-ml centrifuge cup) with one volume of chloroform plus two volumes of methanol and, if necessary, distilled water was added to achieve the stated solvent composition. The mixture was homogenized at room temperature for 2 min after which a further one volume of chloroform was added and homogenized for a further 30 sec; one volume of distilled water was then added and blending continued for another 30 sec. The mixture was next centrifuged to assist separation into three layers: the upper being mainly water and methanol, the middle being that of the tissue or sample residue plus some occluded chloroform and the lower layer being a clear chloroform solution containing a representative fraction of the extracted lipid.

TABLE 1. The analytical results obtained for the range of commodities investigated

Item	Commodity	Storage		Peroxide value	COP assay		
		Time	Temp.		Oxcdiene value	COP value	COP ratio
1	Margarine	as purchased		Nil	Nil	0·5	0·10
2	Pastry mix	as purchased		Nil	C·3	0·5	0·06
3	Salad cream	as purchased		1·2	0·2	2·3	0·01
4	Peanuts (roasted)	as purchased		Nil	C·2	4·5	0·02
5	Soup powder	as purchased		2·4	C·2	1·0	0·40
6	Margarine	120 d	25°C	31·4	C·3	11·9	0·14
7	Pastry mix	147 d	25°C	2·0	C·5	1·9	0·18
8	Salad cream	110 d	25°C	51·0	C·8	23·0	0·05
9	Peanuts (roasted)	112 d	25°C	17·3	C·3	10·3	0·02
10	Soup powder	113 d	25°C	2·3	C·3	1·0	0·20
11	Herring slices +	as purchased		9·9	0·2	3·6	2·0
12	Kipper fillets +	as purchased		4·2	Nil	2·0	1·9
13	Herring slices +	130 d	−18°C	42·6	C·8	14·0	2·5
14	Kipper fillets +	130 d	−18°C	21·3	C·2	8·9	2·9
15	Potato crisps (1)	84 d	room	1·3	C·8	2·6	0·14
16	Potato crisps (2)	84 d	room	1·0	0·8	2·9	0·09
17	Flour	*–	room	0·2	2·2	21·5	0·10
18	Potato powder	*–	room	3·5	6·3	29·5	0·35
19	Potato powder	*–	room	8·0	2·0	5·2	0·70
20	Pork mince +	*–	−18°C	5·0	Nil	1·5	0·28
21	Lecithin	*–	room	15·0	1·2	17·2	0·17
22	Sunflower oil	as purchased		4·9	0·6	5·1	0·05
23	Groundnut oil	as purchased		2·2	0·3	1·9	0·07
24	Wheat germ oil	as purchased		4·8	0·5	3·3	0·05
25	Sunflower oil	8 hr	100°C	212·6	1·2	77·5	0·05
26	Groundnut oil	47 hr	100°C	149·2	0·9	24·3	0·12
27	Wheat germ oil	8 hr	100°C	130·0	1·8	50·7	0·06
28	Wheat germ oil	9 hr	100°C	207·0	1·8	77·6	0·05
29	Lecithin (egg) †	as purchased		1	0·3	2·2	0·60
30	Lecithin (soya)	as purchased		Nil	0·4	8·8	0·19
31	Lecithin (soya) †	as purchased		1	0·1	8·8	0·24
32	Lecithin (egg) †	16 hr	100°C	4	8·0	12·3	0·55
33	Lecithin (soya)	145 hr	100°C	Nil	2·6	13·7	0·31
34	Lecithin (soya) †	94 hr	100°C	3	9·4	19·1	0·47
35	Sunflower + lecithin (1:1)	62 hr	100°C	2·8	3·2	13·3	0·22
36	Groundnut + lecithin (1:1)	94 hr	100°C	1·5	3·3	8·8	0·12

+, A frozen sample.

*, A product of poor quality.

†, Samples mixed with inert methyl laurate to reduce viscosity, results calculated on lecithin basis.

For the present study, no attempt was made to effect a quantitative yield of lipid, although providing care is taken to avoid evaporation or spillage we found that accurate measurement of the volume of chloroform used sufficed to allow a calculation for lipid content. Most of the lower phase was easily aspirated into a stoppered flask from which aliquots were taken for (a) determination of lipid content (dried to constant weight under vacuum < 10 mmHg at 50°C) and (b) experimental examination. Alternatively, aliquots can be removed directly, through the upper layers, using an hypodermic syringe fitted with a long needle. Butylated hydroxytoluene (BHT), at the level of 0·02% of the lipid, was safely added to the chloroform solution after extraction, to ensure that no further oxidation took place when samples were freezer stored before analysis.

The extraction procedure can be applied to most materials, by adjusting the size of 'wet' samples or by adding extra water to 'dry 'materials, providing that the proportions given for chloroform, methanol and water are not altered. In this survey the lipid concentration in the final chloroform layer was never allowed to exceed 5% (w/v).

The assay of peroxides

The peroxide value (British Standard, 1958), which measures all substances liberating iodine from potassium iodide under the conditions of the test, was determined on all lipid samples. The deaeration procedure (method 2) was used with, however, a solvent composition of acetic acid: chloroform, 3 : 2 by volume. Results, calculated as millilitres of 0·002 N thiosulphate per gram of lipid, are therefore in units of μ moles of peroxide per gram.

The assay of conjugable oxidation products (COP assay)

(*i*) *The procedure*, based on that of Fishwick & Swoboda (1976), was as follows. A stock solution of the extracted lipid is prepared, and three equal aliquots of this solution are then used for measurement of absorbance in the ultraviolet after appropriate treatment. Thus, a known weight of lipid (about 0·25 g) was dissolved in a 5·0-ml volume of iso-octane: ethanol (1 : 1 by volume). One millilitre aliquots of this stock solution were transferred to each of three 25·0-ml stoppered volumetric flasks which were designated 'O', 'R' and 'D'.

To Flask code 'O' (= original), 1·0 ml iso-propanol was added and the volume made up to 25·0 ml with ethanol.

To Flask code 'R' (= reduced), 1·0 ml of a filtered, saturated solution of sodium borohydride in iso-propanol* was added and held at 60°C for 30 min, then cooled and made up to 25·0 ml with ethanol.

* 1 g of solid sodium borohydride was dispersed in 100 ml of iso-propanol and freshly filtered before use to yield a 0·4% solution. The suspension was stable for at least a week in a refrigerator.

Flask code 'D' (=dehydrated) was first treated as for flask 'R', and then before final dilution, 5·0 ml of a 20% (w/v) sulphuric acid solution in ethanol was added and the flask again held at 60° for 30 min. Finally, it was cooled and made up to 25·0 ml with ethanol.

The ultraviolet spectrum between 200 and 450 nm was recorded for solutions 'O', 'R' and 'D' using a 1·0-cm cell against a reference cell containing ethanol (Fig 1). Before making measurements, the absorbance zero of the spectrophotometer was set with either air or ethanol in both cells. A Unicam S.P. 800 recording spectrophotometer was used. However, a non-recording instrument would suffice for measurements of absorbance at the three specified wavelengths of 268, 275 and 301 nm.

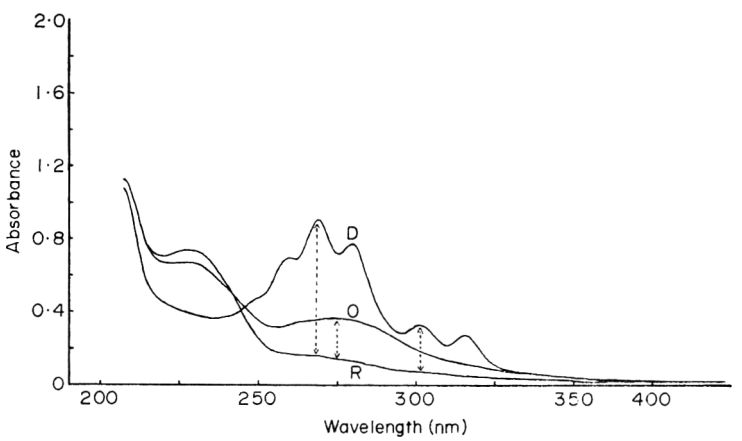

FIG. 1. The ultraviolet spectra for the solutions 'O', 'R' and 'D', obtained using lipid from potato powder, showing absorption differences for the 'COP assay'.

If the absorbance of any solution was too great to measure, further quantitative dilutions, with ethanol, were prepared and the appropriate dilution factor included in the calculations. On a few occasions, particularly with lecithins, cloudiness was observed on making up to volume and was effectively cleared by centrifugation rather than filtration. Both dilution and centrifugation were shown to be sound procedures.

(*ii*) *The calculation of results* from the measurement of absorbance at three wavelengths is based on the change of spectra resulting from the three treatments (Fig. 1).

The measurements required are the absorbance of solution 'O' at 275 nm ($A_{275}{}^{O}$), the absorbance for solution 'R' at 268, 275 and 301 nm ($A_{268}{}^{R}$, $A_{275}{}^{R}$ and $A_{301}{}^{R}$) and the absorbance for solution 'D' at 268 and 301 nm ($A_{268}{}^{D}$ and $A_{301}{}^{D}$). The weight of lipid in grams (w) taken for preparation of the 5-ml stock solution yields a final solution in the 25-ml volumetric coded flask of strength $0{\cdot}8 \times (w)$ per 100 ml, as the stock has been diluted twenty-five-fold. The results obtained from the COP assay are then calculated as follows.

$$\text{Oxodiene value} = (A_{275}{}^{O} - A_{275}{}^{R}) \div 0{\cdot}8w$$
$$\text{COP value} = \{(A_{268}{}^{D} - A_{268}{}^{R}) + (A_{301}{}^{D} - A_{301}{}^{R})\} \div 0{\cdot}8w.$$
$$\text{COP ratio} = (A_{301}{}^{D} - A_{301}{}^{R}) \div (A_{268}{}^{D} - A_{268}{}^{R}).$$

Both the oxodiene and COP values are thereby expressed in units of absorbance measured in a 1-cm cell for a 1% w/v lipid solution. The COP ratio, is, however dimensionless.

Results and discussion

The ultraviolet spectra of the COP assay

Figure 1 shows the spectra of solutions 'O', 'R' and 'D' obtained on analysis of oxidized lipid extracted from old potato powder (item 18 of Table 1). This typical example illustrates the changes in absorbance at the three wavelengths. The decrease at 275 nm from the original ('O') to the reduced ('R') spectrum resulting from treatment with sodium borohydride gives the 'oxodiene value'. The product of the reduction results in an increase at 230 nm which is not used for purposes of the calculation. However, the fact that the 'O' and 'R' curves cross over is consistent with the chemical reactions taking place and confirms that absorbance differences are not due to cloudy solutions. The next step of the COP assay results in the reduced ('R') spectrum changing to the dehydrated ('D') spectrum because of the formation of conjugated chromophoric groups with absorption maxima at 268 and 301 nm, whilst that at 230 nm decreases. The sum of these absorbance changes at 268 and 301 nm yield the 'COP value' whilst their relative proportions define the 'COP ratio'. For the calculation of results the concentration of the final lipid solution has to be taken into account for the oxodiene and COP values. The 'COP ratio' is, however, without dimension and ranges from a value of zero for pure conjugated triene to 2·8 for conjugated tetraene products (Fishwick & Swoboda, 1976).

Figure 2 illustrates more clearly the dissimilar spectra that are obtained for the 'D' solution depending on whether the oxidized lipid being analysed is mainly dienoic fatty acid (e.g. sunflower oil (item 25)), or more highly polyunsaturated (e.g. herring oil (item 13)). Whereas, in the COP assay of these two types of oil the same COP value could occur, indicating the extent of oxidation, a quite different COP ratio must exist. Since the spectrum of the 'D' solution is so characteristic of oxidation, a simplified and quicker assay was developed and will be described later in this paper.

Survey of the extent of lipid oxidation in foods

The choice of samples for this investigation was intended to provide examples in which autoxidation of the polyunsaturated lipids present, might be influenced by other food constituents. These constituents include both pro- and antioxidants, which can be present naturally or as additives or contaminants, and could influence the relative rates of formation and degradation of any oxidation product. Table 1 shows the analy-

tical results obtained using the methods described on oils and on lipids extracted from the various foodstuffs, as purchased and after further storage.

Items 1 to 5 were examined immediately after purchase. The results on the extracted lipid show little correlation between peroxide value and COP assay at these low levels of oxidation, though the assay is more sensitive. Indeed, a measurable COP value was obtained for samples with a zero peroxide value (items 1, 2 and 4).

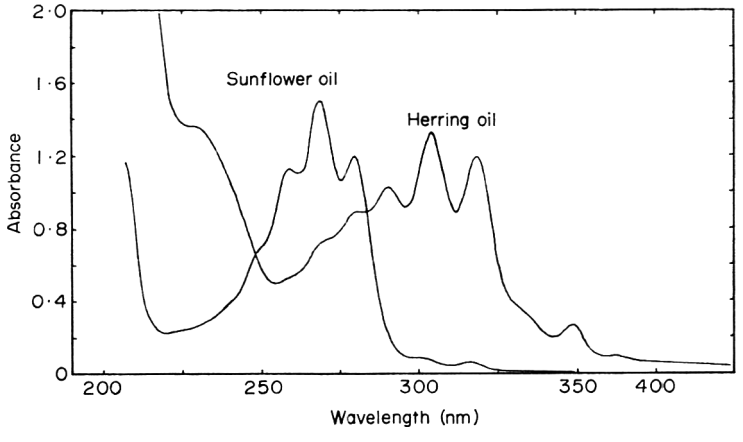

FIG. 2. The ultraviolet spectra for the 'D' solutions showing the dissimilar λ_{max} absorption at the two wavelengths for oxidized lipids of different fatty acid composition.

Items 6 to 10 were results obtained for the above foods after further storage at 25°C and, except for item 10, show considerable increases in both the peroxide and COP values. The COP assay, however, also indicates both (a) a small increase in the oxodiene value and (b) a low COP ratio since the oxidation is essentially of dienoic rather than more highly unsaturated fatty acids.

Items 11 to 14 were packaged frozen fish samples which were extracted for examination of the lipids before and after further freezer storage (-18°C) to provide evidence of oxidation in a system containing highly unsaturated lipids. Again, both the peroxide value and COP value adequately demonstrate oxidative changes. However, the COP ratios are high, indicating the predominant oxidation of the polyunsaturated fish lipids which contain fatty acids with more than two double bonds.

Items 15 to 21 were foods or food materials which were known to have been held in prolonged storage and might not have been of acceptable quality. For some of these, in contrast to stored items 6 to 10, the COP value numerically exceeds the peroxide value. Moreover, in the potato powder and flour, which contain low levels of lipid, there was a significant contribution from the oxodiene value. Under conditions of prolonged storage, therefore, there can be a considerable breakdown of the first formed hydroperoxides into secondary oxidation products which are also measured in the COP assay.

Items 22 to 28 were samples of vegetable oils which were analysed fresh and after accelerated storage at 100°C to give very high levels of oxidation. Nevertheless, the conditions of oxidation are such that peroxides accumulate and there is no excessive decomposition to, for example, oxodienes. In the case of oxidized groundnut oil the peroxide value is numerically six times the COP value, whereas, for wheat germ and sunflower oils the comparative figure is just less than three-fold. These results emphasize the much greater contribution of monoenoic fatty acid oxidation to the peroxide value of groundnut oil; whereas, only oxidation products of polyenoic fatty acids are determined in the COP assay. The analytical results, therefore, reflect the difference in the fatty acid composition of the oils. However, for all three oils the fresh samples exhibit peroxide and COP values which are of comparable magnitude. Thus, there must be, as in the case of fresh food samples (cf. items 1 to 5), a contribution to the COP value from non-peroxidic oxidation products.

Items 29 to 34 were samples of lecithins and were, like the oils, analysed fresh and after accelerated storage at 100°C. Although a significant increase in the COP value and a dramatic increase in the oxodiene value results from the prolonged exposure of the lecithins to elevated temperature, the peroxide value changes only slightly. The relationship between the increases in peroxide and COP values for the lecithins is thus in contrast to that observed for the vegetable oils. Dilution of soyabean lecithin with inert methyl laurate produces a less viscous sample which oxidized more, in a shorter time, but does not appreciably alter the proportions of products.

Items 35 and 36 were mixtures of lecithin and vegetable oils and when oxidized at 100°C showed again a negligible increase of peroxide though exposed to the elevated temperature eight times as long as were the oils alone (items 25 and 26). Thus, the lecithin exhibits considerable antioxidant properties in preventing peroxide accumulation in this system. However, the COP assay does show changes similar to that observed of lecithin alone, particularly in the accumulation of oxodiene.

The relationship between peroxide value and COP value for most of the items analysed are illustrated in Fig. 3. Both axes are plotted on a logarithmic scale showing all experimental values which were greater than one. Both the peroxide value and the COP value, in our experience, have the same level of numerical significance. Values greater than unity can be determined precisely, those below unity are less reproducible.

A yield line has also been drawn in Fig. 3 defining the arbitrary relationship: peroxide value equivalent to twice the COP value. This empirical conversion factor is based on the results of Fishwick & Swoboda (1976) for pure peroxides of polyenoic fatty acid esters. Samples containing appreciable amounts of monoene peroxides would of course yield a lower COP value, whereas the presence of secondary oxidation products would increase the COP value..

The items plotted in Fig. 3 lie scattered above or just below the yield line. All the other results (items 1, 2, 4, 17, 29, 30, 31, 33) with values less than one for either assay, and which are not plotted, also lie above the line. The considerable scatter of results

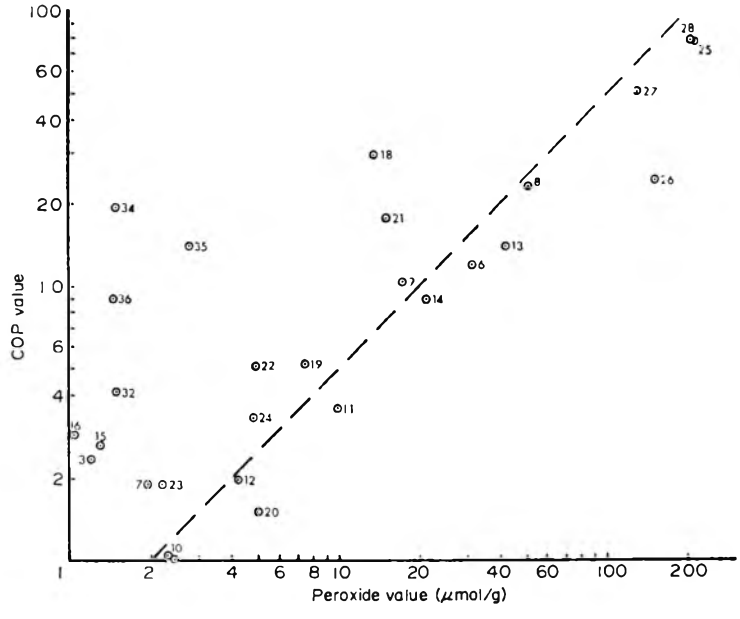

FIG. 3. The relationship between results obtained on lipids tested for the peroxide and 'COP' values. The numbered items are described in Table 1.

above the yield indicate that for foodstuffs appreciable secondary oxidation products of polyenoic fatty acids contribute to the determined COP value, which are not measured by the peroxide value. Only a considerable contribution of monoenoic fatty acid oxidation would greatly depress the experimental result below the line, and this is probably the case with oxidized groundnut oil (item 26).

A simplified assay (Quick COP)

The absorbance of the 'D' solution measured at 268 and 301 nm ($A_{268}{}^{D}$ and $A_{301}{}^{D}$), or just one of these wavelenghs where appropriate, is by itself an indication of the extent of oxidation of the lipid sample being assayed. For quality control the advantage of simplicity possibly outweighs the lack of background correction arising through not subtracting the absorbance of the reduced ('R') spectrum. To achieve this more simple measurement a quick procedure was devised for performing the two reaction steps of reduction and dehydration of the COP assay. The procedure was as follows. Approximately 50 mg of lipid was weighed accurately into a small reaction vessel fitted with a reflux condenser. Five millilitres of iso-octane : ethanol (1 : 1 by volume) was added to effect solution. One millilitre of a filtered, saturated solution of sodium borohydride in iso-propanol* was then added and the mixture allowed to reflux for

* 1 g of solid sodium borohydride was dispersed in 100 ml of isopropanol and freshly filtered before use to yield a 0·4% solution. The suspension was stable for at least a week in a refrigerator.

5 min; 5·0 ml of a 20% sulphuric acid solution in ethanol (w:v) was carefully added through the condenser and reflux was continued for a further 5 min. Finally, the reaction mixture was cooled and made to 25·0 ml with ethanol. The absorbance of this solution was measured at the chosen wavelengths of 268 and/or 301 nm against a reference cell containing ethanol. The results were then calculated, using the weight of sample taken and the final volume of the solution in terms of the specific absorbance (i.e. absorbance for a 1% lipid solution in 1-cm cell).

TABLE 2. Comparison of the specific absorbance (1% 1 cm) of 'D' solutions prepared by the two procedures

Lipid	Full COP		Quick COP	
	268 nm	301 nm	268 nm	301 nm
Groundnut oil	8·9	1·0	8·9	0·7
Margarine	11·5	1·8	13·0	2·5
Salad cream	30·3	2·1	31·9	2·4
Herring oil	2·1	3·2	2·0	2·9
Groundnut oil and lecithin (1:1)	11·7	5·2	10·7	4·6
Sunflower oil and lecithin (1:1)	13·6	3·8	13·1	3·6
Lecithin	13·2	4·5	13·1	4·9

Results obtained on lipids from seven samples of diverse degrees of oxidation, comparing the absorbance measured for 'D' solutions prepared by both the full and quicker versions of the COP assay, are shown in Table 2. It can be seen from the table that the results are comparable whether the reaction steps are carried out in two periods of 30 min at 60°C or for 5 min each under reflux. However, shorter periods of reflux for only 2 min resulted in considerably lower figures. The choice between the full or the quicker procedure is a matter of convenience. The results are in terms of absorbance and measure not only the contribution of oxidation products yielding conjugated chromophoric groups under conditions of the assay, but also include the contribution of pre-existing chromophores in the lipid sample. The latter, however, are corrected for in the determination of the COP value by the full procedure since in this the absorbance of the 'R' solution is subtracted. Again, depending on the fatty acid composition of the sample that is being routinely monitored, sufficient information may be obtained by measurement at either 268 or 301 nm rather than both. Thus specifications for quality control can result from the measurement of the absorbance of only one solution at one wavelength after a relatively quick reaction procedure.

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A pork slurry system for studying inhibition of *Clostridium botulinum* by curing salts

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Summary

A meat slurry system is described for studying the effects of curing agents on growth and toxin production by *Clostridium botulinum*. In developing the system a prime objective was good reproducibility for pre-selected levels of salt (NaCl), sodium nitrite, fat and pH value. This objective was achieved and large numbers of replicate samples were prepared and dispensed with a minimum of technological equipment. Results obtained in the system are compared with those obtained using pork mince packed into bottles (Ashworth, Hargreaves & Jarvis, 1973). Results of preliminary studies show little effect of varying the severity of thermal processes on the inhibitory action of sodium nitrite in the presence of sodium chloride, but some variation in inhibition was observed with different batches of meat used to prepare the slurries.

Introduction

Pasteurized canned cured meat products have an excellent record of microbiological safety and stability. The reasons for this stability are not fully understood but it is believed to depend on many complex interactions in the product (Spencer, 1966; Roberts & Ingram, 1973). The levels of salt (sodium chloride), sodium nitrite and spores in the meat, the degree of thermal processing and the temperature of post-process storage are all believed to play a major role in ensuring stability.

Although based on traditional practices, the use of curing salts, such as sodium nitrite and sodium nitrate, is limited by legislation in many countries. At the present time, the permitted level for use of nitrite is under review because of its implication as a precursor of nitrosamines in cured meat and fish products. Furthermore, there is a trend towards the use of lower levels of salt to satisfy changes in consumer preferences.

It should not be assumed that any equipment, materials or chemicals specifically named in this report are the only items available, or necessarily the most suitable items on the market, for the purpose described.

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For these reasons it is essential to evaluate the various complex interactions which occur in cured meat products so that a potential hazard from nitrosamines is not replaced by a very real hazard from botulism.

During the past few years many studies of the role of sodium nitrite have been made with *in vitro* laboratory media systems (Perigo, Whiting & Bashford, 1967; Johnston, Pivnick & Samson, 1969; Johnston & Loynes, 1971; Roberts & Ingram, 1973; Ashworth *et al.*, 1974b). Although these studies have given some guidance to the interactions which may occur in cured meats, direct comparison of results in meat and media systems do not always give comparable results (Ashworth, Hargreaves & Jarvis, 1973; Ashworth *et al.*, 1974a).

Because of the inherent variability of cured meat products, multifactorial experiments with inoculated packs can become difficult to control in the laboratory. Extensive chemical analysis must be undertaken, because of the inter- and intra-sample variation, and even small variations may have a significant effect on the results obtained in microbiological studies. Inoculation studies on commercial packs of cured meat, such as those made by Christiansen *et al.* (1973), are limited also by the general availability of processing facilities suitable for use with *Cl. botulinum*. In our earlier studies using pork mince (Ashworth *et al.*, 1973) constraints on the number of parameters and replicates which could be tested in any one experiment included the difficulty of packing the mince into bottles, and problems of nonhomogeneous distribution of the various additives in the mince.

For these reasons we considered it desirable to develop a meat-based system which could be used for multifactorial laboratory studies and in which various parameters could be closely controlled. It was considered essential that the system should resemble as closely as possible a typical commercial heat-processed cured meat. This paper describes such a system and gives examples of comparability with the pork mince system used previously (Ashworth *et al.*, 1973).

Experimental

Meat slurry system

The meat slurry consisted of a blend of equal parts by weight of minced pork and a brine containing appropriate quantities of the required additives, at an appropriate pH value.

Preparation of the meat. Commercial prime pork shoulder was trimmed of skin, fat and connective tissue and cut into small pieces. It was twice minced through the 4-mm plate of a Hobart Mixer/Mincer (KP 470). The minced meat was stored at −32°C as pre-weighed 500 g–2 kg aliquots. The trimmed fat was minced and stored similarly in 100-g aliquots. Samples of the meat were analysed for fat and water content.

Preparation of the additives. From the original analysis of the meat the quantity of salt necessary to give a prerequisite salt-on-water level in the final slurry was calculated.

Similar calculations were made for other additives (e.g. nitrite, polyphosphate, ascorbate, etc.). After dissolving the additives in filter-sterilized distilled water, the pH value of the solution was adjusted as required (see Results).

Preparation of the slurry. The minced pork was allowed to thaw for 18 hr at 15°C and additional minced pork fat was mixed in by hand, if required. The solution of curing salts was added to the minced meat, or meat plus fat, in a bottom-drive Hobart homogenizer and blended for 30 sec. Small quantities of slurry were prepared using the blender attachment to the Kenwood Chef Food Mixer.

Preparation of the spore inocula. Spores of proteolytic strains of *Cl. botulinum* Type A (NCTC 7272, NCTC 62A, NCTC 3806) and Type B (NCTC 7273, NCTC 3807, NCTC 13982) were prepared in the Trypticase Peptone Thioglycollate medium of Schmidt & Nank (1960). Spores of *Cl. sporogenes* were prepared by the National Canners Association Research Laboratories (1968) method. Spores were harvested by centrifugation, washed in sterile distilled water at pH 7 and stored at 4°C as concentrated suspensions. The spore suspensions were diluted in sterile distilled water to the level required for inoculation. Aliquots of the working suspensions were heat shocked for 10 min at 70°C and were enumerated using Miles & Misra (1938) counts on Blood Agar Base (Oxoid) containing 5% v/v defibrinated horse blood (Wellcome Laboratories, Beckenham). Plates were incubated for three days at 30°C in BTL anaerobe jars in an atmosphere of 95% hydrogen: 5% carbon dioxide (British Oxygen Ltd), using Gas-Pak room temperature catalysts (BBL Ltd).

Filling and inoculation of the slurries. The slurries were filled into 1-oz Universal bottles and sealed with metal screw caps having a central hole of 4 mm diameter and fitted with new, thoroughly cleaned, rubber liners. Bottles were filled using an Albro single head vacuum-operated filler. The bottle caps were coded using a water-insoluble marking ink.

Spore suspensions (0·5 ml/bottle) were inoculated into the centre of the meat using a sterile 1-ml repeating syringe. Before inoculation the rubber septum was swabbed with 70% v/v alcohol and, after inoculation, with 4% Chloros solution (ICI Ltd).

Heat treatment. Bottles of slurry were processed by total immersion in a specially constructed waterbath (Plate 1) consisting of a lagged hot water reservoir (R), a circulating pump and a heating bath (B) which had two chambers fitted with a weir overflow (W) for return of water to the reservoir. Water was heated by two 2 kW immersion heaters controlled through an ILPC thermoregulator (Leatherhead Food R.A.) to give temperature control within ±0·1°C of the required temperature. Water entered the bath through a series of jets along the bottom of each heating chamber and returned to the reservoir under gravity.

Total immersion of bottles of slurry in the bath at 80°C raised the centre temperature from ambient to 70°C in 7 min. To obtain a longer process, bottles were transferred to a bath at 70°C and held for an appropriate time period. On removal from the bath, bottles were allowed to cool for 1 hr at room temperature and then at 4°C for up to

6 hr to reduce the temperature of the cooked slurry to below the intended incubation temperature.

Incubation of slurry and assessment of spoilage. Bottles were incubated in sealed metal trays at temperatures within the range 15 to 37°C. The frequency of examination of samples for overt spoilage varied according to incubation temperature: every two days at 30°C and above; every fourteen days at 15°C. In some experiments bottles were removed and tested for botulinal toxin only when evidence was obtained for overt spoilage; in other experiments a number of replicate bottles was removed at predetermined intervals. Overt spoilage was detected by a colour change and by granulation of the meat plug, which was accompanied by breakdown and cloudiness of the 'jelly' (Plate 2). Formation of gas within the bottles sometimes produced obvious distension of the rubber septum. At the lower temperatures of incubation spoilage was sometimes difficult to detect since little colour change occurred and only minor 'cracking' could be observed in the meat plug. In all experiments non-inoculated control samples were incubated concurrently. On termination of incubation, all remaining samples were tested for toxin even in the absence of overt spoilage.

Pork mince system

The pork mince system described previously by Ashworth *et al.* (1973) was used in limited comparative studies with *Cl. botulinum* and *Cl. sporogenes*. Full comparisons were not possible because of the handling problems associated with packing large numbers of bottles of minced meat.

Analytical methods

Chemical analyses. Raw meat samples and bottles of non-inoculated pork slurry and of pork mince, were analysed for salt, moisture and fat by the British Standards Institution (1970) methods. Nitrite was determined by the Ashworth & Spencer (1972) modification of the method of Schall & Hatcher (1968). The pH values of the pork, the salts solutions used in preparation of the slurries and the heated and unheated slurries were determined electrometrically using a Radiometer pH meter (Model 29) fitted with a glass spear electrode (GK2311c), or a Pye pH meter (Model 290) with a combined glass electrode (401 E_o7).

Bacteriological analysis. Colony counts on Horse Blood agar were made before and after heating the pork slurry by the method of Miles & Misra (1938). Dilutions were prepared in a quarter strength Ringer solution containing 0·1% w/v Peptone (Oxoid). Plates were incubated aerobically, or in an atmosphere of 95% hydrogen: 5% carbon dioxide with a Gas-Pak catalyst (BBL Ltd), for three days at 30°C.

Toxicity tests. Gas pressure was released from the bottles of meat slurry in a pathological cabinet; the meat plug and/or liquor was decanted, macerated with gelatin phosphate buffer (GPB), pH 6·5 (Lewis & Angelotti, 1964), centrifuged for 25 min at 1200 *g* and stored at −20°C until tested. Botulinal toxin was detected by injection of 0·4 ml

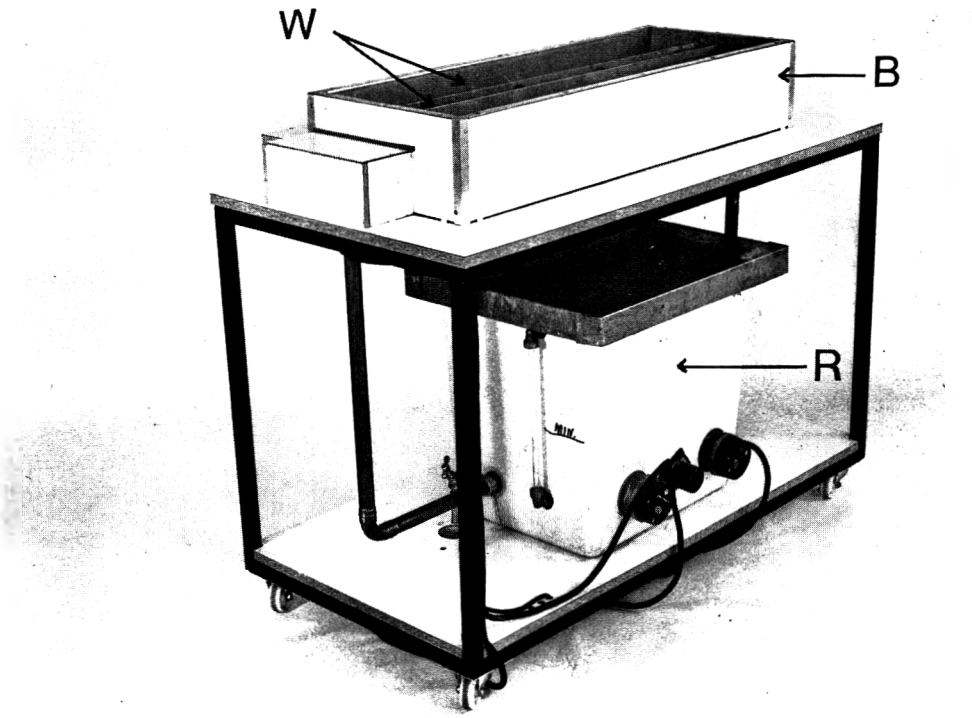

PLATE 1. Specially constructed waterbath for processing bottles of meat slurry. Water is heated in the reservoir (R) and is pumped to the heating bath (B) where water enters through a series of jets along the bottom of each chamber. Water returns to the reservoir over a weir (W) which runs along the centre of the bath.

(*Facing p.* 16)

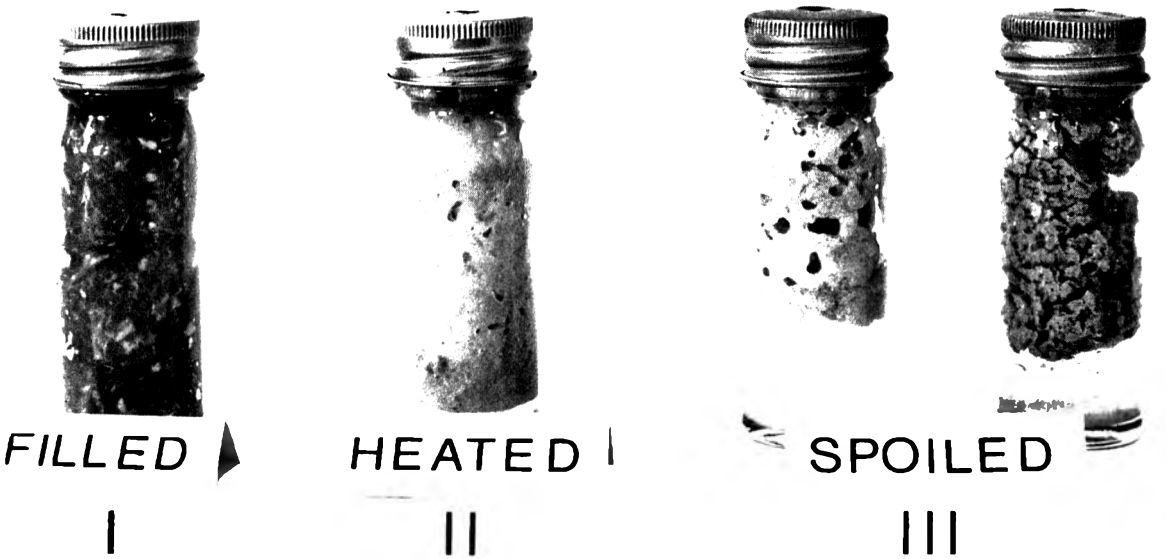

PLATE 2. Bottles of meat slurry after filling (I), after heating (II) and showing evidence of spoilage (III). Splitting of the meat plug and gas formation can be seen in the spoiled samples.

of a suitable dilution of the supernatant in GPB into each of a pair of 18–20 g female Swiss white mice. The mice were observed for typical symptons of botulism over a period of three days. Toxin titres were determined by serial dilution of the sample in GPB. Confirmation of toxin was made by mouse protection tests using either polyvalent antitoxin (State Serum Institute, Copenhagen) or monovalent antitoxins types A to E (Institute Pasteur, Paris).

Results and discussion

Chemical analyses on the slurries

The relationships between calculated and analysed values for salt-on-water and sodium nitrite in slurry are illustrated in Fig. 1. These analyses were made immediately after mixing the slurries, so that reaction between sodium nitrite and the meat constituents was minimal. Results of chemical analyses obtained in a number of experiments,

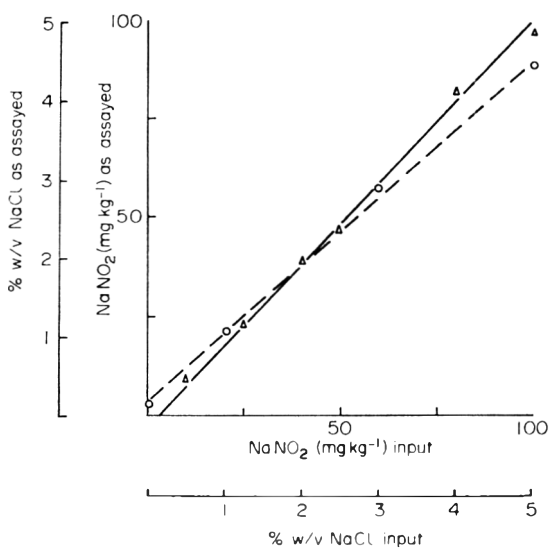

FIG. 1. Chemical analysis of meat slurries immediately after preparation. Meat slurries were prepared (see Methods) to contain various levels of sodium chloride (○; % w/v) and sodium nitrite (△; mg kg^{-1}) and were analysed immediately after filling into 1-oz bottles. In each case, the values are the means of three replicate analyses; the regression coefficients for the lines are both greater than 0·98.

summarized in Table 1, show good reproducibility between experiments. As expected, the analysable quantities of sodium nitrite are somewhat reduced after processing, although probably to a lesser extent than that which would be achieved in a large (i.e. 6 lb) canned pasteurized ham.

TABLE 1. Analytical data on several batches of meat slurry

Parameter	Target level	No. of experiments	Mean (and range) determined
Salt-on-water (%)	1·8	2	1·65 (1·56–1·73)
	3·5	4	3·44 (3·40–3·48)
Sodium nitrite (mg kg^{-1})	40	4	28·7 (20–45)*
	75	6	60·7 (31–84)*
		2	72·0 (71–73)**
	125	4	75·0 (63–92)*
	175	6	132·7 (75–170)*
		2	149 (146–151)**
	225	4	129·5 (88–161)*
	300	6	201·1 (118–290)*
		2	240·0 (234–246)**
pH value	6·0	6	5·90 (5·85–5·95)†
			6·20 (6·13–6·27)††

* Processed for $P_{80°C} = 0·65$; analysed immediately.

** Processed for $P_{80°C} = 12·65$; analysed after 48 hr hold at 4°C (Roberts, Jarvis & Rhodes, 1976).

† Pre-process value.

†† Post-process value.

It was not possible to preset the pH value of the slurry without undertaking preliminary experiments with each batch of meat, portions of which were slurried with brine solutions adjusted to various pH values. Recorded pH levels in a number of experimental batches of slurry are presented in Table 1; in most cases the observed value was within 0·1 pH unit of the required level. It is noteworthy that the post-process pH value of most batches of slurry tested was some 0·2–0·3 pH units higher than the pre-process value. Monitoring of the pH value during storage of non-inoculated slurry showed a further initial increase of about 0·3 pH units, with subsequent stabilization of pH. The difficulty in presetting the pH value of the slurry is considered to be due to the complex buffering capacity of the meat, which varies markedly from batch to batch.

Microbiological changes during processing

Experience has shown that preparation, dispensing, inoculation and heat treatment of the slurries in large experiments (1000–2000 bottles) can conveniently be divided between two days. Growth of contaminating micro-organisms occurs to a limited extent during thawing of the deep frozen minced meat and during subsequent handling procedures. In one typical experiment, storage of the bottles of slurry for 36 hr at 4°C,

before inoculation and heating, resulted in aerobic and anaerobic bacterial counts of about 2×10^8 colony forming units (c.f.u.) g^{-1}; after heating, these counts were reduced to 9×10^3 and < 10 c.f.u. g^{-1}, respectively. Samples which had been stored for a similar time period at $-32°C$ had aerobic and anaerobic counts, before heating, of 9×10^4 and 2×10^4 c.f.u. g^{-1} respectively and < 10 c.f.u. g^{-1} after heating. Although the pre-heat counts were high in refrigerated non-inoculated samples, there was no evidence of overt spoilage at the time of processing. Even in experiments with very low inocula of *Cl. botulinum* spores (1–5 per bottle), spoilage in the heated inoculated slurries always occurred before spoilage in heated non-inoculated controls.

The spore inocula were injected as suspensions in a minimal volume of water; provided that it was adequately mixed into the slurry no significant localized effects on the salt-on-water level would occur, but compensation for the inoculum volume could be made when formulating the slurry. Recovery of *Cl. botulinum* spores from jelly and sectioned meat plugs (obtained by carefully breaking and removing the bottles) showed good distribution of spores. Localized pockets of high spore numbers or of low salt or nitrite are therefore unlikely to occur in the bottles of heated slurry.

Inhibition of Cl. botulinum by sodium nitrite

Comparability of pork slurry and pork mince systems. Previous studies on production of a Perigo-type inhibitor in pasteurized minced pork (Ashworth *et al.*, 1973) showed that *Cl. sporogenes* was more readily inhibited by heated nitrite than by unheated nitrite, but that the inhibitory level varied considerably from one experiment to another. Similar observations have been made with *Cl. sporogenes* inoculated into a meat slurry system (J. Ashworth & B. Jarvis, unpublished). Retrospective comparison of results from a number of experiments with both *Cl. botulinum* and *Cl. sporogenes* (Table 2) in pork mince and in the pork slurry system shows a slight difference, which is not statistically signifi-

TABLE 2. Comparison of inhibitory levels of sodium nitrite in pork mince and in pork slurry

Organism	Temp. (°C)	ED_{100}* sodium nitrite (mg kg^{-1}) after incubation for fifteen days in			
		Pork mince		Pork slurry	
		No. expts	Mean (and range)	No. expts	Mean (and range)
Cl. sporogenes	37	10	325 (150–600)	7	436 (300–600)
Cl. botulinum Types A and B	25/30	4	200 (150–250)	10	223 (175–300)

* ED_{100} is defined as the lowest concentration of sodium nitrite which inhibits growth and/or toxin production in all replicates tested (Ashworth *et al.*, 1973).

cant ($P > 0{\cdot}05$), in the levels of nitrite which prevented growth. In one series of direct comparisons, no differences were seen between the two meat systems; it is possible, therefore, that the overall differences shown in Table 2 may reflect variation associated with the use of different batches of meat.

That such variation can occur in the slurry system is demonstrated in Table 3. Over a six-month storage period, the overall occurrence of toxin (at all nitrite levels) was

TABLE 3. Effect of inter-batch variation in the meat on nitrite inhibition of toxin production by *Clostridium botulinum* in meat slurry at pH 6·0 and 3·5% salt on water

Sodium nitrite (mg kg^{-1})	No. samples toxic/no. tested						
	At 15°C			At 25°C			
	A*	B*	C*	A*	B*	C*	Total
75	7/15	13/15	12/15	15/15	15/15	15/15	77/90
125	4/15	8/15	6/15	14/15	13/15	11/15	56/90
175	5/15	9/15	4/15	7/15	12/15	11/15	48/90
225	2/15	5/15	2/15	9/15	11/15	4/15	33/90
300	0/15	1/15	2/15	4/15	5/15	2/15	14/90
Total	18/75	36/75	26/75	49/75	56/75	43/75	228/450
	80/225			148/225			

*A, B, C refer to three separate batches of pork tested simultaneously.

Total toxic replicates for meat batch A = 67/150 (44·7%); total toxic replicates for meat batch B = 92/150 (61·3%); total toxic replicates for meat batch C = 69/150 (46·0%).

45, 61 and 46% for three different batches of meat, all of which had been treated identically. Since the three meat systems had all been prepared and processed concurrently it is more likely that the differences reflect inherent properties of the meats used than variations in the specific levels of nitrite, salt, or process parameters (cf. Ashworth & Spencer, 1972). However, such variations could confound the effects of differences between meat samples. The results show also the overall reduction in occurrence of toxic samples with increasing initial nitrite levels (86% contained toxin at 75 mg kg^{-1} sodium nitrite cf. 16% at 300 mg kg^{-1} sodium nitrite), and an increase in toxic samples with increasing storage temperatures (35.6% contained toxin at 15°C cf. 65·8% at 25°C). A more detailed investigation of interaction of storage temperature with initial nitrite levels in the slurry system have been reported elsewhere (Roberts, Jarvis & Rhodes 1976).

Effects of thermal process. The extent of the heat treatment received by the bottles of slurry was evaluated by recording the temperature profile at the centre of the slurry

during the heating, holding and cooling periods using thermocouples. Process values were calculated by the method of Shapton, Lovelock & Laurito-Longo (1971) at a reference temperature of 80°C ($P_{80°C}$ values). Raising the centre temperature of the slurry to 70°C by immersion of the bottles for 7 min in an 80°C water bath and then allowing the bottles to cool to ambient temperature gave a total process equivalent to $P_{80°C} = 0{\cdot}65$. By transferring the heated bottles to a water bath at 70°C for various time periods process values of up to $P_{80°C} = 12{\cdot}65$ were achieved. Calculated on the same basis, the centres of large packs of commercial pasteurized hams receive processes varying from $P_{80°C} = 3$ to 7·5 (G. G. Evans, personal communication).

An experiment to assess the effect of thermal process was set up at three nitrite levels (75, 175 and 300 mg kg^{-1}), one salt level (3·5% salt on water) and using one batch of meat (7·5% fat); the replicate bottles were processed at either $P_{80°C} = 0{\cdot}65$ or $P_{80°C} = 12{\cdot}65$ and were subsequently incubated at either 15 or 25°C. Five replicate bottles from each treatment were withdrawn at fixed time intervals up to three months (ninety days) and were examined for evidence of overt spoilage and for botulinal toxin. The results, expressed as time in days to toxin production at either temperature (Table 4),

TABLE 4. Effects of thermal process and nitrite level on time for *Cl. botulinum* toxin production in meat slurry stored at 15 and 25°C

Initial nitrite level (mg kg^{-1})	Thermal process value $P_{80°C}$ (min)	Time (days) to detection of toxin in replicates stored at			
		25°C		15°C	
		One or more replicates	All replicates	One or more replicates	All replicates
75	0·65	8	8	32	> 90
	12·65	8	8	62	> 90
175	0·65	8	15	62	> 90
	12·65	15	15	62	> 90
300	0·65	81	> 90	> 90	> 90
	12·65	81	> 90	> 90	> 90

demonstrate little difference with the two thermal processes. Similar results were obtained with respect to spoilage although the replicates given the higher heat process ($P_{80°C} = 12{\cdot}65$) tended to spoil slightly more slowly than did those given the lower process. Since the higher process would have been expected to reduce significantly the incidence of toxicity more detailed investigations of the effects of thermal processes are now being undertaken.

Conclusions

The meat slurry system provides a simple and convenient method of assessing the interactions which occur in heated cured meat systems. The results of inhibition studies described are used merely to illustrate the applications of the slurry system. Many more detailed investigations have been undertaken, some of which have been reported already (Roberts, Jarvis & Rhodes, 1976).

A potential disadvantage of the slurry system is that the water content is somewhat higher than would be observed in a normal commercial cooked cured meat. Comparison of results in the slurry system with those in pork mince confirms our belief that the high water content does not invalidate the use of the system. Recent experiments using a slurry prepared from four or five parts of minced meat to one part of brine solution have shown that it is possible to prepare such a slurry system which very closely simulates the physical and chemical characteristics of a pumped or 'tumbled' ham. However, the many problems of handling so thick a slurry reduces its appeal for large-scale laboratory experiments.

The studies undertaken to date have been restricted to those using spores of *Cl. botulinum* and *Cl. sporogenes* as inoculum. There is no obvious reason why the slurry system could not be used in studies with other organisms (e.g. faecal streptococci; *Bacillus* spp.) which are known to survive the pasteurization process commonly given to cured meats (Ingram, 1969).

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Inhibition of *Clostridium botulinum* by curing salts in pasteurized pork slurry

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Summary

The interaction of sodium chloride (salt), sodium nitrite and storage temperature on toxin production and spoilage by *Clostridium botulinum* was studied in meat slurry at pH 6·0. Over six months storage greatest inhibition at 1·8% or 3·5% salt (on water phase) was observed at 15°C with less at 17·5°C. Little inhibition occurred at these salt concentrations even in the presence of 300 mg kg^{-1} sodium nitrite at 20, 22·5 and 25°C. A pronounced salt-nitrite-time interaction was observed at the lower incubation temperatures. The level of the spore inoculum (10^1, 10^3 or 10^5) only affected the extent of spoilage or toxicity at 15°C. Toxin formation without overt spoilage was obtained occasionally, especially at 15°C.

Introduction

Pasteurized cured meats contain viable bacterial spores which normally fail to grow in the product. This inability to grow is the result of the interaction of several factors, including pH value and the concentrations of sodium chloride and sodium nitrite (Riemann, 1963). Bacterial cells are more readily inhibited by salt and nitrite at temperatures near the minimum for growth, and storage temperature would be expected to play an important part in the stability of pasteurized cured meats. In reviewing the relevant literature, Spencer (1966) showed that heating, within the range $F_0 = 0{\cdot}1$ to 1·0, has a supplementary effect, and that systems inhibitory when inoculated with 1–10 spores per gram fail when challenged with numbers 100 to 1000 times larger.

Some of the nitrite in cured meats is converted into nitrosomyoglobin, which gives the characteristic pink colour, and nitrite is essential for the characteristic flavour which distinguishes ham and bacon from salt pork, but both these functions require less nitrite than is required to guarantee bacterial stability and safety from botulism (Ingram, 1974, 1975).

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With the commoner use of highly sensitive gas-liquid chromatographic and mass spectrometric analytical methods, low molecular weight nitrosamines have occasionally been detected in cured meats at the $\mu g\ kg^{-1}$ (ppb) level. These substances are carcinogenic for laboratory animals when administered in relatively large ($mg\ kg^{-1}$ or ppm) doses and at such levels must be regarded as potentially carcinogenic for man. The dose–effect relationship for man is unknown and it is not clear whether repeated low doses might have a cumulative effect (see Krol & Tinbergen, 1974). Nitrosamines are formed by the reaction of nitrite with secondary and tertiary amines, and it would seem logical to examine first whether a reduction in the levels of nitrite would lead to a corresponding reduction in the levels of nitrosamines. However, reducing the levels of nitrite would also reduce the stability of the product with respect to bacterial growth, and might also increase the likelihood of supporting the growth of *Clostridium botulinum*. *Cl botulinum* appears to be more common in pork than in lamb or beef, and must be expected to occur from time to time in pork products, although normally the number of spores per gram will be very low.

Such considerations emphasize the need for a fuller understanding of the factors interacting to inhibit the growth of bacteria, particularly *Cl. botulinum*, in pasteurized cured meats. The logical extension of the early work on the inhibitory effects of curing salts reviewed by Spencer (1966) and a relatively small statistically-based factorial experiment described by Riemann (1963) was an attempt to devise a model system to portray the interaction of three factors, which could be expressed in mathematical terms. This has been largely achieved in a laboratory medium by varying the pH value and the concentrations of sodium chloride and sodium nitrite (unheated) against vegetative forms of *Cl. botulinum* types A, B, E and F (Roberts & Ingram, 1973), and the philosophy has been discussed (Roberts, 1974). When heated in certain laboratory media, nitrite becomes more inhibitory to *Clostridium* spp. (Perigo, Whiting & Bashford, 1967; Perigo & Roberts, 1968), but the relevance of these observations to meat is in doubt, since the addition of as little as 1% meat to culture medium in which the nitrite inhibitor had been formed neutralized its activity (Johnson, Pivnick & Samson, 1969). However, Ashworth & Spencer (1972) showed that if the nitrite was added to minced whole pork before, rather than after, heating through a sterilization process, half as much residual nitrite (at the time of inoculation) sufficed to inhibit *Cl. sporogenes*. Although this was taken to indicate a 'Perigo' nitrite inhibitor, higher input levels were required when nitrite was heated in the pork than when it was unheated. Subsquently, Ashworth, Hargreaves & Jarvis (1973) demonstrated the production of a Perigo-type effect in pasteurized whole minced meat; this effect was evident in terms of both input nitrite and residual nitrite levels. Pivnick & Chang (1974) detected anti-bacterial activity in commercially formulated luncheon meat when no free nitrite remained, and interpreted this as indicating the presence of an inhibitor derived from the original nitrite.

Investigators are faced with the problem of trying to determine the minimum effective concentration of nitrite knowing that its effects are modified by pH value,

sodium chloride and incubation temperature. The precise relationship of results obtained in laboratory media to those obtained in pork is not known, and it seems desirable to attempt to define further these interactions in a meat medium. At the same time the range of pork products to which these observations apply is wide and their formulations vary. It is clear from completed studies (Greenberg, 1972) that the properties of pasteurized cured meats which are inhibitory to bacterial spores are heavily dependent upon nitrite concentration, and that nitrite cannot be totally omitted, or substantially reduced, without compensation, e.g. by increasing the salt content thereby reducing the water activity of the product.

The purpose of this study was to attempt to define the inter-relationships between salt, nitrite and incubation temperature against growth and toxin production by *Cl. botulinum* spores in a meat system at a single pH value. A major problem in investigations involving many variables is the difficulty in handling sufficient replicates in any one laboratory. Hence this investigation was planned as a joint exercise between two laboratories: the Meat Research Institute (MRI) and the Leatherhead Food R.A. (LFRA). Preliminary experiments demonstrated the desirability of preparing the meat slurries in one laboratory and of using a common mixed spore preparation.

Materials and methods

Organisms

Three strains each of *Cl. botulinum* type A and B were used: type A, NCTC 3806, NCTC 7272 and 62A and type B, NCTC 3807, NCTC 7273 and NCTC 13982 (all proteolytic strains).

Sporulation media

Spores were prepared in Trypticase-peptone-thioglycollate medium (Schmidt & Nank, 1960) or a similar medium comprising (% w/v) Trypticase (BBL), 5; Bacto-peptone, 0·5; yeast extract ('Oxoid'), 0·2; glucose, 0·4 and cysteine hydrochloride, 0·05, harvested by centrifugation and stored at 1–4°C for at least a month.

Preparation of spore inoculum

Numbers of spores in each crop were determined by colony counts on Blood Agar Base ('Oxoid') containing 5% (w/v) defibrinated horse blood (Wellcome Reagents Ltd, Beckenham) (HBA) and incubated in 95% H_2 + 5% CO_2 or in Reinforced Clostridial Agar ('Oxoid') containing 0·1% (w/v) sodium hydrogen carbonate added to the molten agar at 45°C as a filter sterilized 10% (w/v) solution, incubated in 100% H_2 in BTL anaerobic jars containing a room temperature catalyst ('Deoxo' pellets; Engelhard Industries Ltd, Cinderford, Glos.; or Gas Pak Catalyst, BBL).

Equal numbers of spores of each of the six strains of *Cl. botulinum* were mixed in sterile water, and the stock mixture was retained for both experiments described. In

experiment A, the spore inoculum was not heated but in experiment B spores were heated at 60°C for 90 min before inoculating the slurries.

Preparation of slurry

The preparation of the pork slurry is described by Rhodes & Jarvis (1976). Aerobic and anaerobic bacterial counts (30°C/three days) were made on HBA on both unheated and heated slurry by the method of Miles & Misra (1938). Decimal dilutions were made in quarter strength Ringer's solution containing 0·1% (w/v) peptone ('Oxoid'), and anaerobic counts were made after incubation in 95% H_2 + 5% CO_2.

Chemical analyses

Uninoculated bottles of meat slurry were analysed chemically in both laboratories both before and after the heat process. Salt, moisture and fat concentrations were determined by the British Standards Institution (1970) methods for analysis of meat products. Nitrite was determined by a modification of the Schall & Hatcher (1968) method as described by Ashworth & Spencer (1972) (LFRA) or by the official method of the Society of Analytical Chemistry (1974) (MRI).

The pH values of the meat, the salts solution used to prepare the slurry, and the heated and unheated slurry were determined electrometrically using a Radiometer pH meter (model 29; Radiometer, Copenhagen, Denmark) with a glass spear electrode (NK 2311C).

Toxin tests

The clarified supernatant from each sample was diluted 1 : 5 in gelatin-phosphate buffer (pH 6·5; Lewis & Angelotti, 1964) and was tested for botulinal toxin by intraperitoneal (i.p.) injection of 0·4 ml(LFRA) or 0·5 ml (MRI) into each of a pair of 18–20 g female Swiss white mice. The mice were observed for typical symptoms of botulism for three days. Toxin was confirmed by neutralization tests in mice using either polyvalent antitoxin (State Serum Institute, Copenhagen, Denmark) or monovalent antitoxin types A and B (Institut Pasteur, Paris, France).

Experimental plan

The factors investigated were NaCl, $NaNO_2$, incubation temperature and inoculum level. Details, including replication, are shown in Table 1.

The meat slurry system of Rhodes & Jarvis (1976) was used throughout employing pork shoulder carefully de-fatted by hand before chopping and mincing. For each combination of salt and nitrite a 7 kg batch of slurry was prepared at pH 6·0. This was dispensed into 1 fl. oz Universal bottles, a total of 240 bottles each containing 25 g being prepared from each batch of slurry. After filling, the bottles were divided into two groups for inoculation, heating and incubation. One group was tested at the LFRA and the other was transported and tested at the MRI. To restrict microbial growth

TABLE 1. Concentrations of sodium chloride, sodium nitrite, spore inoculum levels, incubation temperatures and replication

NaCl (% w/v) (on water)	$NaNO_2$ (mg kg^{-1})	Expt	Inoculum (spores/bottle)			Incubation temperature (°C)				
			10^1	10^3	10^5	15	17·5	20	22·5	25
		A		+*	+	+		+		+
	40							20M†		20M
1·8	75					20F				20F
	125									
3·5	175									
	225	B	+	+		+	+	+	+	+
						20F		10F		10F
						10M	10M	10M	10M	

*All combinations of the concentrations of sodium chloride and sodium nitrite listed were tested at the inoculum levels and incubation temperatures shown as +.

†Replicates per lab.: M, MRI; F, LFRA.

during transportation, bottles of slurry were chilled to 4°C in the first experiment (A) or frozen at −32°C in the second experiment (B). Inoculation and heat processing was carried out concurrently in the two laboratories. The bottles of slurry were equilibrated to ambient temperature, inoculated with 10^1, 10^3 or 10^5 spores per bottle (see Table 1) and were then heated in a water bath at 80°C for 7 min which permitted the centre temperature of the meat slurry to rise to 70°C (see Rhodes & Jarvis 1976) and gave a calculated process value $P_{80°C}$ of 0·65 (Shapton, Lovelock & Laurita-Longo, 1971). After heating, the bottles were cooled for 30–60 min at room temperature and then for 2–6 h at 4°C. Ten or twenty replicate bottles per salt and nitrite combination per laboratory were incubated at 15, 17·5, 20, 22·5 or 25°C (see Table 1). Uninoculated bottles were incubated concurrently as spoilage controls. Bottles were examined at weekly intervals for four weeks and then every second week for a total period of six months. Bottles showing evidence of spoilage were removed; the liquor was decanted, centrifuged for 25 min at 1200 × *g* and stored at −20°C until tested for toxin. After six months incubation all apparently unspoiled samples were tested for toxin.

Results

The analytical data for both experiments are given in Table 2 from which it can be seen that there was good agreement between the two laboratories for the analyses of fat, moisture and NaCl. There were larger differences in the analyses of $NaNO_2$, but this is known to be a less reproducible assay, and the differences were acceptable in almost every case. Table 3 and 4 give in full the toxicity results for both experiments.

TABLE 2. Analysis of slurries for fat, moisture, sodium chloride and sodium nitrite before and after pasteurization

Expts A and B calculated		Expt A (pH 5·96)					Expt B (pH 5·94)				
				Post process					Post process		
NaCl (% w/v on water)	$NaNO_2$ (mg kg^{-1})	Fat (%)	Moisture (%)	NaCl (% w/v on water)	$NaNO_2$ (mg kg^{-1}) (F)	$NaNO_2$ (mg kg^{-1}) (M)	Fat (%)	Moisture (%)	NaCl (%w/v on water)	$NaNO_2$ (mg kg^{-1}) (F)	$NaNO_2$ (mg kg^{-1}) (M)
1·8	40	—*	—	1·7	5	2	5·2(M) 7·5(F)	83·9(M)	—	23	28
	75	—	—	1·8	31	4	—	83·5(F)	—	53	60
	125	5·78(F)	—	1·7	81	61	—	—	2·2	63	87
	175	—	83·2(F)	1·8	125	85	—	—	2·0	75	127
	225	5·70(M)	81·9(M)	1·8	153	108	—	—	2·2	88	160
	300	—	—	1·8	209	140	6·4(M)	82·6(M)	—	128	196
3·5	40	—	—	3·3	27	30	5·6(M)	82·3(M)	—	20	35
	75	5·2(M)	82·9(M)	3·4	53	56	—	—	3·5	60	49
	125	5·4(M)	83·7(M)	3·5	92	93	—	—	4·8	64	96
	175	5·4(M)	82·4(M)	3·4	124	127	—	—	—	133	129
	225	—	—	3·6	161	171	—	82·3(F) 82·5(F)	—	115	169
	300	—	—	3·6	210	225	5·6(M) 7·42(F)	82·0(M)	—	118	224

*— = not tested.

TABLE 3. The effects of sodium chloride, sodium nitrite, inoculum level and incubation temperature on spoilage and toxin production by *Clostridium botulinum* types A and B (Expt A)

NaCl (% w/v on water)	$NaNO_2$ (mg kg^{-1})		Each treatment twenty replicates. (a) Incubation temperature °C. (b) Spores/bottle											
			(a) 15*				20°				25°			
			(b) 10^3		10^5		10^3		10^5		10^3		10^5	
			S	T	S	T	S	T	S	T	S	T	S	T
1·8	40	M*					20/20	3/3	20/20	3/3	20/20	3/3	20/20	1/1
		F	20/20	10/16	20/20	8/11					20/20	1/1	20/20	1/1
	75	M					20/20	3/3	20/20	3/3	20/20	3/3	20/20	0/0
		F	20/20	11/18	20/20	19/20					20/20	1/1	20/20	1/1
	125	M					20/20	3/3	20/20	3/3	20/20	3/3	20/20	0/0
		F	20/20	6/7	20/20	7/7					20/20	1/1	20/20	1/1
	175	M					20/20	3/3	20/20	3/3	18/20	3/3	20/20	0/0
		F	20/20	13/20	20/20	11/11					20/20	1/1	20/20	1/1
	225	M					20/20	9/9	20/20	10/10	10/20	3/3	20/20	0/0
		F	1/20	4[a]/20	8/20	18[b]/20					20/20	8/8	20/20	6/6
	300	M					19/20	5/5	20/20	0/0	5/20	5/5	16/20	4/4
		F	1/20	1/20	0/20	0/20					18/20	14/20	17/20	13/17
3·5	40	M					20/20	4/4	20/20	3/3	20/20	2/2	20/20	0/0
		F	20/20	3/15	20/20	5/20					20/20	1/1	20/20	1/1
	75	M					20/20	4/4	20/20	3/3	20/20	3/3	20/20	0/0
		F	7/20	4/20	17/20	3[c]/20					20/20	1/1	20/20	1/1
	125	M					20/20	3/3	20/20	3/3	20/20	3/3	20/20	0/0
		F	0/20	0/20	0/20	0/20					20/20	1/1	20/20	5/5
	175	M					20/20	3/3	19/20	2/2	20/20	3/3	20/20	0/0
		F	0/20	0/0	2/20	4[d]/20					20/20	4/4	20/20	4/4
	225	M					13/20	7/7	18/20	3/3	14/20	3/3	20/20	0/0
		F	0/20	0/0	0/20	2[e]/20					20/20	4/4	20/20	4/4
	300	M					17/20	0/0	16/20	0/0	9/20	8/8	20/20	0/0
		F	0/20	0/0	0/20	0/20					20/20	15/16	20/20	9/9

S, spoilage; T, toxic.

(a), 3 toxic but not spoiled; (b), 11 toxic but not spoiled, 1 spoiled not toxic; (c), 2 toxic but not spoiled, 16 spoiled not toxic; (d), 2 toxic but not spoiled; (e), 2 toxic but not spoiled.

* M, MRI; F, LFRA.

TABLE 4. The effects of sodium chloride, sodium nitrite, inoculum level and incubation temperature on spoilage and toxin production by *Clostridium botulinum* types A and B (Expt B)

Each treatment ten replicates except 15°C—twenty replicates. (a) Incubation temperature °C. (b) Spores/bottle

NaCl (% w/v on water)	$NaNO_2$ (mg kg^{-1})		(a) 15° (b) 10^1 S	10^1 T	15° 10^3 S	10^3 T	17·5° 10^1 S	10^1 T	17·5° 10^3 S	10^3 T	20° 10^1 S	10^1 T	20° 10^3 S	10^3 T	22·5° 10^1 S	10^1 T	22·5° 10^3 S	10^3 T	25° 10^1 S	10^1 T	25° 10^3 S	10^3 T
1·8	40	F*	20/20	16/20	20/20	18/20					10/10	1/5	10/10	2/2					10/10	9/9	10/10	2/2
		M					10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	75	F	20/20	12/20	20/30	18/20					10/10	2/2	10/10	2/2					10/10	2/2	10/10	2/2
		M					10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	125	F	19/20	8/20	20/20	17/20					10/10	3/5	10/10	7/10					10/10	2/2	10/10	2/2
		M					10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	175	F	13/20	4/20	19/20	19/19					10/10	8/9	10/10	4/4					10/10	10/10	10/10	10/10
		M					9/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	225	F	0/20	0/20	12/20	9/20					10/10	8/8	10/10	3/3					10/10	10/10	10/10	10/10
		M					9/10	1/1	10/10	2/2	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	300	F	0/20	0/0	1/20	1/20					8/10	3/5	5/10	6ª/10					10/10	4/4	10/10	10/10
		M					8/10	3/3	10/10	2/2	10/10	1/1	10/10	1/1	10/10	1/1	10/10	2/2				
3·5	40	F	19/20	15/20	18/20	16/20					10/10	6/6	10/10	5/5					10/10	2/2	10/10	2/2
		M					10/10	10/10	9/10	9/9	10/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	75	F	14/20	4/20	17/20	16/20					10/10	7/8	10/10	3/3					10/10	7/7	10/10	3/3
		M					10/10	9ª/9	10/10	10/10	9/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	125	F	0/20	1ª/20	3/20	2ª/20					10/10	5/5	10/10	8/10					10/10	7/7	10/10	9/9
		M					10/10	10/10	10/10	10/10	9/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	175	F	2/20	2ª/20	5/20	4/20					9/10	6/10	10/10	4/6					9/10	8/10	10/10	10/10
		M					7/10	7/7	9/10	9/9	10/10	1/1	10/10	1/1	10/10	3/3	10/10	1/1				
	225	F	0/20	1ª/18	4/20	4/20					7/10	6ª/10	8/10	7/9					7/10	5/10	10/10	9/10
		M					4/10	4/4	8/10	8/8	9/10	1/1	10/10	1/1	10/10	1/1	10/10	1/1				
	300	F	1/20	1/20	1/20	0/20					2/10	3ª/10	6/10	6/10					8/10	6/10	10/10	10/10
		M					1/10	1/1	5/10	5/5	6/10	1/1	10/10	1/1	7/10	1/1	10/10	1/1				

S, spoilage: T, toxic.
* F, LFRA: M, MRI.
a, Toxic but no spoilage.

At a given temperature of incubation the most inhibitory combination of NaCl and $NaNO_2$ was tested first for the presence of toxin. If all, or a high proportion, of the replicates tested were toxic, it was assumed that a high proportion of replicates containing less inhibitory combinations of NaCl and $NaNO_2$ would also be toxic and therefore fewer were tested. Hence, for example, in Table 3 at 1·8% NaCl, 300 mg kg^{-1} $NaNO_2$ and an inoculation level of 10^3 spores per bottle, eighteen replicates spoiled during incubation at 25°C; all twenty replicates were tested for toxin and fourteen of these contained toxin. This explains why a lower number of replicates were tested at lower concentrations of $NaNO_2$. Considering further Table 3, 1·8% NaCl, even with 300 mg kg^{-1} $NaNO_2$, failed to prevent toxin formation at 25 or 20°C. At 15°C only one replicate was toxic and there was some inhibition of toxin production by 225 mg kg^{-1} $NaNO_2$. Most replicates were toxic at 175 mg kg^{-1} $NaNO_2$. Considering next 3·5% NaCl in Table 3, most replicates tested at the highest concentration of $NaNO_2$ used were toxic at 25°C. At 20°, 225 mg kg^{-1} $NaNO_2$ failed to prevent toxin formation but at 15°C no toxin was produced with 10^3 spores per bottle if 125 mg kg^{-1} $NaNO_2$ or more was present. There was little difference in the number of replicates which became toxic after inoculation with 10^3 or 10^5 spores.

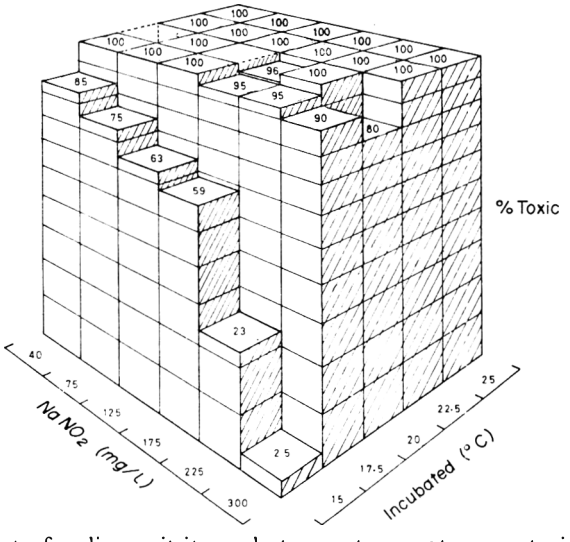

FIG. 1. The effect of sodium nitrite and storage temperature on toxin production by *Clostridium botulinum* in a meat slurry at pH 6·0, containing 1·8% w/v salt (on water phase) over a six-month storage period (data from Expt B, both inoculum levels summated). The presence of a block indicates the production of botulinal toxin at the concentrations of sodium chloride and sodium nitrite indicated after incubation at the stated temperature to spoilage, or up to six months in its absence. The height of the column of blocks shows the percentage of bottles toxic, which number is shown on the top of each column. The total number at each nitrite concentration and incubation temperature was twenty or forty (see Table 4).

TABLE 5. The effect of sodium chloride, sodium nitrite, incoulum level and incubation temperature on spoilage

NaCl (% w/v) on water	$NaNO_2$ (mg kg^{-1})	(a) Incubation temperature °C. (b) Spores/bottle	Experiment A						Experiment B									
		(a)	15°		20°		25°		15°		17·5°		20°		22·5°		25°	
		(b)	10^3	10^5	10^3	10^5	10^3	10^5	10^1	10^3	10^1	10^3	10^1	10^3	10^1	10^3	10^1	10^3
			Days to visual spoilage in any one replicate															
1·8	40		35	35	14	14	7	7	29	29	21	21	7	7	7	7	8	8
	75		35	49	18	14	7	7	29	29	21	21	14	7	14	14	8	8
	125		140	140	14	14	7	7	50	50	21	21	14	14	14	14	8	8
	175		140	140	14	14	11	11	50	50	33	21	14	14	14	14	8	8
	225		175	140	18	18	11	11	183+	76	49	49	14	14	21	21	12	12
	300		175	183+	28	35	11	11	183+	139	49	49	14	14	14	21	15	8
3·5	40		140	140	28	28	7	7	76	76	33	49	12	12	33	33	8	8
	75		150	155	28	28	7	7	50	50	49	49	12	25	33	33	8	8
	125		183+	183+	28	28	7	11	183+	76	49	49	25	25	33	33	8	8
	175		183+	140	35	28	11	11	167	139	49	49	25	25	33	33	12	8
	225		183+	183+	28	28	18	11	183+	76	49	49	33	25	33	33	25	12
	300		183+	183+	42	49	8	18	139	167	183+	97	33	33	33	33	25	15

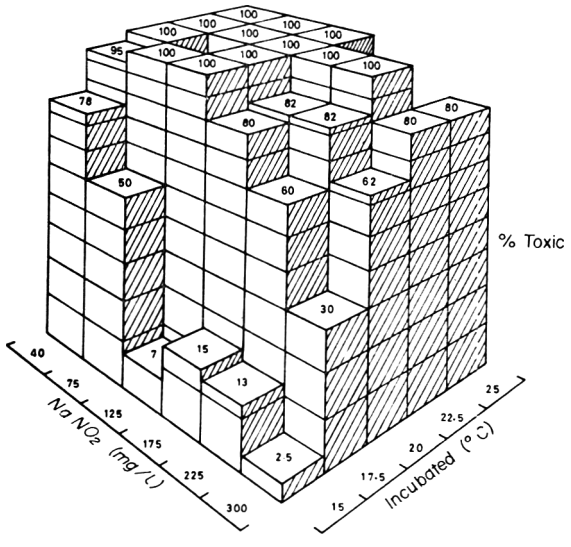

FIG. 2. The effect of sodium nitrite and storage temperature on toxin production by *Clostridium botulinum* in a meat slurry at pH 6·0, containing 3·5% w/v salt (on water phase) over a six-month storage period (data from Expt B, both inoculum levels summated). The presence of a block indicates the production of botulinal toxin at the concentrations of sodium chloride and sodium nitrite indicated after incubation at the stated temperature to spoilage, or up to six months in its absence. The height of the column of blocks shows the percentage of bottles toxic, which number is shown on the top of each column. The total number at each nitrite concentration and incubation temperature was twenty or forty (see Table 4).

In view of the common production of toxin at 20°C, and its frequent absence at 15°C, 17·5°C was included as an intermediate incubation temperature in the second experiment. Data are presented in Table 4. In the presence of 1·8% NaCl and up to 300 mg kg^{-1} $NaNO_2$ almost every replicate tested after spoilage at 25, 22·5, 20 or 17·5°C contained toxin. Even at 15°C toxin production was commonplace, though fewer replicates were toxic at the higher concentrations of $NaNO_2$. Considering 3·5% NaCl, the percentage spoilage at 25, 22·5 and 20°C was high, and almost every replicate tested contained toxin. Incubation at 17·5°C reduced spoilage but most of those replicates which had spoiled contained toxin. Similarly at 15°C spoilage was reduced at the high concentrations of $NaNO_2$, but was almost complete at 40 and 75 mg kg^{-1} $NaNO_2$. Most of those replicates which had spoiled were toxic, but occasionally an unspoiled replicate was found to contain toxin.

For the sake of completeness Table 5 lists the time to spoilage for the first bottle in any group of replicates for each of the combinations of NaCl and $NaNO_2$ used. At all temperatures increasing concentrations of $NaNO_2$ increased the time to spoilage, and this effect was greater at the lowest temperatures of incubation. The results obtained

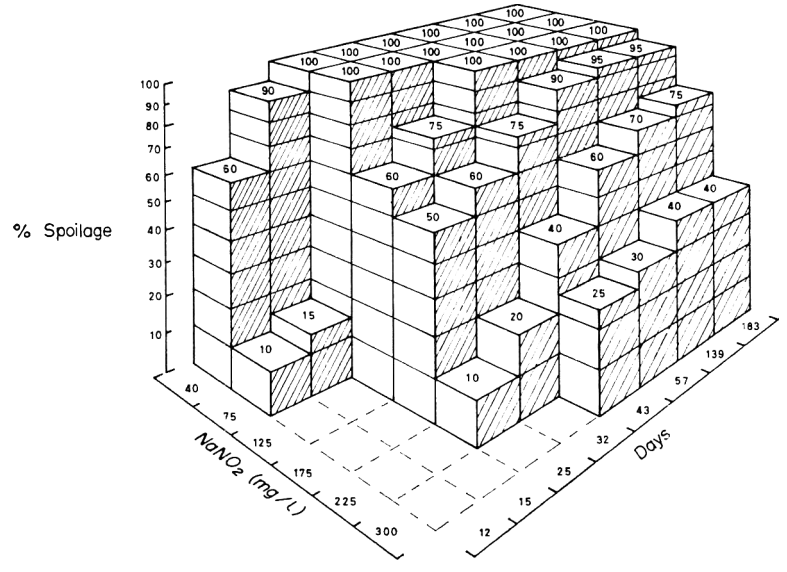

FIG. 3. The effect of nitrite concentration and time to spoilage by *Clostridium botulinum* in a meat slurry, pH 6·0, containing 3·5% salt (on water phase) when stored at 20°C (data from Expt B, both inoculum levels summated). The presence of a block represents visible spoilage at the concentrations of sodium chloride and sodium nitrite shown after incubation at the temperature indicated for the number of days stated. The percentage of bottles showing spoilage is represented by the height of the column and is shown on the top of each column.

in the two laboratories were essentially similar for any one incubation temperature, although slight differences were occasionally noted, probably reflecting the subjective nature of spoilage assessment. It is possible that some 'spoiled' samples may have been removed from storage before significant levels of toxin had been produced. The pasteurization treatment reduced the number of viable bacteria in Experiment A from *c.* 10^8/g to *c.* 10^4/g. The same batch of pork was held frozen for Experiment B and the count on the slurry was only *c.* 10^5/g, and was reduced by pasteurization to < 10/g. On no occasion was spoilage observed in an uninoculated bottle of meat slurry prior to spoilage of all samples under test.

The incidence of both spoilage and toxicity differed with the inoculum level only in replicates incubated at 15°C. In 1·8% w/v NaCl (in Experiment A) 45% of low inoculum and 81% of high inoculum replicates tested contained toxin and in Experiment B 40% and 69% respectively. At 3·5% w/v NaCl toxin was formed in 13% and 12% samples (Experiment A) and in 20% and 35% (Experiment B) respectively.

The overall pattern of the interaction of nitrite and incubation temperature for Experiment B is illustrated in Fig. 1 (1·8% w/v NaCl) and Fig. 2 (3·5% w/v NaCl) for replicates incubated for six months. Since the inoculum level had little effect on the number of replicates becoming toxic results for both levels have been summed. The

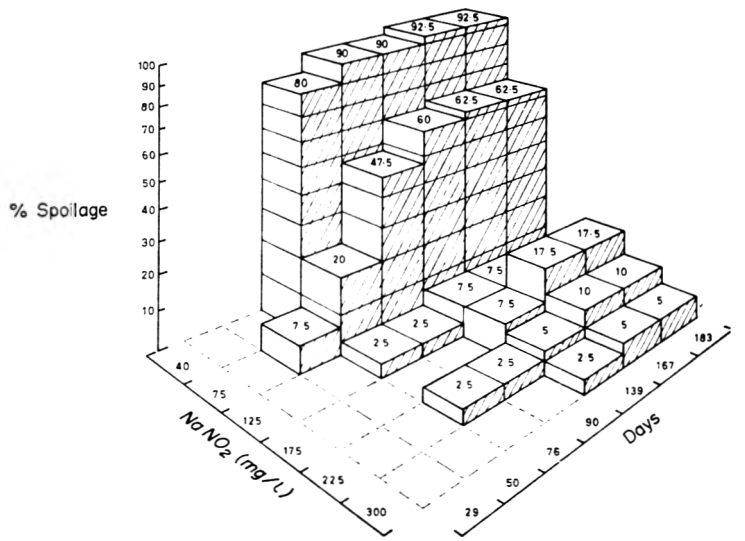

FIG. 4. The effect of nitrite concentration and time to spoilage by *Clostridium botulinum* in a meaty slurry, pH 6·0 containing 3·5% salt (on water phase) when stored at 15°C (data from Expt B, both inoculum levels summated). The presence of a block represents visible spoilage at the concentrations of sodium chloride and sodium nitrite shown after incubation at the temperature indicated for the number of days stated. The percentage of bottles showing spoilage is represented by the height of the column and is shown on the top of each column.

reduction in toxin production with increasing nitrite concentration is clearly seen at 15°C in both Figs 1 and 2 and at 17·5 and 20°C in Fig. 2.

Figures 3 and 4 are drawn similarly, summing the results of the two inoculum levels in Experiment B and show clearly the reduction in spoilage with increasing nitrite concentration, the increase in spoilage with increasing incubation time, and, comparing Figs 3 and 4, the reduction in spoilage by reducing the incubation temperature from 20 to 15°C. Little or no spoilage occurred in meat slurry containing 125 mg kg^{-1} nitrite, at 15°C, but at 20°C significant spoilage occurred even at 300 mg kg^{-1} nitrite.

Discussion

Recent demonstrations of traces of carcinogenic nitrosamines in cured meats, presumably through reaction of the nitrite used in curing with secondary and tertiary amines present in the meat, have led to suggestions to restrict severely, or even to forbid, the use of nitrite and of nitrate which may serve as its precursor (Status Report, 1972). Such evidence has recently been reviewed (Krol & Tinbergen, 1974).

Besides providing the characteristic colour and flavour of cured meats, nitrite is believed to have important microbiological effects on both safety and stability. Botulism

has been relatively common in home-cured pork where nitrite and/or nitrate are not used, or are used without adequate control (Sebald, 1970; Gonzalez & Guttierez, 1972), whereas no botulism has occurred from the huge amount of commercially cured meat where these salts are used under good control, although the presence of *Cl. botulinum* has been demonstrated (Abrahamsson & Riemann, 1971; Jarvis, Rhodes & Williams in preparation; Roberts & Smart, in preparation).

Attempts have been made to understand the microbiological action of nitrite, and to find a means of calculating how much nitrite is necessary in particular circumstances. Much of this work has been done in bacteriological media where a triple interaction between pH, NaCl and $NaNO_2$ has been demonstrated (Roberts & Ingram, 1973). Furthermore, growth of *Cl. botulinum* is known to be inhibited more readily at reduced incubation temperatures (Segner, Schmidt & Boltz, 1966). While, in principle, such information is likely to be generally applicable, differences are to be expected between inhibitory combinations of curing salts in bacteriological media and in meat. Current pressures to reduce nitrite levels make it important to try to determine the extent of these relationships in a meat system. The inhibitory combinations of salt and nitrite reported here were similar to those inhibiting a vegetative inoculum of *Cl. botulinum* at pH 6 in laboratory medium (Roberts & Ingram, 1973) and to levels at which a salt nitrite interaction was observed for mildly heated spores of *Cl. botulinum* type B (Jarvis *et al.*, 1976).

It was anticipated that the inoculum size would be an important factor, but it was shown only to affect the number of replicates which became toxic at 15°C. The similarity in occurrence of toxin production in the two experiments is difficult to explain, since the high inoculum in Experiment B was the same as the low inoculum in Experiment A.

Toxin was occasionally detected in samples which were not obviously spoiled, especially at 25°C. Although toxin production by the proteolytic strains of *Cl. botulinum* types A and B is usually accompanied by marked proteolysis, there have been occasional reports of toxin production without overt spoilage in salted meats with a brine concentration of 6·25–7·12% (Greenberg, Silliker & Fatta, 1959) and in ham containing 3·6–4·5% salt and 93–106 mg kg^{-1} residual nitrite (Pivnick & Barnett, 1965). The latter workers observed that toxin formation at suboptimal incubation temperatures (20–25°C) was less frequently associated with putrefaction than was toxin formation at 30°C. Hence, it is unwise to attempt to define bacteriological safety of cured meats in terms of data obtained solely from studies of spoilage.

The levels of nitrite required to inhibit toxin production in this investigation were somewhat higher than those observed previously in whole minced meat containing 3·5% NaCl (Ashworth *et al.*, 1973), and than those reported by Christiansen *et al.* (1973) in pasteurized comminuted meat. Small variations in experimental methods may cause such differences, and also make it difficult to compare results from different laboratories. In this study the heat process used was lower than that which would be achieved at the

centre of a large (over 16 lb) can of ham pasteurized to a centre temperature of 70°C, but nevertheless reflects the mildest heat treatment which spores might receive in a commercial pack. The heat treatment is not believed to be a highly significant factor since comparison of this low treatment ($P_{80°C} = 0{\cdot}65$) with one more severe ($P_{80°C} = 12{\cdot}6$) than that commonly applied to large cans ($P_{80°C} = 3–7$) had little effect on the level of nitrite required to inhibit toxin formation (Rhodes & Jarvis, 1976), although the more severe thermal process delayed the onset of spoilage and toxicity.

Further work remains to be done to determine the importance of pH value on the inhibitory levels of salt and nitrite, and whether other commonly used additives such as polyphosphate and ascorbate have any effect on toxin production by *Cl. botulinum*. This study, together with others already published, shows that nitrite plays an important role in inhibiting the growth of *Cl. botulinum* at suboptimal incubation temperatures.

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Sensitization of heat-damaged spores of *Clostridium botulinum*, type B to sodium chloride and sodium nitrite

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Summary

Spores of *Clostridium botulinum* type B (NCTC 7273) were sensitized to the inhibitory action of 2·5% and 3·5% w/w NaCl by heating at 85, 90 or 95°C at pH 6·0 and pH 6·5. Spores heated at 70 or 80°C were not significantly sensitized to these concentrations of NaCl but they were sensitized to 4·5% and 5·5% NaCl. A significant salt–nitrite interaction was observed only at NaCl levels of 4·5% and above. Heat-shocked spores and spores heated through a '1-D process' at 70 or 95°C were more sensitive to nitrite heated in meat or in culture medium (121°/10 min) than to unheated nitrite added after heating. However, no differences in sensitivity to the heated nitrite (Perigo factor) were observed with spores heated at 70 and 95°C.

Introduction

Pasteurized canned cured meats occupy a unique position amongst canned foods since the thermal processes applied to such products would be grossly inadequate to ensure the safety and stability of other low-acid canned foods. It is believed that their stability results from the low incidence of clostridial spores and the interactions between the various curing agents and the heat process. Investigations by many workers have shown that salt (NaCl), nitrite, pH value and thermal process all interact to produce a system which is more inhibitory to spores than is any one factor alone (Roberts & Ingram, 1973). A problem in assessing the effective levels of the various ingredients undoubtedly lies in the lack of quantitative data on the interactions. Roberts & Ingram (1966), Roberts, Gilbert & Ingram (1966) and Pivnick & Thacker (1970) have demonstrated that salt and nitrite inhibit germination and outgrowth of heat-damaged spores, but that the presence of these ingredients in the heating menstruum does not reduce the actual heat resistance of the organisms. This was subsequently confirmed by Ingram & Roberts (1971) who discussed the concept of 'apparent D values' in assessing the effects of salt and nitrite as inhibitors of thermally-damaged spores.

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Most laboratory investigations on salt and nitrite have used unheated inorganic nitrite in the recovery medium. However, Perigo, Whiting & Bashford (1967) and Perigo & Roberts (1968) demonstrated that nitrite heated in a culture medium was a more potent inhibitor than was an equivalent concentration of unheated nitrite. These observations were subsequently confirmed and extended by ourselves (Ashworth *et al.*, 1974a, b). The production of a 'Perigo-type effect' has been demonstrated also in a minced whole meat given a sterilization treatment (Ashworth & Spencer, 1972) or a pasteurization treatment (Ashworth, Hargreaves & Jarvis, 1973) and in a pasteurized meat slurry system (Rhodes & Jarvis, 1976).

Ingram & Roberts (1971) showed that spores of *Clostridium botulinum* which had been given a sublethal heat process at 95°C were more strongly inhibited by nitrite which had been heated in the recovery medium for 15 min at 115°C than by unheated nitrite. However, in a pasteurized meat system containing salt and nitrite, Ashworth *et al.* (1973) demonstrated that spores heated in the meat for up to 4 hr at 80°C were inhibited by a similar level of nitrite to that which was inhibitory for unheated spores inoculated into the meat after heating. These observations suggest that although spores heated at, say, 95°C are sensitized to the inhibitory action of salt and nitrite, spores heated at pasteurization temperatures (e.g. 70–80°C) might not be sensitized by heat to the inhibitory action of curing salts. Alternatively, the 'Perigo-type factor' produced in meat from heated nitrite might be an equally effective inhibitor against both heat-damaged and unheated spores (cf. Roberts & Smart, 1974).

The present investigation was undertaken to assess the extent to which thermal sensitization of spores to curing salts occurs over a range of temperatures such as are commonly encountered in meat processing and to investigate further the effects of heated nitrite on heat-damaged and unheated spores of *Cl. botulinum*.

Materials and methods

Preparation of spores

Spores of *Clostridium botulinum* type B (NCTC 7273) were prepared in the trypticase–peptone–thioglycollate medium of Schmidt & Nank (1960). The sporulation medium, inoculated with 1% (v/v) of an 18-hr culture in cooked meat broth (Southern Hospital Group Laboratories, London) was incubated at 30°C until a high proportion of cell-free phase-bright spores was observed microscopically (about seven days). Spores were harvested by centrifugation for 30 min at 22 000 × ***g*** and 4°C and were washed four times in sterile distilled water. The washed spores were stored at 4°C as a concentrated suspension in sterile distilled water.

Preparation of spore ampoules

Heating menstruum. Twice-minced lean pork was homogenized with an equal volume of distilled water using a tubular sealed unit laboratory mixer (Silverson Ltd, London).

The slurry was centrifuged for 30 min at 22 000 × ***g*** and the aqueous supernatant was decanted and heated for 10 min at 70°C to precipitate coagulable protein. The suspension was filtered through Whatman No. 1 filter paper (H. Reeve Angel & Co. Ltd, London) and was divided into three portions, the pH values of which were adjusted to 6·0, 6·5 or 7·0; the extracts were then sterilized by membrane filtration.

Spore suspensions. The spore concentrate was centrifuged for 30 min at 22 000 × ***g*** and the pellet was evenly suspended in a suitable volume of sterile meat extract to give a level of about 10^6 spores ml^{-1}. Aliquots of 0·5 ml were pipetted accurately into sterile freeze-drying ampoules by means of an Agla glass micrometer syringe (Burroughs Wellcome Ltd, Beckenham) and, after sealing, the ampoules were stored at 4°C until required.

Experimental procedure

Media for recovery of heated spores. Preliminary experiments demonstrated that highest recovery of heat-damaged spores of *Cl. botulinum* was obtained on horse blood agar (HBA) containing 1% (w/v) sodium bicarbonate. The recovery media contained: blood agar base (Oxoid), 40 g; sodium bicarbonate, 10 g; defibrinated horse blood (Wellcome Reagents Ltd, Beckenham), 50 ml; NaCl, 5, 25, 35, 45 or 55 g; $NaNO_2$, 0, 50, 100 or 200 mg; distilled water to 1 litre; pH 6·0, 6·5 or 7·0.

The blood agar base was dissolved in a suitable volume of distilled water. After sterilization, the agar was tempered to 50°C and appropriate volumes of horse blood and of filter-sterilized solutions of NaCl, $NaNO_2$ and $NaHCO_3$ were added aseptically to give the required concentrations. The pH value of the medium was adjusted aseptically and sterile distilled water was added as appropriate to standardize the volume of medium. Twenty millilitre quantities were dispensed in Petri dishes and the surface of the agar was dried for 3 hr at 37°C prior to inoculation.

Heat processing and estimation of surviving organisms. Ampoules of inoculated meat extract were weighted with terry clips and immersed completely in a water bath at 70, 80, 85, 90 or 95°C. Duplicate ampoules were removed to beakers of iced water after various times up to 48 hr. The content (0·5 ml) of each ampoule was diluted with 9·5 ml of sterile distilled water in a square-bottomed Universal bottle (MSE Ltd, London) and the mixture was homogenized for 1 min at full speed using the micro-attachment of the MSE top drive homogenizer and an ice-water cooling bath. Ten-fold serial dilutions were prepared in sterile distilled water and duplicate drops (0·02 ml) of the dilutions were plated on each of two plates of each medium, which contained a different combination of NaCl and $NaNO_2$ at the same pH value as the heating menstruum. Plates were randomly distributed to different anaerobe jars (Baird & Tatlock Ltd, London) (to minimize the possible effects of partial or total failure of any one anaerobe jar) and were incubated at 30°C in an atmosphere of hydrogen. Colonies were counted after one, two and six weeks incubation; only on rare occasions was an increase in count observed after incubation for more than seven days.

Analysis of results. Apparent decimal reduction times (D′ values) were determined by regression analysis using the method of least squares. Differences between the slopes of the regression lines obtained at any one heating temperature were analysed by Tukey's method of multiple analysis of variance (Guenther, 1964).

An index for sensitization of spores to NaCl or nitrite was calculated by the following method:

$$\% \text{ sensitization to (e.g.) } 3{\cdot}5\% \text{ NaCl} = \frac{C^t_{0.5} - C^t_{3.5}}{C^t_{0.5}} \times 100$$

where $C^t_{0.5}$ = mean colony count on media containing 0·5% NaCl and $C^t_{3.5}$ = mean colony count on media containing 3·5% NaCl, after heating for t min at any one specific temperature.

Sensitivity of spores to heated nitrite

In culture media. Nitrite (10–50 ppm) was heated in the medium of Perigo *et al.* (1967) for 10 min at 121°C, or was added as filter-sterilized solutions at levels of 50–150 ppm to medium sterilized without the addition of nitrite. Five bottles of medium were prepared at each nitrite level and these were inoculated with *c.* 1000 spores/bottle. The cultures were incubated at 25°C and were examined for evidence of growth at daily intervals for up to one week and then at weekly intervals for up to ten weeks.

In a meat slurry system. Meat slurry was prepared as described by Rhodes & Jarvis (1976) at a salt (NaCl) level of 3·5% (as salt on water). The slurry had a fat content of 7·5% and a pH value of 6·2. Sodium nitrite was added at initial levels of 0, 125, 250, 375 and 500 ppm to 25 g quantities of the meat slurry in 1 oz Universal bottles and the bottles were heated for 4 hr at 70°C. A series of control experiments were prepared concurrently in which filter-sterilized solutions of nitrite were added to heated (4 hr at 70°C) meat slurry. The bottles were stored for 16 hr at room temperature before inoculation with *c.* 800 spores *Cl. botulinum* per bottle (i.e. *c.* 30 spores/g meat slurry). The inoculated bottles were incubated at 25°C and were tested for toxin when evidence of spoilage was observed, or after a total incubation period of ten weeks. Toxin was detected by injecting 0·4 ml of a 1 in 5 dilution of the meat in gelatin–phosphate buffer (pH 6·5) into 18–20 g female Swiss white mice. Botulinal toxin was confirmed by observation of clinical symptoms and by antitoxin protection tests on mice.

Spore inocula. For studies of sensitivity to heated nitrite, spore suspensions in the aqueous meat extract at pH 6·0 were treated as follows: heat-shocked for 10 min at 70°C; heated to 10% survivors at either 70 or 95°C.

Nitrite analyses. Residual nitrite at the time of inoculation was determined in non-inoculated replicate bottles of medium or meat for each input nitrite level. The method used was that described previously by Ashworth & Spencer (1972).

Interpretation of results. The number of replicate bottles in which growth/toxin formation had occurred was recorded for each nitrite level and each incubation period.

Results are expressed in terms of the ED_{100} level which has been defined previously (Ashworth *et al.*, 1973) as the lowest concentration of nitrite which inhibited growth and toxin production, for a defined time period, in all replicates of a series of tests. ED_{100} levels were calculated for both heated and unheated nitrite in the medium and the meat systems in terms of initial nitrite content and of residual nitrite at the time of inoculation.

Results

Heat resistance of Cl. botulinum spores recovered in the presence of salt and nitrite

The 'apparent decimal reduction times' (D′ values) for spores heated and recovered at pH 6·5 on HBA containing from 0·5 to 3·5% salt and 0 to 200 mg kg^{-1} nitrite are presented in Table 1. These data were derived from the thermal death curves of spores heated at 70 to 95°C obtained by least-squares analysis of the experimental data. The correlation coefficients of the lines were all better than 0·85. The D′ values obtained over a more restricted range of temperatures at pH 6·0 are presented in Table 2. At pH 7·0, the D′ values were almost identical to those observed at pH 6·5, but since a shoulder was frequently observed in the pH 7·0 death curves, the D′ values were derived from the linear portions of the curves.

The D′ values indicate that spores were sensitized to NaCl when heated at 85, 90 or 95°C. At pH 6·5, sensitization of spores heated at 70 or 80°C was not observed, but there was some sensitization at pH 6·0. For spores heated at 90 and 95°C and recovered on 0·5% NaCl compared with recoveries on 2·5% and 3·5% NaCl statistical analysis demonstrated significant differences ($P < 0·01$) in the slopes of the thermal death curves. The differences were also significant ($P < 0·05$) for spores heated at 85°C. For these

TABLE 1. Apparent decimal reduction times for spores of *Cl. botulinum* type B at pH 6·5

Temperature of heating (°C)	Apparent D value (min) at pH 6·5 when recovered on						
	0·5% NaCl	2·5% NaCl			3·5% NaCl		
		50	100	200	50	100	200
		mg/kg nitrite			mg/kg nitrite		
70	3515*	3635*	4123†	3555*	3132*	3389*	3544*
80	960‡	829‡	830†	899‡	892†	996†	982‡
85	248†	136‡	247†	220*	115*	180*	136†
90	86‡	53†	50†	34*	—	41*	—
95	19·5*	6·8†	7·1*	6·7*	3·7*	3·4*	5·1†

Correlation coefficients of the thermal death curves scored: * $\geqslant 0·95$; † $\geqslant 0·90 < 0·95$; ‡ $\geqslant 0·85 < 0·90$.

TABLE 2. Apparent decimal reduction times for spores of *Cl. botulinum* type B at pH 6·0

Temperature of heating (°C)	Apparent D value (min) at pH 6·0 when recovered on						
	0·5% NaCl	2·5% NaCl			3·5% NaCl		
		50	100	200	50	100	200
		mg/kg nitrite			mg/kg nitrite		
70	3174†	2406†	2633*	2669*	2770*	—	3412‡
80	994*	899*	1364‡	645†	535*	540*	704†
90	76*	43*	44*	48*	32*	—	31*

Correlation coefficients of thermal death curves as Table 1.

three temperatures, the differences in D′ values of spores recovered on 2·5% or 3·5% NaCl were also significant ($P < 0{\cdot}01$). The effect of nitrite on the apparent heat resistance of spores recovered on either 2·5% or 3·5% NaCl was not statistically significant ($P > 0{\cdot}05$).

Sensitization of heated spores to inhibition by salt and nitrite

The inhibitory effects of salt on spores damaged by heat for various time periods at 70–95°C are illustrated in Figs 1 and 2. The sigmoid inhibition curves obtained at both pH 6·0 and pH 6·5 for spores heated at 85°C and above, demonstrate increased

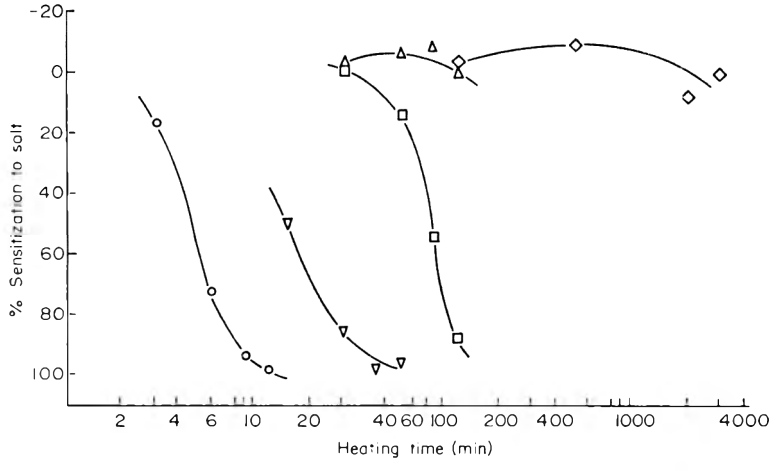

FIG. 1. Effect of heat process on the sensitization of *Cl. botulinum* spores to 3·5% NaCl at pH 6·5. For calculation of sensitization see 'Materials and methods'. ○, 95°; ▽, 90°; □, 85°; △, 80°; ◇, 70°.

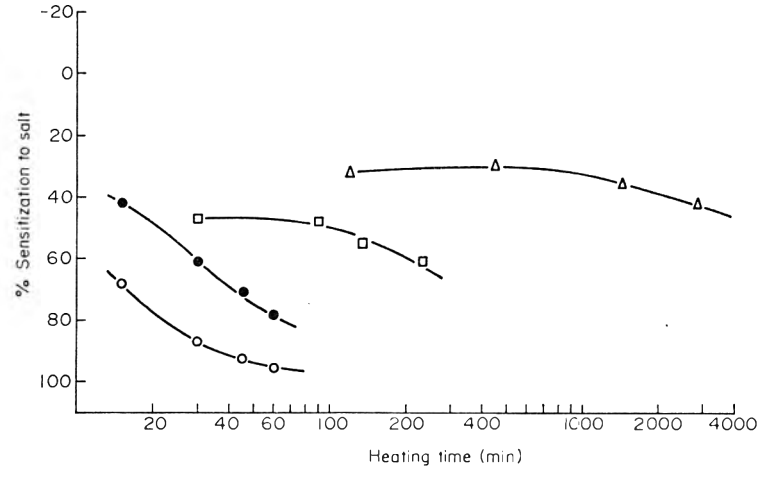

FIG. 2. Effect of heat process on the sensitization of *Cl. botulinum* spores to 3·5% NaCl (○, 90°; □, 80°; △, 70°) and 2·5% NaCl (●, 90°) at pH 6·0.

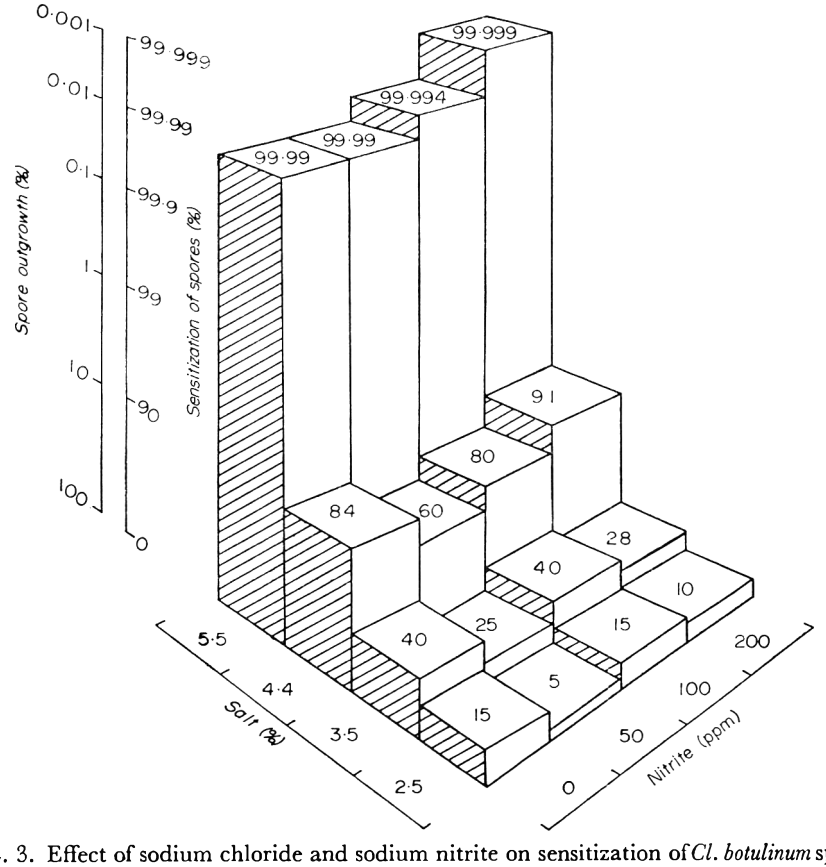

FIG. 3. Effect of sodium chloride and sodium nitrite on sensitization of *Cl. botulinum* spores heated for 2 hr at 70°C, pH 6·5.

sensitization of spores with increased heat damage. Although spores heated at 70 and 80°C were not sensitized significantly over the periods of heating, there is a suggestion of a slight increase in sensitization after heating for 48 hr at 70°C. Comparison of the response line (Fig. 1) for spores heated at 70°C and pH 6·5 with that for spores heated at 70°C and pH 6·0 (Fig. 2) demonstrates a salt–pH interaction on the heated spores. Similar interactions were seen also at the higher temperatures of heating.

Because of the low level of sensitization of spores heated at 70°C, further experiments were undertaken with recovery on media containing up to 5·5% salt. With relatively mild heating (i.e. up to 2 hr at 70°C) little sensitization of spores was observed at 2·5% salt but spores were markedly sensitized to salt concentrations of 4·5% and above. Indeed 99·99% of spores capable of growth at pH 6·0 in 0·5% salt were inhibited by 5·5% salt whereas only about 80% inhibition occurred at 4·5% salt. Some interaction of salt and nitrite was observed also at 4·5% and 5·5% salt (Fig. 3). At pH 6·5 and pH 7·0 the salt–nitrite interaction was much reduced. It is noteworthy that at low salt (i.e. 2·5%) and low nitrite (50–100 ppm), spore recovery was sometimes enhanced at the higher pH values.

Sensitivity of heated spores to heated and unheated nitrite

The relative sensitivities of heated and unheated spores to both heated and unheated nitrite in culture medium and pork slurry are presented in Table 3. As expected, the spores were more sensitive to heated nitrite than to unheated nitrite in Perigo medium and no differences were seen between the different spore treatments. In pork slurry, the spores were all equally sensitive to heated nitrite and were more sensitive to heated nitrite than to unheated nitrite.

TABLE 3. Inhibition of *Cl. botulinum* type B spores by heated and unheated nitrite

Spore treatment	ED_{100} residual nitrite* (mg/kg) after ten weeks in			
	Perigo medium		Pork slurry†	
	Heated nitrite	Unheated nitrite	Heated nitrite	Unheated nitrite
Heat shocked (10 min at 70°C)	20	150	210	385
Heated 70°C‡	20	150	210	338
Heated 95°C‡	20	150	210	310

* Assayed at time of challenge.

† Pork slurry contained 3·5% salt (on water phase).

‡ Heated to 10% survivors and inoculated at same level of viable spores as 'heat shocked' spores.

Spores heated at 95°C appeared to be slightly more sensitive to unheated nitrite than were spores heated at 70°C which were themselves slightly more sensitive than were the heat shocked spores. However, this may reflect merely the increased sensitivity of the spores heated at 95°C to the salt (3·5% on water phase) in the pork slurry.

Discussion

Several workers (e.g. Roberts *et al.*, 1966; Pivnick & Thacker, 1970; Ingram & Roberts, 1971) have demonstrated that heating sensitizes clostridial spores to the inhibitory effects of salt and nitrite. The present investigation confirms these earlier observations. Data are presented on the degree of inhibition by various salt levels for spores of one strain of *Cl. botulinum* heated at various temperatures. Although both Pivnick & Thacker (1970) and Ingram & Roberts (1971) have shown that the degree of sensitization increases with increasing exposure to heat, their studies were restricted to temperatures at the higher end of the range of temperatures used in commercial processing of canned cured meats. Pasteurization of large cans of cured meats frequently occurs in conditions where the centre temperature of the meat is raised to about 70°C over a prolonged period. Further the average salt content of many commercial cured meats may be as low as 3–3·5% (salt on water) and in extreme cases may be as low as 2% (salt on water). In conditions such as these, spores of *Cl. botulinum* would neither be inhibited by the salt and nitrite levels *per se*, nor would they be sensitized by the mild heat process. However, even relatively short exposure of spores to a temperature of 70°C or above would result in sensitization to higher salt concentrations (i.e. 4·5% salt on water).

The results of this study on the effects of heated nitrite on spores of *Cl. botulinum* type B confirm the recent observations of Roberts & Smart (1974) who showed that heat and radiation damaged spores of *Cl. botulinum* types A and E and of *Cl. sporogenes* were no more sensitive to heated nitrite than were undamaged spores. They also extend our earlier observations with *Cl. sporogenes* in meat systems (Ashworth *et al.*, 1973) and demonstrate the lower sensitivity of spores to heated and unheated nitrite in a meat system compared with the sensitivity in culture media. The comparison also indicates the caution needed in interpretation of data obtained in laboratory media in relation to potential effects in meat systems. In the present work, growth and toxin formation in the meat system was inhibited by about 300–400 ppm unheated nitrite or by about 200 ppm heated nitrite. By contrast only 20 ppm heated nitrite was required to inhibit a similar inoculum of spores in culture medium. Consequently in any attempt to quantify the effects of thermal process, salt, nitrite and other factors on the safety of heated cured meat products it is essential to undertake large-scale studies of these interacting parameters in meat systems. For this reason long-term storage tests have been undertaken in inoculated meat systems stored at various temperatures (Roberts, Jarvis & Rhodes, 1976).

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Meat loaf type canned products based on milk or plant proteins

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Summary

A simple method for the manufacture of meat loaf type canned products, based on milk or plant protein, is described. The process relies on preparing a suitable emulsion of the proteins, fats, water and flavour, followed by retorting to form a suitable gel with chewy characteristics. Of all the proteins tested only a low calcium type of milk co-precipitate was found to be a suitable emulsifier. When combined with a water binder, i.e. textured vegetable proteins, granulated co-precipitates or wheat proteins, a suitable simulated meat product is formed after retorting, the characteristics of which resemble those of meat in chewiness, sliceability, appearance and flavour. The products can be used sliced in sandwiches, salads or fried. No change was observed after storage for six months at 45°C.

Sensory evaluation of these products in comparison to a commercial sample, based on texturized vegetable protein, showed no significant differences in the degree of general acceptability, but their sliceability was considered better than the control.

Introduction

One of the unique developments in protein foods is that of simulated meat products. These products, which possess to a remarkable extent, the chewiness, moistness and texture of cooked meat, are gaining in popularity among people who, for a variety of reasons, do not eat meat (Pinkaton & Claydon, 1971).

From previous work on simulated meat over the last two or three decades, a massive volume of literature has emerged on the preparation of materials for the processing and manufacturing of simulated meat products. A few of the references most directly related to the basis of the current study are discussed.

The types of protein materials used from various sources have included soya, casein, milk powder, peanut and wheat glutens (Anson & Pader, 1957, 1958, 1959; Kende & Ketting, 1959; MacAllister & Finucane, 1963; Wrenshall, 1951).

The development of simulated meats is based on altering the physical characteristics

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of these proteins to resemble those of meat. Changes needed depend on the characteristics of the original proteins, their method of extraction and treatment and the characteristics desired in the final product (Wrenshall, 1951).

Wrenshall (1951), for example, used skim milk solids as the basic matrix in the simulation of several types of comminuted meat preparations, such as country sausage, bologna, salami and hamburger. The process involved mixing texturizing agents, which were mainly of cereal origin, with an aqueous suspension of skim milk solids and heating until coagulated. The product was said to possess the texture, flavour and appearance of comminuted meat.

Kende & Ketting (1959) used pH to control the characteristics of casein so as to approximate those of meat. The product, which they called 'milk meat', is prepared by using 30–40 parts of casein, mixed with 6–8 parts of cereals, fat, flavourings and adjusting the pH to 4·9–5·2 and moisture content to 55–60%.

MacAllister & Finucane (1963) used soya flour and wheat gluten together with minor amounts of albumen, starch and meat flavour to form a dough which is extruded and dried to the desired shape. When cooked in water the granules develop a flavour and chewy texture which is characteristic of cooked meat. These granules are suitable for such entrees as meat patties, hamburgers and sausages.

Anson & Pader (1957, 1958, 1959) used the gelling properties of proteins extracted from such materials as soya, casein, peanut and groundnut to simulate meat. The steps included separating the protein, adjusting the pH, adjusting protein content of the suspension to form a gel precursor and, by subsequent appropriate treatment, usually including controlled heating, forming discrete particles of chewy gel. It is claimed that when pieces of substantial size are chewed in the mouth they have the physical properties of resilience, elasticity and resistance to shear. These chewy protein gels are suitable for use in protein food products simulating meat.

Materials and methods

Development of current plan of study

From the previous studies described above and from those conducted at the Dairy Research Centre, Richmond, New South Wales (Thomas, Baumgartner & Hyde, 1974a, b) it was apparent that two physical functions were required of the protein or other colloidally active material used in simulated meat products (S.M.P.). This, of course, is in addition to other desirable characteristics such as flavour and nutritive value.

One of these essential characteristics is that of emulsion formation and stability between the aqueous and fatty phases considered essential in this type of food.

The work of Thomas *et al.* (1974a, b) had already established that certain milk protein preparations had excellent properties in this regard.

The other important characteristic is that of binding water in gel formation to provide

the chewiness and general mouth feel similar to that of cooked meat. The studies already described revealed that some of the milk proteins were suitable for this purpose and it was recognized that other preparations of vegetable origin (generally rich in protein, but often containing appreciable amounts of carbohydrate material) might also provide the characteristics required.

In considering commercially available products special emphasis was paid to the use of calcium co-precipitates as a base material, calcium co-precipitate, a precipitate of casein and whey proteins, in which 95–97% of the milk proteins are recovered (Buchanan, Snow & Hayes, 1965; Muller, Hayes & Snow, 1967). Their functional properties are reported by Thomas *et al.* (1974b).

Ingredients studied

Milk proteins. 'Sod. Cas.' (91% protein), a sodium caseinate obtained from Halcyon Proteins Pty Ltd, Lilyfield, N.S.W.; 'Low Cal' (91% protein), a low calcium co-precipitate; 'High Cal 2' (83% protein), a high calcium co-precipitate; 'High Cal 6' (82% protein), a high calcium co-precipitate; granulated co-precipitate (90% protein), a medium type co-precipitate, 30–40 mesh. All were obtained from Colac Dairying Co. Ltd, Colac, Victoria. Also studied were whey protein powder (82% protein), a soluble and heat coagulable protein supplied by New Zealand Dairy Research Institute, Palmerston North, N.Z.; a spray dried sweet whey (12% protein), manufactured by Kraft, Division of Kraft Corporation, Chicago, Ill., U.S.A.; and a medium heat skim milk powder (36% protein) supplied by Producers Co-op. Distributing Society Ltd, Sydney.

Two types of soya proteins were studied: a soy concentrate 'Promosoy' (65·3% protein) and an isolate 'Promine D' (91·8% protein), manufactured by Central Soya Co. Inc., Chicago, Ill., U.S.A.

A texturized soybean product (TVP) extruded type, 'Mira Tex 210' (50% protein) was used, manufactured by A. E. Staley Manufacturing Co., Decatour, Ill., U.S.A.

Three types of wheat protein were studied. These were a superfine gluten, an undenatured protein (73% protein), 'Cerebind' a denatured protein (15% protein) and granulated protein, a physically modified protein with coarse granules (80% protein). All three were manufactured by Fielder's Starches Pty Ltd, Leichhardt, N.S.W.

Fat. A commercial polyunsaturated blend was used (manufactured by Crisco Vegetable Oil Co., Brunswick, Victoria).

Other ingradients. These included salt, colouring and flavourings.

Methods

Emulsion formation. Mixing and emulsification was done in a silent cutter, a two-speed model with 13·5 kg bowl capacity Type K21 Rasant Cutter, manufactured by Maschinenfabrik Seydelmann, 708 Aalen (Wurtt), Verlangente Schulstrasse 48–50, Germany).

The following sequence of addition of ingredients to the silent cutter was found to give the best results:

place three-quarters of the water, salt, flavourings, colour and all the minor ingredients in the bowl;
run the silent cutter at low speed and add all the protein and the rest of the water in small amounts;
mix for 30 sec. The fat is then slowly added, followed by one or more of the binders. The silent cutter is then run at high speed for a period of 2 min to form the emulsion. The product is then packed in an 8-oz lacquered can and retorted for 60 min at 121°C.

Product development. Two parameters were found to be important in the manufacture of this type of simulated meat product. These include the formation of a stable emulsion during manufacturing and retorting, and the formation of a suitable chewy gel when the product is cooled.

To accomplish this, it was anticipated that a protein with a high capacity to form water/fat emulsion was needed for this product. Thomas *et al.* (1975b) found that of

TABLE 1. Characteristics of the emulsion formed with various proteins, fat and water and the sensory properties of the product obtained by canning the emulsions

Type of protein	Characteristics of raw emulsion	Sensory properties of canned emulsions
Low Cal.	Good viscosity similar to meat sausage, slightly sticky	Firm, good sliceability, good texture and chewability, no disintegration during frying
High Cal 2	Sloppy	Very poor body, very soft and sticky, oil separation
High Cal 6	Good viscosity similar to meat sausage	Very poor body, very soft and sticky, oil separation
Sod. Cas.	Dry and very thick viscosity, sticky and elastic	Soft and sticky, off-flavour, complete disintegration when frying
Skim milk powder	Watery, no emulsion formed	
Whey powder	Watery, no emulsion formed	
Whey protein	Very thin and runny	Very dry and crumbly, separation of the ingredients
Promosoy	Sloppy	Very soft, very sticky and pasty
Promine D	Thin and runny, increased viscosity with time	Gritty mouthfeel, dry feeling in the mouth, pasty
Sod. Cas. + Whey protein*	Less viscosity than normal sausage	Very sticky and pasty, separation of the ingredients

* Proportions used are the same as in co-precipitates.

the three types of milk co-precipitates (High Cal 2, High Cal 6 and Low Cal), 'Sod. Cas' and two types of soya (an isolate and a concentrate), only 'Sod. Cas' and 'Low Cal' showed the capacity to form emulsions when they were used as emulsifiers. It was also found that the emulsions formed with 'Sod. Cas' and 'Low Cal' had a fairly high level of water separation. It was anticipated, therefore, that one or more proteins with a high water binding capacity could be used to absorb the separated water. In the initial trials TVP was used. This product, in addition to its capacity to hydrate and become chewable, can remain unchanged when undergoing retort conditions.

After several trials using 'Low Cal' and/or 'Sod. Cas' as emulsifiers and TVP as the binder, it was found that 'Sod. Cas' always gave a sticky and soft product. Thereafter, all subsequent trials were conducted with 'Low Cal'. The following formulation was considered to give the best results: 'Low Cal', 14%; TVP, 13%; fat, 6%; water, 67%.

This formulation was used in trials to test the suitability of other proteins as emulsifiers. This was done by replacing the 14% 'Low Cal' with other proteins. Results presented in Table 1 show that 'Low Cal' is the only emulsifier to give a satisfactory emulsion and the required body and texture in the canned product.

Results

Having established 'Low Cal' as being the most effective emulsifier, an attempt was made to replace the TVP with other binders. Some of the successful results are presented in Table 2.

It can be seen from Table 2 that all products have a fairly high water concentration and a fairly constant protein content. A variation in the amount of any of the ingredients greatly affected the body and texture. The only variations we were able to

TABLE 2. Formulations used to evaluate efficiency of binders in the 'low cal'–fat–water binder systems

Ingredients	Formulations (as percentages)						
	1	2	3	4	5	6	7
'Low Cal'	14	14	14	14	14	14	14
Water	67	67	67	67	67	60	60
Fat	6	6	6	6	6	13	13
Cerebind			6	4			
Fine gluten			7	4	3	13	6·5
Granular co-precipitate		13			5		
TVP	13						
'High Cal 2'							6·5
Granulated gluten				5	5		

obtain were when we used fine gluten in a high proportion (13%), or fine gluten (6·5%) in combination with 'High Cal 2' (6·5%). The fat content was increased to 13% and water content decreased to 60%.

It should be emphasized that the success of these binders (Table 2) indicates that other ingredients, e.g. rice, corn or legume, flour or granules, might also be useful binders.

Discussion and conclusions

The product can be flavoured to suit the taste of consumers from different ethnic groups and localities, e.g. to resemble meat or other flavours. The flavour and colour of some of the canned formulations is described below:

'Low Cal' and TVP

The product has a toasted flavour which was nevertheless acceptable to many people. The toasted flavour could not be masked by adding other flavourings or masking agents. However, we have since been advised that it may be avoided by using TVP products of other manufacturers.

'Low Cal' and granulated co-precipitate

This product has a slightly bitter flavour which appears as an after-taste. The bitterness seems to come from the granulated co-precipitate and not from the 'Low Cal'. Masking agents are very successful in masking this bitterness. The colour of this product is slightly pink so it is appropriate to use reddish colour.

'Low Cal' and cerebind, fine and granulated gluten; 'High Cal 2' and granulated co-precipitate

These products were white with a bland flavour and are suitable for addition of any suitable colour or flavour.

Sensory evaluation and statistical procedure

Three products, formulations 1, 2 and 6, were chosen for sensory evaluation. All three were flavoured with 1% beef flavour (supplied by International Flavours and Fragrances (Australia) Pty Ltd, Dee Why, N.S.W.). Sensory evaluation of these products was carried out in comparison with a commercial sample of loaf type canned product, containing basically texturized soya and with beef flavour.

Tests were carried out in a hospital cafeteria one week after the products, which resembled meat loaf, were manufactured. The tasters received approximately 10 g of each of the four samples. Orange juice was provided for palate clearing.

The simulated meat products were assessed by a panel of approximately sixty tasters, all but one of whom were consumers of simulated meat products on a daily, or at least a weekly, basis. Participants were asked to judge three characteristics using the following scoring system.

Appearance	5 like very much	1 dislike very much
Sliceability	5 very good	1 very poor
Texture (mouthfeel)	5 like very much	1 dislike very much

They were also asked to state whether they considered the samples acceptable.

A complete block design was used, in which the four samples were judged at one session.

The responses for appearance, sliceability and texture were subjected to separate Analyses of Variance. The acceptability responses were analysed using the Non-Parametric Cochran Q Test (Siegel, 1959).

The mean rating scores for the sensory tests are presented in Table 3.

TABLE 3. Means of scores on hedonic scales of four simulated canned meat loaf products using hedonic scale of 1 (least liked) to 5 (most liked)

Samples	Appearance	Sliceability	Texture
'Low Cal' + granulated co-precipitate	3·7	4·4	3·8
Commercial	3·9	3·7	3·5
'Low Cal' + TVP	3·9	4·4	3·9
'Low Cal' + Gluten	3·7	4·5	3·7
No. of tasters	60	60	55*
Estimate SE for comparison	†	0·08	†

* Five tasters omitted to score.

† No significant difference.

Results of sensory evaluation

Appearance

There were no significant differences in the appearance ratings of the samples, nor between tasters.

Sliceability

There was a highly significant difference between the mean ratings of sliceability. The sliceability of the control sample was rated as significantly inferior to the other three samples. The difference between tasters when scoring sliceability was significant at the 5% level of probability.

Texture

There was no significant difference between the texture of the four products as assessed by this panel. There was a slight trend toward the TVP being the most liked and the control the least liked and this would correlate with the sliceability results. The difference between tasters was significant at the 5% level of probability.

Acceptability

There were no significant differences in the degree of acceptability of the four samples. The majority of tasters found them all acceptable.

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The automated determination of volatile bases (trimethylamine, dimethylamine and ammonia) in fish and shrimp

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Summary

The automated method for the determination of trimethylamine (TMA) in fish and shrimp extracts, which is based upon the colour change of an indicator solution into which TMA vapours are injected, was further improved. Methods for the automated determination of dimethylamine (DMA) and ammonia in extracts were developed and elaborated. The accuracy and precision of the automated TMA and DMA determination equal those of the gas chromatographic determination of these amines described previously.

Both TMA and DMA can be analysed at a speed of thirty samples in an hour, whereas the rate of analysis for NH_3 is sixty per hour.

Introduction

In 1964, Murray & Burt introduced the first automated determination of trimethylamine (TMA) in fish extracts. In this method TMA was vapourized by warm alkali treatment and the vapour brought into a stream of indicator solution, the colour of this solution changing proportionally to the amount of TMA absorbed.

The authors could perform their analyses at a rate of forty per hour. Interference by ammonia, which is always present in fish extracts, and by primary and secondary amines, was overcome by the addition of formaldehyde prior to evaporation from the extract after it has been made alkaline. It was claimed that the total amount of volatile bases could also be determined by omitting the formaldehyde and diluting the samples.

In 1973, Kato & Uchiyama stated that evaporation at 75°C, the temperature chosen by Murray & Burt, may lead to erroneously high values and for this reason they proposed a bath temperature of 60°. With the aid of a somewhat modified equipment they were able to handle twenty extracts in an hour.

During our first attempts to adapt this TMA determination to an AutoAnalyzer II system we found it desirable to introduce some new changes in the method. Furthermore we felt a need for determining the dimethylamine (DMA) content of some fish extracts as well. Finally, in some cases information on the ammonia content of the extracts was

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thought to be useful. For these reasons we have studied the automated determination of TMA, DMA and ammonia in fish extracts.

Equipment, materials and methods

Fish and shrimp extracts were made with the aid of a 5% trichloroacetic acid (TCA) solution as described previously (Ritskes, 1975a).

A Technicon AutoAnalyzer II system was used throughout the work. The gas chromatographic determination of TMA and DMA (Ritskes, 1975a) was used as a reference method. As direct injection on the column resulted in somewhat greater accuracy (Ritskes, 1975b), the liner was omitted.

All reagents used were A.R. grade except the trichloroacetic acid which was of Ph. Ned. VII quality. (This grade almost equals the B.P. 1973 grade.)

Determination of trimethylamine (TMA)

The diagram of the automated TMA analysis is given in Fig. 1. According to Kato & Uchiyama (1973), a Technicon B1 trap is used as a gas trap. The temperature of the oil bath is maintained at 70±1°C. The rate of analysis is thirty per hour.

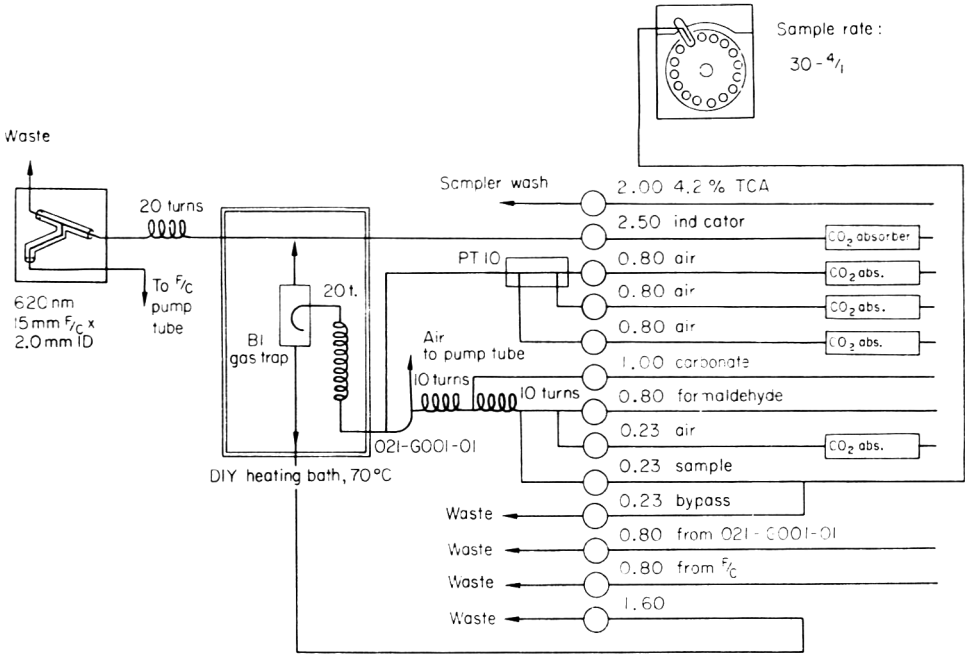

FIG. 1. Flow diagram for the automated determination of trimethylamine (TMA) in fish extracts.

Reagents. (1) Carbonate/thiourea solution: 225 g of K_2CO_3 is dissolved in water and diluted to 250 ml, and 25 g of thiourea is dissolved in 250 ml of warm distilled water. The thiourea solution is added to the carbonate solution, the mixture left overnight and filtered.

(2) Formaldehyde solution. Commercial formalin is shaken with magnesium carbonate, filtered and diluted with an equal volume of water. To 200 ml of the final solution three drops of Rhodorsil Antimousse 426 R (Rhône-Poulenc, Paris) are added.

(3) Indicator solution. The stock solution is prepared by dissolving 400 mg of bromothymol blue in 1 ml of 0·5 N NaOH. This solution is diluted to 100 ml with distilled water. From this stock solution 10 ml is diluted with 1% NaCl in water to about 900 ml, 0·25 ml of wetting agent (Levor IV) is added, the pH is adjusted to 6·2 and the solution is made up to 1000 ml.

(4) Wash solution. 4·2% w/v TCA in water.

(5) Standard TMA solution. 136·4 mg of trimethylamine hydrochloride (equivalent to 20 mg of TMA nitrogen) is dissolved in 100 ml of 4·2% TCA. Working standards are obtained by diluting 1–5 ml of this stock solution to 100 ml with 4·2% TCA solution.

Determination of dimethylamine (DMA)

The automated DMA determination is based upon the reaction with carbon disulphide and ammoniacal copper sulphate solution first described, as a spectrophotometric method, by Dowden (1938). In this reaction the yellow copper salt of dimethyl dithiocarbamic acid is formed:

$$2(CH_3)_2NH + 2CS_2 + Cu^{++} + 2OH^- \rightarrow ((CH_3)_2N{-}\overset{\overset{S}{\|}}{C}{-}S{-})_2Cu + 2H_2O.$$

The copper salt is insoluble in water but can be extracted by organic solvents. The reaction is specific for secondary amines; primary and tertiary amines do not interfere.

Figure 2 gives the diagram for the automated analyses, which can be performed at a rate of thirty samples in an hour. The extractant, chloroform, contains one of the reagents, i.e. carbon disulphide.

The equipment is started up by pumping ethanol into the Acidflex tubes and water in the $CuSO_4$ tube. The ethanol is then replaced by CS_2/EtOH/$CHCl_3$ in the tubing in question, and finally the water is replaced by $CuSO_4$/ammonia.

After the determinations have been finished, $CuSO_4$/ammonia is replaced by water and, after a few minutes, CS_2/EtOH/$CHCl_3$ is replaced by ethanol.

Reagents. (1) Carbon disulphide/ethanol/chloroform solution. Fifty millilitres of carbon disulphide is added to 100 ml of ethanol. This mixture is made up to 1000 ml with chloroform.

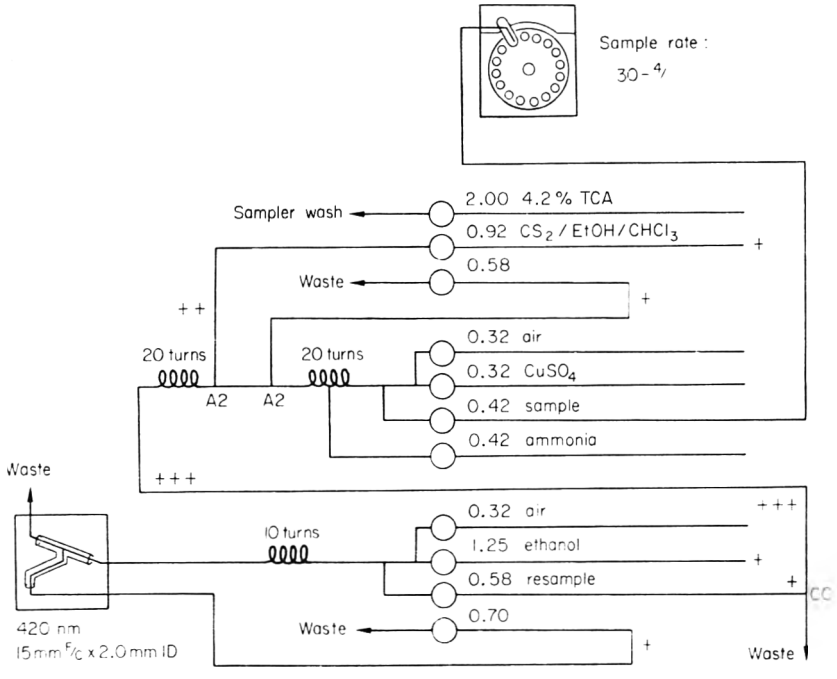

FIG. 2. Flow diagram for the automated determination of dimethylamine (DMA) in fish extracts. +, Acidflex tubing; ++, 0·051-inch silicon tubing, 4-inch long; +++, glass tubing connected with Acidflex.

(2) Ammoniacal copper sulphate solution. Twenty grams of ammonium acetate is dissolved in 100 ml of water. 0·2 g of $CuSO_4.5H_2O$ is added and allowed to dissolve (solution A). Twenty millilitres of concentrated ammonium hydroxide is added slowly to 100 ml of cooled 10% w/v NaOH (solution B). Solution A is added slowly to solution B, under continuous agitation. The combined solutions were made up to 300 ml with distilled water.

(3) Ammonia solution, 25%.

(4) Wash solution: 4·2% w/v TCA in water.

(5) Standard DMA solution: 116·4 mg of dimethylamine hydrochloride (equivalent to 20 mg of DMA nitrogen) is dissolved in 100 ml of 4·2% TCA. Working standards are obtained by diluting 1–5 ml of this stock solution to 100 ml with 4·2% TCA solution.

Determination of ammonia

The automated procedure for the determination of ammonia in sea water was used, this method being based upon the well-known reaction with phenol and hypochlorite in the presence of sodium nitroprusside (Technicon Industrial Systems, 1971). The

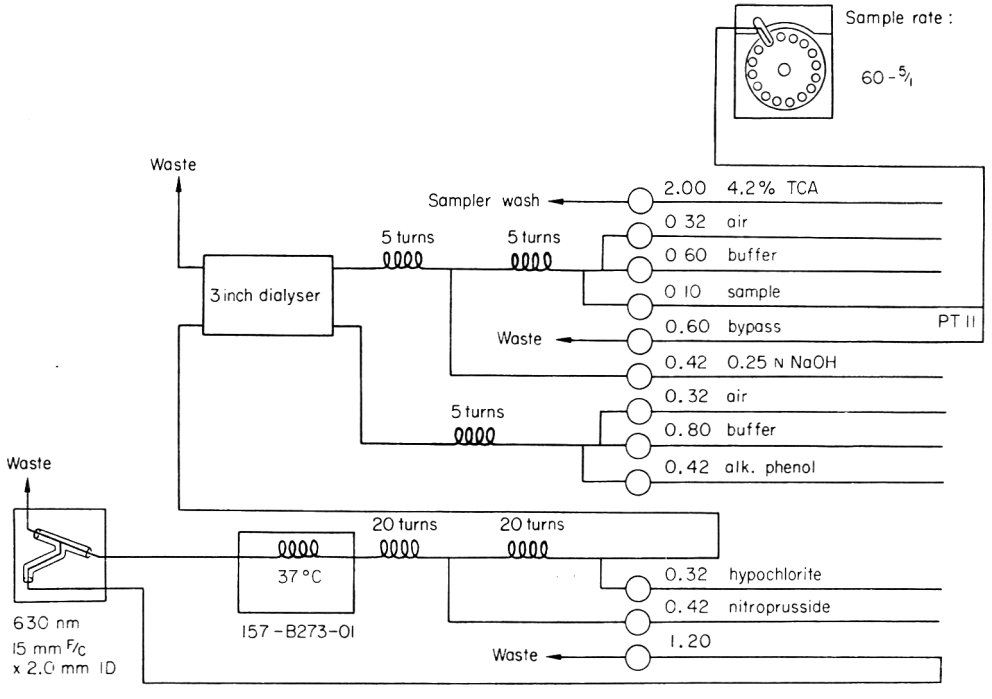

FIG. 3. Flow diagram for the automated determination of ammonia in fish extracts.

manifold is modified in such a way that the alkalified extracts are dialysed prior to colour development, which requires some adaptation of the tubing parameters. The scheme is given in Fig. 3. The rate of analysis is sixty per hour.

Reagents. (1) Tartrate/citrate buffer solution.* Thirty-three grams of sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) and 24 g of sodium citrate ($Na_3C_6H_5O_7.2H_2O$) are dissolved in 950 ml of distilled water. The pH is adjusted to 5·0 with concentrated sulphuric acid, the solution is diluted to 1000 ml and 0·5 ml of wetting agent (Brij- 35) is added.

(2) NaOH. 0·25 N in water.

(3) Alkaline phenol reagent. Eighty-three grams of phenol is dissolved in 50 ml of distilled water. One hundred and eighty millilitres of 5 N NaOH is cautiously added, in small amounts, under continuous agitation, and the solution is made up to 1000 ml with distilled water.

(4) Sodium hypochlorite solution. The stock solution should contain not less than 5·25% of available chlorine. The working solution is obtained by diluting the stock solution 1 to 5 with distilled water.

* Apart from the buffering action this solution also prevents precipitation of the hydroxides of Ca, Mg and other metals, which is of particular use in the analysis of sea water.

(5) Sodium nitroprusside ($Na_2Fe(CN)_5NO.2H_2O$). 0·05% in water.

(6) Wash solution. 4·2% w/v TCA in water.

(7) Standard NH_3 solution. 47·1 mg of ammonium sulphate (equivalent to 10 mg of NH_3 nitrogen) is dissolved in 100 ml of 4·2% TCA, this solution being used directly as a working standard. Dilutions of 1–5 ml to 10 ml may be made for additional standards.

Discussion

TMA determination

Serious drift in the blank value, which was often observed, may be due to pH instability of the indicator solution. This problem was largely overcome by the addition of 1% NaCl to this solution.

In order to minimize the time required for obtaining the steady state level, the volume of the gas trap as well as the connection line to the indicator solution should be kept as small as possible. For that reason the Technicon B1 gas trap as used by Kato & Uchiyama was chosen, the connection tubing to the indicator stream being not longer than 1·5 cm. Creeping up of the alkaline solution, which may result in severe contamination of the indicator solution, was avoided by the antifoaming agent present in the formaldehyde solution. Both gas trap and connection tubing included the T joint where the vapour is injected into the indicator stream should preferably be immersed in the heating bath.

At higher temperatures the steady state will be reached faster and the steady state itself seems to be somewhat more stable. However, Kato & Uchiyama found that, at a bath temperature of 75°C, too high values were observed which, in their opinion, might be due to the presence of ammonia and other volatile bases. In order to establish this assumption the response for an ammonia solution at two different bath temperatures, with or without formaldehyde in the system, was measured, the results being given in Table 1. It is shown that the response for ammonia, as compared to that of TMA, is rather small. However, the amount of NH_3 in fish usually exceeds that of

TABLE 1. Response to ammonia in the TMA determination under different conditions, expressed as percentages of the response for an equimolar amount of TMA; solutions tested: (a) 2 mg NH_3 nitrogen in 100 ml 4·2% TCA and (b) 2 mg TMA nitrogen in 100 ml 4·2% TCA

Base used	Formaldehyde added	60°C	70°C	75°C
KOH, 30% w/v	Yes	No response	1·5%	4·4%
KOH, 30% w/v	No	3·4%	3·3%	4·5%
K_2CO_3, 67% w/v	Yes	No response	No response	No response
K_2CO_3, 67% w/v	No	4·7%	5·2%	6·6%

TMA by many times, and for that reason serious interference of NH_3 in the TMA determination, when performed at 75°, can be expected.

It is also shown from Table 1 that the formaldehyde–ammonia complex does not resist the KOH treatment at 75°. No other components were found that could replace formaldehyde as a NH_3 binder. Sodium hypochlorite, which readily forms chloramine (NH_2Cl) from ammonia, seemed to be successful in concentrations of about 0·1%. However, the response for TMA was lowered if NH_3 was originally present, thus suggesting a reaction between NH_2Cl and TMA.

The problem could be resolved by replacing the KOH solution with a potassium carbonate solution. As is shown in Table 1, ammonia evaporation was inhibited completely at 75°, even if concentrated solutions of K_2CO_3 (over 50%) were applied. No response was observed for concentrations of up to 20 mg of NH_3 nitrogen in 100 ml of 4·2% TCA.

In most fish extracts TMA and NH_3 are the only volatile bases present in more than trace amounts. In cod and cod-like fishes DMA is found as well, in particular when these fish are kept for a long time in the frozen state. For this reason some additional attention has to be paid to possible interference by DMA.

Formaldehyde is able to bind DMA to some extent, but interference in the TMA determination still may occur. Tozawa, Enokihara & Amano (1971), who studied the DMA interference in the picrate method for TMA, found this interference to be more serious if K_2CO_3 is used instead of KOH. The same was observed by Murray & Gibson (1972), and also by Keay & Hardy (1972). We also observed this effect in the automated TMA method. However, the response for DMA at 75° was not more than 18% of the response for an equimolar amount of TMA. The signal for DMA could be further decreased by reducing the K_2CO_3 concentration, by lowering the bath temperature and by the addition of thiourea to the K_2CO_3 solution. The latter component also has a favourable effect on the steady state stability. It was observed that a lowering of the bath temperature from 75° to 70° hardly affected the time required for reaching the steady state level. In combination with a reduction of the K_2CO_3 concentration to 45% w/v and the addition of 5% of thiourea the response for DMA could be reduced to 2–4% of that for TMA, which is low enough to permit accurate TMA determinations in extracts containing some DMA. Synergistic effects were not observed.

No response was observed for trimethylamine oxide (TMAO), even at a concentration of 100 mg of TMAO nitrogen in 100 ml of extract.

Linearity: under the conditions described, the determination is linear over the range of 0–2·5 mg% TMA nitrogen in the extract which, in the case of lean fish, corresponds to 0–12·5 mg% TMA nitrogen in the flesh.

DMA determination

Up till now, no interferences from other components present in the fish extract have been observed. The linearity of the automated method is more than sufficient for a

correct determination of the DMA concentrations usually found in cod and related species.

The extraction step involved in the procedure sometimes leads to difficulties in the phase separation. These difficulties were overcome by the addition of 10% ethanol to the carbon disulphide/chloroform solution, and by mixing the solution, after the extraction procedure, with ethanol.

In some cases a small amount of precipitate is produced during the extraction which obviously disturbs the determination. An additional filtration of the extracts, e.g. over a Millipore filter, will prevent this disturbance.

Attempts to automate other reactions that might be used for a DMA determination in fish extracts remained unsuccessful. The reaction between secondary amines, phenothiazine and bromine (Bröll & Fischer, 1962), in our hands, did not lead to any result. The reaction of secondary amines with acetaldehyde and sodium nitroprusside (Feigl & Anger, 1937) could be easily automated, but extracts of fish and, in particular, of shrimp produced extremely high blank values which made the method unsuitable for this purpose. Possibly it may be applied to a DMA determination in total volatile base distillates.

NH_3 determination

The manual method of determining ammonia directly in fish and shrimp extracts suffers from interferences possibly due to the reactivity of the intermediate, i.e. NH_2Cl, towards components in the extract. In order to obtain reliable values the NH_3 has first to be isolated by distillation and then is determined in the distillate.

These interferences did not occur in the automated procedure. It is thought by us that the dialyser removes the material that may react with NH_2Cl, thus eliminating the need for distillation. No other interferences were observed.

TABLE 2. Recovery and standard deviation in the automated *v.* the gas chromatographic TMA and DMA determinations; analyses performed in tenfold

	TMA–N, mg/100 ml	DMA–N, mg/100 ml	NH_3–N, mg/100 ml
Composition			
Solution I	2·00	0·404	2·01
Solution II	0·200	0·0404	2·02
Analysis by AA II			
Solution I	2·02 ± 0·8%	0·416 ± 0·9%	2·00 ± 0·7%
Solution II	0·200 ± 3·0%	0·036 ± 8·4%	
Analysis by GC			
Solution I	1·98 ± 2·3%	0·410 ± 2·6%	
Solution II	0·192 ± 2·0%	0·037 ± 7·3%	

Precision and accuracy

Two different solutions of TMA, DMA and NH_3 in 4·2% TCA were prepared, and submitted to analysis for TMA and DMA in tenfold, both on the AutoAnalyzer II by the procedures described here, and by the gas chromatographic method (Ritskes, 1975a). The results are given in Table 2. The conclusion has to be drawn that both accuracy and precision of the automated methods are comparable to those of the gas chromatographic method.

Accuracy and precision of the automated NH_3 determination, which were also tested, were much better than those of the manual method. This part of the investigation was not examined in detail.

In another experiment known amounts of TMA, DMA and NH_3 were added to a fish or a shrimp extract, and the original as well as the spiked extracts were submitted to analysis. Recovery figures are given in Table 3. It was found that the recoveries in all cases are adequate.

TABLE 3. Recovery of TMA, DMA and NH_3 from spiked fish and shrimp extracts

	TMA–N added, mg/100 ml	TMA–N, determined mg/100 ml	DMA–N added, mg/100 ml	DMA–N, determined mg/100 ml	NH_3–N added, mg/100 ml	NH_3–N, determined mg/100 ml
Cod extract	—	0·07–0·07	—	0·22–0·22	—	2·26–2·26
Cod extract	0·2	0·27–0·29	0·2	0·41–0·42	—	—
Cod extract	1·0	1·09–1·07	1·0	1·24–1·24	2·0	4·36–4·33
Cod extract	2·0	2·17–2·18	0·5	0·70–0·74	—	—
Shrimp extract	—	0·06–0·06	—	—	—	0·92–0·93
Shrimp extract	0·2	0·22–0·23	—	—	0·2	1·13–1·13
Shrimp extract	2·0	2·16–2·19	—	—	2·0	2·89–2·88

Conclusions

By the methods described, several hundreds of fish or shrimp samples may be analysed for TMA, DMA and NH_3 in one week, this amount being largely dependent on the speed with which the extracts can be prepared.

Since both precision and accuracy of the automated TMA and DMA methods are equal to those of the gas chromatographic determination, the described methods should be preferred for routine purposes. If, however, the presence of volatile bases other than TMA, DMA and NH_3 is expected, if unusual products have to be investigated, or if only small numbers of samples have to be analysed, the gas chromatographic method should be chosen.

Acknowledgments

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Control of thaw rigor by manipulation of temperature in cold store

I. McDONALD* AND N. R. JONES†

Summary

Fillets cut from pre-rigor codling and frozen singly were observed to shrink rapidly whilst thawing. This paper describes a series of experiments investigating this phenomenon of thaw rigor and its control by manipulation of temperature in cold storage.

Introduction

The phenomenon of thaw rigor has been known to flesh technologists for many years (e.g. Jones, 1969). In fish it can cause serious loss of weight in tissue water and water solubles, particularly in fillets (rather than whole fish) frozen pre-rigor. With pre-rigor frozen fish blocks, the problem is exacerbated after sawing and cook thawing, as, for instance, in fish fingers, where it can be accompanied by gross distortion. Textural effects that appear to be of a fundamentally similar nature have been reported, for instance in trap caught cod (MacCallum *et al.*, 1968). From preliminary observations (McDonald & Jones, 1967) it became apparent that such faults in quality could be minimized or even eliminated by a period of cold storage at temperatures somewhat above those commonly accepted to be of good practice in terms of avoidance of denaturation reaction.

This paper describes a series of experiments illustrating the general principle of the approach.

Experimental

Codling between 350 and 650 mm were caught on the Faroe ground and filleted by hand. Single fillets were blast frozen pre-rigor. Fillets from successive batches were randomized to avoid batch to batch variation. They were 'glazed' with fresh water, wrapped in foil and maintained at −29°C on the vessel and ashore (ten days) before separating into four groups. They were stored respectively at −7, −14, −22 and −29°C.

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Thawing was carried out in air blast at 18°C, 100% relative humidity, fillets being laid on waxed paper. Change of length was measured by reference to the pencilled outline of the original frozen fillet. Fillets were weighed before and after thawing.

Results and discussion

Tables 1 and 2 show percentage changes in length and weight respectively following storage at the four different storage temperatures and thawing.

TABLE 1. Mean per cent weight loss and standard deviation within each batch of seven fillets

Zero time control	Storage temp. (°C)	Storage time (days)						Significance	
		2·5	7	14	30	92	434	Between storage times	Storage versus control
	−7	+0·35 (±0·85)	−0·45 (±1·05)	−0·38 (±1·02)				NS	***
	−14		1·63 (±1·55)	1·39 (±1·23)	1·23 (±1·0)			NS	***
5·16 (±1·85)									
	−22			2·40 (±2·87)	3·49 (±4·02)	2·20 (±1·52)		NS	*
	−29				2·41 (±1·58)	0·87 (±0·65)	3·12 (±3·57)	NS	**

NS, not significant; *, significant at the 5% level; **, significant at the 1% level; ***, significant at the 0·1% level.;

Shrinkage remains roughly constant after ninety-two days storage at −29°C, with only a slight fall at 434 days. A slight fall only was observable in −22°C stored fish. By comparison, shrinkage in the −14°C fish was almost eliminated after thirty day's storage and within seven days at −7°C. Weight changes behaved somewhat similarly although relatively erratically (some gains were recorded as a consequence of condensation). It was recognized, however, that shrinkage did not exactly parallel weight loss.

On the evidence available, it is not possible to explain all aspects of the phenomenon. It may be expected that shrinkage may well be affected (1) by the metabolism of glycolytic intermediates and ribomononucleotides (ATP, etc.) in the frozen state,

TABLE 2. Mean per cent contraction and standard deviation within each batch of seven fillets

Zero time control	Storage temp. (°C)	Storage time (days)						Significance	
		2·5	7	14	30	92	434	Between storage times	Storage versus control
	−7	2·69 (±2·29)							***
	−14		6·46 (±3·37)	4·50 (±3·64)	0·61 (±1·05)			*	***
10·40 (±5·09)									
	−22			12·20 (±5·45)	11·64 (±8·20)	7·61 (±3·62)		NS	NS
	−29				13·23 (±3·31)	10·92 (±2·44)	10·89 (±8·31)	NS	NS

NS, not significant; *, significant at the 5% level; **, significant at the 1% level; ***, significant at the 0·1% level.

energy being essentially dissipated as heat rather than mechanically, and hence unavailable for contraction at thawing; and (2) that protein aggregation reactions may to some degree affect contractility.

Water relations would be affected also, for instance through lactic acid accumulation and from loss of binding capacity resulting from aggregation reactions.

While it is interesting to postulate the interrelationship between such factors, it is clear that at the level of practical thaw rigor control such approaches offer an alternative to slow thawing, with its attendant demands on space and equipment. Judgment is needed, however, to balance their desirability against any adverse effects of denaturation, which could, theoretically, occur if cold storage times are over extended.

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Comparison of methods of freshness assessment of wet fish

II. Instrumental and chemical assessments of boxed experimental fish

J. R. BURT, D. M. GIBSON, A. C. JASON AND H. R. SANDERS

Summary

Samples of cod were obtained from different fishing grounds at different seasons. They were stored in boxes with ice for periods of up to 20 days. At regular intervals measurements by Torry Fish Freshness Meter and Intelectron Fish Tester V and determinations of hypoxanthine and trimethylamine concentrations were made.

Linear relationships with length of time of storage were established and calibrations with sensory tests are presented.

Ground and seasonal effects were found in the relationships with days of storage and with sensory assessment. The amounts of spoilage measured by the different tests are correlated.

Introduction

In Part I (Burt *et al.*, 1975) results of sensory assessment on cod from a number of fishing grounds at various seasons were reported. The same fish were tested by four non-sensory methods; the results from these and comparisons with the sensory test results are given here.

Two chemical compounds were chosen as indicators of spoilage, trimethylamine which is produced by the action of bacteria on trimethylamine oxide, and hypoxanthine which is produced by tissue enzymes from nucleotides. The extensive literature on trimethylamine and the quality of fish was reviewed by Ruiter (1971). Burt, Stroud & Jones (1969) list references to the determination of hypoxanthine concentrations in nearly twenty species of fish, including cod, during periods of chill storage; since then many further reports have been published and it appears that hypoxanthine may be of value in assessing the quality of over fifty species of fish (marine and freshwater) and shellfish (molluscs and crustaceans).

Two types of instruments, both measuring dielectric properties, were used. The Intelectron Fish Tester V has been commercially available for some time (Hennings,

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1963, 1965). Graphite electrodes are placed on either side of the fish and the impedance is measured at two frequencies. A meter displays the value $Q=(R_L/R_H-1)\times 100$, where R_L and R_H are the impedances at the lower and higher frequencies respectively. Q decreases from high values for fresh fish as spoilage progresses.

To overcome some of the disadvantages of this instrument, particularly the requirement to pick a fish out of the box and hold it up while applying measuring forceps, a new instrument, the Torry Fish Freshness Meter, was developed. This measures the power factor of the intact fish by a four-electrode technique and can be used without removing the fish from the box (Jason & Richards, 1975).

Materials and methods

Details of catching, storage and sampling procedures were given in Part I.

Torry Fish Freshness Meter

The Torry Fish Freshness Meter (TFM) used was a prototype instrument with a linear scale from 0 to 100. Readings decrease as spoilage progresses. The electrodes were contained in a separate probe head which was connected by flexible cable to a box containing the measuring circuits and meter. Fish were tested in their undisturbed position in the box. The probe head was applied to a precisely defined position on the fish, above and parallel to the lateral line just behind the belly cavity.

Intelectron Fish Tester V

Two models of the Intelectron Fish Tester V (IFT) were used. One had been modified by the manufacturers to include temperature compensation for fish above 0°C (Gibson & Shewan, 1971). Both instruments were graduated from 0 to 100. Readings decrease as spoilage progresses. The fish to be tested was lifted from the box by its head and held up vertically. The electrodes, held by measuring forceps, were applied one on either side of the body of the fish on the lateral line just below the end of the belly cavity.

Hypoxanthine

Hypoxanthine determinations were carried out on the fish selected for analysis by removing a portion of the fish muscle from behind the head. A 5-g sample was weighed ($\pm$ 0·05 g), ensuring that it was not contaminated with any blood, dark muscle or skin. Extracts were prepared by homogenizing the sample with 50 ml 0·6 M perchloric acid for 1 min (MSE Nelco homogenizer). The homogenate was filtered and 5 ml of the filtrate neutralized with 5 ml of 0·557 M KOH containing 0.2 M phosphate buffer (Burt *et al.*, 1969). Analysis of the samples was carried out either by the manual enzymatic analytical method (Jones *et al.*, 1964) or by the automated enzymatic method using the Technicon Auto-Analyser (Burt, Murray & Stroud, 1968). Hypoxanthine concentrations (HXC) were expressed as weight in milligrams per 100 g of fish. In order

to linearize the scale with respect to age-in-ice and to equalize standard deviations, a logarithmic transformation to a hypoxanthine index (HXI) was made, where HXI = $\log_{10}$ (HXC + 5), (Jones *et al.*, 1964).

Trimethylamine

Trimethylamine-nitrogen (TMA-N) was determined on fish muscle extracts by the automated procedure of Murray & Burt (1964), also described by Murray & Gibson (1972). After the taste panel had made their examination of the raw fish fillet, the fillet was quartered and diagonally opposite quarters were taken, being cut if necessary to give 100 g. For smaller fish the whole fillet and sometimes the other fillet still attached to the backbone of the fish were needed to give 100 g of fish muscle. The weighed portions were cut into small pieces with a pair of scissors and homogenized with 300 ml 5% (w/v) trichloracetic acid in an Atomix bottom drive homogenizer for 1 min at half speed and 1 min at full speed. The slurry was filtered through Whatman No. 12 fluted filter paper, and a Universal bottle filled with filtrate. The extracts were stored at 2°C until analysed. For purposes of calculation it was assumed that the water content of cod was 80 ml/100 g. Standard solutions of trimethylamine were run in the analyser, their nitrogen content being determined by micro-Kjeldahl procedure. Samples were run in duplicate and the mean of the two results was taken; if, as happened very occasionally, the difference between the results was greater than 5% of the mean, further replicates were run. Trimethylamine concentrations (TMC) were expressed as weight in milligrams of nitrogen per 100 g of fish. In order to linearize the scale with respect to age-in-ice and to equalize standard deviations, a logarithmic transformation to a trimethylamine index (TMI) was made, where TMI = $\log_{10}$ (TMC + 1). Ehrenberg & Shewan (1955) multiplied this index by 10. They used a micro-diffusion (Conway) procedure and quote their results for concentration as milligrams of nitrogen per 100 m of extract; these results must be multiplied by 0·8 for comparison with the present results.

Results and discussion

The results of the non-sensory tests were analysed in the same way as those of the sensory tests in Part I.

Preliminary calculations confirmed the validity of expressing the hypoxanthine and trimethylamine concentrations by their logarithmic transformations and results are quoted as the respective indices.

When fish is fresh the rate of trimethylamine production is low and results from fish stored for less than 7 days in ice were not used in the subsequent analysis.

The results from the two models of the Intelectron Fish Tester were sufficiently similar for the results of only one to be quoted. Although in the present experiments, when all fish were at the temperature of melting ice, temperature compensation was not required, the results from the temperature-compensated model were used so as to

be compatible with subsequent experiments on fish markets where the temperature of the fish is often higher.

Analysis of variance showed a marked effect of layer for the instruments, the top layer appearing fresher by the equivalent of $1\frac{1}{2}$ days when measured by TFM and nearly 1 day by IFT. This effect was not found in the chemical tests; amongst the sensory tests it had been observed only for general appearance, and then to a smaller degree. It seems to be a surface effect, probably caused by the additional pressure on the fish in the lower layer.

The effect of fish size was non-significant for TFM and equivalent to less than $\frac{1}{4}$ day for IFT. The chemical tests showed a larger effect, $\frac{3}{4}$ day for TMI and 2 days for HXI; in the sensory tests the effect was $\frac{1}{2}$ day. In all cases the larger fish appeared fresher; this effect was observed throughout the range of ages-in-ice.

Within-box-layer standard deviations were pooled over all runs and are shown in Table 1 for separate age-in-ice ranges and pooled over all ages. There is a general increase in standard deviations for longer stored fish, indicating variations in the rate of spoilage of individual fish in a box. Between-box standard deviations pooled over all runs and ages are also given in Table 1. There were significant between-box standard deviations for TFM, IFT and TMI but not for HXI. The reasons for this are not known.

TABLE 1. Within-box-layer and between-boxes standard deviations

Days-in-ice		TFM	IFT	HXI	TMI
Within-box-layer	2–6	6·9	6·6	0·039	—
	7–9	6·9	7·2	0·048	0·117
	10–12	6·8	8·7	0·068	0·148
	13–16	7·8	10·3	0·088	0·163
Pooled		7·1	8·4	0·063	0·144
Between-box pooled		3·3	3·3	ns	0·056

ns, not significant at the 5% level.

Within-box correlation coefficients pooled over all runs and ages are shown in Tables 2 and 3. The highest correlations between tests are for the two instrumental methods. The correlations between length and chemical tests have already been noted in the analysis of variance. pH was most strongly correlated with HXI. The small, though significant, correlations between pH and instruments are opposite in effect though not in sign to those of pH and the chemical and sensory tests; low pH is associated with indications of greater spoilage.

Regression analyses with age-in-ice as independent and instrument reading or chemical index as dependent variable were carried out. No consistent departures from linearity were found. Linear regression parameters (intercept a, regression coefficient b and standard deviation from regression s_r) pooled over all runs are shown in Table 4. For compatibility with the results from sensory tests, only batches for which assessments

TABLE 2. Pooled within-box correlation coefficients

TFM			
0·5	IFT		
ns	ns	HXI	
−0·1	−0·1	0·2	TMI

ns, not significant at the 5% level.

TABLE 3. Pooled within-box correlation coefficients

	Length	pH
TFM	ns	0·1
IFT	0·1	0·1
HXI	−0·3	0·3
TMI	−0·2	0·1

ns, not significant at the 5% level.

TABLE 4. Linear regression parameters with days-in-ice

Test	a	b	s_r
TFM	64	−3·0	8·4
IFT	110	−4·3	9·9
HXI	0·80	0·042	0·069
TMI	−0·45	0·112	0·179

on cooked fish had been carried out were included in the analysis. Box means and regression lines with age-in-ice are plotted in Figs 1–4. The standard deviation from regression for HXI was obtained from separate regressions for each size group pooled over all runs. Separate values for the regression coefficient and the intercept are not quoted, as differences between sizes are considerably lower than differences between runs.

The sensitivity of each method (Baines & Shewan, 1965) was calculated as the ratio

of its standard deviation and its regression coefficient with days-in-ice (s/b). These ratios express the standard deviations in units of equivalent days-in-ice and enable direct comparisons to be made between the methods.

When the mean instrumental reading or chemical index within the sampling unit is to be estimated from the results on a sample, a combination of within-box-layer and between-box standard deviations is used. For HXI where the between-box standard deviation is non-significant the sampling unit may extend to any number of boxes of

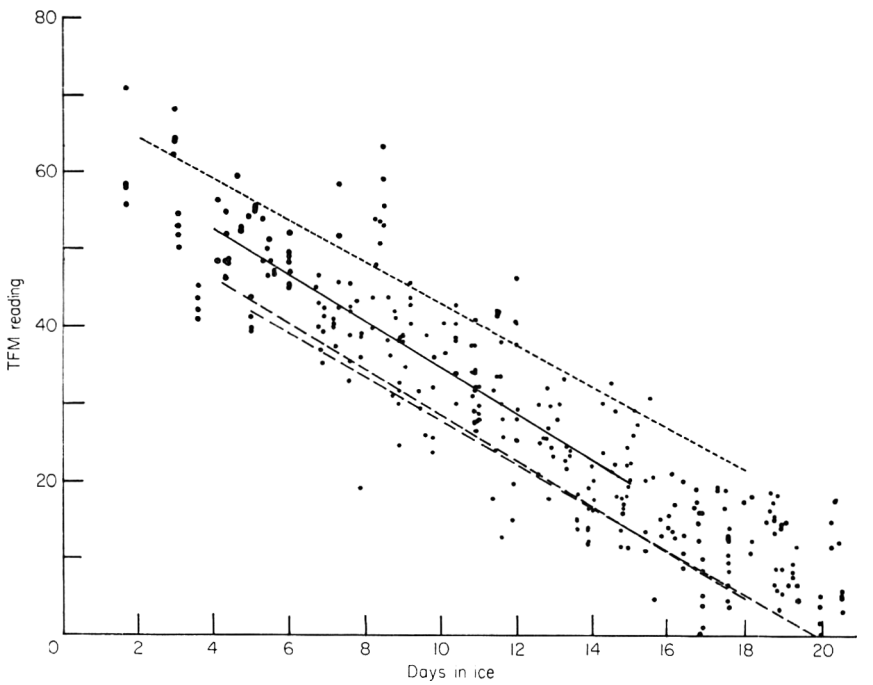

FIG. 1. Relationship between Torry Fish Freshness Meter readings and time of iced storage. Box means and fitted regression lines. ———, Pooled over all runs; — — —, November Iceland; ------, February North Sea.

the same size group and age-in-ice. When there is a significant between-box standard deviation samples must be taken from more than one box and the standard deviation entering the calculation depends on the number of fish taken from each box. If enough boxes are available it is advantageous to take only one fish from each box to be tested. The standard deviation s for the calculation of sensitivity in this case is obtained from $s^2 = s_b^2 + s_w^2$ where s_w is the within-box-layer and s_b the between-box standard deviation. The appropriate standard deviations for TFM, IFT and TMI become 7·8, 9·0 and 0·154 respectively. The corresponding sensitivities are shown in Table 5. Sample numbers required to estimate the mean instrumental reading or chemical index to the

equivalent of ± 1 day-in-ice are given in Table 6. If not enough boxes are available, or if all boxes are not readily accessible, more fish must be taken from each box. This, however, increases the total number of fish to be tested; for TFM instead of one fish from each of twenty-seven boxes, three fish may be taken from each of twelve boxes requiring an increase in effort of one-third for the same degree of precision.

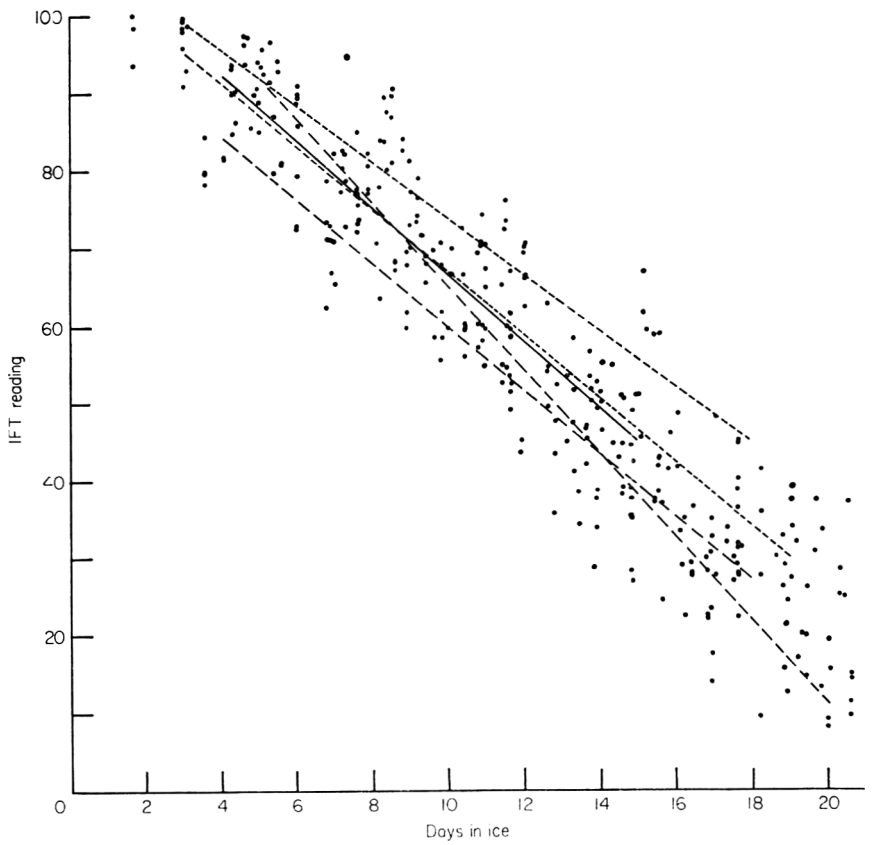

FIG. 2. Relationship between Intelectron Fish Tester V readings and time of iced storage. Box means and fitted regression lines. ———, Pooled over all runs; — — —, November Iceland; – – – – –, February North Sea.

The differences between layers has not been considered in these calculations. It is assumed that in practice only the top layer is tested and a suitable correction applied where appropriate.

Sensitivities were also calculated for the prediction of true days-in-ice from instrumental readings or chemical indices (Table 5); in this case the standard deviations from regression (s_r in Table 4) were used.

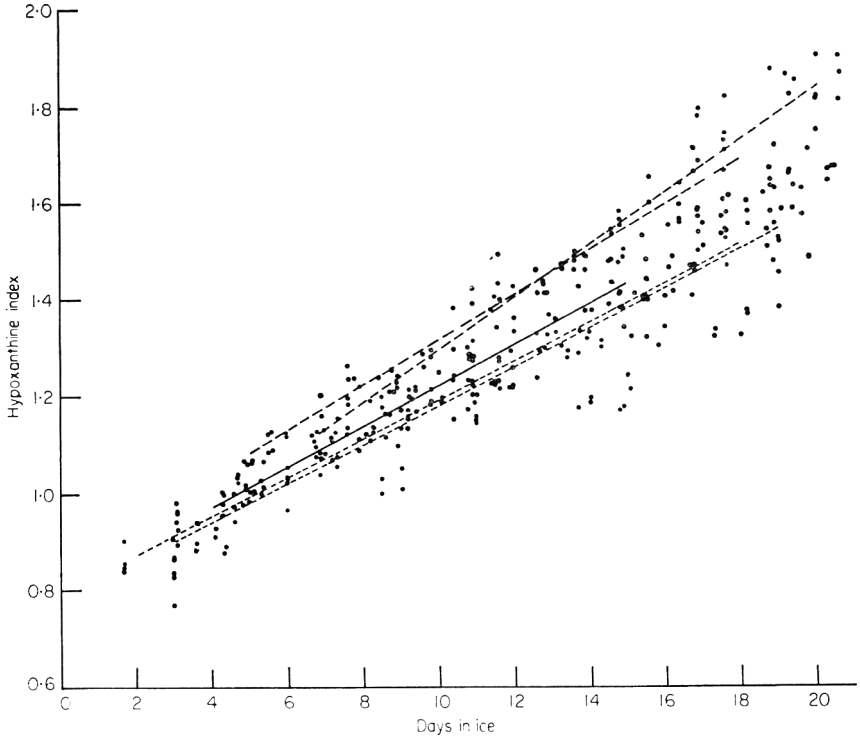

FIG. 3. Relationship between hypoxanthine index and time of iced storage. Box means and fitted regression lines. ———, Pooled over all runs; — — —, November Iceland; ------, February North Sea.

TABLE 5. Sensitivity of test, days-in-ice

Test	Estimate	Prediction
TFM	2·6	2·8
IFT	2·1	2·3
HXI	1·5	1·7
TMI	1·4	1·6

TABLE 6. Sample numbers, ± 1 day-in-ice, 95% confidence

Test	Estimate	Prediction
TFM	27	31
IFT	17	20
HXI	9	11
TMI	7	10

The sample numbers required to predict the true age-in-ice to ± 1 day with 95% confidence are given in Table 6.

Table 7 shows the ages-in-ice predicted using regression lines obtained for different runs and the regression lines pooled over all runs at values of meter readings and chemical indices corresponding to about 6½ and 14 days-in-ice. The regression lines were obtained for fish of all ages-in-ice (TMI from 7 days upwards). Run 1 has been omitted

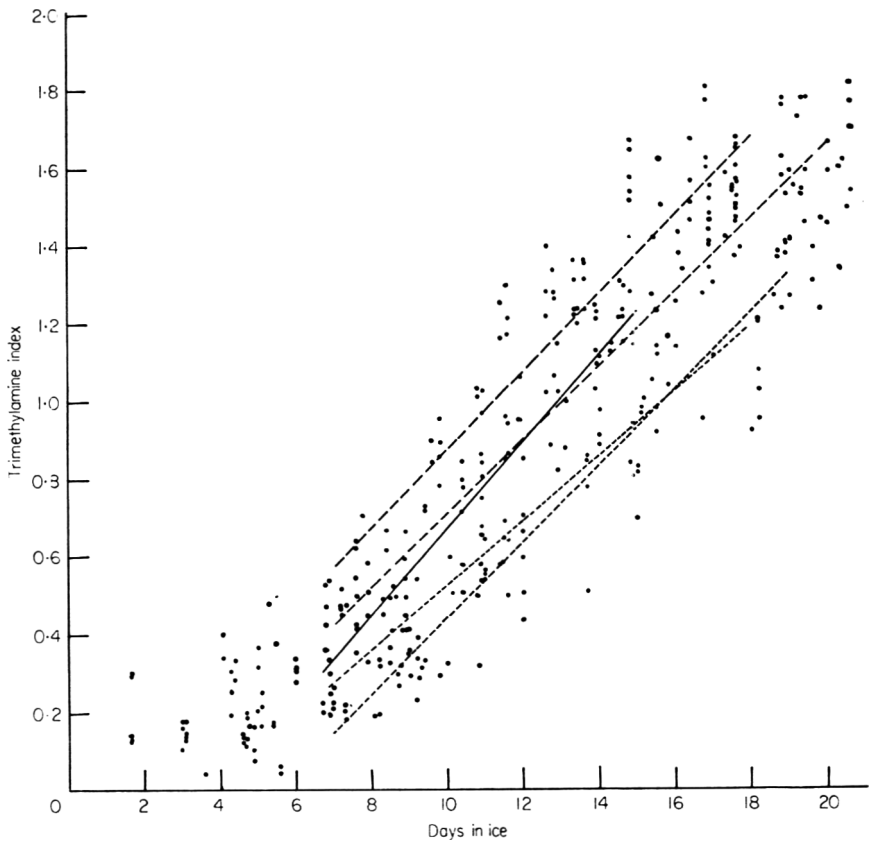

FIG. 4. Relationship between trimethylamine index and time of iced storage. Box means and fitted regression lines. ———, Pooled over all runs; — — —, November Iceland; – – – – –, February North Sea.

for TFM because the instrument's calibration was different for that run. During part of run 2 the Intelectron Fish Tester was faulty and IFT results were not used. Only large fish were obtained for run 12 and HXI results were not used because of the strong size effect on this test. Runs 12 and 14 had too few samples for the inclusion of TMI results. The use of the pooled regression equation instead of that appropriate to the particular run (which is generally not known) can introduce a bias of up to the equivalent of 2½ days for TMI and 3½ days for the other tests. The range of ages-in-ice

predicted by the different methods for any one run is on average 2 days but can be as great as 4 days.

In terms of these tests, keeping time can be defined as the time taken to reach a reading or index corresponding to 14 days-in-ice for the pooled regression. Keeping

TABLE 7. Days-in-ice predicted in different runs

			Nominal days-in-ice							
			6·5				14·0			
Run	Month	Ground	TFM*	IFT	HXI	TMI	TFM	IFT	HXI	TMI
			45†	82	1·07	0·31	23	48	1·41	1·08
1	2	NS	–	6·5	7·1	8·7	–	14·8	15·5	16·4
2	6	I	6·1	–	5·6	7·4	14·4	–	14·5	15·1
3	9	BI	5·5	5·8	6·2	6·1	13·9	12·8	13·3	12·3
4	11	I	4·4	4·7	4·6	4·3	11·8	13·0	11·9	11·9
5	2	L	6·3	5·9	8·3	8·4	13·6	15·2	17·7	15·5
6	2	NS	9·3	8·0	6·9	7·3	17·5	17·3	15·4	16·4
7	4	L	7·2	6·7	6·9	6·0	14·4	13·6	12·7	13·4
8	4	I	7·4	6·8	6·4	6·6	14·3	13·0	13·0	13·1
9	6	I	5·9	5·7	7·6	6·3	14·1	13·2	14·6	13·9
10	6	I	8·7	8·1	5·7	5·6	16·6	15·2	14·9	14·0
11	9	BS	5·8	5·3	4·8	4·5	12·7	14·5	14·0	12·3
12	9	BI	3·3	3·6	–	–	11·2	11·9	–	–
13	11	I	3·9	7·0	5·8	5·7	11·7	13·2	12·0	13·8
14	11	NS	8·1	7·7	7·2	–	14·7	17·3	17·0	–
Pooled			6·4	6·4	6·4	6·5	13·9	13·9	14·0	14·0
Range			6·0	4·4	3·7	4·4	6·3	5·4	5·8	4·5

* Test; † reading/index.

BI, Bear Island; BS, Barents Sea; L, Lofoten; I, Iceland; NS, North Sea.

times are generally highest for February/North Sea runs, and lowest for the September/Bear Island, September/Barents Sea and November/Iceland runs. The HXI keeping times (time to reach 1·41) range from 12 days for November/Iceland to nearly 18 days for February/Lofoten; they are higher than the times of $11\frac{1}{2}$ to $12\frac{1}{2}$ days calculated from the data of Jones *et al.* (1964) for North Sea, Iceland and Faroes fish. The TMI keeping times (time to reach 1·08) range from 12 days for November/Ice-

land to 16½ for February/North Sea and are within the range of 11 days (September/Spitzbergen) to 18 days (February/North Sea) calculated from the data of Ehrenberg & Shewan (1955).

Differences in keeping times between corresponding runs in successive years were up to 3 days for TFM, 2½ days for IFT, 2 days for TMI and ½ day for HXI.

Biases can in most cases be reduced by the use of the relationship for the particular ground and season combination instead of the pooled relation. The calculated regression lines for the runs with the longest (February/North Sea) and the shortest (November/Iceland) keeping times are shown in Figs 1–4.

TABLE 8. Pooled within-box correlation coefficients

	GA	RO	CO	CF
TFM	0·1	ns	ns	0·1
IFT	0·1	ns	ns	ns
HXI	−0·1	−0·2	−0·3	−0·3
TMI	−0·1	−0·1	−0·2	−0·2

ns, not significant at the 5% level.

TABLE 9. Linear calibration parameters with sensory scores

	GA		RO, CO, CF	
	a	*b*	*a*	*b*
TFM	−6	15	−18	9
IFT	8	22	−9	13
HXI	1·79	−0·215	1·95	−0·125
TMI	2·17	−0·57	2·60	−0·33

TABLE 10. Calibration

RO, CO, CF	GA	TFM	IFT	HXI	HXC	TMI	TMC
8	3·9	54	95	0·950	3·9	–	–
7	3·3	45	82	1·075	6·9	0·29	0·9
6	2·7	36	69	1·200	10·8	0·62	3·2
5	2·1	27	56	1·325	16·1	0·95	7·9
4	1·5	18	43	1·450	23·2	1·28	18·1

TABLE 11. Correlation coefficients of keeping times

GA							
0·5	RO						
ns	0·6	CO					
0·5	0·5	0·9	CF				
0·5	0·5	0·5	0·5	TFM			
ns	0·6	0·8	0·7	0·7	IFT		
ns	0·6	0·8	0·7	0·5	0·8	HXI	
0·5	ns	0·6	0·6	0·6	0·7	0·7	TMI

ns, not significant at the 10% level.

TABLE 12. Range of keeping times, equivalent days-in-ice

	TFM	IFT	HXI	TMI
Age-in-ice	6·3	5·4	5·8	4·5
GA	5·5	5·0	6·0	3·8
RO	4·7	3·8	5·5	4·7
CO	5·2	4·2	5·4	4·8
CF	5·7	4·5	5·4	4·5

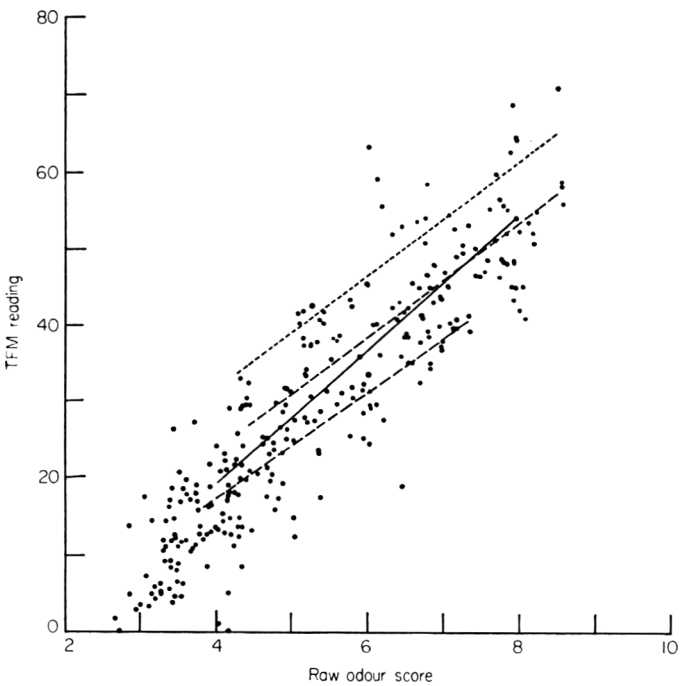

FIG. 5. Relationship between Torry Fish Freshness Meter readings and raw odour scores. Box means and calibration lines. ———, Pooled over all runs; — — — , November Iceland; – – – – –, February North Sea.

In order to follow seasonal variations closely, the intervals between catches from the same ground have to be much smaller than in the present experiment. Jason & Lees (1971) have shown that there is a seasonal cyclical variation for TFM and sensory scores. This cycle, which presumably is a reflection of biological variations, is not necessarily repeated at the identical calendar time in successive years. Thus the method

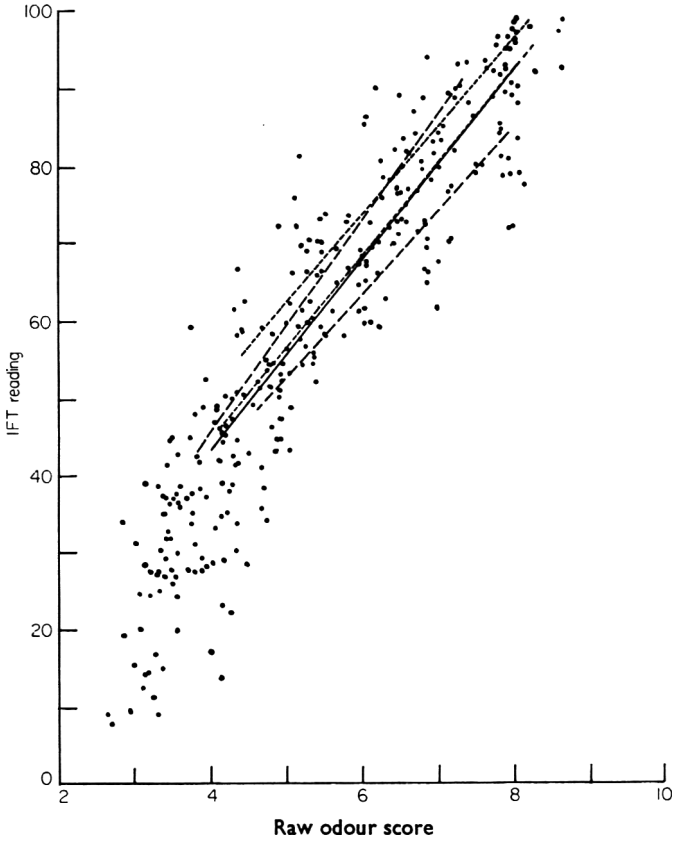

FIG. 6. Relationship between Intelectron Fish Tester V readings and raw odour scores. Box means and calibration lines. ———, Pooled over all runs; — — —, November Iceland; ------, February North Sea.

of chronological sampling described in this paper does not ensure obtaining fish in the same biological condition in different years.

To test the association between non-sensory and sensory tests (GA: general appearance; RO: raw odour; CO: cooked odour; CF: cooked flavour) pooled within-box correlation coefficients were calculated (Table 8). The highest association is between HXI and cooked assessments. The instruments are associated mainly with GA, indicating the importance of the state of the surface on instrument readings.

In Table 9 linear calibration parameters are given in the form (reading or index) $= a + bx$ (sensory score). They were obtained from the linear regression of each test on age-in-ice; the values for the non-sensory tests relate to all ages (TMI from 7 days), those for sensory tests for ages when cooked assessment was carried out. Calibration lines with RO are shown in Figs 5 and 8. The regressions of RO, CO and CF were sufficiently close for a single calibration relation to be used for all three tests. Table 10

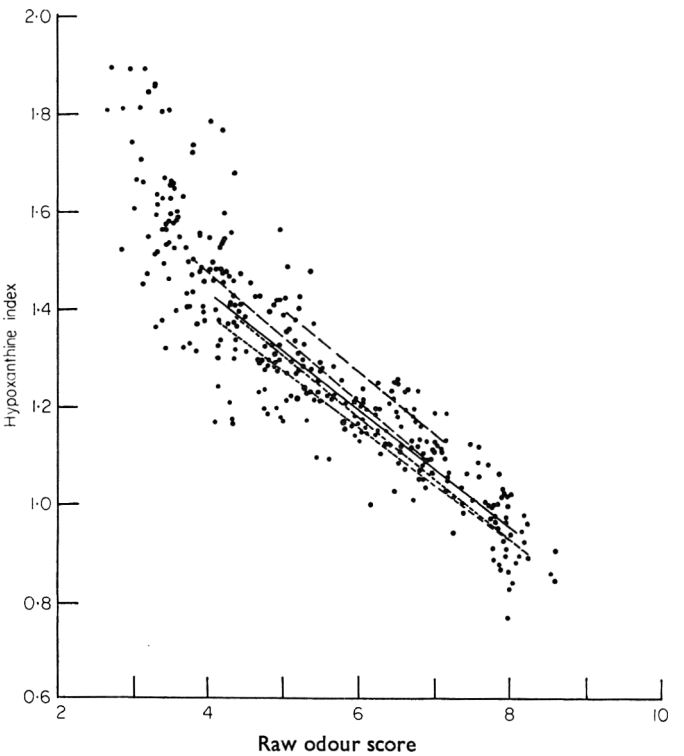

FIG. 7. Relationships between hypoxanthine index and raw odour scores. Box means and calibration lines. ———, Pooled over all runs; — — —, November Iceland; ------ February North Sea.

lists calibration relations between all tests in the range of linearity. HXI and TMI values have also been re-converted to concentrations (HXC and TMC).

To test whether the different keeping times found in the different runs reflect a true difference in spoilage rates measured by all tests, correlation coefficients were calculated for the keeping times obtained from the different tests (Table 11) and most were found to be significant. GA and TFM which measure mainly surface effects have the lowest correlation with other tests. The non-sensory tests show higher correlations with cooked than with raw assessments.

Calculations similar to those giving rise to Table 7 have been carried out to determine the range of sensory scores corresponding to a given instrument reading or chemical index in different runs. If the two tests being compared are similarly affected by any ground or seasonal variation, the range will be considerably less than when the true age-in-ice is considered. In the ideal case the correspondence would be perfect and a

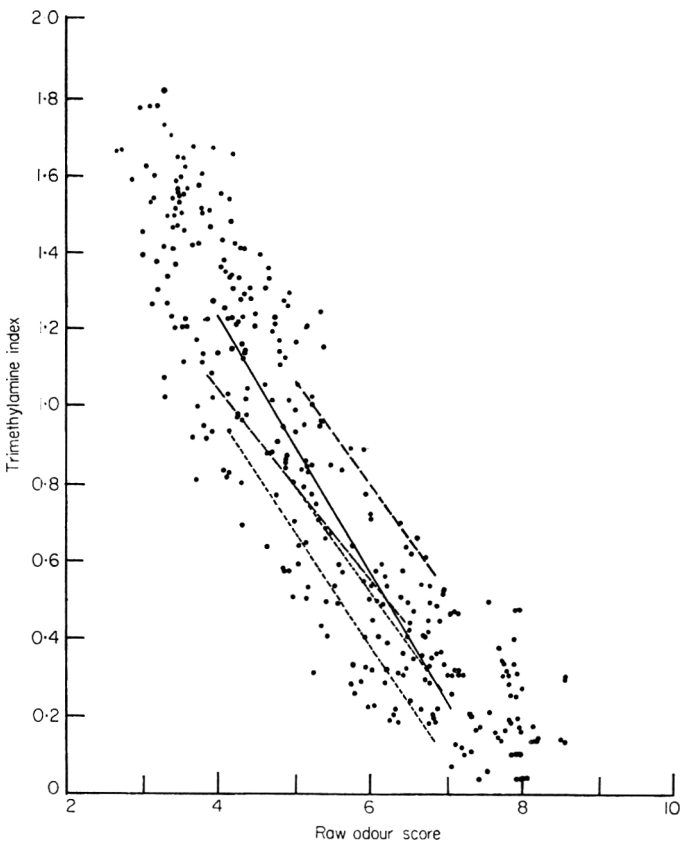

FIG. 8. Relationship between trimethylamine index and raw odour scores. Box means and calibration lines. ———, Pooled over all runs; — — —, November Iceland; ------, February North Sea.

given reading or index would always correspond to the same sensory score, whatever ground or season is chosen.

The ranges found have been converted from sensory scores to equivalent days-in-ice and are shown in Table 12. In most cases there is a reduction from the values for true age-in-ice but considerable variations remain.

The ranges of keeping times determined by the non-sensory tests (Table 7) are greater than the ranges determined by the sensory tests (2·5 to 3·4 days). A given

reading or index will therefore correspond to a range of sensory scores even for perfect correlation; in a run with a low spoilage rate a given reading or index will be associated with a lower sensory score than in a run with a high spoilage rate. The present results show a general trend in this direction. Calibration lines are shown in Figs 5–8 for the runs with the highest and lowest spoilage. The closest relationship between corresponding runs in successive years was found for HXI and CF, where the maximum difference was less than 1 equivalent day-in-ice.

Shewan & Ehrenberg (1957) found no consistent relationship between TMI and sensory score. The relationship between trimethylamine concentration and sensory assessment of cod has also been studied by Hoogland (1958), Castell, Elson & Giles (1961) and Antonacopoulos (1971) and between the Intelectron Fish Tester V and sensory assessment by Wittfogel & Schlegel (1971), but their different methods of sensory assessment preclude a direct comparison with the present results.

Conclusion

For iced boxed fish stored for 4 to 20 days (7 to 20 for trimethylamine index) linear relationships with age-in-ice exist for all four tests. Differences between fish from different grounds at different seasons are considerable but can be reduced, though not eliminated, by using separate relationships for each ground and season combination.

Conversion of results from non-sensory methods to equivalent sensory scores is possible on the basis of average relationships and some allowance can be made for ground and season. However, each test is best used in its own right.

The instruments require larger sample numbers than the chemical tests for the same precision and are sensitive to the position of the fish in the box. On the other hand, they are non-destructive and even allowing for the larger sample numbers are faster to apply and simpler to operate.

Of the two instruments, the Torry Fish Freshness Meter requires larger sample numbers but is faster and more convenient to operate than the Intelectron Fish Tester V. Hypoxanthine is more closely correlated with sensoy scores but requires larger sample numbers, though smaller weights of fish, than trimethylamine. It is also useful over a wider range of storage time. When results on individual fish are not required, it is possible to reduce the analytical labour for both chemical methods by the use of a single extract from a composite of the individual samples.

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Thermodynamics of water vapour sorption by sugar beet root

HÉCTOR A. IGLESIAS, JORGE CHIRIFE AND PASCUAL VIOLLAZ

Summary

Previously obtained water sorption isotherms were used for the calculation of thermodynamic functions of water vapour sorption in sugar beet root and its main components, namely the water insoluble fraction and sucrose. Integral molar enthalpy, entropy and free energy were calculated as a function of moisture content and several hypotheses were advanced in order to explain the values observed. These hypotheses were mainly concerned with configurational modifications of the adsorbents during the course of sorption.

Introduction

Water sorption isotherms in foods are of great importance in several aspects of food preservation by dehydration. There is a well established relationship between water sorption isotherms and chemical, physical and stability characteristics of dehydrated food products (Loncin, Bimbenet & Lenges, 1968; Labuza, Tannebaum & Karel, 1970; Karel, 1973). The determination of equilibrium moisture contents for dehydrated foods also provides valuable information on the thermodynamics of water sorption, since from existing theories, thermodynamic functions can be readily calculated from the measured sorption isotherms. Thermodynamic functions allow for the theoretical interpretation of experimental results in accordance with the statements of theory. For instance, the free energy required for the transference of a water molecule from the vapour state to the solid is a quantitative measurement of dry food/water affinity. The variation of entropy might be related to the order/disorder concept, which is useful for the interpretation of such processes as dissolution, crystallization and swelling, which usually occur during water sorption by food products (McLaren & Rowen, 1951; Berlin, Anderson & Pallansch 1968; Karel, 1973, Iglesias, Chirife & Lombardi, 1975b,c). Finally, the variation of enthalpy indicates up to which level the interaction water/substrate is greater than the interaction of water molecules.

In previous articles (Iglesias *et al.*, 1975b,c), the water sorption isotherms of sugar beet root and its main components were reported. Sorption isotherms of sugar beet

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root, its water insoluble fraction and amorphous sucrose, were determined at several temperatures and an attempt was made to describe the experimental data using some existing theories on physical adsorption. It is the purpose of the present work to calculate thermodynamic functions of water vapour sorption on sugar beet root and its main components from the already reported sorption isotherms.

Results and discussion

In order to calculate thermodynamic functions the spreading pressure must be first evaluated. Its calculation has been thoroughly described by Boyd & Livingston (1942) and it will not be discussed here. The final expression for the spreading pressure, π, is:

$$\pi = \frac{kT}{A_m} \int_0^{a_w} \frac{\theta}{a_w} da_w \qquad (1)$$

where k = Boltzmann's constant, T = absolute temperature, A_m = area of water molecule, $\theta = X/X_m$, X = moisture content, per cent dry basis, X_m = moisture content value at the monolayer same units as X and a_w = water activity = p/p_0.

The evaluation of the area of water molecule, A_m, requires the knowledge of the density of the sorbed water. In view of the uncertainties about the physical state of the sorbed water, it is usually assumed that its density is that of liquid water (Gregg & Sing, 1967), and A_m is calculated from

$$A_m\ (Å^2) = 1{\cdot}09\ (M/\rho N)^{2/3}.\ 10^6 \qquad (2)$$

where M = molecular weight, N = Avogadro's number and ρ = density of the liquid water.

However, Gur-Arieh, Nelson & Steinberg (1967) measured the density of water sorbed in low-protein flour and found that in the monolayer region, the density of the sorbed water was greater than that of the liquid one. Considering that the density value reported by Gur-Arieh *et al.* (1967) might be more representative of reality than that of the liquid water (Ngoddy & Bakker-Arkema, 1972) we adopted the former for the calculation of the area of the sorbed water molecule. In order to calculate the integral shown in Eqn (1) Hill, Emment & Joyner's (1951) procedure was followed. The integral was evaluated by Simpson's method taking as lower limit $a_w = 0{\cdot}05$; the value of the integral between $a_w = 0$ and $a_w = 0{\cdot}05$ was obtained assuming a linear relationship between θ and a_w (Henry's law). The values for the spreading pressure for sugar beet root, its water insoluble fraction, and amorphous sucrose are shown on Figs 1, 2 and 3.

As mentioned, the evaluation of the spreading pressure included the graphical integration of Eqn (1). However, if an analytical expression were known for the sorption isotherm—that is a relationship between θ and a_w—it might be possible to solve the above integral analytically. This is attempted in the following way. Recently, Iglesias *et al.* (1975a,b) showed that a multilayer adsorption equation, originally developed by

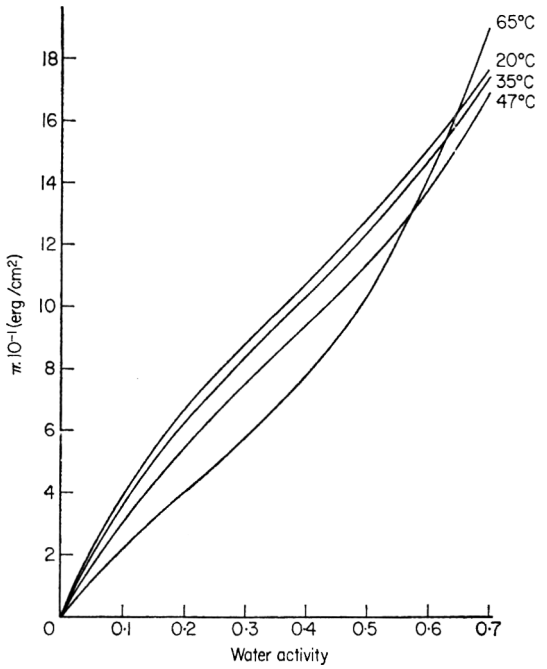

FIG. 1. π isotherms of sugar beet root at different temperatures.

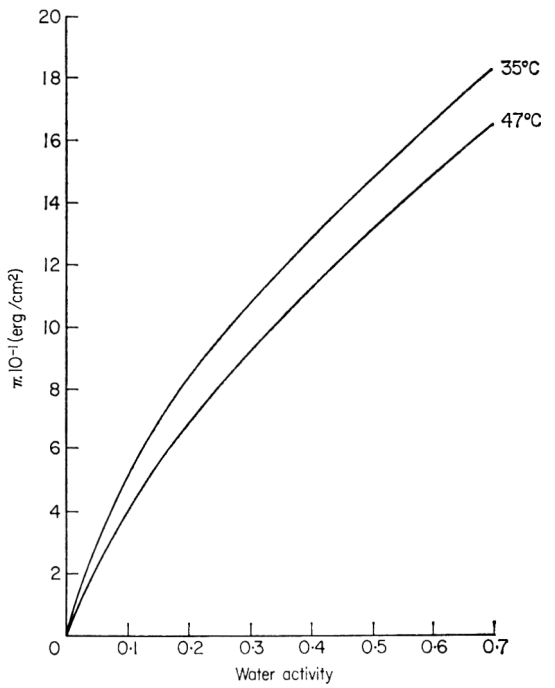

FIG. 2. π isotherms of the water insoluble fraction of sugar beet root at 35 and 47°C.

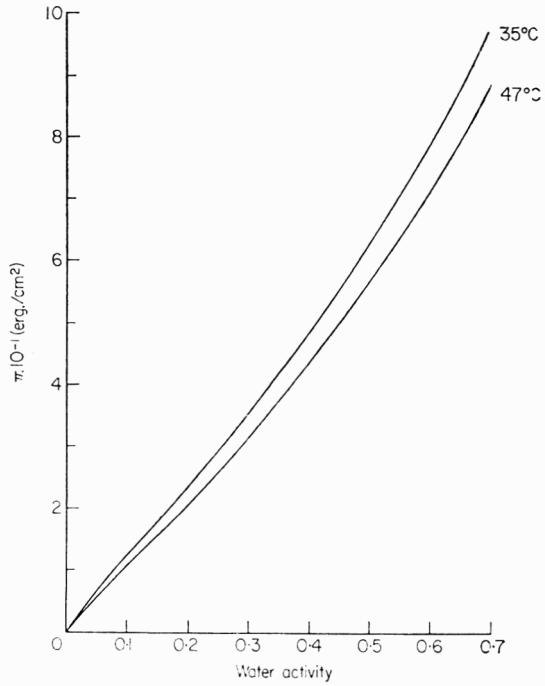

FIG. 3. π isotherms of amorphous sucrose at 35 and 47°C.

Halsey (1948), could be used to describe the water sorption behaviour of sugar beet root as well as a great variety of food materials. Halsey's equation is

$$p/p_0 = \exp\ (-a/RT\ \theta^r) \tag{3}$$

where a, r = parameters.

Equation (3) may be put in the form

$$\ln \ln\ (1/a_w) = -r \ln \theta + \ln\ (a/RT) \tag{4}$$

defining

$$\ln\ (1/a_w) = z;\ a' = a/RT$$

then

$$\ln z - \ln a' = -r \ln \theta$$

and

$$\ln \theta = \frac{\ln\ (z/a')}{-r}$$

defining

$$1/(-r) = b$$

then

$$b.\ \ln\ (z/a') = \ln \theta$$

and

$$\theta = (z/a')^b$$

Therefore

$$\int_{a_{w_0}}^{a_w} \frac{\theta}{a_w} da_w = \int_{a_{w_0}}^{a_w} \frac{(z/a')^b}{a_w} da_w$$

$$= (1/a')^b \int_{a_{w_0}}^{a_w} \frac{(z)^b}{a_w} da_w = (1/a')^b \int_{a_{w_0}}^{a_w} \frac{[\ln (1/a_w))^b}{a_w} da_w$$

integrating

$$\int_{a_{w_0}}^{a_w} \frac{\theta}{a_w} da_w = (1/a')^{-1/r} \int_{a_{w_0}}^{a_w} \frac{da_w}{(-\ln a_w)^{1/r}. a_w}$$

$$= (a')^{1/r} \left[\frac{1}{\left(\frac{1}{r} - 1\right) (-\ln a_w)^{1/r-1}} \right]_{a_{w_0}}^{a_w}$$

Substituting in Eqn (1)

$$\pi = \frac{kT}{A_m} (a')^{1/r} \left[\frac{1}{\left(\frac{1}{r} - 1\right) (-\ln a_w)^{1/r-1}} \right]_{a_{w_0}}^{a_w} \tag{5}$$

Equation (5) was calculated using computation techniques; values of the parameters r and a' were those previously reported (Iglesias *et al.*, 1975b). The lower limit of

TABLE 1. Comparison of the graphical and analytical methods for the evaluation of the spreading pressure
Sugar beet root

Water activity	20°C		35°C	
	π_A	π_B	π_A	π_B
0·05	22·38	22·38	20·45	20·45
0·10	40·09	40·72	37·18	37·50
0·15	54·47	54·37	51·24	50·37
0·20	66·86	66·05	63·51	61·53
0·25	77·90	76·73	74·44	71·84
0·30	88·12	86·89	84·73	81·75
0·35	97·88	96·82	94·57	91·52
0·40	107·54	106·74	104·20	101·38
0·45	117·34	116·84	113·87	111·51
0·50	127·52	127·30	123·87	122·09
0·55	138·48	138·30	134·50	133·34
0·60	150·54	150·09	146·13	145·51
0·65	163·88	162·95	159·30	158·94
0·70	178·28	177·32	175·06	174·13

π_A, spreading pressures calculated graphically, erg/cm^2; π_B, spreading pressures calculated analytically (Eqn (5)), erg/cm^2.

Eqn (5) was also taken as $a_w = 0{\cdot}05$, so to the computed value was added the value corresponding to the interval, $a_w = 0$ to $a_w = 0{\cdot}05$, which was calculated assuming a lineal relationship (Henry's law) between θ and a_w.

Table 1 shows the values of the spreading pressure for sugar beet root obtained by both the graphical and the analytical methods. The results show conclusively that the use of Halsey's equation leads to accurate results in the calculation of spreading pressures with the method here developed.

The thermodynamic analysis of water sorption in amorphous sucrose deserves a special comment. The water sorption isotherms of sucrose reported earlier (Iglesias *et al.* 1975c) represent only 'apparent' equilibrium moisture content values (Makower & Dye, 1956) because of the phase transformations of sucrose. It is clear that if these sorption values are not true equilibrium ones, the laws of classical thermodynamics are no longer applicable. However, we considered that it may be interesting to see the nature of the thermodynamic functions so obtained. The integral molar enthalpy was calculated in each case from the temperature dependence of the π isotherms,

$$\Delta H = H_L' - H_S = RT^2 \, (\partial \ln a_w / \partial T)\pi \qquad (6)$$

where H_L' = integral molar enthalpy of the liquid state at the reference temperature T and H_S = integral molar enthalpy of the sorbed state.

A least squares analysis was performed to obtain $(\partial \ln a_w/\partial T)\pi$ as the slope of the straight line representing $\ln a_w$ vs. T at constant π. The reference temperature used throughout this work in the calculation of the thermodynamic functions is 35°C. Figure 4 shows the integral molar enthalpy plotted as a function of moisture content for sugar beet root, its water insoluble fraction and amorphous sucrose. In the same figure, and for the purpose of comparison, are plotted the isosteric heat curves which were previously obtained (Iglesias, 1974; Iglesias *et al.* 1975b). It can be seen that the isosteric heat is always greater than the integral molar enthalpy change, as it might be expected from a theoretical point of view (Hill *et al.*, 1951; Ross & Olivier, 1964). In the case of amorphous sucrose, the total enthalpy decreases as the moisture content increases until 6% of moisture content is reached, and then it remains practically constant; the same behaviour is observed with the isosteric heat. It seems as if the water already sorbed could have given certain mobility to the amorphous structure, generating new active sites for sorption.

It must be pointed out that the variation of enthalpy as it was defined, $H_L' - H_S$, has a positive value in spite of the fact that, adsorption being an exothermic process, the enthalpy change must be negative. Nevertheless, traditionally it has been done in this way (Hill, 1949; Hill *et al.*, 1951; Hill, 1952) and consequently the change of enthalpy has the same sign as that given to the isosteric heat.

It is worth noticing, that for the calculation of the change of integral molar enthalpy it is not necessary to define a standard state because enthalpy is only a function of temperature. This is not the case for entropy, which is a function of temperature and

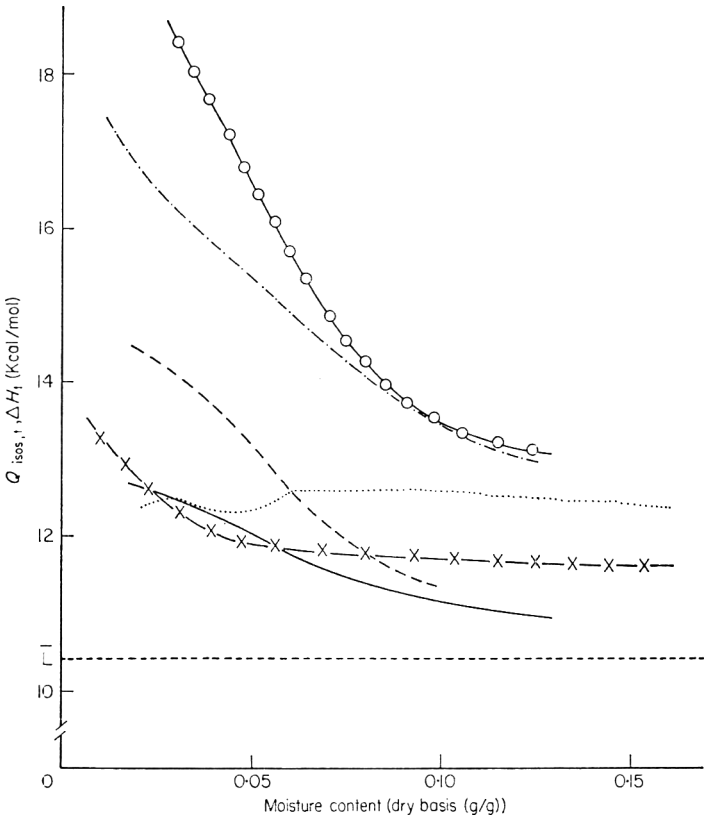

FIG. 4. Variation of the total integral molar enthalpy and isosteric heat as a function of moisture content. Sugar beet root: $Q_{isos,t}$, – – –; ΔH_t, —·W_Q insoluble fraction: $Q_{isos,t}$, ○—○; ΔH_t, —·—·—·Sucrose: $Q_{isos,t}$, · · · ·; ΔH_t, ×—×.

pressure. Consequently, the Gibb's molar free energy is generally related to a standard state which is usually water vapour at 760 mm of mercury and at the temperature at which the variation occurs. Therefore, the variation of molar free energy in an isothermal expansion of water vapour from 760 mm of pressure to the equilibrium pressure, p, is given by,

$$\Delta G_T = \int_{760}^{p} v.\ \mathrm{d}p.$$

where v = molar volume.

If an ideal gas behaviour is supposed,

$$\Delta G_T = RT \ln (p/760).$$

Analogously, the molar free energy change from 760 mm of pressure to p_0 (vapour pressure of pure water at temperature T) is given by

$$\Delta G_T = RT \ln (p_0/760).$$

Therefore, the net free energy change at temperature T, is

$$\Delta G_{n,T} = RT \ln (p/p_0) = RT \ln a_w$$

and, since

$$\Delta G = \Delta H - T.\ \Delta S$$

then,

$$\Delta S = \Delta H/T - R \ln a_w$$

therefore,

$$S_S - S_L' = -\frac{H_L' - H_S}{T} - R \ln a_w = \frac{H_S - H_L'}{T} - R \ln a_w$$

where S_L' = molar integral entropy of the liquid at temperature T and pressure p_0 (Young & Crowell, 1962) and S_S = integral molar entropy of sorbed state.

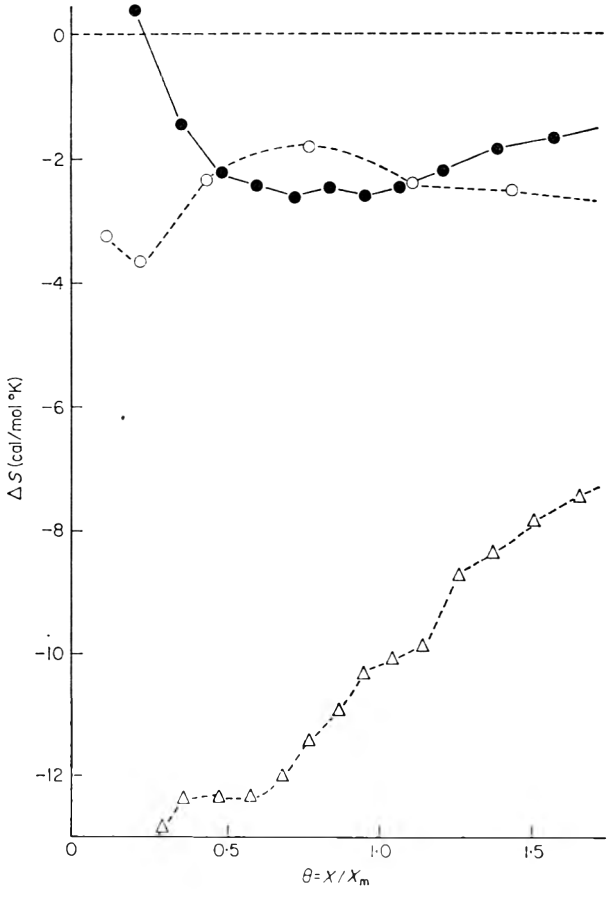

FIG. 5. Variation of the integral molar entropy with coverage (as number of monolayers). ●--●, sugar beet root; ○--○, sucrose; △--△, water insoluble fraction.

Figure 5 shows the integral molar entropy change plotted as a function of coverage expressed as numbers of monolayers. The analysis of the variation of the integral molar entropy with coverage suggests several hypotheses. With respect to the water insoluble fraction of the sugar beet root it can be seen that the values of $-\Delta S$ are very high, and although they continually decrease, the function never takes positive values. As it has been stated by Pauling (1945) and Cassie (1948), this behaviour may be attributed to the existence of chemical adsorption. The fact that water is so strongly adsorbed can not be explained assuming that it is an 'ice-like' state, because the entropy of freezing is only 6 cal/mol °K at 30°C (McLaren & Rowen, 1951). Another possible explanation is assuming that the change in the integral molar entropy is taking into account some structural modifications of the adsorbent. The water insoluble fraction of sugar beet root is mainly composed of the polysaccharides which constitute the cell wall, that is, cellulose, hemicellulose and pectic substances. Shimazu & Sterling (1961) measured the crystallinity of dehydrated cellulose (containing some hemicellulose) and calcium pectinate and found a considerable percentage of amorphous regions, which amount to about 75% for calcium pectinate and 55% for cellulose. Consequently, it may be stated that the dried water insoluble fraction of sugar beet root also contains a significant percentage of amorphous regions. This allows us to assume that during the adsorption process, some of the amorphous regions undergo a crystallization giving a change in the integral molar entropy which will increase the change of entropy already taking place due to the adsorption process by itself (Bettelheim & Volman, 1957). Experimental evidence which may support this hypotheses is the fact that once the adsorption-desorption cycle was concluded and a second one was performed, the moisture content values obtained were less than those corresponding to the first run (Iglesias *et al.*, 1975b). This may indicate a decrease in the number of active sites, that is to say, a decrease of the amorphous regions (Shimazu & Sterling, 1961). It is difficult to give an explanation of the apparent step changes in the entropy versus coverage as it is shown in Fig. 5. Perhaps, a structural study (i.e. X-ray diffraction) of the adsorbent during the course of sorption may throw some light on the phenomenon. With respect to the amorphous sucrose, the ΔS, curve shows a first section where the integral molar entropy increases as the moisture content decreases. This section corresponds to a range of water activity which goes from, $a_w = 0$ to $a_w = 0{\cdot}10$ and where no crystallization was previously observed (Iglesias *et al.*, 1975c). This behaviour is unexpected because the adsorption process should theoretically show a decrease in the entropy function as less active sites are being occupied. Nevertheslss, it must be recognized that adsorption is taking place on a surface which does exhibit configurational modifications as local solubility or incipient solution. These modifications may result in an increase of entropy. It may be concluded that what is observed is the result of a decrease of entropy as the moisture content increases due to the adsorption process itself, and an increase of entropy due to solubility. Once the monolayer is formed, the variation of integral molar entropy is practically constant.

In the case of sugar beet root, it is observed that the entropy change decreases as the moisture content increases up to the monolayer value. This first section of the curve shows a balance of the effects attributed to the water insoluble fraction and the sucrose. Once the monolayer is completed, there is an increase in the entropy function which resembles that which is observed for the water insoluble fraction. This effect maybe

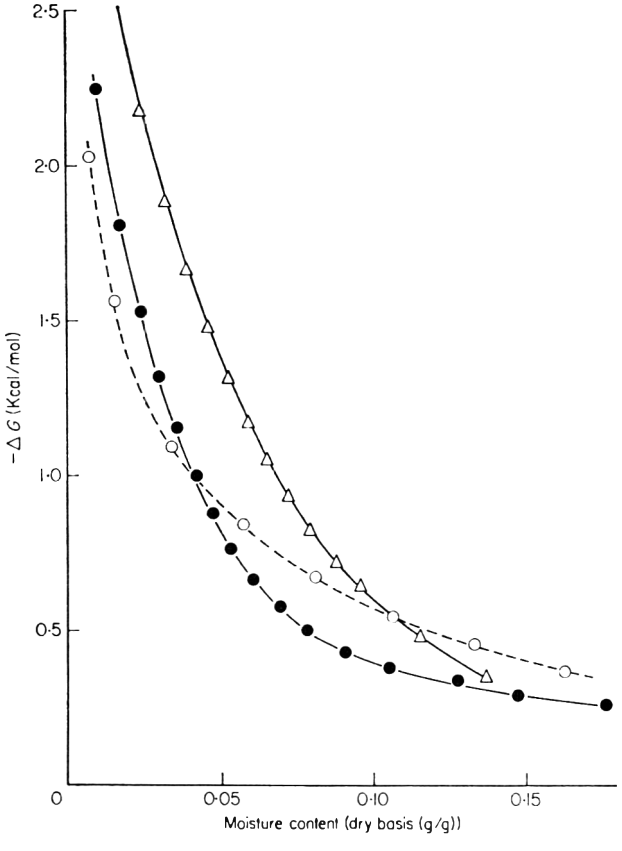

FIG. 6. Variation of the integral molar free energy as a function of moisture content. ●——●. Sugar beet root; △——△, water insoluble fraction; ○- -○, sucrose.

attributed to a mixing process of all the constituents of the sugar beet root, because in this range of water activity a greater mobility of the molecules might be expected.

Figure 6 shows the change in the integral molar free energy as a function of moisture content for sugar beet root, water insoluble fraction and amorphous sucrose. In each case the integral molar free energy decreases gradually with the increase of sorbed water. These curves also show the contribution of each constituent of the sugar beet root to the entire process.

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Book Reviews

Plants Consumed by Man. By B. Brok.
London: Academic Press, 1975. Pp. ix + 479. £14.80.

The aim of this book is to provide a reasonably comprehensive survey of plants used for human consumption; over 300 plants are described. They include primary food plants as well as those which yield non-nutritive ingredients such as flavours, colours, thickening agents and the bacteria and fungi which produce edible materials or which are themselves consumed. Plants used for smoking and chewing as well as for preparation of alcoholic and alkaloidal drinks are also included. The plants are defined in terms of the strict botanical nomenclature of their edible parts and also for convenience they are grouped according to common usage, i.e. as cereals, vegetables, fruits or nuts, etc. A brief history of each plant is given with a note of the important factors regarding cultivation and suitable varieties for processing. There are numerous excellent line drawings of the edible parts. The book is intended for use by agriculturists, food scientists, botanists, in particular by students of these sciences and by all who wish to acquire knowledge of food plants.

There are no specific references in the text, but an appendix lists over 100 books and other literature sources, though virtually none to recent reviews or papers. The importance of, for example, cereals, vegetables and fruits as foods is stressed and detailed information on their chemical composition in terms of proteins, fats, carbohydrates and vitamins is given as tables, but, surprisingly, there is no reference to the important book by McCance & Widdowson, *Composition of Foods*.

The inclusion of beverage plants, 'fumitories and masticatories', fermentative microorganisms, cellulose ethers and esters, etc. may detract somewhat from the main emphasis of the work. The fact that certain microorganisms contribute to foodstuffs in the form of their metabolic products but are not themselves consumed except in very small amounts may not have been made sufficiently clear. A serious fault lies in the errors in the chemical components of plants on which their use as flavours depends and which are now assuming increasing importance in taxonomy. This is understandable, because errors in the literature have been copied and re-copied by several authors, but will prove misleading to students and non-specialist readers. The statements on the nature and origin of the lachrymatory substance of onion are completely wrong, the corresponding information on garlic has minor errors, the enzymic reaction leading to a mustard oil in horseradish is reversed and there is no attempt to distinguish between the principal mustard oils of that plant and that of black mustard on the one hand and of white mustard on the other, which are important in determining the properties of the mustard products of commerce.

The book may fail by falling between the two extremes of individual readers, who

may be deterred by its high price, and libraries, which already possess or have access to the authoritative sources listed in the appendix, but it will serve a useful purpose if it directs the attention of readers to those sources and to specialized reviews. There is an index of scientific and common plant names, which does not, however, include plant components.

G. G. FREEMAN

Lactic Acid Bacteria in Beverages and Food. Ed. by J. G. CARR, C. V. CUTTING and G. C. WHITING.
London, New York and San Francisco: Academic Press, 1975. Pp. xv + 415. £10.80.

This book is the proceedings of a Symposium held at Long Ashton Research Station, University of Bristol, in 1973. It is the only comprehensive review available of this important group of organisms in beverages and food and will be of interest to all scientists and technologists concerned with these products. The title of the book is somewhat misleading as the emphasis has been placed on the lactobacilli, to a less extent on *Leuconostoc* and *Pediococcus*, with little information being provided concerning the important streptococci which are also generally included as 'lactic acid bacteria'. At a time when all organisms tend to be considered as harmful and therefore need to be eliminated from food, this book serves to remind us of the important role the lactic acid bacteria play in the fermentation, preservation and flavour enhancement of many foods.

The book is divided into six sections. The first two sections are mainly concerned with beverages and in particular the metabolism of organic acids and the significance of the malo-lactic fermentation in wines and other alcoholic beverages. The next three sections contain chapters on fish, meat and dairy products, the fermentation of sauerkraut, brined cucumbers, olives, soy sauce, sour-dough bread and Parisian barm. In many of these products particular types of lactobacilli are an essential part of a fermentation process whilst others are mainly involved in spoilage. The various stages of each process are discussed and often serve to emphasize how little is known about traditional processes some of which have been in use for thousands of years.

The last section of the book concerns the classification and identification of these organisms. The present classification is based mainly on those lactobacilli which are important in dairying and the evidence supplied throughout the book emphasizes that different types of lactobacilli occur in different habitats. Many of these organisms cannot be related to existing species. As more detailed studies are made in order to understand and improve these fermented products, further research will be needed concerning the nature of the organisms themselves. Anyone embarking on such a project would be well advised to make a careful study of the information contained in this book.

ELLA M. BARNES

Bread. Ed. by A. SPICER.
London: Applied Science Publishers, 1975. Pp. x + 358. £14.00.

Shortly before his death the late Lord Rank set up the Rank Prize funds to encourage research and make awards in a number of fields including human and animal nutrition. The first symposium, organized by Professor Arnold Spicer, was on the social, nutritional and agricultural aspects of wheaten bread and was attended by a selected audience of international standing. It was held at Selsdon Park Hotel, Croydon in the autumn of 1974, when twenty three papers were presented and this book is a record of them and the discussions which took place.

It is divided into seven sections which follow precisely the order of the various sections, prefaced by a brief history of wheat and bread and written, as is befitting, by the organizer. The first section then deals with fundamentals, the sociology of bread, the origins of wheat, the structure and biochemistry of the wheat grain and the crop, the environment and the genotype.

Quality aspects are then considered, the nutritive value of wheat proteins, milling and baking quality, cultural, genetic and other factors affecting wheat quality followed by a paper on the structural aspects of wheat starch, containing a number of most informative photographs taken on the scanning electron microscope of starch granules in wheat and bread.

Wheat yields are then discussed, the limitations to productivity by diseases and pests in the field being dealt with (a) in North America and (b) in Europe. Pre-harvest and post-harvest losses are then considered, followed by some effects on the yield and quality of English wheat as affected by crop nutrition and soil fertility, this section closing on a consideration of plant diseases and nutritive value.

There then follow two papers on extraction rates, the first technical and discussing milling processing implications and the second the nutritional implications of such techniques.

The next section under the heading of Improvement deals with the fortification of white flour by added nutrients followed by advances in breadmaking technology. It is a pity that a little more time was not spent on the question of flour and bread improvers, one of the things dear to the consuming public when considering its attitude to the nutritional content, quality and acceptability of wheaten bread. This subject and the actual baking of bread, equally of public concern where technology has also much changed in recent years, are unfortunately skimped in an otherwise authoritative and informative publication, *Wheat and Bread*, for there is so much more information on wheat that it is fair to say the title is misleading, and that just given more appropriate.

The book then concludes with discussions on wheat agronomy in North America, the Netherlands, Australia and India and finally a summing up and last discussion. In this emerged the idea of a 'Selsdon Wheat' on which a specification was prepared stating the necessary properties wheat should have to provide the right kind of British bread so as to assist the wheat breeders and farmers of the future. What a fitting tribute

this is to the late Lord Rank, particularly in the foresight and planning which has given us this memorable volume. It should find a place on the bookshelf of all those institutions which are interested in improving the yields and quality of the wheats required to give the world its daily bread. Nor should it elude students studying food science and technology for it provides an excellent blue print of the spectrum of disciplines involved in the growth of primary products and their conversion into an acceptable, digestible and nutritionally valuable food. However, the cost is such that it will only be consulted in libraries, a matter becoming of very great concern to those who realise still yet another aspect of the effects of inflation on the student population and not necessarily only on undergraduates. Perhaps this is something for the administrators of the Rank Prize funds seriously to consider?

J. B. M. Coppock

Freeze Drying and Advanced Food Technology. Ed. by S. A. Goldblith, L. Rey and W. W. Rothmayr.
London: Academic Press, 1975. Pp. xxxi + 730. £17.00.

This book contains forty-one papers presented at the 'International Course on Freeze Drying and Advanced Food Technology' held in June 1973 under the joint chairmanship of the editors. The papers, each with a chapter number, are divided into nine sections; the first two dealing with the freezing process, the next four dealing with freeze drying followed by two sections on concentration and a final one on intermediate moisture foods.

Two papers are presented in French, the remainder being in English. A useful subject index is included at the end of the book.

Eleven papers are presented on freezing and its applications and these include a review on the crystallization of water, modern methods of freezing, mainly direct refrigerant contact techniques, and specific applications to vegetables, fruit juices and biological materials. The bulk of the papers, in the book, are contained in the sections on freeze drying, eighteen in all, and these cover the basic heat and mass transfer problems, industrial equipment and freeze drying of coffee, marine and meat products as well as pharmaceutical and biological products. The concentration techniques discussed include freeze concentration, membrane processing and gel filtration and the product applications covered are vegetable juices, whey and skim milk and enzymes. The final four chapters deal with low and intermediate moisture foods and their safety and stability. The last chapter deals with the principles and application of radio frequency energy to food preservation with particular reference to concentration and dehydration.

Food technologists wishing to keep abreast of the literature of recent developments

will find this book stimulating and rewarding. Many of the chapters consist of excellent reviews in which the principles and applications are clearly presented. There is a good balance between theoretical discussion of basic mechanisms and practical applications of the techniques to food as well as other products. Most of the papers have a good list of references covering the individual topics adequately; some are, however, limited and omit key references whilst others have no references at all. The last category includes some of the freezing papers and one on heat and mass transfer on freeze drying. Although the accounts of freezing techniques are adequate in themselves, the omission of references must be regarded as a serious shortcoming in a work of this nature.

There is inevitably some overlap with other recent publications, for example, the discussion of aroma recovery has been dealt with in *Advances in Food Research* (1973), freeze drying fundamentals and also intermediate moisture foods in *Critical Reviews of Food Technology* (1971 and 1973 respectively) and freeze concentration and membrane processes in the proceedings of the symposium on 'Advances in preconcentration and dehydration of foods' (1974). Despite the overlap there is much useful information on many other topics, especially the practical applications of the techniques.

In general the book can be recommended to all those who are concerned with food processing in industry as well as students and university workers. The editors and publishers are to be congratulated on the high standard of publication.

S. D. HOLDSWORTH

Meat. Proceedings of the Twenty-first Easter School in Agricultural Science, University of Nottingham, 1974. Ed. by D. J. A. COLE and R. A. LAWRIE. London: Butterworths, 1975. Pp. II + 596. £14.50.

Meat as a subject of science is still very new, even among the food sciences. In the standard works on food composition and analysis of twenty-five years ago the proximate composition of meat was discussed and applied, but its other properties enjoyed hardly a mention, let alone a chapter. Those twenty-five years have seen such an enormous growth in knowledge of the subject that the writers of books have only just begun to catch up with it. There is now an acute need for a good comprehensive textbook to cover and consolidate this ground for the ordinary practitioner and student of meat science and technology, a need which has not yet been satisfactorily met by any one of the several books which have appeared in English in recent years. Against this clear need a book which presumes to bear the bold title of *Meat* must be judged, and it is a joy to report that it appears to be the best attempt yet.

As its sub-title makes clear, the book is, like so many others, a compendium of the papers presented by specialist speakers at a particular meeting. Authorship in this form is inherently likely to lack the continuity of thought and the unified style which

comes from a single author or small group. In this case the editors are to be congratulated on their success in minimizing this problem. Though there are differences in level and approach among their authors, the general homogeneity of the complete work is above average.

The book commences with an introduction on 'The meat-eating habit in man', then five chapters on 'Meat production', covering the growth, breeding, nutrition and disease and post mortem changes, carcass grading and cutting, processed meat and microbiological questions. Then four chapters on 'Composition of meat' cover variability, physico-chemical parameters, analytical and legal aspects: here and in the previous section the emphasis is placed directly on practice in the UK. 'Eating quality' comprises chapters on water holding capacity, tenderness, flavour, colour and the cooking process and 'Nutritional aspects' includes a general survey, a chapter on meat lipids and one on its effects in the human alimentary tract. Finally a section entitled 'Future prospects' deals with conventional meat animals, other animals especially the water buffalo, meat analogues and methods of assessment of alternatives.

Each topic is treated by an acknowledged expert but is clearly written for the competent workman in that field or the other fields adjacent. Each covers the general state of knowledge in his area, with apposite references to original work, and each has resisted any temptation to deal otherwise than modestly and in context with his own significant contributions and opinions. There is a good index. A reviewer can certainly point to small exceptions and deficiencies, measured against his own interests, but they detract little from an excellent book to be recommended to scientists and technologists and all serious students of meat. The printing is clear, the binding is good and in a couple of years time we will probably regard the price as very reasonable.

M. D. Ranken

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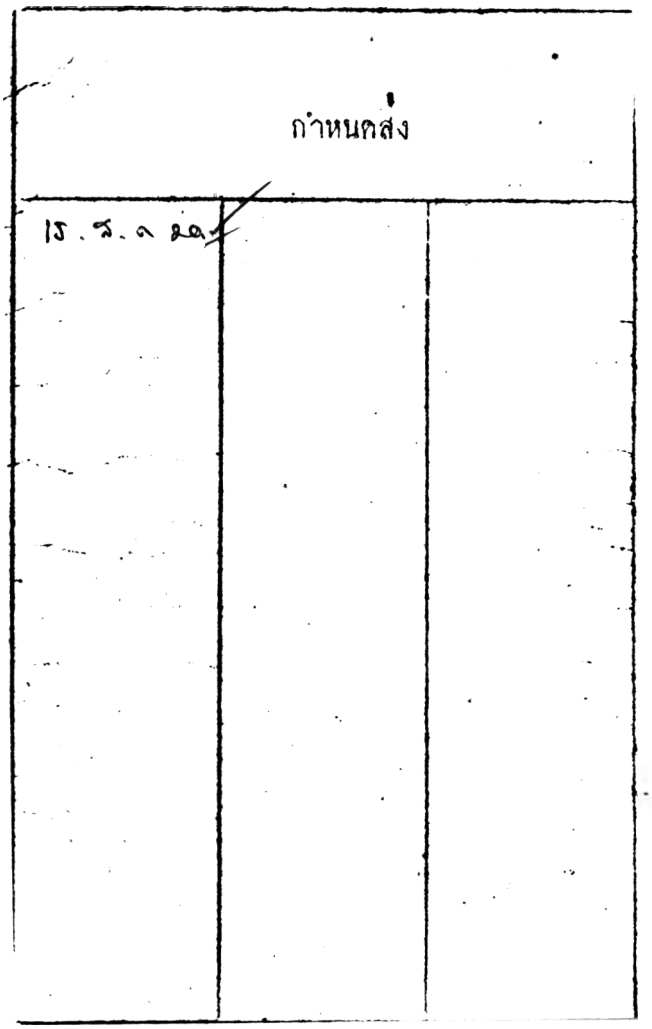
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Arrangement. Papers should normally be divided into: (a) Summary, brief, self-contained and embodying the main conclusions; (b) Introduction; (c) Materials and methods; (d) Results, as concise as possible (both tables and figures illustrating the same data will rarely be permitted); (e) Discussion and conclusions; (f) Acknowledgments; (g) References.

References. Only papers closely related to the author's work should be included; exhaustive lists should be avoided. References should be made by giving the author's surname, with the year of publication in parentheses. When reference is made to a work by three authors all names should be given when cited for the first time, and thereafter only the first name, adding *et al.*, e.g. Smith *et al.* (1958). The '*et al.*' form should always be used for works by four or more authors. If several papers by the same author and from the same year are cited, a, b, c, etc., should be put after the year of publication, e.g. Smith *et al.* (1958a). All references should be brought together at the end of the paper in alphabetical order. References to articles and papers should mention (a) name(s) of the author(s); (b) year of publication in parentheses; (c) title of journal, underlined, abbreviated according to the *World List of Scientific Publications*, 4th edn and supplements; (d) volume number; number of first page of article. References to books and monographs should include (a) name(s) and initials of author(s) or editor(s); year of publication in parentheses; (b) title, underlined; (c) edition; (d) page referred to; (e) publisher; (f) place.

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Abbreviations. Abbreviations for some commoner units are given below. The abbreviation for the plural of a unit is the same as that for the singular. Wherever possible the metric SI units should be used unless they conflict with generally accepted current practice. Conversion factors to SI units are shown where appropriate.

SI UNITS

gram	g	Joule	J
kilogram	kg = 10^3 g	Newton	N
milligram	mg = 10^{-3} g	Watt	W
metre	m	Centigrade	°C
millimetre	mm = 10^{-3} m	hour	hr
micrometre	μm = 10^{-6} m	minute	min
nanometre	nm = 10^{-9} m	second	sec
litre	l = 10^{-3} m^3		

NON SI UNITS

inch	in	= 25·4 mm
foot	ft	= 0·3048 m
square inch	in^2	= 645·16 mm^2
square foot	ft^2	= 0·092903 m^2
cubic inch	in^3	= 1·63871 × 10^4 mm^3
cubic foot	ft^3	= 0·028317 m^3
gallon	gal	= 4·5461 l
pound	lb	= 0·453592 kg
pound/cubic inch	lb in^{-3}	= 2·76799 × 10^4 kg m^{-3}
dyne		= 10^{-5} N
Calorie (15°C)	cal	= 4·1855 J
British Thermal Unit	BTU	= 1055·06 J
Horsepower	HP	= 745·700 W
Fahrenheit	°F	= 9/5 *T*°C + 32

Figures. In the text these should be given Arabic numbers, e.g. Fig. 3. They should be marked on the backs with the name(s) of the author(s) and the title of the paper. Where there is any possible doubt as to the orientation of a figure the top should be marked with an arrow. Each figure must bear a reference number corresponding to a similar number in the text. Photographs and photomicrographs should be unmounted glossy prints and should not be retouched. Line diagrams should be on separate sheets; they should be drawn with black Indian ink on white paper and should be about four times the area of the final reproduction. Lines and lettering should be of sufficient thickness and size to stand reduction to one-half or one-third. Whenever possible, the originals of line diagrams, prepared as described above, should be submitted and not photographs. The legends of all the figures should be typed together on a single sheet of paper headed 'Legends to Figures'.

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