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Micro-organisms in dried foods: their significance, limitation and enumeration*

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Summary

Good manufacturing practice (GMP) should form the basis of microbiological quality control in the processing of foods, especially dried products with their extremely heterogeneous microflora. Dried foods can be separated into different classes each carrying specific microbiological risks. The significance of a microbiological criterion for a particular product must be deduced by studying the intrinsic properties, mode of processing and anticipated fate of the food before reconstitution and its usual culinary preparation and use. Standards should therefore be limited to those species, genera or groups of organisms that are relevant for a given category of dried food.

Reliable and reproducible methods and the correct interpretation of results are essential elements in microbiological monitoring. There is an urgent need for proper evaluation and standardization of methodology, paying particular attention to the resuscitation of sublethally impaired cells of various micro-organisms occurring in dried foods. Once criteria and methods have been chosen, acceptable levels (specifications) can be determined for all pertinent groups or species. These levels should be derived from surveys of similar commercially available products whose manufacture by GMP has been previously verified.

Introduction

The microbial ecology of dried foods

It is often thought that there are no microbiological problems associated with dried foods. In comparison with fresh proteinaceous foods the microbiological hazards are certainly trifling. Microbial spoilage is to a great extent precluded and, in most dried foods, the isolation of pathogenic organisms is a rare, but by no means non-existent, occurrence. The mere presence of some micro-organisms may, however, greatly impair the quality of dried foods.

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The significance of the organisms recovered from food samples cannot be assessed adequately without considering the microbial ecology of the product from which they have been isolated. It is essential to know whether these organisms, in the numbers encountered, are in fact unavoidable even when the food product in question has been processed following good manufacturing practices. It is also necessary to study the fate of these organisms during storage, distribution and culinary preparation of the product.

Spore forming bacteria are the most common contaminants of dried foods. Most species of the *Bacillaceae* are harmless but dried products can contain pathogenic types, e.g. *Bacillus cereus* and *Clostridium perfringens*.

Spoilage of canned foods due to *Bacillaceae* strains of unusually high heat resistance can occur if these organisms are present in sufficiently large numbers in raw materials subsequently incorporated into 'commercially sterilized' products destined for tropical regions. Many such 'ultra-thermoresistant' strains have been isolated and identified since the first report by Jensen, Wood & Jansen in 1934 of microbial spoilage of canned meats resulting from spore forming bacteria contaminating the spices used in their preparation. The numbers that may be considered hazardous depend on the quantity of the ingredient in the final product and the lethality of the processing treatment. The characteristics of the most important bacteria in this class are summarized in Table 1.

TABLE 1. Sporogenous bacteria with a thermal resistance exceeding that of *Clostridium botulinum* often involved in the spoilage of canned foods (Mossel, 1975a)

Type of spoilage in canned foods	Trivial name	Systematic name	Optimum temp. (°C)	Mode of dissimilation of glucose	Formation of H_2S from protein	Maximal thermo-resistance* (minutes at 120°C)
Flat sour	Flat sour	*Bacillus stearother-mophilus*	50	a	–	45
Putrid swell	Putrefactive anaerobe	*Clostridium sporogenes*	37	a+g	+	15
Sulphide spoilage	Sulphur stinker	*Clostridium nigrificans*	55	–	+	10
Hard swell	Thermo-philic anaerobe	*Clostridium thermosaccharo-lyticum*	55–62	a+g	–	>80

a, acid; g, gas; –, inert.

* Determined at pH 7 and $a_w \geq 0{\cdot}98$; maximal thermoresistance for *Clostridium botulinum* under these conditions is 4 min at 120°C.

Enterobacteriaceae can survive for long periods of time in dried products (Haines & Elliot, 1944; Rishbeth, 1947; Mossel & Koopman, 1965; Licari & Potter, 1970; Ray, Jezeski & Busta, 1971c; Miller, Goepfert & Amundson, 1972; Christian & Stewart, 1973) and are consequently of considerable significance. Various members of this family, occasionally even *Salmonella*, have often been encountered in dried foods, including products which have been 'fully' processed by heat in the course of their manufacture, i.e. by exposure to thermal energy equivalent in lethality to heating for 30 min at 65°C. *Enterobacteriaceae* have been reported particularly in dried egg products (Gibbons & Moore, 1944; Solowey *et al.*, 1947; Schroeder, Aserkoff & Brachman, 1968), meat and bone meal, fish meal and cotton seed flour (Rohde, 1955; Rutqvist, 1961; van der Schaaf & Mossel, 1963), to a lesser extent in dried milk (Collins *et al.*, 1968; Meister, 1968; Hilker & Solberg, 1973; Hoben, Ashton & Peterson, 1973) and occasionally even in cocoa (Werner *et al.*, 1974).

Outbreaks of staphylococcal enterotoxicosis followed by the detection of staphylococcal enterotoxins have, somewhat unexpectedly, been traced to dried milk. During the process of milk concentration, the prevailing conditions of reduced water activity (a_w) allow *Staphylococcus aureus* to grow in the absence of competition (Mossel, 1975b). In the subsequent drying process the bacterial cells are killed but the preformed heat stable toxins remain unaffected (Anderson & Stone, 1955; Denny, Ran & Bohrer, 1966; Read & Bradshaw, 1966; Denny, Humber & Bohrer, 1971; Fung *et al.*, 1973). Similar conditions for the uninhibited outgrowth of *S. aureus* can also occur during the manufacture of pasta products where a drying temperature of 45°C and a gradual decrease of water activity are involved (Mossel, 1975a).

Finally, dried foods may often contain mycotoxins formed during mould growth on raw materials while they are still relatively moist, i.e. a situation similar to the formation of staphylococcal enterotoxins. The dried product may itself become mouldy if the water activity of the food is marginal. In this case sufficient water vapour may migrate towards the colder parts of a packaged product to allow a local increase of a_w to just above 0·65 (Heiss, 1957; Hazeu & Hueck, 1966) permitting spore germination of the more zerophilic moulds (Mossel, 1975b).

Quality assurance

In dried foods as in other products, quality control is often conceived as a suppressive system in which samples are drawn, examined and consignments rejected if they are found to be defective. The statistical reliability of such a system is, however, extremely low (Mossel, 1975a) and, in practice, substandard consignments are seldom banned from the market. Perhaps the most serious drawback of this approach is that it does not generate the information required to identify and remedy any deficiencies uncovered (Mossel, 1975a).

Quality control of foods should instead be tackled with a preventative system (Wilson, 1964) involving the following steps (Mossel, 1975a).

(a) Systematic monitoring of the microbiological quality of raw materials to control the general level of plant and equipment contamination.

(b) Optimum selection of processing conditions during all thermal treatments, e g. enzymatic digestion, pasteurization and desiccation, so that a significant microbiocidal effect is obtained, being especially careful to avoid combinations of temperature and water activity that might permit microbial growth.

(c) Frequent monitoring of the microbiological condition of all apparatus and equipment used in the manufacture of food, immediate cleaning and disinfection being carried out when results warrant this, even although some loss of production may be entailed.

(d) The maintenance of a clean air supply. During drying processes the air supply can be a serious source of contamination for food products (Hedrick & Heldman, 1969). The risks attendant on the aerosolization of manure deposits, especially poultry droppings, are well known (Zottola, Schmeltz & Jezeski, 1970). Sawdust, particularly from tropical woods, has been found to contain more than 10^6 cells/g of *Enterobacteriaceae* (Mossel, 1975a). If entrained in an airflow such types of sawdust would form a potent source of contamination.

(e) Constant vigilance against recontamination of processed products with organisms originating from raw materials.

(f) Microbiological testing of packaging materials and the substitution of higher quality materials if necessary.

(g) Systematic checking of the condition of the final packaged product for piercing or other damage to the packaging by biological, chemical, physical or mechanical means.

Providing such a system of preventative quality control has been established and is assiduously carried out, it is then logical to draw and examine samples of the final product. Good manufacturing practices have been shown to reduce the variability of microbiological quality in the final product, thus greatly increasing confidence that a sample drawn will be truly representative of the consignment under examination (Cheftel, 1955; Cranston, 1971). Reliability of sampling is particularly important in dried foods where the contamination that occurs, even under reasonable manufacturing conditions, is extremely heterogeneous (Rishbeth, 1947; Silverstolpe *et al.*, 1961; Jacobs *et al.*, 1963; van Schothorst *et al.*, 1966; Ray, Jezeski & Busta, 1971b).

The scope of routine monitoring

The significance of the micro-organisms isolated from dried foods depends, among other factors, on the manufacturing conditions. There are two main types of product to be considered. Firstly, those that have been fully processed by heat in the course of production to such an extent that all non-sporing pathogens will have been eliminated. This category includes dried milk, dried egg products, animal feed ingredients, dehydrated puddings and some types of dried soup. The second group of products,

which have undergone less rigorous heat processing or have had no thermal treatment, comprises all raw cereal flours, certain potato preparations, lightly dried vegetable products, e.g. onion rings, and dried soup mixes containing these ingredients.

Obvious requirements for dried foods of both classes are the absence of *Salmonella* from 10 g to 50 g aliquots, small numbers only of *Staphylococcus aureus* and low counts of *Bacillus cereus* and *Clostridium perfringens*.

The *Enterobacteriaceae* group is the key criterion used to monitor dried products. Fully heat processed products should be examined for all types of *Enterobacteriaceae*. These bacteria are less useful as index organisms in the second group of products as these may naturally contain some innocuous types of vegetable origin, e.g. species of *Enterobacter*, *Serratia* and *Erwinia*. Such products should therefore be monitored by enumerating *Escherichia coli*, as in the examination of potable water (Mossel & Krugers-Dagneaux, 1963).

Only dried foods of completely unknown history require the entire battery of tests described below. As the total number of different tests carried out should always be strictly limited to the ecologically justified minimum, it would be unreasonable to test a given product routinely for, e.g. *Bacillus cereus* or *Clostridium perfringens*, if many consecutive samples had been shown to contain acceptably low levels of these organisms; instead, a total spore count would give adequate protection. This relaxation of testing can, of course, only be considered in manufacturing plants operating with constant and vigilant microbial quality control.

Methods for routine monitoring

Standard aerobic mesophilic plate count

Aliquots (1 ml) of suitable decimal dilutions of the food suspended in peptone saline solution are plated in tryptone soya peptone glucose phosphate agar, tempered at approx. 47°C and incubated at 31 ± 1°C for two to five days (Mossel, Shennan & Vega, 1973).

Aerobic spore count

The above procedure is used after exposing serial decimal dilutions to a pasteurization treatment for 1 min at 80°C (Mossel, Meursing & Slot, 1974). Tubes containing the dilutions are heated in a boiling water bath until the thermometer inserted in a parallel tube containing water indicates 79°C, then immediately transferred to a second water bath held at 80°C. After 1 min at 80°C the tubes are chilled rapidly to approx. 10°C in a beaker containing equal volumes of tap water and ice.

Enterobacteriaceae

When selective media are used for the enumeration or detection of bacteria in dried foods, the food dilutions should not be incubated directly with such media, since this

will result in the inhibition of sublethally impaired cells by the inhibitors present in the media (Mossel & Ratto, 1970; Ray, Jezeski & Busta, 1971a; Gomez *et al.*, 1973; Mossel, Harrewijn & van Zadelhoff, 1974). To restore the normal functioning of these stressed cells, the food sample is suspended (1:10) in tryptone soya peptone glucose phosphate broth and shaken at approx. 20°C for 2 hr (Mossel & Ratto, 1970).

After this 'resuscitation' treatment, 1 ml quantities of serial decimal dilutions are inoculated into 10 ml volumes of buffered brilliant green bile glucose broth, pretested for absence of inhibitory properties. The toxicity of certain triphenylmethane dyes and bile salts preparations towards non-impaired cells of *Enterobacteriaceae* has been repeatedly demonstrated (Mossel & Harrewijn, 1972; Mossel, Harrewijn & van Zadelhoff, 1974). As a consequence many of the *Enterobacteriaceae* enrichment broth media on the dehydrated media market are unsuitable for use (Busse, 1968; Moussa *et al.*, 1973). This problem can be overcome by using only dyes certified for inclusion in selective culture media and by replacing bile salts by analytical reagent grade sodium dodecylsulphate which is completely non-toxic (Mossel, Harrewijn & van Zadelhoff, 1974).

Enterobacteriaceae enrichment tubes are incubated overnight at 30°C and the contents streaked on to dried MacConkey agar plates. After overnight incubation at 30°C, adequate numbers of lactose-negative and lactose-positive colonies are examined for the criteria delineating the *Enterobacteriaceae*, i.e. fermentative attack on glucose and the absence of oxidase activity. This can be done rapidly and reliably by stabbing into Gram-negative diagnostic tubes (Mossel, Harrewijn & van Zadelhoff, 1974). These tubes contain a lower 8 cm layer of violet red bile glucose agar topped by a 2 cm layer of motility test agar. The mode of attack on glucose can be read directly and the oxidase reaction tested on the biomass on top of the tube. Motility and pigmentation can also be read directly.

Staphylococcus aureus

Suitable aliquots of the food are added to Giolitti and Cantoni's tellurite glycine broth, sealed with paraffin and incubated at 37°C for up to 48 hr. The presence of *S. aureus* in blackened tubes is confirmed by streaking on to Baird-Parker's agar followed by testing typical egg-yolk positive and negative isolates for coagulase activity.

Lancefield group D streptococci

Suitable decimal dilutions of the food are streaked in 0·1 ml quantities on to plates of aesculin azide kanamycin agar (Mossel *et al.*, 1976). Following overnight incubation at 37°C, a representative number of colonies with black haloes is examined for the characteristics of this group of streptococci, i.e. catalase-negative cocci, usually in short chains, capable of rapid growth at $45 \pm 0{\cdot}1$°C, and in the presence of 40% bile, attacking glucose by a fermentative pathway without the formation of gas. This confirmation procedure will distinguish *Aerococcus* species and occasional *Lactobacillus* strains which can grow on this medium.

Clostridium group

Suitable decimal dilutions are mixed with sulphite iron polymyxin agar, tempered at approx. 47°C, in plastic pouches (de Waart & Smit, 1967). These are sealed, incubated for 24–48 hr at 31 ± 1°C and black colonies counted.

Moulds

Suitable decimal dilutions are plated in oxytetracycline glucose yeast extract agar, tempered at approx. 47°C, followed by incubation for up to five days at 22 ± 2°C. A neutral medium containing a broad-spectrum antibacterial antibiotic is preferred to the frequently used acidified malt or potato extract agar since acid-intolerant moulds may be suppressed by the latter media (Mossel, Vega & Put, 1975). The addition of 5μg/ml of rose bengal to this medium will restrict the spread of rapidly growing mould mycelia from such contaminants as *Neurospora* (Mossel *et al.*, 1975).

Foods that have been subject to mould growth may, following heat processing, show very low counts of mould propagules while yet containing appreciable levels of mycotoxins. Direct microscopic inspection of foods is therefore occasionally required. The preparatory technique of Warnock (1971) is recommended to facilitate this task: samples are treated at 80°C for 5 min in the presence of 5% sodium hydroxide to dissolve starchy, proteinaceous and lipid structural material thus making mycelial fragments more readily visible.

Escherichia coli

In the second category of foods (those not subjected to full heat processing), the presence or absence test for *Enterobacteriaceae* described above is not applicable and should be replaced by a similar test for *E. coli*.

Suitable aliquots of resuscitated suspensions are enriched in ten-fold volumes of brilliant green bile lactose broth at 30°C for 18–24 hr. Non-gassing cultures are discarded. Gas-positive enrichments are subcultured on to MacConkey agar slopes contained in screw-capped bottles and incubated overnight at 44 ± 0·1°C. Typical lactose-positive colonies so obtained are further examined by a modified Eijkman test, namely for formation of indole from tryptone and gas from lactose at 44°C within 48 hr (Mossel & Vega, 1973).

Additional methods

When the results of routine testing of dried foods suggest that a more comprehensive examination is required, the following additional methods can be considered (Mossel, Shennan & Vega, 1973).

Salmonella

Suitable aliquots of resuscitated suspensions are enriched in Muller-Kauffmann's tetrathionate broth, incubating at 43°C for up to 48 hr. These cultures are then streaked

on to brilliant green phenol red lactose sucrose agar. After incubation at 37 ± 1°C for 24–30 hr, suspect colonies, i.e. those surrounded by red haloes, are examined for fermentative attack on glucose, H_2S formation in Kligler agar, absence of β-galactosidase and urease activities and positive lysine decarboxylase and specific agglutination reactions. When examining dried milk, in which lactose-positive salmonellae may be found, plating should be carried out on dulcitol brilliant green agar, the β-galactosidase test omitted and testing for H_2S formation carried out in lysine iron agar (Mossel, 1975a).

Clostridium perfringens

Serial decimal dilutions are inoculated into sulphite iron cycloserine broth, covered with paraffin and incubated at 46 ± 0·1°C for 24–48 hr (Mossel & Pouw, 1973). Tubes showing blackening are streaked on to Willis and Hobbs' lactose egg-yolk neomycin agar and incubated anaerobically at 46 ± 0·1°C for 24–48 hr. Egg-yolk positive colonies are examined for lack of motility.

Bacillus cereus

Aliquots (0·1 ml) of appropriate serial dilutions are spread on to mannitol egg-yolk polymyxin agar (Mossel, Koopman & Jongerius, 1967). Following overnight incubation at 31 ± 1°C the number of typical irregularly shaped mannitol-negative, egg-yolk positive colonies are counted.

When the identity of suspect colonies is in doubt, isolates are tested for growth at 42°C and development without acidification in the depth of Gram-positive diagnostic tubes containing mannitol purple agar (Mossel, Harrewijn & van Zadelhoff, 1974).

Pseudomonas aeruginosa

Suitable aliquots are enriched overnight in nitrofurantoin broth (Thorn *et al.*, 1971) at 42°C (Mossel *et al.*, 1973). Turbid cultures are streaked on to slants of either nitrofurantoin agar to confirm growth at 42°C, or glycerol mannitol acetamide cetrimide agar (Mossel & Indacochea, 1971) on which a positive result is indicated by the presence of colonies with red haloes after overnight incubation at 42°C.

Examination for process resistance spores

Dried foods that are to be incorporated into appertized or sterilized products should be examined for spores that may be resistant to the processing treatment (Knock & Baumgartner, 1947). The methods recommended by the U.S. National Canners Research Laboratory (1968) are commonly used, comprising plate counts for *Bacillus stearothermophilus* spores and presence or absence tests for *Clostridium nigrificans*, *Cl. sporogenes* and *Cl. thermosaccharolyticum*.

An alternative method, the thermophilic spore titre (Mossel, Harrewijn & van

Sprang, 1973) is useful when a more general investigation into the degree of contamination of dried foods by process-resistant spores is required. Aliquots of 25 g are suspended in ten-fold quantities of brain heart infusion broth with added yeast extract (0·3%) and starch (0·1%). This suspension is heated rapidly to 120°C in a pressure cooker, maintained at that temperature for exactly 4 min (cf. Table 1), and then chilled immediately. Under rigorously aseptic conditions, two aliquots each of 100, 10 and 1ml are withdrawn and incubated under aerobic and anaerobic conditions at 48 ± 2°C. Tubes not showing obvious turbidity after three days' incubation are discarded. Positive cultures are first checked microscopically and then, if necessary, by physiological tests. For this purpose the organisms are isolated by heating the 'positive' tubes for 4 min at 120°C as described above, followed by aerobic and anaerobic subculture at 48°C on tryptone soya peptone glucose phosphate agar with added starch (0·1%). The resulting 'titre' of aerobic or anaerobic process resistant spores is defined as the negative logarithm of the highest decimal dilution of the material found to contain such spores.

Microbiological specifications

General guidelines for microbiological specifications applicable to foods processed for safety are summarized in Table 2. These recommendations are derived from some ten

TABLE 2. Microbiological specifications for dried foods

Class	Group	Organisms	Limits*	
			Aimed at	Maximum ever tolerated†
I‡	1	*Salmonella, Arizona, Edwardsiella, Shigella*	Not detected in a representative aliquot of (i.e. absent in) 25 g	
	2	*E. coli*	Absent in 10 g	Absent in 1 g
	3	*Staph. aureus*	Absent in 1 g	
	4	*Bacillus cereus*/1 g	10^2	10^3
	5	Mould spores/1 g	10	10^2
II§	6	*Enterobacteriaceae*¶	Absent in 1 g	Absent in 0·1 g
	7	Total aerobic count/1 g	10^4	10^5
	8	Aerobic spore count/1 g¶	Approximately equal to aerobic count	
	9	Lancefield D streptococci/1 g	10^2	10^3
	10	*Clostridium perfringens*/1 g	10	10^2

* By prescribed methods.

† Not to be encountered in more than two out of ten samples.

‡ Pathogenic or sometimes pathogenic organisms.

§ Indicator organisms.

¶ Applicable only to dried foods which have been subjected to a full pasteurization treatment in the course of manufacture.

years' experience in the examination of dried foods produced under good processing conditions and satisfactory distribution practices (Mossel & Krugers-Dagneaux, 1963; Harrewijn, Mossel & de Groote, 1972; Mossel, Shennan & Vega, 1973; Mossel, Harrewijn & van Sprang, 1973; Mossel, Meursing & Slot, 1974; Mossel, Harrewijn & van Zadelhoff, 1974; Drion & Mossel, 1976). These specifications are in agreement with similar data obtained elsewhere (Powers *et al.*, 1971; Heidelbaugh *et al.*, 1973).

By carrying out surveys into the microbiological conditions of a selection of similar products, the levels of micro-organisms that are technologically attainable for any particular foodstuff can be assessed. For example, in an investigation into animal feeds (Mossel, Shennan & Vega, 1973), a hundred samples of a representative assortment of commercially available high quality products of the same type were examined for pathogenic, toxinogenic and indicator organisms and minimum, maximum and mean values for each criterion were determined. The outcome of a typical survey can be represented as a curve illustrating the frequency distribution of sample means for a particular criterion in a product manufactured to acceptable quality limits (Fig. 1).

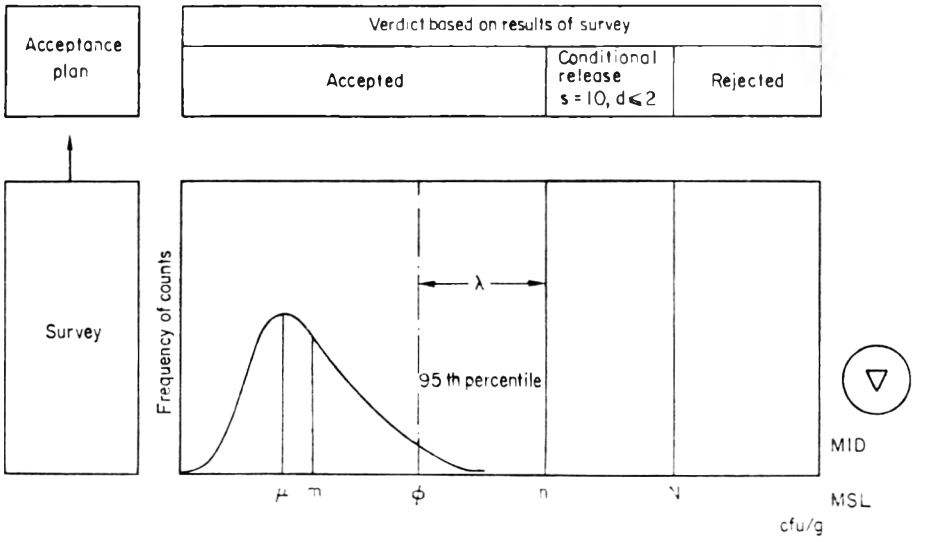

FIG. 1. Typical frequency distribution of samples means for a product manufactured to acceptable quality standards. s, Sample size; d, number of samples that may show a count greater than n but less than N; λ, margin; μ, mode; m, median; ϕ, count not exceeded by 95% of sampling; n, specification; N, absolute maximum count; MID, minimum infectious dose; MSL, minimum spoilage level; cfu, colony forming units.

The number of tests applied to any product should be limited to the minimum required to determine risk to health or danger of spoilage. The choice of tests is decided by the ecological parameters of the food product investigated (Murphy, 1973). These include intrinsic properties such as pH and a_w, the mode of processing, the method of

culinary preparation and the consumer groups at risk, e.g. adults, babies, invalids, etc.

In the specific instance of dried ingredients for appertized or sterilized foods, microbiological requirements have been laid down by the US National Canners Association (1968) and, in a slightly modified form, by Knock & Baumgartner in Great Britain (1947). Both standards are useful, depending on the ultimate mode of processing of the food into which the ingredients are to be incorporated (Mossel, Meursing & Slot, 1974).

The significance of testing for Enterobacteriaceae

There has been speculation as to whether monitoring foodstuffs for the *Enterobacteriaceae* group (Mossel, Shennan & Vega, 1973; Powers, Silverman & Rowley, 1973), as outlined above, gives sufficient consumer protection against contamination with specific enteric pathogens (Hobbs, 1973). This question has, however, never been posed in the context of the 'coli-aerogenes' test, a procedure which is less sensitive than that for the total *Enterobacteriaceae*, as only 0·1 g instead of 1·0 g of the food is usually examined, and particularly as the 'coli-aerogenes' bacteria, being lactose-positive, are taxonomically a more restricted group. Therefore the 'coli-aerogenes' tests customarily used confer less protection to the consumer than does examination for *Enterobacteriaceae*. That this situation has never given rise to problems in the monitoring of dried milk is probably due to the relatively low frequency of lactose-negative *Enterobacteriaceae* and the weak epidemiological pressure of *Salmonella* in this environment. The use of the 'coli-aerogenes' test may be more difficult to justify as the sole criterion for monitoring egg products, for example, as these contain greater numbers of lactose-negative *Enterobacteriaceae*.

The extent to which the *Enterobacteriaceae* test can give adequate consumer protection has been determined by ecological studies. An evaluation was made (Drion & Mossel, 1976) of the risk of accepting *Salmonella*-contaminated consignments of dried foods, despite the negative outcome of (i) examination of 1·5 kg aliquots for *Salmonella*, (ii) monitoring two 1 g aliquots for *Enterobacteriaceae* and (iii) the simultaneous application of both tests. The computations were based on the examination of some 6500 samples of dried foods, processed for safety (Mossel & Krugers-Dagneaux, 1963; Harrewijn *et al.*, 1972; Mossel, Shennan & Vega, 1973; Mossel, Harrewijn & van Sprang, 1973; Mossel, Meursing & Slot, 1974; Mossel, Harrewijn & van Zadelhoff, 1974; Drion & Mossel, 1976). It was found that levels of colony forming units, (cfu)/g, for both criteria individually and the so-called ϵ-factor, defined as cfu/g *Enterobacteriaceae* ÷ cfu/g *Salmonella*, varied widely between commodities (Table 3) and also between different consignments of the same product.

The general conclusion of this investigation was that, because $\epsilon > 10^3$ in the vast majority of cases, examination of two 1 g aliquots for *Enterobacteriaceae* and accepting no positive results, would eliminate *Salmonella* positive samples. In the rare instances where $\epsilon < 10^3$, additional testing for *Salmonella* gave only a minute improvement in consumer

TABLE 3. Summary of data on enteric bacteria in dried foods (Drion & Mossel, 1976)

Type	Approx. number of samples examined	*Enterobacteriaceae* $\log_{10}$/g		*Salmonella* $\log_{10}$/g		ε-factor*	
		Min.	Max.	Min.	Max.	Min.	Max.
Processed cereals	4000	−1	4	−3	1	20	10^5
Pelletized animal feeds	1500	0	3	−3	0	10^2	4×10^5
Egg products	300	−1	4	−3	−1	2	3×10^6
Gelatin, plasma, etc.	300	1	3	−1	1	3	6×10^2
Soups	200	..†	3	θ‡		..	..
Nuts	200	−1	4	−3	−2	2×10^2	10^6

* ε, cfu/g *Enterobacteriaceae* / cfu/g *Salmonella*.
† .., Not determined or undefined.
‡ θ, None detected.

protection compared with testing for *Enterobacteriaceae*, while examination for both groups led to consistent *Salmonella* quality control.

These calculations have as their baseline the strict scheme of *Salmonella* testing used in the USA in which 1·5 kg aliquots are required to be examined. However, the ε-factor approach is valid at all levels. If the baseline adopted is, instead, the customarily used requirement for absence of *Salmonella* in 100 g aliquots, then the *Enterobacteriaceae* testing scheme can be proportionately relaxed: in this example testing two 0·1 g aliquots would suffice.

It is obvious that the monitoring of dried foods for other enteropathogenic organisms, e.g. enteropathogenic strains of *E. coli*, *Shigella*, etc., can be considered in a similar manner. Unfortunately there are few published data on the numbers of enteric pathogens other than salmonellae occurring in heat processed foods and the gathering of results is hampered by the often inadequate methodology available.

The need for rigorous standardization of methods

The use of microbiological specifications in international trade is well-nigh impossible unless reliable methods of examination are available and agreed upon. In attempting to achieve standardization the three primary parameters involved in any microbiological method of examination should be recognized.

(i) Precision, determined by (a) reproducibility between different laboratories and (b) repeatability within a single laboratory. The latter factor is in turn compounded of the variability of results obtained by the same worker carrying out the test, for example, in triplicate, and the spread of results from different workers within the same laboratory, each performing the same test.

(ii) Accuracy, assessed by applying the method to samples artificially contaminated with known numbers of the relevant organism.

(iii) Sensitivity, defined as the lowest number of cfu/g that can be detected in the presence of the usual background flora belonging to different foods. It is often necessary to determine counts as low as one cell in 10^5–10^8 of the total microbial population.

The choice of method can be influenced by practical considerations, not every laboratory having the same resources of skill, effort and facilities. The rapidity with which results can be obtained, the simplicity of operation of methods and the cost of carrying out a finite number of tests are all important factors.

The proper sampling of consignments is of primary importance (Silverstolpe *et al.*, 1961) especially in view of the heterogeneous microflora of dried foods, both in quantity and in the types of organism concerned (Rishbeth, 1947; Jacobs *et al.*, 1963; van Schothorst *et al.*, 1966; Ray *et al.*, 1971b). Consequently, careful consideration should be given to the intensity and randomization of sampling in every instance (Murphy, 1973). Comprehensive guidelines are available for this purpose (Harrewijn *et al.*, 1972; Ingram *et al.*, 1974).

The importance of resuscitation in testing for the *Enterobacteriaceae* and species within this group has already been emphasized. In addition, there are several other factors of importance which are seldom mentioned in the published descriptions of procedures. For instance, strict observation of the method of pasteurization to obtain true spore crops (Mossel, Meursing & Slot, 1974) will reduce the number of false positive results in aerobic spore counts. The optimum incubation period for most tests should not be exceeded: firstly, if there is no growth or reaction within the specified time, there is nothing to be gained by pursuing the test further and secondly, the slow growth of inhibited species in complex inhibitory media may ultimately become apparent and lead to confusing results (Mossel, 1975a).

The levels of pass criteria set for given groups of organisms in dried foods may be low, i.e. 10/g or 100/g. Unless enrichment methods are used, entailing a marked loss of reproducibility, this may mean that critical counts fall close to the practical limits of accurate enumeration and may also be significantly affected by normal experimental error. Hence efficient sampling and adequate replication in testing are again of paramount importance.

Specifications are not, of course, intended to be rigidly applied in every case. In the setting of microbiological standards it is essential to apply tolerances in order to accommodate two factors which have already been emphasized, namely the heterogeneous nature of contamination in dried foods and the inherent variation in the enumeration procedures themselves. Such tolerances generally accept that up to two out of every ten samples examined for a given group of organisms may show an excess in counts, but this should not exceed the standard by more than a factor of ten (Mossel, 1975a).

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Effect of suspending and plating media on the recovery of *Salmonella gallinarum* following freezing and thawing*

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Summary

Salmonella gallinarum suspended in distilled water was frozen at a rate of 22°C/min, 86% of the cells were inactivated, as determined from differences in numbers of colony-forming units observed on TSA medium before and after freezing. About 35% of the viable survivors were injured, as determined by their failure to form colonies on a minimal plating medium (MA) but their success on a 'complete' medium (TSA). Following 1 hr incubation of the frozen-thawed cells in water or in 0·3 mM monopotassium phosphate, the percentage survival was reduced from 14 to 8% whereas in selenite cystine or tetrathionate broths, the percentage of survivors was reduced to about 3%. Higher recoveries of the frozen-thawed cells were obtained when nonselective suspending media such as nutrient broth and lactose broth were employed.

The possibility of obtaining better recovery by the addition of specific compounds to plating media was investigated. Filter-sterilized catalase or vitamin-free hydrolyzed casein, when added to the minimal agar medium improved the recovery of frozen-thawed *S. gallinarum* by 32 and 34% respectively. Sodium pyruvate, heat-sterilized catalase or ethylenediaminetetraacetic acid had no significant effect, but the addition of haemin or cysteine reduced the rate of recovery by 48 and 57% respectively.

Introduction

In a population of bacterial cells after freezing and thawing, some cells will require an enriched medium for growth (Straka & Stokes, 1959; Arpai, 1962; Moss & Speck, 1963). Those cells which can no longer grow on a minimal medium are usually referred to as metabolically injured. Minimal agar media, being deficient in required nutrients, do not support, by definition, repair of nonlethal metabolic injury. A complete medium, on the other hand, contains the nutrients necessary to promote repair.

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Another manifestation of freeze-injury to bacterial cells is their increased sensitivity to many selective agents, such as those added to media for use in their detection and enumeration (Maxcy, 1970; Raccach & Juven, 1973; Ray & Speck, 1973).

An approach to the investigation of freeze-injury which may be of great practical value, is the study of the repairing effect achieved through the addition of specific compounds to plating media. Some findings regarding the influence of the composition of media on the recovery of *S. gallinarum* from freezing and thawing are reported herein.

Materials and methods

Organism

Salmonella gallinarum K_{74} was originally obtained from the National Salmonella Centre, Ministry of Health, Jerusalem, and maintained on slants of nutrient agar. Inocula for the experimental media were prepared by growing the organism in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C. Stationary phase cells were harvested by centrifugation and washed three times either in distilled water or in 0·065 M phosphate buffer (pH 7·0).

Chemicals

The following compounds were used in this study. A 1 M solution of sodium pyruvate (BDH Chemicals Ltd, Poole, England) was prepared in distilled water and sterilized by membrane filtration. Vitamin-free hydrolyzed casein (Oxoid Ltd, London), crystalline bovine haemin (Sigma Chemical Co., St Louis, Mo.), DL-cysteine HCl (Sigma), and ethylenediaminetetra-acetic acid (EDTA) tetrasodium salt (BDH) were sterilized by autoclaving (121°C for 15 min). Aqueous solutions of crystalline catalase (Worthington Biochemical Corp., Freehold, N.J.) were sterilized by either membrane filtration or by autoclaving.

Freezing and thawing procedures

Bacterial suspensions (1·2 ml) in distilled water were placed in 75 × 10 mm culture tubes and frozen in a model BF4 Biological Freezer System (Linde Division of Union Carbide) down to −25°C; the mean cooling rate was of 22°C/min. In less than 5 sec after the freezing cycle, the suspensions were thawed to a final temperature of approximately 4°C, at a warming rate of 15–20°C/min.

Survival, injury and repair

After serial dilutions the frozen and thawed samples were surface-plated on tryptic soy agar (TSA, Difco) and on the minimal agar medium of Sorrells, Speck & Warren (1970) which had the following composition: K_2HPO_4, 7·0 g; KH_2PO_4, 3·0 g; trisodium citrate.$2H_2O$, 0·1 g; $MgSO_4.7H_2O$, 0·1 g; $(NH_4)_2SO_4$, 1·0 g; glucose, 2·0 g; leucine, 3 mg; thiamine, 2 mg; distilled water, 1000 ml; pH 7·0. Percentage survival was calcu-

lated from TSA counts before and after freezing and thawing, and percentage injury was calculated from TSA and MA counts after freezing and thawing. When the effects of incubation in nonselective and selective media were evaluated, any increase in MA count, without a concurrent increase in counts on TSA, was assumed to be due to repair of the injured cells. When the increase in counts occurred both on TSA and on MA, it was assumed to be due to cell multiplication.

Results

Cell suspensions of *Salmonella gallinarum* ($8{\cdot}5 \times 10^8$/ml) in distilled water were frozen at a cooling rate of 22°C/min and then thawed at a warming rate of 15–20°C/min. Several suspending media were then inoculated with approximately equal numbers of survivors and incubated at 25°C for 1 and 2 hr. Cells suspended in distilled water were used as controls. Colony counts on TSA and MA at 0 hr were $1{\cdot}2 \times 10^8$ and $7{\cdot}0 \times 10^7$ per ml respectively. After incubation, serial dilutions were made in 0·3 mM monopotassium phosphate (pH 7·2) and aliquots were surface-plated both on TSA and MA. Table 1

TABLE 1. Effect of selective and nonselective suspending media* on the recovery of frozen-thawed *Salmonella gallinarum*

Suspending medium	Colony counts** ($\times 10^{-6}$)/ml after 1 and 2 hr incubation at 25°C on					
	MA***			TSA***		
	1 hr	2 hr	Statistical comparison of MA counts	1 hr	2 hr	Statistical comparison of TSA counts
Water	70	70		70	70	
0·3 mM monopotassium phosphate, pH 7·2	45	45		70	105	$P<0{\cdot}05$
Nutrient broth	100	100		120	140	N.S.
Lactose broth	70	90	N.S.	120	95	N.S.
Selenite cystine broth	20	5	$P<0{\cdot}05$	20	10	$P<0{\cdot}01$
Tetrathionate broth	5	0·1	$P<0{\cdot}01$	25	0·1	$P<0{\cdot}005$
LSD: at 0·05 level		9·9			13·4	
at 0·01 level		13·8			18·8	

P is the probability that counts after 1 and 2 hr are equal.

* Nutrient broth, lactose broth, selenite cystine broth and tetrathionate broth were obtained from Difco Laboratories.

** Results are the average of three replicas.

*** TSA, tryptic soy agar; MA, minimal agar medium; for composition see 'Materials and methods'.

shows the effect of selective and nonselective suspending media on the recovery of frozen and thawed *S. gallinarum*.

Counts on TSA show that after 1 hr incubation of the frozen-thawed cells in water or in the phosphate diluent, the percentage of survivors decreased from 14 to 8%. In selenite cystine or in tetrathionate broths the percentage of survivors was reduced to about 3%. After 2 hr a significant further reduction in counts was observed in selenite cystine and tetrathionate broths. Improved cell recoveries were obtained on MA plates after incubation of the frozen-thawed cells either in nutrient broth or in lactose broth for 1 or 2 hr respectively. On TSA improved recoveries relative to water and phosphate were achieved after 1 hr incubation in nutrient broth or in lactose broth, or after 2 hr in the phosphate diluent.

The possibility of obtaining better recovery of *S. gallinarum* upon the addition of specific compounds to plating media was studied. Stationary phase cells grown on tryptic soy broth at 37°C were centrifuged, washed and resuspended in 0·065 M phosphate buffer (pH 7·0). Cells (3×10^9/ml) were frozen and thawed, and aliquots were then serially diluted and surface-plated on TSA, MA and MA supplemented with selected compounds. The colony counts on TSA and MA were $1{\cdot}1 \times 10^9$ and $7{\cdot}9 \times 10^8$ per ml respectively. The effect of the addition to MA of specific compounds on the rate of recovery of the frozen-thawed cells is summarized in Table 2. The addition of filter-sterilized catalase, or vitamin-free hydrolyzed casein to the minimal agar medium improved the recovery of frozen-thawed *S. gallinarum* by 32 and 34% respectively.

TABLE 2. Relative efficiency of several plating media on the recovery of survivors of frozen-thawed *Salmonella gallinarum*

Plating medium*	Addition to the plating medium		Count** ($\times 10^{-7}$)/ml
		Conc.	
TSA		None	112
MA		None	79
MA	Catalase, heat-sterilized	1000 units/ml	88
MA	Catalase, filter-sterilized	1000 units/ml	104
MA	Sodium pyruvate	0·1 M	76
MA	Haemin	0·1 mM	41
MA	Casein	1% (w/v)	106
MA	EDTA	0·01 mM	89
MA	Cysteine	1 mM	34
		LSD: at 0·05 level	14·3
		at 0·01 level	19·8

* TSA, tryptic soy agar; MA, minimal agar medium.

** Results are the average of three replicas.

Sodium pyruvate, heat-sterilized catalase or EDTA had no significant effect whereas the addition of either haemin or cysteine reduced the rate of recovery 48 and 57% respectively.

Discussion

When *Salmonella gallinarum* suspended in distilled water was frozen at a rate of 22°C/min, 65 to 85% of the cells were inactivated, as determined from differences in numbers of colony-forming units observed on TSA medium before and after freezing. About 35% of the viable survivors were injured, as determined by their failure to form colonies on a minimal plating medium (MA) but their success on a 'complete' medium (TSA). Following 1 hr incubation of the frozen-thawed cells in water or in 0·3 mM monopotassium phosphate the percent of survival was reduced by about 40%, whereas in selenite cystine or tetrathionate broths the percent of survival was reduced by about 80%.

Improved cell recoveries were obtained after incubation of the frozen-thawed cells in nutrient broth (MA and TSA, 1 h), lactose broth (MA, 2 hr; TSA, 1 hr) or phosphate diluent (TSA, 2 hr). These results are explainable on a basis of cell multiplication rather than repair of injured cells, since it is assumed that, when repair is concerned, there is an increase in MA but not in TSA counts.

Monopotassium phosphate aqueous solution (0·3 mM, pH 7·2) has been recommended as a diluent in the microbiological examination of frozen foods (APHA, 1966). The results reported here support other workers' views that the number of bacterial cells may be reduced significantly when suspended in the phosphate diluent after freezing and thawing (Straka & Stokes, 1957).

The possibility, that a significant portion of the microorganisms present in foods may be injured by freezing, has been widely discussed in the literature. In analysing frozen foods for *salmonellae* by conventional methods, the microbiologist depends on a variety of selective media such as selenite cystine broth, tetrathionate broth, etc. Raccach & Juven (1973) showed that on a selective plating medium, recovery of frozen-thawed *Salmonella gallinarum* was only 60% of that on a non-selective medium, and decreased to 5% after 1 week of storage at −20°C. Therefore, the conventional analytical procedures may fail to detect injured salmonellae cells in frozen foods, and thus lead to a false evaluation of the microbiological quality of these foods.

The use of non-selective pre-enrichment media, as a means of repairing those injured cells, has been recommended by a number of workers (North, 1961; Thatcher & Clark, 1968). The results presented here show that freeze-injured *S. gallinarum* is recovered to a higher degree after inoculation in non-selective medium such as lactose broth or nutrient broth. However, the use of a non-selective pre-enrichment to allow recovery of injured salmonellae from frozen foods may be doubtful, since other bacteria present in the food sample may antagonize the salmonellae in the non-selective medium and make their subsequent detection by selective media difficult or even impossible (Speck, 1970).

By definition, minimal agar media do not support growth of cells with non-lethal metabolic injury. A compound is regarded as aiding in repair of freeze-injury if, when added to the minimal medium, the colony count of the frozen-thawed cells is increased and approaches that obtained with a complete medium. The addition of filter-sterilized catalase or vitamin-free hydrolyzed casein to the minimal agar medium improved the recovery of frozen-thawed *S. gallinarum* (Table 2). Sodium pyruvate or heat-denatured catalase had no significant effect whereas the addition of haemin reduced the rate of recovery. Lewicki & Silverman (1968) reported higher recoveries of *S. typhimurium* after freeze-drying, upon the addition of catalase (100–1000 units/ml), sodium pyruvate (0·01–0·1 M), or haematin (0·1 mM) to a minimal agar medium: they obtained increased recoveries with either filter-sterilized or heat-denatured catalase, the effect of the latter being half of that of the active catalase. Similar results were obtained with pyruvate and catalase for *Staphylococcus aureus* (Baird-Parker & Davenport, 1965); the improved recoveries were explained by the ability of those compounds to inactivate peroxide, on the assumption that the enzyme catalase of *Staph. aureus* might be destroyed or its activity reduced after freeze-drying. *In vitro* inactivation of catalase subjected to freezing and thawing has been reported by Ashwood-Smith & Warby (1972). Our findings on the effect of casein do not support those reported by Straka & Stokes (1959), who found that vitamin-free acid-hydrolyzed casein, when added to a minimal agar medium at a concentration of 2%, was ineffective in the recovery of *Ps. fluorescens* which had been exposed to subzero (°C) temperatures.

The possibility that toxic elements, present in the water used for dilutions, may increase death of bacteria after freezing, has been considered by several authors. Two of the compounds tested, cysteine and ethylenediaminetetra-acetic acid (EDTA), chelate toxic ions and remove them from the bacterial environment. When added to a minimal salts glucose plating medium, cysteine (1–10 mM) was found to give a significant increase in the recovery of the frozen cells of both *A. aerogenes* and *E. coli* (MacLeod, Smith & Gelinas, 1966). EDTA (0·01 mM) was also effective on *A. aerogenes*, although less than cysteine; for *E. coli*, EDTA was almost ineffective. In the present work, addition of EDTA to the minimal plating medium had no significant effect on the recovery of the frozen-thawed *S. gallinarum*, but the addition of cysteine reduced recovery by 57%.

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The occurrence and growth of *Clostridium* spp. in vacuum-packed bacon with particular reference to *Cl. perfringens* (*welchii*) and *Cl. botulinum*

T. A. ROBERTS AND J. L. SMART

Summary

Presumptive *Clostridium* spp. were detected in 234 of 263 25 g samples of vacuum-packed bacon, sixty-four of which contained *Cl. perfringens* and eleven *Cl. botulinum* (ten confirmed as type B and 1 type A). Growth of presumptive *Clostridium* spp. occurred in vacuum packs stored at 15°, 20° and 25°C but growth of *Cl. perfringens* or *Cl. botulinum* never occurred. Growth of clostridia was not related to any particular curing process, though some of the results suggested that the role of nitrate in their control should be further investigated.

In one subsequent batch of unsliced collar bacon, of twenty-six 175 g samples, nineteen contained *Cl. botulinum* type A.

Introduction

Clostridium spp. occur naturally in meat, but neither their distribution nor frequency is well documented owing mainly to technical difficulties in enumeration. Most *Clostridium* spp. in meat are harmless putrefactive mesophiles resembling *Cl. sporogenes* (Riemann, 1963) but from time to time the food poisoning species *Cl. perfringens* and *Cl. botulinum* occur and the processing and storage of meat and meat products must take this into account. *Clostridium botulinum* has been demonstrated so rarely in some foods that there is a temptation to infer that it is absent, because no outbreaks of botulism have been traced to them. Changes in technology are sometimes proposed which raise the question of the risk from botulism. If *Cl. botulinum* were truly absent from a food, the process and storage conditions need no longer be so severe as to control it. If it is present, even at a low level of incidence, the food could occasionally become toxic unless growth of the organism is prevented, e.g. by adequate heat processing, curing salts, reduced storage temperature or by combinations of these factors.

The desirable level of nitrite in cured meats is at present being scrutinized because nitrosamines, many of which are carcinogenic for animals and almost certainly for

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humans, have occasionally been detected in cured meat products (Sebranek & Cassens, 1973). They are formed by the reaction of nitrite with secondary and tertiary amines and it seemed reasonable to assume that if less nitrite were used the amounts of nitrosamines would be reduced; on the other hand it may be argued that such a reduction in nitrite concentration would increase the risk of botulism, particularly in products such as bacon which are often stored unrefrigerated. Cured meats in general enjoy a commendable record of safety with respect to public health, but 'farm-cured hams', which are essentially dry-salted pork and, as far as we can ascertain (R. Buttiaux, personal communication; C. Gonzalez, personal communication), contain no nitrite, have been responsible for numerous outbreaks of human botulism in France (Sebald, 1970) and Spain (Gonzalez & Guttierez, 1972). Thus sodium nitrite appears to help in the control of *Cl. botulinum*, a conclusion supported by much experimental evidence (Riemann, 1963; Spencer, 1966; Greenberg, 1972; Ingram, 1973) and since the amount of nitrite needed to control the growth of *Cl. botulinum* is considerably greater than that needed to develop the characteristic colour and flavour of cured pork, any reduction will impinge, firstly, on the question of microbiological safety.

The numbers of *Clostridium* spores in non-sterile cured meats are reputedly low and very few demonstrations of *Cl. botulinum* have been reported (Riemann, 1963; Steinkraus & Ayres, 1964; Taclindo *et al.*, 1967; Mol & Timmers, 1970; Abrahamsson & Riemann, 1971). These data refer to countries abroad and none related to the UK have hitherto been published. In this study vacuum-packed (vp) bacon produced in experimental cures in a commercial curing cellar, and using pigs taken from the slaughter line of a Wiltshire curing factory, has been examined for the presence of *Clostridium* spp. in general, and for *Cl. perfringens* and *Cl. botulinum* in particular.

Materials and methods

Curing and sampling procedures

Bacon was prepared in a factory, over a period of ten months, using the injection and immersion brines listed in Table 1. Pigs of bacon weight were chosen randomly at an abattoir attached to a large meat processing plant. Cures were compared using opposite sides of the same carcases. After curing and maturation at the factory, portions of the sides as required, were transported to the laboratory under refrigeration, for boning, slicing and packaging. Slices were allocated to the packs such that after storage, the samples examined for *Clostridium* spp., *Cl. perfringens* and *Cl. botulinum* represented all the pigs from a single cure (Taylor & Shaw, 1975). When available, additional samples of unsliced collar and back bacon, from individual sides, were examined for *Cl. botulinum*.

Media

(a) The medium of Mossel & de Waart (1968) (LSUP) comprising (% w/v) Bacto

Table 1. Composition of curing brines

Comparison	Curing code	Curing brines						No. of pig sides represented in samples examined
		Injection			Immersion			
		NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	
Hand pumped								
1	13X	26	1000	5000	26	1000	5000	2 Left (L)
	13Z	26	1000	0	26	1000	0	2 Right (R)
2	13X	26	1000	5000	26	1000	5000	2 L
	13Z	26	1000	0	26	1000	0	2 R
3	14X	26	1000	5000	26	1000	5000	2 L
	14Z	26	1000	0	26	1000	0	2 R
4	15X	26	1000	5000	26	1000	5000	6 L
	15Z	26	1000	0	26	1000	0	6 R
5	16X	26	1000	5000	26	1000	5000	6 L
	16Z	26	2000	0	26	2000	0	6 R
Multineedle								
6	17X	17	700	0	26	1000	0	7 L
	17Z	17	700	2700	26	1000	4200	7 R
7	19X	16	700	0	26	1000	0	7 L
	19Z	16	700	2100	26	1000	4200	7 R
8	20X	16	700	0	26	1000	0	7 L
	20Z	16	1500	0		Dry salted		7 R
9	21X	16	700	2100	26	1000	4200	7 L
	21Z	16	1500	4000		Dry salted		7 R
10	22X	16	1500	0		Dry salted		7 L
	22Z	16	1500	4000		Dry salted		7 R

Comparison 1 samples stored aerobically. All other comparisons vacuum packed.

Tryptone (Difco), 1·5; Yeast Extract Powder (Oxoid), 1·0; Lab Lemco Powder (Oxoid), 0·3 adjusted to pH 7·0. Single strength (SS) LSUP was distributed in 100 ml lots in 250 ml polypropylene centrifuge bottles and double strength (DS) medium in 10 ml lots in 1 fl oz screw-capped (s.c.) bottles and autoclaved at 121°C for 15 min. After cooling, 5 ml of each of the following freshly prepared filter sterilized solutions (Millipore 0·22 μ membrane filter) was added to 100 ml SS medium or 1 ml to 10 ml DS medium: (% w/v) $FeSO_4.7H_2O$ (BDH Analar), 0·4; $Na_2SO_3.7H_2O$ (BDH Analar), 1·0; Polymyxin B Sulphate (Burroughs Wellcome & Co., London), 0·02.

(b) Lactose egg yolk agar (Willis & Hobbs, 1958, 1959). A basal medium consisting of 400 ml Hartley's digest broth, 1·2% w/v New Zealand agar, 1·2% w/v lactose and 1·3 ml of a 1% solution of neutral red was autoclaved at 121°C for 20 min. After cooling to 50°C, 15 ml of freshly prepared egg yolk emulsion (equal volumes egg yolk and sterile 0·9% w/v sodium chloride solution) and 60 ml of sterile milk were added and the plates poured immediately. One half of each plate was coated with a mixture of equal volumes of *Cl. perfringens* type A and *Cl. oedematiens* type A antisera.

(c) Robertson's Cooked Meat Medium (CMM).

(d) Blood agar (BA): Hartley's digest broth containing 2·4% w/v New Zealand agar and 5% v/v oxalated horse blood (Wellcome Reagents Ltd, Beckenham, Kent).

Enumeration of Clostridium *spp.*

Presumptive *Clostridium* spp. counts were determined by a Most Probable Number (MPN) technique using LSUP medium. Twenty-five grams of bacon, minced through a 3·5 mm diameter screen (Moulinex Mark III meat grinder), were macerated in an Atomix jar (M.S.E. Ltd, London) for 0·5 min at half-speed and 1 min at full speed, with 100 ml diluent comprising 0·1% w/v Bacto-peptone (Difco) and 0·85% w/v sodium chloride. Decimal dilutions of the macerate were prepared in sterile water, and three 10 ml replicates of each dilution added separately to 3×10 ml DS LSUP in 1 fl oz s.c. bottles and incubated at 35°. The number of presumptive *Clostridium* cells present in the original sample was determined from the number of cultures exhibiting sulphite reduction by reference to probability tables (Parnow, 1972).

Caution must be used in the interpretation of presumptive *Clostridium* spp. counts by the above method. The shortcomings of the technique are clearly stated by Mossel & de Waart (1968). In our study, microscopic examination of blackened cultures invariably showed a mixture of cocci, rods and occasionally a few spores. To date, cocci isolated from such cultures have failed to reduce sulphite to sulphide. Attempts were made to confirm that the blackening observed was due to sulphite-reduction by clostridia. Samples of blackened medium were pasteurized by totally immersing 1 ml in a 1 fl oz s.c. bottle at 78°C for 30 min, cooling, and adding 20 ml of freshly prepared SS LSUP. If, after incubation at 35°, sulphite reduction again occurred, the growth was plated on BA and incubated aerobically and anaerobically. If no growth resulted under aerobic incubation, but growth occurred under anaerobic incubation, the

sulphite reduction was taken to be due to *Clostridium* spp. Experience showed that pasteurization frequently inactivated the bacteria causing the blackening, probably indicating that *Cl. perfringens* failed to form spores in LSUP. Furthermore, if blackened LSUP was tested as soon as blackening had occurred (24–48 hr), *Cl. perfringens* was commonly shown to be present, but if incubation was continued for five to seven days before confirmation was attempted, *Cl. perfringens* was less frequently shown to be present. Hence the counts listed are likely to be an underestimate of the actual numbers of *Cl. perfringens* present. Where it is necessary to examine large samples of meat because the numbers of *Cl. perfringens* present are low there is no alternative to the methods used. (There are good alternative methods where numbers are high.)

Detection and enumeration of Cl. perfringens

All cultures exhibiting sulphite reduction were streaked on lactose-egg yolk agar (Willis & Hobbs, 1958, 1959), incubated anaerobically at 35° for 24 hr and examined for the characteristic reactions of *Cl. perfringens.*

Detection of Cl. botulinum

Twenty-five gram samples of sliced, minced vp bacon were added to 100 ml LSUP and incubated at 35°C until sulphite reduction was observed (with a few exceptions within six days). Samples of the cultures were centrifuged at 4000 rpm for 20 min at 4°C and the resulting supernatants stored at − 10°C to await toxin testing. Supernatants were not trypsinized. Additional samples of unsliced, unpackaged collar and back bacon were examined similarly, but samples were chopped (1 cm^3) then mixed before adding weighed quantities to medium.

Toxin test

Two hours prior to the injection of the test samples, mice were passively immunized by intraperitoneal (i.p.) injection with 0·3 ml of a mixture of one part each *Cl. welchii* type A and type B; *Cl. oedematiens* type A and type B; *Cl. septicum* and *Cl. chauveoi* Wellcombe diagnostic antisera and four parts *Cl. tetani* Wellcome concentrated antitoxin. This treatment effectively eliminated non-specific deaths in an earlier survey for *Cl. botulinum* (Roberts, Thomas & Gilbert, 1973). Samples (0·5 ml) of the LSUP supernatants were injected (i.p.) into 20–24 g immunized mice which were then observed over a period of 96 hr for clinical symptoms of botulism.

Isolation of Cl. botulinum *and toxin typing*

One millilitre of the toxic LSUP cultures was subcultured into CMM in 1 fl oz s.c. bottles and incubated at 35°C for 48 hr. The subsequent bacterial growth was streaked on BA plates and incubated anaerobically at 35°C for 48 hr. Colonies typical of *Cl. botulinum* were cut from the agar and grown in CMM at 35°C for toxin testing. These procedures were repeated until pure cultures of *Cl. botulinum* were obtained. Isolates

were typed using *Cl. botulinum* monovalent antitoxins types A–F (types A–E, Pasteur Institute, Paris; type F, N.C.D.C., Atlanta, Georgia). The technique described above was used basically to isolate *Cl. botulinum* but since the CMM cultures proved more toxic than the original LSUP enrichment cultures of bacon, they were used in the neutralization tests, thereby avoiding the necessity of concentrating the LSUP cultures by dialysis.

Results

The presumptive *Clostridium* spp. counts and the samples in which *Cl. perfringens* and/or *Cl. botulinum* were detected are shown in Tables 2–10. Data on the incidence of *Cl. botulinum* in various foods, compiled by several workers and including our data are summarized in Table 11.

TABLE 2. Comparison 1: presumptive *Clostridium* spp. (cells/g) in aerobically stored bacon cured with and without nitrate

Curing code	Bacon type	Analytical data					Storage conditions		
		pH	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	Water (%)	0	5° — 7	15°(c) — 4 days
13X	C*	6·2	4·0	66	462	64	55	55	2·3
	BL	5·9	3·0	56	436	69	75	W12·5	1·3
	BF	6·4	2·1	53	894	13	+	0·4	–
13Z	C	6·1	4·0	81	31	61	12·5	12·5	17·5
	BL	5·7	3·6	62	28	69	1·0	0·8	0·2
	BF	6·2	2·4	72	36	19	W4·8	0·5	0·3

	Brines (hand pumped)		
	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)
13X	26	1000	5000
13Z	26	1000	0

* C = collar; BL = back lean; BF = back fat.
\+ = Clostridia detected in 25-g sample.
– = Clostridia not detected in 25-g sample.
W = *Cl. perfringens* detected in 25-g sample.

Presumptive Clostridium *spp. counts*

Of a total of 263 samples examined, 234 contained clostridia. Prior to storage the counts ranged from zero in 25 g sample to 75/g; the mean being about 5/g. *Clostridium* spp. grew in some of the stored samples but growth was not meaningfully related to the

TABLE 3. Comparison 2: presumptive *Clostridium* spp. (cells/g) in vacuum-packed bacon cured with and without nitrate

Curing code	Bacon type	Analytical data					Storage conditions					
								15°			20°	25°(C)
		pH	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	Water (%)	0	7	13	19	12	9 days
13X	C*	6·2	5·0	66	455	67	12·5	B 1·0	125	700	+	+
	G	6·2	3·9	76	592	74	0·2	—	—	+	0·2	12·5
	BL	5·8	3·5	66	472	69	W 1·3	B 1·0	—	+	12·5	—
	BF	6·4	2·4	78	529	19	1·0	0·5	—	0·5	0·5	0·2
13Z	C	6·4	5·1	57	25	67	12·5	1·3	—	2·3	7×10^4	700
	G	6·3	3·0	62	21	72	0·8	1·8	1·25	700	225	700
	BL	5·8	3·2	65	1	69	12·5	B 0·5	—	1·3	0·2	W 4·8
	BF	6·4	2·5	73	30	19	10·0	1·3	—	0·8	0·6	100

Brines (hand pumped)

	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)
13X	26	1000	5000
13Z	26	1000	0

* C = collar; G = gammon; BL = back lean; BF = back fat.

\+ = Clostridia detected in 25-g sample.

— = Clostridia not detected in 25-g sample.

W = *Cl. perfringens* detected in 25-g sample.

B = *Cl. botulinum* detected in 25-g sample.

TABLE 4. Comparison 3: presumptive *Clostridium* spp. (cells/g) in vacuum-packed bacon of higher pH cured with and without nitrate

Coding cure	Bacon type	Analytical data					Storage conditions					
								15°			20°	25°(C)
		pH	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	Water (%)	0	7	13	19	12	9 days
14X	C*	6·4	5·6	101	503	64	0·5	+	700	W700	700	700
	G	6·0	3·3	67	515	72	—	1·3	700	7·0	4·8	12·5
	BL	6·4	4·6	129	629	68	0·5	—	B +	+	700	0·2
	BF	6·5	2·8	93	450	22	2·3	0·5	55	+	0·2	2·3
14Z	C	6·4	5·8	96	30	65	0·8	+	700	W700	700	700
	G	6·15	3·6	67	28	72	—	+	700	700	700	700
	BL	6·3	5·0	143	34	68	+	0·8	—	+	4·8	—
	BF	6·5	2·8	10	14	22	4·8	1·3	2·3	W225	12·5	+

Brines (hand pumped)

	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)
14X	26	1000	5000
14Z	26	1000	0

* C = collar; G = gammon; BL = back lean; BF = back fat.

\+ = Clostridia detected in 25-g sample.

— = Clostridia not detected in 25-g sample.

W = *Cl. perfringens* detected in 25-g sample.

B = *Cl. botulinum* detected in 25-g sample.

TABLE 5. Comparisons 4 and 5: presumptive *Clostridium* spp. (cells/g) in vacuum packed bacon cured with and without nitrate and with increasing nitrite to the legally permitted (200 ppm) maximum in the absence of nitrate

Curing code	Bacon type	Analytical data					Storage conditions								
								5°		15°			20°	25° (C)	
		pH	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	Water (%)	0	20	33	9	14	19	12	6 days	
15X	C*	6·0	5·4	126	746	63	W2·3	1·3	NT	W0·8	2·3	—	W75	W7000	
	G	5·7	4·5	90	670	68	2·3	1·3	NT	W0·2	+	NT	—	W	+
	BL	5·7	3·1	64	432	69	B 2·3W	NT	0·2	NT	NT	NT	+		+
15Z	C	5·9	5·0	105	186	67	W55	W0·8	NT	W4·8	550	W0·2	$5·5 \times 10^5$	W	$2·8 \times 10^6$
	G	5·7	4·8	88	63	71	W2·3	0·5	NT	0·2	0·2	NT	0·2		0·2
	BL	5·6	3·1	81	64	69	B 4·8W	NT	1·3	NT	NT	NT	55	W	+
16X	C	5·9	5·2	110	756	66	W0·2	0·2**	NT	0·2	0·2	NT	0·2		0·2
	G	5·7	3·2	47	508	73	W1·3	+ **	NT	0·2	+	NT	0·5		+
	BL	5·7	3·1	65	466	69	1·2	NT	1·3	—	NT	W+	—		+
16Z	C	6·0	5·3	225	97	67	B 0·4W	W0·2**	NT	0·5	W +	NT	+	B 7500	
	G	5·7	3·4	101	101	73	W1·3	+ **	NT	W +	0·2	NT	—		—
	BL	5·7	3·7	102	104	68	W0·5	NT	W0·2	NT	NT	W0·2	W +		1·3

Brines (hand pumped)

	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	
15X	26	1000	5000	Comparison 4
15Z	26	1000	0	
16X	26	1000	5000	Comparison 5
16Z	26	2000	0	

* C = collar; G = gammon; BL = back lean.
\+ = Clostridia detected in 25-g sample.
— = Clostridia not detected in 25-g sample.
NT = Not tested.
** = Stored at 5°C for twenty-one days.
W = *Cl. perfringens* detected in 25-g sample.
B = *Cl. botulinum* detected in 25-g sample.

TABLE 6. Comparison 6: presumptive *Clostridium* spp. (cells/g) in vacuum packed bacon cured with and without nitrate using multi-needle injection

Curing code	Bacon type	Analytical data					Storage conditions				
								15°		20°	25° (C)
		pH	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	Water (%)	0	14	19	12	6 days
17X	C*	6·1	4·3	70	22	71	W2·0B	2500	NT	W2300	1·3
	BL	5·8	5·3	93	25	72	W1·3B	NT	+	W0·2	W0·4
17Z	C	6·1	4·6	76	573	71	+	W+	NT	1·3	W0·2
	BL	5·7	4·5	78	655	73	+	NT	+	—	—

Brines

	Injection (multineedle)			Immersion		
	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)
17X	17	700	0	26	1000	0
17Z	17	700	2700	26	1000	4200

* C = collar; BL = back lean.
\+ = Clostridia detected in 25-g sample.
— = Clostridia not detected in 25-g sample.
NT = Not tested.
W = *Cl. perfringens* detected in 25-g sample.
B = *Cl. botulinum* detected in 25-g sample.

TABLE 7. Comparisons 7–10: presumptive *Clostridium* spp. (cells/g) in vacuum packed bacon cured by multineedle injection and dry salting processes in the presence and absence of nitrate

Curing code	Bacon type	Analytical data					Storage conditions				
								15°		20°	25°(C)
		pH	NaCl (%)	$NaNO_2$ (ppm)	$NaNO_3$ (ppm)	Water (%)	0	15	19	11	5 days
19X	C	6·2	4·8	96	26	68	W +	7300	NT	W +B	70 000
	BL	5·8	5·8	128	0	72	0·2	NT	W +	—	+
19Z	C	6·2	4·7	93	568	70	W0·2	W1·3	NT	W0·2	2500
	BL	5·9	5·1	100	689	72	+	NT	—	0·2	—
20X	C	6·0	4·6	83	4	67	+	10000	NT	—	W70 000
	BL	5·8	4·9	84	18	72	+	NT	+	+	+
20Z	C	6·0	4·4	114	2	72	+	+	NT	+	0·6
	BL	5·7	3·9	100	20	74	+	NT	+	—	0·5
21X	C	6·2	4·9	111	623	70	W0·2	W +	NT	0·2	120
	BL	5·9	5·2	96	586	72	W +	NT	W +	+	W +
21Z	C	6·2	4·7	127	408	69	1·3	W +	NT	7000	W +
	BL	5·8	4·0	92	381	73	W +	NT	+	0·2	W +
22X	C	6·1	4·6	123	32	71	W +	+	NT	W2·3 × 10^5‡	W +**
	BL	5·8	4·0	92	27	73	+	NT	W0·2	W0·2‡	+**
22Z	C	6·2	5·0	165	526	69	1·3	47	NT	0·2‡	W0·2**
	BL	5·7	3·6	89	374	73	+	NT	—	W +	—**

Brines as Table 1.
C = collar; BL = back lean.
+ = Clostridia detected in 25-g sample.
— = Clostridia not detected in 25-g sample.
B = *Cl. botulinum* detected in 25-g sample.
W = *Cl. perfringens* detected in 25-g sample.
NT = Not tested.
** = Stored at 25°C/6 days.
‡ = Stored at 20°C/12 days.

TABLE 8. Growth of *Clostridium* spp. in vacuum-packed bacon stored at 15°, 20° and 25°C

Increase over initial count	Collar	Gammon	Back lean	Back fat	Totals
X10	*29/76	9/40	2/69	3/24	43/209
X100	23/76	8/40	1/69	1/24	33/209

* An X10 increase in *Clostridium* count occurred in twenty-nine of seventy-six samples tested.

TABLE 9. The effect of nitrate on growth of *Clostridium* spp. in vacuum-packed bacon stored at 15°, 20° and 25°C

Increase over initial count	Nitrate added brines				Nitrate free brines			
	Collar	Gammon	Back lean	Back fat	Collar	Gammon	Back lean	Back fat
Hand pumped								
X10	*8/23	2/20	1/20	1/12	10/23	7/20	1/19	1/12
X100	5/23	1/20	1/20	1/12	8/23	7/20	0/19	1/12
Multineedle								
X10	2/9	—	0/9	—	6/9	—	0/9	—
X100	2/9	—	0/9	—	6/9	—	0/9	—
Multineedle and dry salt								
X10	2/6	—	0/6	—	1/6	—	0/6	—
X100	1/6	—	0/6	—	1/6	—	0/6	—
Totals								
X10	12/38	2/10	1/35	1/12	17/38	7/20	1/34	1/12
X100	8/38	1/20	1/35	1/12	15/38	7/20	0/34	1/12

* An X10 increase in *Clostridium* count occurred in eight of twenty-three samples tested.

curing processes. If growth was taken to be a hundred-fold increase in MPN count, growth occurred more frequently in collar bacon (23/76) than in back (1/69) or gammon (8/40) (Table 8), the highest count (5×10^5/g) being in a sample of vp collar bacon cured in nitrate free brine and stored at 20°C for twelve days (Table 5). The pH of collar bacon was higher than that of back bacon. Additionally 'Collar' comprises a large number of small muscles, some of which are of particularly high pH. This tendency to high pH probably promotes growth of *Cl. perfringens*. Growth (hundred-fold increase) occurred more frequently on bacon cured in nitrate free brines (23/104) than that cured in brines containing nitrate (11/105) (Table 9), and numbers were generally greater on bacon from nitrate-free brines.

TABLE 10. The detection of *Cl. botulinum* in bacon

Number of samples	Sample weight (g)	*Cl. botulinum*	
		Present in	Type
(1)			
96 (collar)	25	5	B(4) A(1)
48 (gammon)	25	0	—
119 (back)	25	6	B
(2)			
68 (collar)	50	3	B
42 (back)	50	3	A
(3)			
26 (collar)	175	19	A

(1) Each sample was a homogenized preparation from two to seven sides from different pigs.

(2) Collar samples from each side of thirty-four pigs. Back samples from forty-two sides of same batch of thirty-four pigs. *Cl. botulinum* was detected in back samples from opposite sides of one pig but was not detected in the collar samples from this pig.

(3) Each sample from individual sides of a batch of sixteen pigs (six sides not sampled). Sides from fifteen of the sixteen pigs contained *Cl. botulinum*.

Detection of Cl. perfringens

Clostridium perfringens was detected in sixty-four of 263 × 25 g samples of bacon but their presence was unrelated to the curing method (Tables 2–7). *Cl. perfringens* was detected more frequently in collar bacon than in back lean, back fat or gammon: initial samples collar 9/20, back lean 4/20, back fat 1/6 and gammon 2/8; samples incubated at 15°, 20° or 25°, collar 22/70, back lean 14/63, back fat 1/22, gammon 3/36. No evidence was obtained of growth of *Cl. perfringens* during storage in vacuum-packs at 15°, 20° or 25°C.

Detection of Cl. botulinum

Two hundred and thirty-four of 263 25 g bacon samples which exhibited sulphite reduction in LSUP were examined for *Cl. botulinum*. Ten contained *Cl. botulinum* type B and one *Cl. botulinum* type A. Pure culture isolates have been made from eight of the samples. The presence of *Cl. botulinum* was not related to the concentrations of nitrite and nitrate used in the brines. There was no indication that *Cl. botulinum* grew in this vp bacon whether stored at 5°, 15°, 20° or 25°C. In similar investigations sixty-eight 50 g samples of collar and forty-two 50 g samples of back bacon from the same batch of pigs and twenty-six 175 g samples from another batch of pigs were examined for *Cl. botulinum*.

TABLE 11. Incidence of *Cl. botulinum* in various foods

Reference	Food type	Weight of sample (g)	Number of samples	Total weight of samples/kg	No. of samples containing *Cl. botulinum*	Toxin type	Spores/kg sample*
This report	Vacuum-packed bacon	25	263	16·525	36	A(1) B(10)	2·17
		50	108			A(3) B(3)	
		175	26			A(19)	
Abrahamsson & Riemann (1971)	Cooked ham	30	100	3·00	5	A	1·66
Abrahamsson & Riemann (1971)	Smoked turkey	30	41	1·23	1	B	0·81
Insalata *et al.* (1969)	Vacuum-packed frankfurters	150	10	1·50	1	B	0·66
Taclindo *et al.* (1967)	Luncheon meat	12 × 2 g	73	1·75	1	A	0·57
Greenberg *et al.* (1966)	Chicken	6 × 0·5 g	1078	3·23	1	C	0·31

* Assuming one spore only in each sample shown to contain *Cl. botulinum*.

Type A *Cl. botulinum* was detected in three of the forty-two 50 g back samples and in nineteen of the twenty-six 175 g collar samples. Type B *Cl. botulinum* was detected in three of the sixty-eight 50 g collar samples (Table 10).

Discussion

The level of *Clostridium* spp. found in the initial unstored bacon samples was higher than that found by Riemann (1963) in pork products and is more in accord with the data obtained by Mol & Timmers (1970) in lean pork and ham trimmings. Riemann (1963) reported a maximum in raw pork trimmings of 51/g similar to our figure (maximum 55/g) in unstored vp bacon. Of considerable public health importance is the demonstration that presumptive *Clostridium* spp. grew in some of the vacuum packs stored at 15°, 20° and 25°C reaching a maximum of 5×10^5/g. In fourteen samples clostridia reached levels exceeding 10^3/g, all of which were collar bacon, eleven cured without nitrate and three with nitrate, suggesting that the role of nitrate in vp bacon should be investigated more thoroughly.

Twenty-four per cent of the samples examined contained *Cl. perfringens*, comparable to that reported by Hobbs *et al.* (1953) (24·1%) and by Hall & Angelotti (1965) (37%) in raw pork and 11·1% in bacon and hams. Most surveys for *Cl. botulinum* in raw meat and in non-sterile meat products have indicated a lower incidence than reported here (Table 11). In the surveys conducted in the USA or Canada differences in methodology, type, size and number of samples all contribute to the evident variation. Further variation might be attributed to seasonal and geographical distribution of *Cl. botulinum* (see Greenberg *et al.*, 1966; Kravchenko & Shishulina, 1967). That the numbers apparently increase in chronological order probably reflects improvements in methodology.

This survey has demonstrated, that *Cl. botulinum* occurs in pigs in the UK and with relatively high frequency. The possibility of growth must therefore be considered when modifications to processes involving pork are suggested. However, no growth of *Cl. botulinum* or *Cl. perfringens* was observed in bacon containing lower levels of nitrite than are used commercially, stored at relatively high temperatures. Although this survey was fairly extensive in the number and size of samples for bacteriological analysis, its limitations in relation to the size of the UK cured meat industry must be emphasized. Samples were obtained at random from one factory and in only one batch was a relatively high incidence of *Cl. botulinum* shown. *Clostridium botulinum* is found in the soil and is likely to occur from time to time in the gut of pigs but the routes by which the musculature of pigs becomes contaminated with it are essentially unknown. Pre-slaughter stress and stress susceptibility of pig breeds might be involved since it has been established that the musculature of cattle slaughtered while under stress contains more clostridia than that of rested animals (Narayan & Takács, 1966).

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Changes in quality of cassava roots during storage

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Summary

Changes that occurred during the storage of fresh cassava roots and their effect upon acceptability of the roots both for human consumption as a fresh vegetable and for animal feed purposes are reported. During storage there was a rapid accumulation of total sugars accompanied by a small decline in starch content. In those roots showing internal discolouration and deterioration the percentage of sucrose declined very dramatically. Although roots softened during storage they required a longer cooking time for human consumption. In most cases roots remained of acceptable eating quality over an eight-week period although none of the stored roots were as good as freshly harvested roots. All stored roots had a sweet flavour and frequently an uneven texture not present in fresh roots. Cassava intake by pigs was lower for stored than for freshly harvested roots: this reduction was more marked for sweet than for bitter varieties which suggests that hydrocyanic acid content is not the only factor limiting consumption, texture and organoleptic changes may also be important. Despite all the changes that occurred during storage the feeding quality of cassava meal in rat feeding trials was not noticeably affected, thus for practical purposes the preparation of cassava meal for diets for domestic animals, notably chicken and pigs, might eliminate the limitations observed in texture and eating quality of stored roots.

Introduction

A large percentage of the world's cassava (*Manihot esculenta* Crantz) production, estimated at around 105 million tons of fresh roots from a cultivated area of 11 million ha in 1972 (FAO, 1972), is used as human food either as a fresh vegetable or in some simple processed form (Phillips, 1974). Cassava is also used for animal feed, more than

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one million tons of dried cassava being imported annually into European Economic Community (EEC) countries for this purpose (Coursey & Halliday, 1974; Phillips, 1974), and industrial uses, mostly based on the production of starch.

Although information exists on the chemical composition of cassava roots and on some of the changes in carbohydrate constituents that occur during growth (Ketiku & Oyenuga, 1972), little information is available as to the specific composition and quality requirements for the different uses.

The rapid deterioration that normally occurs within days of harvesting cassava roots (Booth, 1975a) renders them completely unacceptable for human consumption, reduces their acceptability for animal feed, and lowers the quality of starch obtained from them. As a result of studies on the dehydration of cassava, Kuppuswamy (1962) stated that, 'If there is a delay of three days before the material is dried, its keeping quality is adversely affected as shown by organoleptic evaluation and reflected in the rapid increase in water soluble acidity'. Pacheco (1952) noted that storage of roots for 24, 48 and 72 hr gave starch of progressively worse quality in terms of colour and type of sedimentation and that these changes preceded the occurrence of any visual deterioration. He concluded that for the manufacture of starch, storage of cassava roots for longer than 24 hr was not advisable.

As long-term storage of fresh cassava has hitherto not been generally practised except to a limited extent by waxing and in high cost systems such as refrigeration (Ingram & Humphries, 1972), little information is available as to the biochemical and quality changes that occur during storage. During a four-week storage period at 5°C, Czyhrinciw & Jaffe (1951) recorded a continuous rise in starch content in close parallel to the rise in dry weight or loss of moisture, a considerable drop in vitamin C content and little change in pH. No information was presented on changes in sugar contents. Booth & Coursey (1974) reported that roots stored in field clamps consistently tasted sweeter and less bitter than fresh roots of the same variety and that preliminary trials indicated that a slight conversion of starch to sugars and reduction in hydrocyanic acid level occurred during storage

For both human and animal consumption, particularly when used fresh, it is desirable that the hydrocyanic acid content be as low as possible. The toxicity implications of the cyanide content of cassava in human (Coursey, 1973) and animal (Maner & Gomez, 1973) nutrition have recently been reviewed together with other considerations relating to cyanogenesis in cassava (Nestel & MacIntyre, 1973). It has been shown that when fed free-choice together with a protein supplement, daily consumption by growing pigs of fresh sweet cassava (i.e. varieties of low cyanide content) was three times higher than that of bitter cassava (i.e. varieties of high cyanide content) (CIAT, 1973). Although the differences when fed cassava meal were considerably less, meal prepared from sweet varieties was still preferred by pigs, to that made from bitter varieties. For industrial purposes, high starch content is the major requirement.

Two simple techniques that allow the storage of fresh cassava roots for up to three

months have recently been developed (Booth, 1975b). As well as providing a weight of produce as near as possible to the original weight placed in store at the end of required storage period, a further requirement of any system is that the stored produce be of acceptable quality. This paper reports some chemical, biochemical and biophysical changes that occurred during the storage of fresh cassava roots and the effect these changes had on the acceptability of the roots both for human consumption as a fresh vegetable and for animal feed purposes.

Materials and methods

All of the storage work, animal feeding trials and associated biochemical tests reported in this paper were undertaken at the Centro Internacional de Agricultura Tropical, Cali, Colombia and the studies on carbohydrate changes and the organoleptic trials at the Instituto de Investigaciones Tecnologicas, Bogota, Colombia.

Storage systems

For biochemical and biophysical tests and for human acceptability trials, roots of the cultivar 'Llanera' harvested at twelve to thirteen months were used. For animal feeding trials the sweet cultivar (M. Col. 22) and the bitter cultivar (CMC. 84) were used in the pig experiments and the sweet cultivar (M. Col. 1148) and the bitter (CMC. 84) for the rat experiments.

Two root storage systems were used: field clamps and storage boxes (Booth, 1975b). Each field clamp contained approximately 300 kg of roots and was constructed with a 5-cm thick straw cover, a 30-cm thick soil cover and with four basal radial and one central vertical straw ventilators. Each storage box contained 16–20 kg of roots packed in moist sawdust and was stored in a small ventilated shed. Roots were placed in storage on the same day as they were harvested and with the exception of very small roots, all harvested roots were utilized. Sound roots were removed from the clamps and/or boxes as required for the various trials.

Biochemical tests

(i) Moisture content was determined weekly following storage in field clamps and bi-weekly following storage in boxes, over an eight-week period. One-third portions of each of six roots were peeled, chopped into small pieces and duplicate samples dried to a constant weight in a forced draught oven at 100°C.

(ii) Soluble carbohydrate determinations were made, at weekly intervals following storage in field clamps and at bi-weekly intervals following storage in boxes, over an eight-week period. One-third portions of each of six roots were peeled, dried in a forced draught oven at 50°C and ground (so as to pass a 60-mesh grid) using a Wiley mill. Determinations of starch, total sugars, reducing sugars, and individual sugars were made.

In a second storage trial carbohydrate determinations were made on samples taken on alternate days over a ten-day period. In the case of the roots kept at ambient temperature, samples taken after six days showed symptoms of primary deteriorations and sampling was discontinued. At each sampling time a central peeled portion of a single root was blended with distilled water and vacuum-freeze-dried in the case of the stored roots but oven dried at 65°C in the case of the roots kept at ambient temperature. These dried samples were ground and five subsamples taken for starch, total sugars, reducing sugars, and individual sugars determinations.

For the determination of starch, method number 22045 of the Association of Official Agricultural Chemists (AOAC, 1965) recommended for grains and stock feeds was used. For the determination of total and reducing sugars AOAC methods 13028 and 13029 recommended for use with flours were used.

Individual sugars were extracted for identification and quantification using the method recommended by AOAC (1965) and derivatives prepared for gas chromatography using methods one and four of the *Handbook of Silylation* (1970). The sugars were identified using a Perkin Elmer gas chromatograph (model 3920) and quantified by calculating the area of the peaks as a percentage of the total area of recorded peaks.

Biophysical tests

An estimate of the texture of uncooked roots was made by measuring the force required to penetrate 1·5 cm into a 3 cm thick transverse root slice using a 3 mm diameter flat ended probe attached to an Instron Universal Tensile Tester.* The press was adjusted to a crosshead speed of 10 cm per minute and a chart speed of 20 cm per minute. Measurements were made on individual transverse slices from six roots in both central tissue, 1 cm from the centre and 1·5 cm apart, and in peripheral tissues, 1 cm from the outside and 1·5 cm apart. The number of measurements per sample therefore varied with root diameter and is presented together with the average force required for penetration.

This method of texture determination could not be used on cooked samples, as a single measurement caused the sample to disintegrate. An evaluation of texture of cooked samples was therefore included in the organoleptic tests.

Organoleptic tests

Evaluations of colour, smell, taste and texture of roots stored over an eight-week period in field clamps and boxes were made and compared with those of freshly harvested roots. On each occasion one-third peeled samples from each of six roots were used and from these portions a test piece approximately 7 cm long was taken and cut longitudinally into pieces of approximately 3 cm thick. These pieces were cooked for 20–30 min in 700 ml of water to which 7 g of table salt was added. Two or three of the

* Instron Corporation, Canton, Mass., U.S.A.

cooked root pieces were examined by a minimum of four trained taste panelists and scored according to the following scale for colour, smell and taste: 5, excellent; 4, good; 3, fair/acceptable; 2, poor; 1, bad.

The number of panelists and their average score for each character is presented. The texture of the cooked samples is described since it could not be measured.

Animal feed tests

Chemical analysis. Chemical composition of freshly harvested and stored cassava roots as well as of their dried meal samples were performed by routine proximate analyses (AOAC, 1965). Hydrocyanic acid determination was performed by the method described by Wood (1965), with the addition of a crude homogenate preparation of linamarase (Wood, 1966) during the hydrolysis period.

Pig feeding trial. Twelve weaned pigs were distributed according to sex (one castrated male and two females) and body weight (approx. 30 kg) to the following four treatment groups: fresh sweet cassava; stored sweet cassava; fresh bitter cassava; stored bitter cassava. Cassava roots were stored in field clamps for at least two weeks before being fed to pigs. All experimental groups had free access, in a separate feeder, to a protein supplement supplying 40% crude protein and were allowed to drink water *ad libitum.* The composition of the protein supplement expressed in percentages was: soybean meal, 79·6; corn, 8·4; bone meal, 10·0; mineral premix, 1·25; vitamin premix, 0·75. Cassava roots were chopped daily and supplied to pigs in open-troughs every morning. Daily cassava and protein supplement consumptions were recorded throughout the twenty-eight day experimental period. Dry-matter determination of cassava samples were performed daily during the third week of the experimental period and the average values were used to calculate the cassava dry-matter consumption during the entire experimental period. Body weight changes of all pigs were determined weekly.

Rat feeding trial. Fresh roots and roots stored for two weeks in field clamps and storage boxes of both sweet and bitter cassava cultivars were chipped, oven-dried (65°C) and ground into meals. Because of the wide variation in nitrogen content of the meal samples, as analysed by micro-Kjeldahl, a total of three cassava starch-soybean meal control diets were included in the rat feeding experiment. Fifty-four weanling albino rats (Sprague-Dawley) with an overall average body weight of $53{\cdot}2 \pm 0{\cdot}6$ g were randomly allotted to nine experimental groups of six rats (three males and three females) per group. The composition of the experimental diets for growing rats is shown in Table 1. Rats were individually housed in cages with raised screen bottoms and were allowed to consume the diets and water *ad libitum.* Weight changes as well as feed consumption were obtained twice weekly during the twenty-eight day experimental period.

Results

Biochemical tests

Very little loss of moisture from cassava roots took place during storage in field

TABLE 1. Composition of the experimental diets for rat feeding trial

Ingredient	Control diets % crude protein in diet*			Cassava meal† diets (%)
	10·7	12·5	14·9	
Cassava meal	—	—	—	75·0
Cassava starch	70·4	66·8	62·0	—
Soybean meal	21·6	25·2	30·0	17·0
Minerals‡	4·0	4·0	4·0	4·0
Vitamins§	2·0	2·0	2·0	2·0
Corn oil	2·0	2·0	2·0	2·0
DL-methionine	0·2	0·2	0·2	0·2

* Protein content: N × 6·25.

† Cassava meals were prepared from fresh and two-week stored (field clamps and storage boxes) sweet (M. Col. 1148) and bitter (CMC. 84) cassava roots.

‡ Salt Mixture USP XIV, Nutritional Biochemical Corporation, Cleveland, Ohio.

§ Vitamin Diet Fortification Mixture, Nutritional Biochemical Corporation, Cleveland, Ohio.

TABLE 2. Effect of storage on moisture content of cassava roots (cultivar Llanera)

Weeks in storage	Percentage of moisture content of roots stored in	
	Field clamps	Storage boxes
0 (Fresh)	62·2	62·2
1	60·2	—*
2	61·2	66·7
3	60·7	—
4	63·2	67·1
5	60·8	—
6	61·2	59·2
7	59·9	—
8	62·1	59·8

* — No samples taken.

clamps (Table 2). During the first four weeks of storage in boxes the moisture content of the roots increased slightly but subsequently fell to slightly below the original level.

In roots stored in both field clamps and storage boxes there was a rapid two to three-fold increase in root total sugars (Table 3). This sharp rise in sugar content was accompanied by an approximate 10% drop in starch content. Following these initial rapid changes, which appeared to occur largely during the first one or two weeks of storage, the levels of total sugars and starch remained relatively constant although the starch content continued to decline slowly. The content of reducing sugars in the roots rose, reaching a maximum of 3·6 times the original level after four weeks storage in field clamps, and 2·9 times the original level after two weeks storage in boxes, after which the levels declined to approximately their original levels (Table 3). The total content of carbohydrates (starch plus total sugars) remained relatively constant throughout the eight-week storage period.

TABLE 3. Effect of storage on the carbohydrate content of cassava roots (cultivar Llanera)

Weeks in storage	Storage system					
	Total sugars (% dry wt)		Reducing sugars (% dry wt)		Starch (% dry wt)	
	Clamps	Boxes	Clamps	Boxes	Clamps	Boxes
0 (Fresh)	5·2	5·2	1·6	1·6	84·4	84·4
1	13·9	—*	3·7	—	76·8	—
2	15·9	14·2	4·0	2·6	75·1	75·3
3	14·3	—	4·4	—	76·1	—
4	15·9	12·0	5·8	3·1	73·3	74·1
5	15·0	—	4·2	—	74·9	—
6	16·4	11·4	3·3	2·2	74·9	79·0
7	15·8	—	3·2	—	74·5	—
8	14·7	12·2	2·2	1·2	71·6	69·3
Std deviation (N=5)	±0·22		±0·02		±0·7	

* — No samples taken.

These changes in the content of reducing sugars were paralleled by a similar variation in the proportion of the reducing sugars (α- and β-glucose) to total sugars (Table 4). During the first four weeks of storage in field clamps and the first two weeks of storage in boxes there was a decline in the percentage of sucrose and an increase in the percentage of fructose and α- and β-glucose in the total sugars. This situation was reversed during the final weeks of the eight-week storage period such that the final percentage of sucrose in the total sugars was higher and that of fructose and α- and β-glucose was lower than the original percentages in the fresh roots (Table 4).

TABLE 4. Effect of storage on the sugar constituents of cassava roots (cultivar Llanera)

Weeks in storage	Storage system							
	Sucrose (% total sugars*)		Fructose (% total sugars*)		α Glucose (% total sugars*)		β Glucose (% total sugars*)	
	Clamps	Boxes	Clamps	Boxes	Clamps	Boxes	Clamps	Boxes
0 (Fresh)	73·1	73·1	13·6	13·6	6·6	6·6	6·8	6·8
1	67·3	—†	14·5	—	9·8	—	10·0	—
2	66·3	62·5	14·2	14·5	8·4	11·5	9·1	11·5
3	63·0	—	16·5	—	6·6	—	13·9	—
4	55·3	82·4	21·0	6·4	11·3	5·4	12·5	5·7
5	70·5	—	13·2	—	6·2	—	10·0	—
6	76·5	87·1	8·5	5·3	7·1	3·6	7·9	4·0
7	83·2	—	7·3	—	4·0	—	5·6	—
8	83·5	88·6	7·3	4·4	3·9	3·4	5·3	3·5

* Calculated as a percentage of total area of recorded peaks.

† — No samples taken.

TABLE 5. Changes in carbohydrate content of cassava roots (cultivar Llanera) during the first ten days of storage

Days in storage	Storage system								
	Reducing sugars (% dry wt)			Total sugars (% dry wt)			Starch (% dry wt)		
	Clamps	Boxes	Ambient	Clamps	Boxes	Ambient	Clamps	Boxes	Ambient
0 (Fresh)	1·9	1·9	1·9*	7·2	7·2	7·2*	82·5	82·5	82·5*
2	3·2	1·9	3·6*	11·2	10·2	11·6*	80·9	81·3	78·6*
4	3·3	1·6	4·1*	14·5	7·4	8·4*	81·7	80·4	79·4*
6	3·5	1·4	4·1**	12·3	7·2	8·9**	79·8	81·6	77·6**
8	4·5	1·9	—†	15·5	8·4	—	74·6	81·6	—
10	3·6	2·6	—	14·0	9·1	—	74·5	81·7	—
Std deviation (N=5)	±0·02			±0·22			±0·7		

* Roots with no symptoms of post-harvest deterioration.

** Roots showing symptoms of primary deterioration.

† — No samples taken.

N.B. Samples prepared from roots stored in boxes or clamps were vacuum-freeze-dried whereas those from roots kept at ambient were oven dried at 65°C.

TABLE 6. Changes in the sugar constituents of cassava roots (cultivar Llanera) during the first ten days of storage

Days in storage	Storage system											
	Sucrose (% total sugars*)			Fructose (% total sugars*)			α Glucose (% total sugars*)			β Glucose (% total sugars*)		
	Clamps	Boxes	Ambient	Clamps	Boxes	Ambient	Clamps	Boxes	Ambient	Clamps	Boxes	Ambient
0 (Fresh)	80·9	80·9	80·9	5·5	5·5	5·5	7·1	7·1	7·1	6·5	6·5	6·5
2	74·8	88·8	72·0	10·2	4·2	8·6	6·9	3·5	8·4	8·1	3·5	10·5
4	75·3	79·7	62·6	9·1	7·0	14·0	6·8	6·5	11·2	8·3	6·8	12·2
6	71·8	84·5	8·3‡	10·2	6·4	39·8‡	8·4	4·4	25·6‡	9·7	11·6	26·2‡
8	76·7	83·2	—†	8·3	6·5	—	6·7	5·0	—	8·4	5·2	—
10	82·6	76·4	—	6·9	9·5	—	4·9	7·3	—	5·5	7·4	—

* Calculated as a percentage of total area of recorded peaks.

† — No samples taken.

‡ Roots showing symptoms of primary deterioration.

N.B. Samples prepared from roots stored in boxes and clamps were vacuum-freeze-dried whereas those from roots kept at ambient were oven dried at 65°C.

Since the changes in starch and sugar contents essentially occurred during the first two weeks of the storage period, more frequent observations were made in a second storage trial and the results obtained indicated that metabolic changes occur during the first two to four days of storage (Table 5). During the ten-day period a continuous slow decline in the starch content and an increase in reducing sugars content occurred. The magnitude of these changes was not so great in the roots stored in boxes as in those stored in field clamps. Similar changes were also observed in roots kept for six days under ambient conditions in the laboratory (24°C) although the starch content declined more rapidly (Table 5). However, there were very obvious differences in the individual sugar components of roots stored in boxes or clamps compared with those of roots kept at ambient, especially those which showed symptoms of primary deterioration. In the latter the sucrose level declined from 62·6 to 8·3% and the fructose and glucose levels rose from 14·0 to 39·8 and 23·4 to 51·8% of the total sugars, respectively, between the fourth and the sixth day (Table 6).

Biophysical tests

The average maximum force required to penetrate the peripheral tissues of root slices declined by about 30% during the first six weeks of root storage in field clamps but returned to nearly the original levels during the final two weeks of storage (Table 7). The force required to penetrate the central tissues was always considerably less, and declined throughout the eight-week period of the trial to approximately 50% of its

TABLE 7. Effect of storage on the texture of cassava roots (cultivar Llanera); average maximum force (kg) required to penetrate 1·5 cm into a 3 cm thick transverse root slice using a 3 mm diameter flat ended probe attached to a Universal Instron Press

Weeks in storage	Peripheral tissue		Central tissue	
	Clamps	Boxes	Clamps	Boxes
0 (Fresh)	4·9 (30)*	4·9 (30)	3·7 (19)	3·7 (19)
1	3·9 (43)	—†	2·4 (29)	—
2	3·7 (39)	4·0 (26)	2·3 (24)	2·6 (16)
3	3·7 (50)	—	2·3 (32)	—
4	3·5 (56)	6·1 (47)	2·2 (29)	3·6 (27)
5	3·5 (54)	—	2·1 (30)	—
6	3·4 (49)	3·1 (36)	2·0 (26)	1·3 (21)
7	4·7 (52)	—	2·2 (33)	—
8	4·1 (56)	4·6 (45)	1·9 (30)	1·6 (23)

* Number of penetrations.

† — Sample not taken.

original value (Table 7). A similar pattern of softening occurred in roots stored in boxes. Also a similar pattern can be seen in the texture index (Culpepper & Magoon, 1924), i.e. the ratio of starch to moisture content of the roots (Table 8).

TABLE 8. Effect of storage on the starch: moisture content ratio in cassava roots (cultivar Llanera)

Weeks in storage	Texture Index (Starch content/moisture content)	
	Clamps	Boxes
0 (Fresh)	1·36	1·36
1	1·28	—*
2	1·23	1·13
3	1·25	—
4	1·16	1·10
5	1·23	—
6	1·22	1·33
7	1·24	—
8	1·15	1·16

* — Sample not taken.

Organoleptic tests

The eating quality, when boiled and tested as a fresh vegetable, of the freshly harvested roots was excellent from all points of view: colour, smell and taste, and the texture was normal although slightly fibrous (Table 9). The cooking time required to prepare the stored root samples was slightly longer (30 min for roots stored in field clamps, 25 min for roots stored in storage boxes) than required for fresh roots (20 min). Although the eating quality of none of the stored samples was as good as that of the fresh roots, their colour and small remained of acceptable quality throughout the eight-week storage period in both boxes and clamps. The taste quality of all the stored roots declined immediately but remained acceptable throughout the eight-week storage period in boxes; following storage in clamps it remained acceptable for four weeks and after five to eight weeks was poor (Table 9). All stored roots had a sweet flavour not present in fresh roots and in occasional samples there was also a residual bitter flavour. The texture of the cooked samples prepared from stored roots was less good than that of the freshly harvested roots: they were in general harder and more fibrous, but the loss of quality of texture was largely due to loss of uniformity and the development of an irregular texture. It was noted that some areas of the roots became somewhat translucent, semicrystalline and of an undesirable texture. These texture changes in the cooked samples were less severe in roots stored in boxes than in those stored in clamps.

TABLE 9. Effect of storage on the quality of cassava roots (cultivar Llanera) for human consumption as a fresh vegetable; organoleptic tests

	No. of panelists	Cooking time (min)	Observations on cooked samples*			
			Colour†	Smell†	Taste†	Texture
Weeks in storage clamps						
0 (Fresh)	7	20	4·8	4·7	4·7	Normal, slightly fibrous
1	7	30	3·3	3·1	2·7	Slightly hard and fibrous
2	6	30	4·2	4·2	3·2	Slightly hard and fibrous
3	4	30	3·6	3·5	2·7	Hard and fibrous
4	6	30	3·9	3·5	3·0	Slightly hard and fibrous
5	6	30	3·7	3·7	2·3	Hard and slightly fibrous
6	6	30	3·2	3·2	2·4	Hard and slightly fibrous
7	6	30	2·8	3·3	2·7	Slightly hard and fibrous
8	6	30	3·5	3·3	2·3	Hard and fibrous
Weeks in storage boxes						
0 (Fresh)	7	20	4·8	4·7	4·7	Normal, slightly fibrous
2	6	25	4·5	4·2	3·1	Normal, slightly fibrous
4	6	25	3·7	3·3	2·4	Slightly hard and fibrous
6	6	25	4·2	4·2	3·8	Normal, slightly fibrous
8	6	25	3·9	3·5	3·4	Slightly hard and fibrous

* Average of all panelists.

† Panelist's scale: excellent, 5; good, 4; acceptable, 3; poor, 2; bad, 1.

Animal feed tests

Chemical changes. The effects of storage systems and length of storage on the proximate chemical composition of sweet and bitter cassava flours are shown in Table 10. Chemical

composition is expressed on dry-matter basis, however residual moisture content of cassava flours (prepared by oven-drying cassava chips at 65°C) decreased with the

TABLE 10. Effect of storage on the chemical composition of sweet and bitter cassava flours*

System and period of storage	Apparent protein (N × 6·25) (%)	Ether extract (%)	Crude fibre (%)	Ash (%)	N free extract (%)
Sweet cassava (M. Col. 1148)					
Fresh	2·68	1·22	4·47	3·01	88·62
Field clamps, 2 wk	2·48	1·47	3·77	3·97	88·31
Field clamps, 4 wk	3·89	5·24	3·74	3·04	84·09
Stor. boxes, 2 wk	2·48	1·47	3·77	3·97	88·31
Stor. boxes, 4 wk	1·98	4·88	4·29	5·33	83·52
Bitter cassava (CMC. 84)					
Fresh	8·48	1·06	3·72	3·76	82·98
Field clamps, 2 wk	8·22	2·49	4·15	3·92	81·22
Field clamps, 4 wk	8·08	2·93	3·64	3·31	82·04
Stor. boxes, 2 wk	5·43	1·02	3·73	3·66	86·16
Stor. boxes, 4 wk	7·58	2·28	3·68	5·65	80·81

* Cassava roots were sliced, oven-dried in chips at 65°C and then ground. Values expressed on dry-matter basis.

length of the storage period. For instance, cassava flours prepared from fresh, two-week and four-week stored (field clamps) sweet cassava roots showed moisture contents of 4·3, 4·2 and 2·6%, respectively. A similar trend was observed for both cassava varieties stored under the two experimental storage systems.

All bitter cassava samples exhibited higher nitrogen, and therefore apparent protein (N × 6·25) contents than the sweet cassava flours (Table 10). Some differences were expected since nitrogen determination by micro-Kjeldahl, includes all nitrogen-containing compounds, and among these hydrocyanic acid, but the magnitude of the difference cannot be accounted for by the differences in the hydrocyanic acid content alone. Cassava flours from the bitter and sweet cultivars had hydrocyanic acid contents in the ranges of 250–350 ppm and 50–100 ppm respectively, on dry-matter basis. A considerable proportion of nitrogen content in cassava appears to be undefined (Calderon, 1973). Nitrogen content tended to decrease with length of storage but the results were not consistent (Table 10).

The ether extract fraction appears consistently to increase along with the length of the storage period. The increments were higher for the sweet than for the bitter cassava flours (Table 10). Crude fibre and ash contents did not change appreciably during storage. Nitrogen-free extract values were obtained by difference and therefore reflect

TABLE 11. Effect of storage (field clamps) of sweet and bitter cassava cultivars on their nutritive value for growing pigs

Parameter*	Experimental variable			
	Sweet cassava (M. Col. 22)		Bitter cassava (CMC. 84)	
	Fresh	Stored	Fresh	Stored
Cassava dry-matter (%)†	40·0	38·7	30·5	31·7
Hydrocyanic acid content in cassava (ppm DM basis)	280·2	114·3	574·5	687·9
Av. daily intake				
Cassava as fed (kg)	1·90	1·68	1·61	1·43
Protein supplement (kg)‡	0·51	0·91	0·81	0·87
Total dry feed (kg)	1·27	1·56	1·30	1·33
Percent dry matter fed as cassava	59·8	41·7	37·7	33·8
Av. intial body wt (kg)	30·5	30·9	31·0	30·6
Av. daily gain (kg)	0·57	0·75	0·63	0·66
Feed/gain (dry matter basis)	2·23	2·06	2·07	2·03

* Means of three animals, fed the experimental diets for a twenty-eight day period.

† Dry-matter determinations of cassava samples were performed daily during the third week of the experimental period.

‡ The protein supplement supplied 40% crude protein and contained 88·2% dry matter.

part of the changes of other constituents previously reported; in general, they do not appear to be appreciably affected by storage systems or by the length of the storage period, at least up to four weeks.

Pig feeding experiment. The results of the pig feeding trial are summarized in Table 11. The dry-matter content of cassava did not significantly change after two weeks of storage in field clamps. The hydrocyanic acid contents of whole cassava roots of the sweet cultivar stored for two weeks decreased to approximately 40% its initial value whereas in the bitter cultivar there was an apparent increase after storage. This difference probably also reflects the wide root to root variation observed in hydrocyanic acid levels.

Cassava intake by the pigs was lower for the stored roots than for the freshly harvested roots. There was also a consistent lower intake of the bitter cassava than of the sweet roots for both fresh and the stored roots. However, these differences in consumption were not as striking as those previously reported (CIAT, 1973). This discrepancy can be explained, at least partially, by the initial body weight of the experimental animals

and the sweet cassava variety used previously; in the previous experiment (CIAT, 1973) initial body weight of pigs was around 60 kg, whereas in the experiment herein reported it was 30–31 kg and the cultivar 'Llanera' was used. Initial body weight of experimental animals appears to be an important factor for adaptation to fresh cassava-based diets.

The low consumption of stored sweet cassava roots and of fresh and stored bitter cassava roots was compensated by a large intake of the protein supplement (Table 11) so that the quantity of the total dry feed consumption was similar for all four experimental groups. Consequently, the supply of dry matter from cassava was practically 60% for pigs fed sweet fresh cassava and dropped to 42% for pigs fed the two-week stored sweet cassava. This difference due to storage was greater for the sweet (60 *v.* 42%) than for the bitter (38 *v.* 34%) cassava varieties. These results suggest that hydrocyanic acid content is not the only factor limiting consumption of cassava by growing pigs: texture, structural and organoleptic changes may also be important.

The body gain of pigs mainly reflects protein supplement consumption Pigs fed the stored sweet cassava consumed a little more than 900 g of protein supplement per day and gained the most (750 g/day). Feed/gain was more efficient for the bitter cassava and for the stored sweet cassava fed pigs, but economically more expensive due to the higher protein supplement consumption.

TABLE 12. Effect of storage on the nutritive value of sweet and bitter cassava meals for the growing rats*

Dietary variable	% apparent prot. in diet	Total body gain (g)	Total feed intake (g)	Feed/ gain
Control diets				
Cassava starch + soybean meal (SBM)	10·7	100·2	405·4	4·08
Cassava starch + soybean meal	12·5	118·2	418·4	3·58
Cassava starch + soybean meal	14·9	120·0	421·1	3·58
Sweet cassava (M. Col. 1148) meals + SBM				
Fresh	10·8	98·7	418·5	4·24
Field clamps, 2 wk	10·7	99·3	497·0	4·20
Stored boxes, 2 wk	12·2	100·6	417·5	4·16
Bitter cassava (CMC. 84) meals + SBM				
Fresh	14·9	99·1	383·9	3·89
Field clamps, 2 wk	12·7	103·9	378·5	3·65
Stored boxes, 2 wk	14·7	105·4	411·3	3·91

* Means of six rats per group, maintained individually in cages for a twenty-eight day experimental period.

Rat feeding trial. The results of the rat experiment are presented in Table 12. Total body gain in a twenty-eight day experimental period was similar for all cassava meal-based diets, despite the apparently higher protein (N × 6·25) content of the bitter cassava meal-based diets. Feed intake however was lower for the bitter cassava meal-based diets and consequently the feed conversion was better for the rats fed these diets. Storage of cassava in field clamps or in boxes did not adversely affect the palatability of the meals as evidenced by a similar or higher consumption by the growing rats of all the cassava meal-based diets.

Discussion

It is well known that during the storage of other root crops such as potatoes and sweet potatoes continuous interconversions of starch and sugars occur. These interconversions reach a state of equilibrium at levels of the different substances which are determined by environmental conditions, particularly temperature and relative humidity, and by the response of the variety and individual root or tuber to them (Burton 1966).

During the storage of cassava roots in both field clamps and storage boxes there was a rapid accumulation of total sugars accompanied by a decline in starch content. Early in the storage period there was an increase in reducing sugar content which was followed by a decrease and a simultaneous increase in the percentage of sucrose in the total sugars. These conversions are similar to those known to occur in potatoes (Burton, 1966) and sweet potatoes (Sistrunk, Miller & Jones, 1954).

Although the gross carbohydrate changes in the cassava roots kept at ambient temperature were similar to those stored in boxes and clamps, the balance of the individual sugars changed very markedly as soon as the roots showed symptoms of primary deterioration, in particular the percentage of sucrose declined very dramatically.

The changes in texture index (starch to moisture content ratio) and the changes in pressure required to penetrate root slices, particularly in the central tissues, confirms the earlier subjective observation that roots soften during storage. This softening may in part be caused by the changes in starch and moisture content levels but may also reflect changes in starch quality. Changes in starch properties, such as granule diameter density, water holding capacity, and the substances absorbed within the granule, are known to occur during the curing and storage of sweet potatoes (Barham & Wagoner, 1946).

Although the stored roots were softer, they required a longer cocking time than fresh roots for their preparation as a fresh vegetable for human consumption. The cooked samples prepared from stored roots were generally harder and more fibrous than those prepared from fresh roots and they had an uneven texture associated with the development of firm semitranslucent and semicrystalline areas, which caused a decline in their acceptability. A similar hardening in cooked samples has on occasions been observed in potatoes following prolonged storage (Burton, 1966).

None of the stored root samples were of as good an eating quality as the freshly harvested roots. There was an immediate decline in the taste quality of stored roots,

but whereas those roots stored in boxes remained of acceptable quality for the eight-week duration of the trial, those stored in field clamps became of poor taste after five and more weeks storage. All stored roots had a sweet flavour not present in the fresh roots, reflecting increases in total sugar levels. It is similarly stated, that in sweet potatoes the chief factor causing the differences in sweetness of the cooked roots is the amount of sucrose formed in storage (Culpepper & Magoon, 1926).

Experimental data on feeding trials indicated that bitterness of cassava roots affected the consumption by the growing pigs, but apparently other factors may be as important as their content of hydrocyanic acid. The texture differences and the lowered eating quality of stored cassava roots as compared to freshly harvested roots observed and recorded in the organoleptic tests undoubtedly affected the results of the pig experiments.

Observations in our laboratories (G. Gomez, personal communication) would suggest that some of the sweet cassava cultivars exhibit a relative high hydrocyanic acid content in their peels in spite of their relative low content in whole root samples; this fact may, at least partially, explain the differences in results obtained in animal feeding trials when different cassava cultivars are used. The results of the rat feeding trial clearly demonstrated that despite all chemical, biochemical and texture changes that occurred during storage, the feeding quality of cassava meal is not noticeably affected. Actually, for practical purposes the preparation of cassava meal for diets in domestic animals, notably chicken and pigs, would eliminate the limitations observed in texture and eating quality of stored roots. The results of chemical analyses and nutritive evaluation with growing rats of cassava meals prepared from either sweet or bitter cassava roots suggest the need for better characterization of nitrogen-containing fractions of cassava roots.

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Freeze dehydrated compressed sour cherries

II. Stability of anthocyanins during storage

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Summary

The stability of anthocyanin in freeze dehydrated compressed sour cherries during storage at 21 and 38°C were studied by extraction of pigments, thin-layer chromatography and spectrophotometry. Total anthocyanin contents of freeze-dried compressed sample decreased from 9·28 mg/100 g to 5·04 and 1·10 mg/100 g, respectively, after six months at 21 and 38°C. Cyanidin-3-(2G-xylosylrutinoside) was the least stable pigment among the seven pigments identified.

Introduction

The red colour of sour cherries is attributed to the presence of several water-soluble anthocyanin pigments (Dekazos, 1970; Shrikhande & Francis, 1973). Anthocyanins are generally unstable and may change easily if not properly protected. Certain physical and chemical factors influence the degradation of these pigments while in the fresh tissue and/or during processing and storage of anthocyanin-containing products (Pratt *et al.*, 1954; Lukton, Chichester & Mackinney, 1956; Grommeck & Markakis, 1964; Goodman & Markakis, 1965; Dekazos, 1966; Daravingas & Cain, 1968; Erlandson & Wrolstad, 1972).

Recently, not only canning and freezing, but also freeze dehydration methods of preservation have been applied to sour cherries. Freeze dehydrated cherries retain better colour, flavour, texture and nutritive value than those preserved by other dehydration methods. In addition, freeze dehydration can decrease or retard the rates of biochemical, enzymatic and microbiological deterioration during subsequent storage. However, discoloration may still occur during subsequent storage of the dehydrated fruits under high temperature and high relative humidity.

In this paper we discuss the stability of anthocyanins of freeze dehydrated, compressed sour cherries with respect to storage temperature and duration.

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Materials and methods

Mature, fresh and wholesome sour cherries (*Prunus cerasus* L., var. Montmorency) were mechanically harvested from a commercial orchard of Payson Fruit Growers in Payson, Utah. The cherries were freeze dehydrated and compressed into discs 8·2 cm ($3\frac{1}{4}$ in) in diameter and 1·3 cm ($\frac{1}{2}$ in) thick by the method reported previously (Do *et al.*, 1975).

The compressed cherry discs were packed in number $2\frac{1}{2}$ size tin cans with packages of desiccant and sealed under a vacuum of 20 in Hg. The canned discs were stored at 21 and 38°C for 0, 2, 4 and 6 months, and then were removed for anthocyanin evaluation studies.

Extraction of anthocyanins

Pigments were extracted from fresh and freeze-dehydrated, compressed sour cherries by using modified procedures of Wrolstad & Putnam (1969) and Wrolstad, Putnam & Varseveld (1970).

Ten grams of fresh sour cherries or 1·72 g of freeze-dehydrated product with 8·23 g of distilled water added were homogenized and extracted three times with 50 ml portions of absolute methanol. The extract was filtered through two layers of Whatman No. 1 filter paper on a Buchner funnel and diluted one-fold with distilled water.

A water slurry of purified polyvinylpyrrolidone (PVP) was poured into a Buchner funnel containing two layers of Whatman No. 1 filter paper. Suction was applied until no surface water was visible on the PVP, which was about $\frac{1}{2}$ in thick. The anthocyanin extract was poured on the damp PVP layer. The PVP anthocyanin adsorbate was washed three times with 50 ml of methanol. The washings were checked spectrophotometrically to ensure no loss of anthocyanins. The coloured PVP anthocyanin adsorbate layer was scraped off and extracted with 0·1% methanolic HCl. The extract was filtered through Whatman No. 1 filter paper and concentrated to dryness in a flash-evaporator at 40°C and taken up again with 1% methanolic HCl.

Determination of anthocyanins

Each of the above sample solutions was diluted with 1% methanolic HCl to 1000 ml and the adsorption spectra of these pigment extracts were measured in the range of 230–630 nm with a Beckman DB-G spectrophotometer at slit width 0·05 mm, using a 1 cm cuvette, against a blank solution of 1% methanolic HCl. The results were recorded by a Beckman Linear-Log Ten-Inch Potentiometric Recorder.

Calculations of anthocyanin content were based on the molar extinction coefficient for Idaein, cyanidin-3-galactoside ($C_{12}H_{21}O_{11}.2{\cdot}5H_2O$) at 530 nm ($E = 3{\cdot}4 \times 10^4$), in 1% methanolic HCl (Siegelman & Hendricks, 1958).

The absorption spectra of the individual pigments obtained from the thin-layer chromatograms were measured in the same manner. The $AlCl_3$ shift was determined by dissolving the purified pigment in 0·01% methanolic HCl and adding 3 drops of 5% ethanolic $AlCl_3$ solution to 3 ml of the sample (Harborne, 1958).

Thin-layer chromatography of anthocyanins

The concentrated, partially purified pigments present in the 0·1% methanolic HCl extract used in the total anthocyanin assay were subjected to thin-layer chromatography on 20 × 20 cm precoated microcrystalline cellulose glass plates, 0·1 mm thick coating. The plates were developed in two directions. The solvent system for the first direction was BHClW (*n*-butanol:concentrated HCl:water = 5:2:1), and for the second direction, WHClF (water:concentrated HCl:formic acid = 8:4:1) (Nybom, 1968).

One microlitre of the concentrated extract of fresh and freeze-dehydrated sour cherries was spotted 2 cm from the bottom edge of the plate. Tanks containing solvents were equilibrated before the introduction of the plates. Ascending chromatography was used in all cases. All runs were performed at 21°C in the dark. All plates were allowed to develop to a solvent front distance of 18 cm from the bottom edge. The average R_f value of each pigment from four thin-layers was reported. The thin-layer chromatograms were examined for fluorescence under long-wave ultraviolet light.

From the thin-layer chromatograms, each pigment spot was scraped off and eluted with 1% methanolic HCl. The amount of each pigment was estimated by measurement of optical density at 530 nm and calculated in absolute quantities with the aid of the molar extinction coefficient of Idaein.

Results and discussion

Quantitative estimation of anthocyanins

The total anthocyanin content of the fresh sour cherries (13·2 mg/100 g) was higher

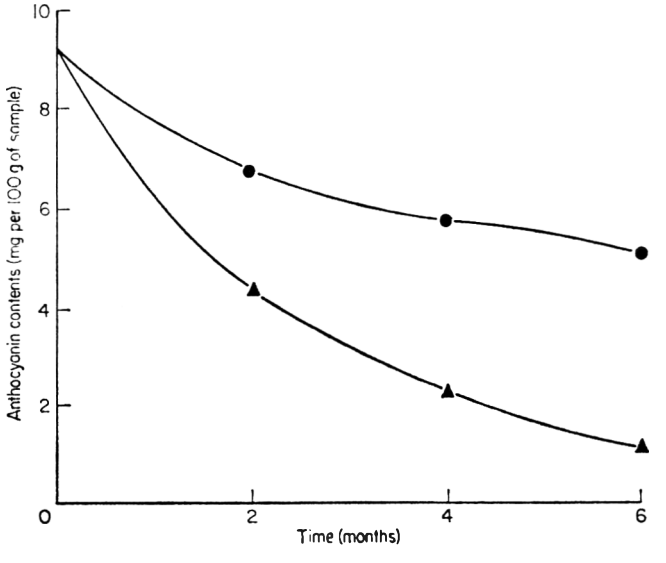

FIG. 1. Effects of storage durations and temperatures on total anthocyanin contents (calculated as Idaein) in freeze-dried compressed sour cherries. ● at 21°C; ▲ at 38°C.

than that of the freeze-dried, compressed samples (9·28 mg/100 g). Freeze-dried, compressed samples stored at 21°C retained more anthocyanin than did comparable samples stored at 38°C. The anthocyanin contents were reduced to 5·04 mg/100 g and 1·10 mg/100 g, respectively, after six months at 21°C and 38°C (Fig. 1). Since the retention of anthocyanin decreased as the temperature (Decareau, Livingston & Fellers, 1956; Adams, 1973) and storage period increased, degradation of some of the pigments must have occurred during freeze-dehydration and subsequent storage at or above room temperature.

Two maxima, 530 and 298 nm, were noticed in the absorption spectra of the anthocyanin extracts of fresh and freeze-dried, compressed sour cherries (Fig. 2). The

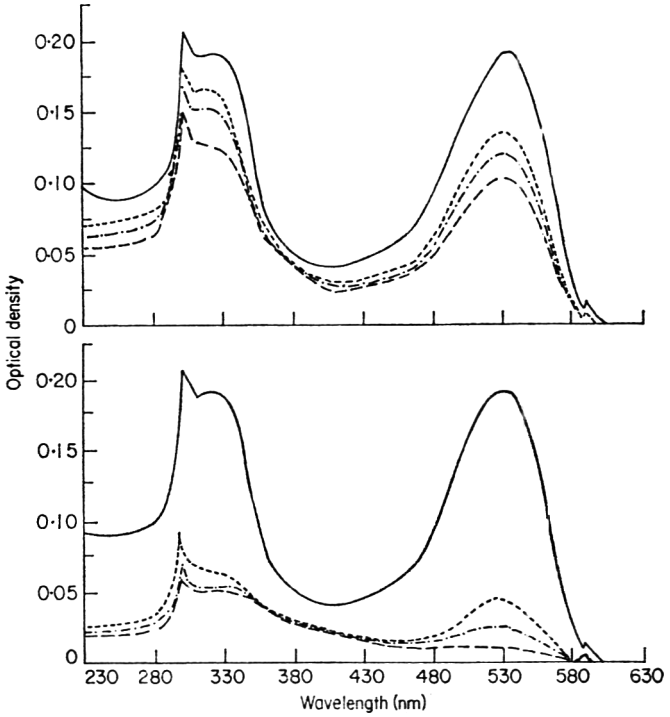

FIG. 2. The absorption spectra of anthocyanin extract of freeze-dried, compressed sour cherries after storage of 0 (—), 2 (. . . .), 4 (·—·—) and 6 months (— —) at 21°C (top) and 38°C (bottom).

absorption spectra of the anthocyanin extracts of freeze-dried samples stored at 21°C were similar to that of fresh sour cherries except that the magnitudes of optical density were less than that of the fresh samples. The absorption spectra of anthocyanin extracts from the 38°C samples showed greater decrease in optical density as the storage duration increased (Fig. 2).

TABLE 1. R_f and spectral data for anthocyanins of fresh sour cherries

Pigments‡	R_f values (× 100)*		Spectral data†				
						Appearance	
	BHClW	WHClF	λ_{max} (nm)	OD_{440}/OD_{max} (as %)	$AlCl_3$ shift	Visible	UV
A Cn	50	4	535	20	+	Light pink	Weak fluorescence
B Cn-3G	26(26) §	13(11) §	529(528)¶	32(35)¶	+(+)¶	Light pink	Dull magenta
C Cn-3RG	39(38)	31(29)	525(525)	23(20)	+(+)	Dark pink	Dull magenta
D Pn-3RG	42	37	524(524)	26(26)	−(−)	Light pink	Dull magenta
E Cn-3GG	39(39)	61(60)	526(523)	28(32)	+(+)	Light pink	Dull magenta
F Cn-3G(Xyl, R)	44(46)	75(74)	525(525)	27(27)	+(+)	Light pink	Dull magenta
G Cn-3G(G, R)	49(47)	83(82)	523(523)	29(31)	+(+)	Dark pink	Dull magenta

* Two-dimensional thin-layer chromatography. Mean value of four determinations.
† 0·01% methanolic HCl.
‡ Notation: Cn = cyanidin, Pn = peonidin, G = glucose, R = rhamnose, Xyl = xylose.
§ Reported by Nybom (1968).
¶ Reported by Shrikhande & Francis (1973).

Spectral properties

The ratio of optical density at 440 nm to optical density at the maximum is useful for distinguishing anthocyanins substituted at the 3- and 3-, 5-positions (Harborne, 1958). The spectral data for OD_{440}/OD_{max} (Table 1) indicated that all seven pigments had the 5-position free and the 3-position blocked by sugars.

Addition of $AlCl_3$ to the sample caused a positive shift in the maximum absorption peak of pigments A, B, C, E, F, and G, but a negative shift was observed with pigment D. A positive shift suggested cyanidin as the aglycone whereas a negative shift suggested peonidin (Table 1). The results concurred with the findings of Harborne (1967) and Shrikhande & Francis (1973).

Thin-layer chromatography of anthocyanins

The results of the two-dimensional thin-layer chromatography, the chemical composition of the anthocyanins in this study and the R_f data are shown in Fig. 3 and summarized in Tables 1 and 2. The same amount of each sample was applied during thin-layer chromatography.

Two-dimensional thin-layer chromatography of the anthocyanin extract of fresh sour cherries, with BHClW and WHClF as developing solvents, yielded seven distinct spots (Fig. 3a). The R_f values of the anthocyanins reported in Table 1 agreed with those observed by Nybom (1968). According to the R_f values, pigment B is cyanidin-3-glucoside, pigment C is cyanidin-3-rutinoside, pigment E is cyanidin-3-sophoroside, pigment F is cyanidin-3-(2G-xylosylrutinoside) and pigment G is cyanidin-3-(2G-

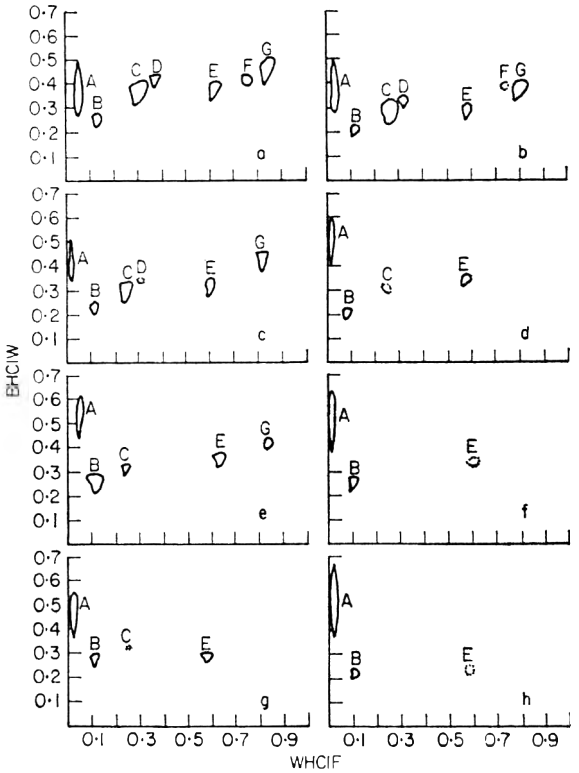

FIG. 3. Two-dimensional thin-layer chromatograms of the anthocyanins of freeze-dried compressed sour cherries stored under various conditions. a, Fresh fruit; b, freeze-dried compressed, 0 month; c, 21°C, 2 months; d, 38°C, 2 months; e, 21°C, 4 months; f, 38°C, 4 months; g, 21°C, 6 months; h, 38°C, 6 months.

glucosylrutinoside). Pigments A and D were eluted with 0·01% methanolic HCl, filtered and concentrated by flash evaporation. The R_f values obtained from cochromatographing with the synthetic cyanidin, and the spectral properties suggested that pigment A was cyanidin. It had been said that anthocyanidins are uncommon to exist in free state in plant tissues. However, the presence of cyanidin and peonidin in sour cherries has been reported by Dekazos (1970). It is possible that cyanidin may have come from the chemical degradation of anthocyanins. Pigment D was characterized

TABLE 2. R_f values of anthocyanins of freeze-dried compressed sour cherries separated on two-dimensional thin-layer chromatography

Pigments*	R_f (×100)†													
	21°C storage								38°C storage					
	BHClW				WHClF				BHClW			WHClF		
	0	2	4	6	0	2	4	6	2	4	6	2	4	6
	(months)				(months)				(months)			(months)		
A Cn	50	52	53	54	3	2	4	3	52	52	52	3	3	3
B Cn-3G	24	26	27	27	12	12	12	11	23	27	24	12	12	12
C Cn-3RG	33	34	33	32	29	27	26	26	32	—	—	26	—	—
D Pn-3RG	35	36	—	—	34	32	—	—	—	—	—	—	—	—
E Cn-3GG	35	36	38	31	59	60	62	59	37	36	27	61	61	60
F Cn-3G(Xyl, R)	40	—	—	—	74	—	—	—	—	—	—	—	—	—
G Cn 3G(G, R)	41	46	44	—	83	82	83	—	—	—	—	—	—	—

* Notation: Cn = cyanidin, Pn = peonidin, G = glucose, R = rhamnose, Xyl = xylose.
† Mean value of four determinations.

by its spectral properties and R_f values on Whatman No. 1 filter paper, being developed in four different solvent systems. They were BAW (*n*-butanol:glacial acetic acid: water = 4:1:5, u/layer), 1% HCl, HOAc-HCl (water:glacial acetic acid:HCl = 82:15:3), and 15% HOAc. The R_f values and the spectral properties (Table 1) indicated that pigment D was peonidin-3-rutinoside.

The anthocyanin extract of freeze-dried compressed sour cherries stored for 0 month was separated into seven components on the thin-layer chromatogram (Fig. 3b). The thin-layer chromatogram of the extract of freeze-dried compressed samples stored at 21°C for two months contained six pigment spots (Fig. 3c). No pigment F was found in the sample after two months at 21°C. Five pigments were found, with pigments D and F absent, in the thin-layer chromatogram of the extract of freeze-dried compressed samples stored at 21°C for four months (Fig. 3e). Four pigments were separated on the thin-layer chromatogram of the extract of freeze-dried compressed samples stored at 21°C for six months (Fig. 3g) with the disappearance of pigments D, F and G.

Four pigments were found on the thin-layer chromatogram of the extract of freeze-dried compressed samples at 38°C for two months (Fig. 3d). Pigments D, F and G had disappeared. Three pigments were separated on the thin-layer chromatogram of

freeze-dried compressed samples stored at 38°C for four months (Fig. 3f). Pigments C, D, F and G were not present after four months at 38°C. Thin-layer chromatography of the extract of freeze-dried compressed samples held at 38°C for six months yielded three pigment spots—A, B and E (Fig. 3h).

All seven pigments of freeze-dried compressed sour cherries observed on thin-layer chromatography showed different degree of degradation as storage time and temperature increased. Each pigment from samples stored at 38°C had decreased faster than that from samples stored at 21°C. Pigment F was the least stable pigment followed by pigments D, G, C, A, E and B, respectively.

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Corrosion of tinplate by citrus juices

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Summary

Juice from the Satsuma Mandarin variety of oranges which was free from bitterness with less acidity gave more corrosion as compared with the juice from Coorg Mandarin, Mandarin Imperial, Tangerine Dancy and Washington Navel. Bitter principles present in the orange juice were found to act as inhibitors of corrosion. Limonin acted as inhibitor of corrosion, while hesperidin did not show any effect. Among different fractions of orange juice, the organic acid fraction contributed towards corrosion.

Introduction

Several instances of heavy corrosion in canned orange products were brought to the notice of the authors by a number of canning factories in India. The production of orange products is increasing every year both for internal consumption and export. Experiments were, therefore, undertaken to study the corrosion behaviour of different citrus juices and their constituents. Heavy corrosion was noticed in canned orange segments by Mahadeviah *et al.* (1969) while studying the effect of the phosphorus content in the base plate on the suitability of Indian hot dipped tinplates. High tin content in canned orange juice as a result of heavy corrosion caused by the presence of nitrate was reported by Horio, Iwamoto & Shiga (1967).

Materials and methods

Cans

Jam size (301 × 309) and A 2½ size (401 × 411) cans fabricated from electrolytic tinplate E100, carrying 11·2 g/m² tin on each side were used in these experiments.

Raw material

Citrus fruits collected from the Athur orchard, Coorg, during one season were used in these experiments.

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Preparation and canning

Orange juice: oranges were washed, peeled, the segments were separated and the juice was extracted using a screw type juice extractor in the case of loose jacketed oranges. Tight skinned oranges were cut into halves and the juice was extracted in a burring machine. The extracted juice was passed through a finisher having a 24-mesh sieve. The brix of the juice was adjusted to 15° using cane sugar and the original acidity (0·5%) was maintained. The juice was heated to 85°C, filled into cans and processed for 20 and 25 min in boiling water (98°C) for jam size and A $2\frac{1}{2}$ respectively.

Methods of analysis

The flavanones in different components of orange were estimated by the method of Davis (1947) and the results expressed as per cent hesperidin. Limonin was estimated by the thin layer chromatographic method of Chandler & Kefford (1966).

Model systems

To confirm the results obtained by the canning trials model experiments were carried out in a few cases. To study the corrosion behaviour in the model systems tinplate was cut into strips of 8 × 2 cm and the edges were protected by coating with lacquer. The strips were placed in test tubes covered with the respective solutions heated to 89°C and the tubes sealed immediately. The sealed tubes were processed in boiling water (98°C) for 25 min and cooled in running cold water.

Corrosion rate

The extent of corrosion was expressed by (i) determining the tin content in duplicate in a composite sample of six cans by the volumetric method described by Mckenzie (1945) and (ii) by determining the percentage loss in weight of the tinplate strips used in the model systems.

Influence of variety on corrosion

The corrosion behaviour of tinplate with the following eight varieties (Singh Daljit, 1969) of oranges was investigated: Coorg Mandarin (*Citrus reticulata Blanco*), Satsuma Mandarin (*C. unshiu*), Mandarin Imperial (*C. reticulata*), Tangerine Dancy (*C. reticulata*), Sour oranges (*C. aurantium* Linn), Red Blood Malta (*C. sinensis*), Washington Navel (*C. sinensis*) and Grape fruit (*C. paradisi*). Juice from these varieties of oranges was separately extracted and canned in 1 lb jam size (301 × 309) cans and stored at 37°C.

Influence of bitter principles on corrosion

Juice from Coorg Mandarin orange segments was extracted either after lyepeeling to remove the bitter principles which are present in the albedo or without lye treatment (to retain the bitter principles) in a screw type juice extractor. The brix of these juices

was adjusted to 15° with cane sugar and the acidity to 0·5% with citric acid and canned in A $2\frac{1}{2}$ size (401 × 411) cans and stored at 37°C.

Influence of hesperidin and limonin

0·05 and 0·1% of pure hesperidin was added to sugar syrup of 15° Brix and 0·5% acidity.

Crystalized limonin was prepared from the orange seeds by following the procedure described by Kefford & Chandler (1961) and was incorporated in the sugar syrup in two different concentrations of 25 and 50 ppm. These were packed separately in test tubes along with tinplate strips and stored at 37°C.

Effect of anionic and cationic fractions

Different fractions from Coorg Mandarin orange juice were separated by passing through anionic and cationic resins as given below and used in the model systems.

Orange juice was mixed with double the quantity of 95% ethyl alcohol and blended in a Waring blendor, strained through nylon cloth and then filtered under suction. The filtrate was concentrated about four-fold in a flash evaporator under vacuum at 40–45°C. The concentrated extract was passed through Dowex-50 cation exchange resin in hydrogen form and washed with water. The eluate consists of organic acids and sugars. A portion of the eluate from the cation exchange resin was passed through Dowex-1 anion exchange resin. Eluate coming out of this resin was free from organic acids but contained only sugars. Another portion of concentrated extract was passed through anion exchange resin to collect fractions consisting of amino acids and sugars.

Elution of different fractions (Hussain, El-mansy & Walker, 1969)

Cationic fraction (*Amino acids*). Amino acids retained in the cation exchange resin were eluted with 2N ammonium hydroxide. The column was then washed with distilled water. The eluate and the washings were combined and heated on a water bath to expel ammonia. The residual solution was used for studies in model systems.

Anionic fraction (*organic acids*). Organic acids retained in the anion exchange resin were eluted using 6N formic acid and washed with distilled water. The eluate and the washings were evaporated on a water bath to expel formic acid and used in the model systems.

The following fractions were collected by the above procedure: organic acid, amino acid, sugar, organic acid + sugar, amino acid + sugar. These fractions were made up to a known volume with distilled water and packed in test tubes along with tinplate strips to study their effect on corrosion.

Results and discussion

Influence of variety

The physico-chemical characteristics of the juices are given in Table 1. The acidity

TABLE 1. Chemical composition and physical characteristics of different varieties of oranges

Composition and physical characteristics	Coorg Mandarin	Satsuma Mandarin	Mandarin Imperial	Tangerine Dancy	Sour Orange	Red Blood Malta	Washing-ton Navel	Grape fruit
Moisture (%)	86·55	88·32	84·68	86·68	91·2	89·15	85·0	91·77
Acidity (as anhydrous citric acid)	0·7	0·5	0·9	0·83	0·96	1·51	0·89	2·68
pH	3·7	4·0	3·5	3·8	3·2	3·4	3·9	3·3
Total soluble solids	11	10	15	12	6	8	14	7
Ascorbic acid (mg%)	49	50	65	65	54·9	65	52·3	48·5
Colour	LOY	DY	LOY	LOY	PY	PY	PY	PY
Taste	Slightly bitter	No bitterness	Slightly sour	More bitter	Too bitter	Sour and bitter	More bitter	Too bitter

LOY, light orange yellow; DY, deep yellow; PY, pale yellow.

was greater in grape fruit and Red Blood Malta as compared to other varieties and the pH varied from 3·2 to 4·0. The Satsuma Mandarin variety of orange was less acidic and free from bitterness. The bitterness was greater with grape fruit, Sour orange, Tangerine Dancy and Washington Navel. The total soluble solids were less than 10° in the case of Sour orange, Red Blood Malta and grape fruit varieties.

Taste and colour of the canned juice from all the eight varieties of oranges were characteristic of the original juice. As shown in Table 2, up to nine months of storage there was no metallic taste in the juice of all the varieties except in the case of Satsuma Mandarin which had a slight metallic taste and the tin content was more than the permissible limit of 250 ppm (Fruit Products Order, 1973). After twelve months,

TABLE 2. Tin content (ppm) in different varieties of canned orange juice

Storage period (months at 37°C)	Variety							
	Coorg Mandarin	Satsuma Mandarin	Mandarin Imperial	Tangerine Dancy	Sour Orange	Red Blood Malta	Washington Navel	Grape fruit
Initial	27	43	35	19	38	49	39	42
3	60	92	76	65	81	98	82	112
6	106	197	126	113	132	180	122	130
9	122	256	138	120	163	206	152	183
12	156	300	190	146	230	343	162	232

Sour orange, Red Blood Malta and grape fruit juice showed a slight metallic taste but this was masked by the sour and bitter taste of the juice. The tin content of Satsuma Mandarin juice was higher than in all the other varieties with the exception of Red Blood Malta juice throughout the storage period.

The juices of Red Blood Malta and Satsuma Mandarin are highly corrosive as compared with the juices of Coorg Mandarin, Mandarin Imperial, Tangerine Dancy or Washington Navel while the juices of grape fruit and sour orange are moderately corrosive.

Influence of bitter principles

The effect of lyepeeling on total soluble solids and acid content is indicated in Table 3. Lyepeeled orange juice, free from bitterness, caused very heavy corrosion as compared to controls (juice prepared without lyepeeling) (Table 4) while in the control corrosion

TABLE 3. Effect of lyepeeling on total soluble solids and acid content

	Lyepeeled orange juice	Control
Original Brix (°)	7·0	8·5
Original acidity (%)	0·3	0·5
Final Brix (°) (adjusted)	15·0	15·0
Final acidity (%) (adjusted)	0·5	0·5
Taste	Sweet No bitterness	Bitter
Colour	Pale orange yellow	Orange yellow

was less after twelve months of storage and the tin content was below 250 ppm.

In the case of lyepeeled orange juice the tin content gradually increased with an increase of storage period and after twelve months storage the extent of corrosion was almost double as compared with that of the control. Heavy corrosion in the canned juice prepared from orange segments after lyepeeling, which removes the bitter principles, confirms their inhibitory effects.

The content of flavonoid compounds and limonin in the albedo, flavedo, juice (with and without lyepeeling) and seeds of Coorg Mandarin oranges are given in Table 5. The flavanones were more in the albedo than in the other components and juice. Lyepeeling of orange segments to remove bitterness reduced the flavanone content from 0·06 to 0·046% and lyepeeled juice was free from limonin.

The bitterness in canned Mandarin has been reported to be due to limonin (Siddappa & Bhatia, 1959) which is present in the juice in concentrations up to 25 ppm. In contrast to the bitterness caused by flavonoids, the bitterness in citrus products containing

TABLE 4. Influence of bitterness on corrosion cut-out analysis of canned orange juice

Storage period (months) (37°C)	Product	Vacuum (inch. Hg.)	Tin (ppm)	Can interior
Initial	OJC	15	25	VLF
	LPOJ	16	30	LF
3	OJC	13	40	LF
	LPOJ	14	120	MF, SD
6	OJC	11	120	LF
	LPOJ	11	196	MF, MD
9	OJC	9	143	MF, SD
	LPOJ	9	239	HF, MD
12	OJC	7	168	MF, SD
	LPOJ	4	383	VHF, HD

OJC, juice from unlyepeeled orange segments (control); LPOJ, juice from lyepeeled orange segments; VLF, very light feathering; LF, light feathering; MF, medium feathering; VHF, very heavy feathering; HF, heavy feathering; SD, slight detinning; MD, medium detinning; HD, heavy detinning.

TABLE 5. Flavanone and limonin contents in different components of Coorg Mandarin orange

Component	Flavanones (as hesperidin %)	Limonin (ppm)
Orange juice (control)	0·06	23
Orange juice from lyepeeled segments	0·046	0
Albedo	0·533	50
Flavedo	0·15	5
Seeds	0·0133	6500 (0·65% wet wt basis)

the comparatively insoluble limonin appears only after heat treatment and storage (Kefford & Chandler, 1970).

Influence of hesperidin

There was no appreciable difference in the rate of corrosion between control and

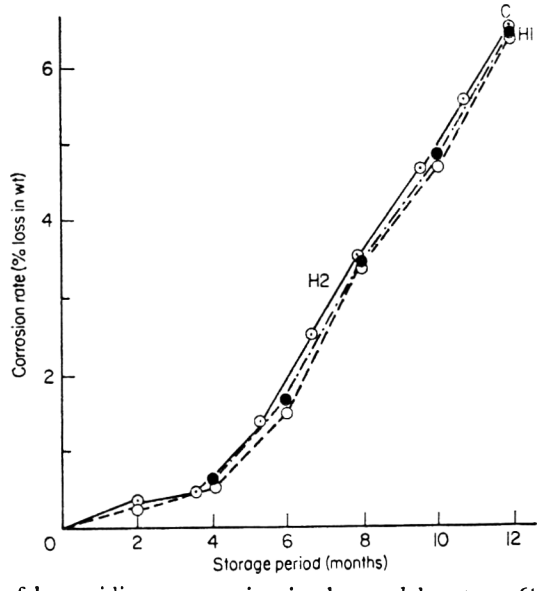

FIG. 1. Influence of hesperidin on corrosion in the model system. C, sugar syrup only (control); H_1, sugar syrup + 0·05% hesperidin; H_2, sugar syrup + 0·1% hesperidin.

treated samples (Fig. 1). An increase in the concentration of hesperidin did not affect the rate of corrosion. The observations were similar when naringin was used in place of hesperidin.

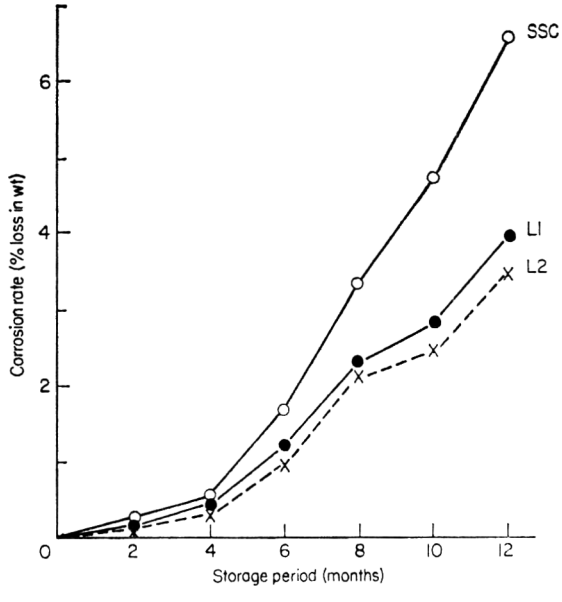

FIG. 2. Influence of Limonin on corrosion in the model systems. SSC, sugar syrup (control); L_1, sugar syrup + 25 ppm limonin; L_2, sugar syrup + 50 ppm limonin.

Influence of limonin

A rapid increase in the corrosion rate was observed in the case of the control. On the other hand 25 and 50 ppm limonin considerably reduced the rate of corrosion (Fig. 2). In these two cases, the loss in weight was 4 and 3·5% respectively as compared to 6·6% in the case of control after twelve months of storage at 37°C. Correspondingly, feathering and detinning of tinplate strips were less in the presence of limonin than in its absence.

Effect of anionic and cationic fractions

The fraction containing organic acids caused more corrosion than other fractions (Fig. 3). The tinplate strips showed heavy feathering with heavy detinning. In the

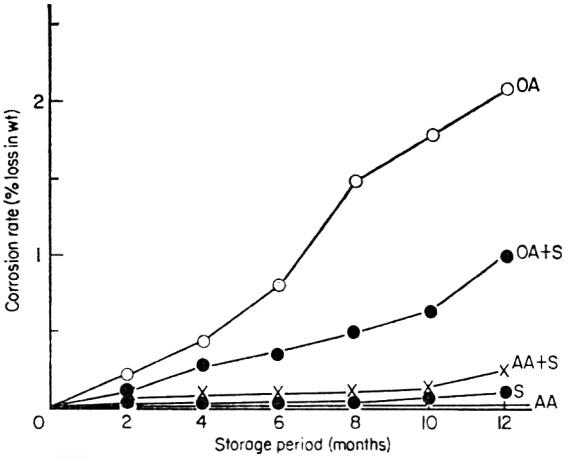

FIG. 3. Influence of different fractions of orange juice in the model systems. OA, organic acid fraction; OA+S, organic acid+sugar fraction; AA+S, amino acid+sugar fraction; S, sugar fraction; AA, amino acid fraction.

presence of sugar, the corrosion of tinplate strips by organic acids was similar. The amino acid fraction did not cause even feathering of tinplate strips. Corrosion caused by sugar alone or sugar+amino acid was insignificant and even after twelve months storage at 37°C, only light feathering was noticed. These observations indicate that only the organic acid fraction of orange juice contributes to corrosion of tinplate.

Acknowledgment

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Recovery of anthocyanins from wine distillation wastes

I. An adsorption-desorption process

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Summary

Adsorption of anthocyanins from red wine distillation wastes was carried out using an insoluble polyvinylpyrrolidone resin (Polyclar AT) and a nylon formulation. Comparison of adsorption data which were plotted according to the Freundlich isotherm show that Polyclar is a stronger adsorbent than nylon and that adsorption capacity is higher at lower temperature and concentration. Study of the kinetics shows that adsorption on Polyclar is almost immediate, while adsorption on nylon requires more than 24 hr to reach completion. This is probably due to the slow penetration of the solution into the finely porous structure of nylon. Different operating modes for desorption show that higher yields were obtained with multiple extraction from Polyclar and with batch countercurrent extraction from nylon. In any case desorption from nylon gives higher yields than from Polyclar. Qualitative and quantitative analyses show that both extracts contain, together with monomeric anthocyanins, considerable amounts of simple phenclics and, in the case of nylon, tannins and anthocyanin-tannin copolymers.

Introduction

Several patents and research papers in the recent literature describe methods for the recovery and purification of anthocyanins from vegetable materials (Pourrat, Tronche & Pourrat, 1966; Chiriboga & Francis, 1973; Bourzeix, 1974; Philip, 1974). This is due to the fact that anthocyanin preparations which differ in composition and purity, are increasingly required as food grade colouring additives and for pharmaceutical formulations. The purpose of this work was to study some technological aspects related to the recovery and purification of anthocyanins from red wine distillation wastes.

Two different methods have been applied for the separation of anthocyanins:

(a) an adsorption-desorption method based on the use of selective adsorbents and
(b) a permeation process using ultrafiltration and hyperfiltration membranes.

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This paper reports the results of the sorption experiments, while the results of the permeation experiments will appear in a following publication. Adsorption of anthocyanins was carried out using an insoluble polyvinylpyrrolidone (Polyclar AT) and a polyamide (Nylonplast). These resins have been extensively studied as detanning and decolourizing agents in wine and beer technology (Peri & Cantarelli, 1963) and for purification of anthocyanins for analytical purposes (Chandler & Swain, 1959; Fuleki & Francis, 1968; Wrolstad & Putnam, 1969; Bourzeix, Heredia & Natchkov, 1973; Hrazdina, 1975). Several investigations have been devoted to the study of the mechanism of adsorption of different phenolics by these resins (Singleton, 1967; Andersen & Sowers, 1968; Mennet & Nakayama, 1969, 1970). However, if these resins are to be used for anthocyanin separation in an industrial process, it is necessary to know much more with regard to operating modes and conditions, especially in the desorption phase, which has often been neglected in previous work. In this investigation we have defined the adsorption capacity and kinetics of our systems and we have compared different technological modes of operation for desorption. Qualitative comparison of the extracts obtained from the two resins is also presented.

Materials and methods

The solution used for the adsorption experiments was obtained as still bottom from an industrial distillation unit operating under vacuum, so that the product's temperature did not exceed 45°C. In this solution the anthocyanins are associated with other phenolic compounds and with non-volatile constituents of wine, especially organic acids and salts.

The adsorbents were:

(i) an insoluble polyvinylpyrrolidone (Polyclar AT, Antara Chemicals, GAF Italiana distr.) and

(ii) a polyamide (Nylonplast, Rhodiatoce) which will simply be called 'nylon' in this paper.

The polyclar AT was used as such, while the nylon was subjected to a regeneration treatment before use. Commercial nylon has a very low adsorbing capacity, due to intramolecular hydrogen bonding which leads to complete inactivation of adsorbing sites (Singleton, 1967). The regeneration of the resin was carried out by dissolution in formic acid followed by coagulation in water, and then drying and grinding according to the procedure described elsewhere (Pompei, Peri & Montedoro, 1971). Pure methanol 0·1% HCl was used as solvent for desorption as it was the most effective in preliminary experiments carried out with methanol and ethanol, either pure on in aqueous mixtures (Prandoni, 1975).

Routine analytical methods were applied for the dry matter, ash and acidity determinations. Phenolic fractions were determined colourimetrically using the Folin-

Ciocalteu reagent (Singleton & Rossi, 1965). Fractionation of phenolic groups was carried out by selective precipitation of tannins and flavonoids (Peri & Pompei, 1971). Results are expressed as gallic acid equivalents, in mg/100 ml.

Phenolic groups are indicated as follows:

Simple phenolics. Derivatives of hydroxybenzoic and hydroxycinnamic acids.

Non-tannin flavonoids. Monomeric anthocyanins and catechins.

Tannins. Polymers and copolymers of catechins, leucoanthocyanins and anthocyanins.

Anthocyanins were determined colourimetrically by applying a method based on SO_2 decoloration (Ribereau-Gayon *et al.*, 1972), using as reference the same standard curve obtained by these authors from an anthocyanin mixture isolated and purified from Vitis vinifera grapes. For the qualitative comparison of the extracts obtained from the two resins, chromatographic separation of the anthocyanins was carried out as follows. Thin layer chromatography on 20 × 20 cm cellulose plates (DC-Fertigplatten, Cellulose F, Merk), 0·10 mm thick, using a mixture of *n*-pentanol-acetic acid-water (2:1:1, v/v) (PAW) as solvent (Wrolstad & Heatherbell, 1974). Spectra of the anthocyanin solutions were also determined for comparison in the visible range from 400 to 600 nm.

Adsorption isotherms were determined as follows. Fractions of 10 ml of the test solution were transferred to 50 ml flanks containing different amounts of the resin (usually 50, 100, 200, 500, 1000 and 1500 mg). The flasks were stoppered and slowly shaken in a thermostatic bath (at 20°C or 5°C) until the equilibrium concentration was attained (usually 4 hr for the Polyclar and 48 hr for the Nylon). At the end of this period the content of each flask was filtered on Whatman no. 4 filter paper and the residual concentration of anthocyanins was determined in a portion of the clear filtrate. By difference with the original content the amount of adsorbed anthocyanins was calculated.

The adsorption data were analysed according to the Freundlich isotherm:

$$\frac{x}{m} = \mathrm{K} \times c^{\mathrm{n}}$$

in which x = mg of anthocyanins adsorbed; m = mg of resin; c = concentration of residual anthocyanins in the filtrate (equilibrium concentration), mg/l; and K and n = constants.

The equation may also be written in the form:

$$\log \left(\frac{x}{m}\right) = \log \mathrm{K} + \mathrm{n} \log c$$

thus a plot of x/m versus c on a log-log scale gives a straight line with a slope equal to n, and x/m is equal to K when c equals 1.

In practice K is a measure of the adsorbing capacity of the resin, whereas n is a measure of the characteristic type of adsorption (Freundlich, 1926). To investigate the

kinetics of adsorption, equal amounts (500 mg) of resin were exactly weighed in 50-ml flasks and 10 ml of the test solution added; the flasks were then stoppered and shaken at constant temperature as for the isotherms determination. After intervals of 2, 5, 10, 30 and 60 min, 2, 6 and 24 hr the contents of each flask was filtered and the residual anthocyanin concentration was determined in the filtrate. Adsorbed quantities were determined by difference from the initial content of the solution.

For the desorption experiments a sample of the resin-anthocyanin complex was prepared by letting the resin equilibrate for 24 hr in an excess solution. This was then dried under vacuum at room temperature over P_2O_5.

Three different operating modes were applied for desorption.

Simple extraction. A weighed amount of the resin-anthocyanin complex was brought into contact with a known volume of methanol-0·1% HCl.

The suspension was shaken until equilibrium was attained (usually 1 hr at 35°C for both resins) and the amount of desorbed anthocyanins was determined as usual on the clear filtrate.

Multiple extraction. The same procedure was followed as above but the volume of solvent to be used in the extraction was first divided into several equal portions. These were used in successive extraction operations.

The total amount of desorbed anthocyanins resulted from the sum of the amounts desorbed by single portions of solvent.

Batch countercurrent extraction. In this experiment a battery of three extractors was used following the classical countercurrent mode (Brown, 1966). In this process, each time a batch of solution is separated from an extractor, it is used to treat a batch of resin which has previously been extracted with a richer solution. Thus the resin is treated with batches of solvent of progressively decreasing anthocyanin content until it is finally extracted with fresh solvent and discharged, while the solvent is brought into contact with batches of resin of progressively increasing anthocyanin content until it finally encounters fresh resin and is then discharged as the finished solution. In this way the solution is brought out at a uniformly high anthocyanin content.

We have given a schematic representation of the countercurrent extraction process in Fig. 5.

Results and discussion

Adsorption

The composition of the crude anthocyanin solution used in this work is given in Table 1. The solution is very concentrated in comparison with a normal red wine, with a high content of titrable acidity and a very high concentration of all phenolic substances. Among these the greater proportion is represented by tannins, while the monomeric anthocyanins represent only a few percent of the total phenolic content. Note that the value of 'anthocyanin' in the table is not directly comparable with the values of the phenolic fractions, having been obtained with a different analytical procedure and

TABLE 1. Composition of the crude anthocyanin solution used for all experiments in this work

Dry matter (% by weight)	4·82	
Ash (% by weight)	0·72	
Total acidity (g of tartaric acid per litre)	11·66	
Phenolics (as mg of gallic acid/100 ml):		
		% of total
Simple phenolics	138	38·3
Non-tannin flavonoids	34	9·4
Tannins	188	52·3
Total phenolics	360	100
Anthocyanins (mg/100 ml)	38·5	

expressed with reference to a different standard. Figures 1 and 2 show the adsorption isotherms obtained with nylon and Polyclar, respectively. To verify the effect of concentration on adsorption three solutions were used, having a different anthocyanin content as follows.

Solution (*a*). The test product, with composition as specified in Table 1 (anthocyanin content = about 40 mg/100 ml).

Solution (*b*). A four-fold dilution of solution (*a*) (anthocyanin content = about 10 mg/100 ml).

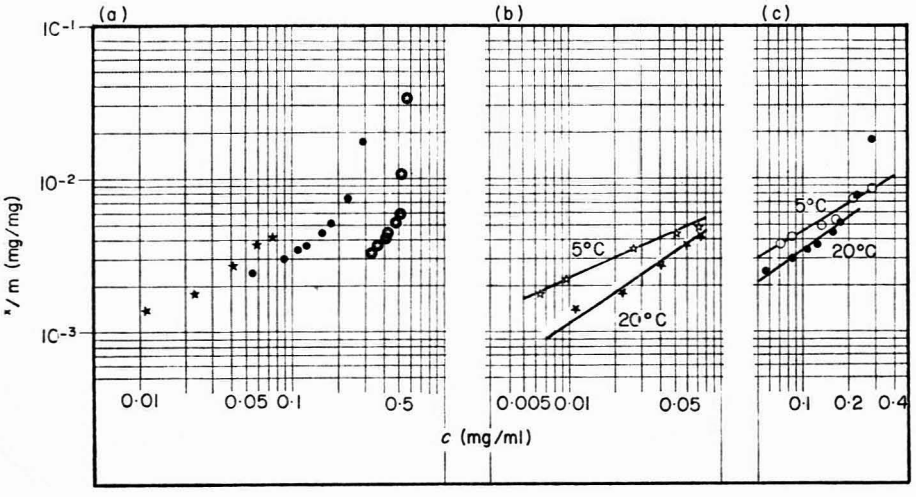

FIG. 1. Freundlich plot of adsorption data for nylon. (a) Isotherms at 20°C of solutions with different anthocyanin content. ● Solution (a) (see text, 'Results and discussion'); ★ solution (b); ✪ solution (c). (b) Isotherms of solution (b) at two temperatures. ★ 20°C; ☆ 5°C. (c) Isotherms of solution (a) at two temperatures. ● 20°C; ○ 5°C.

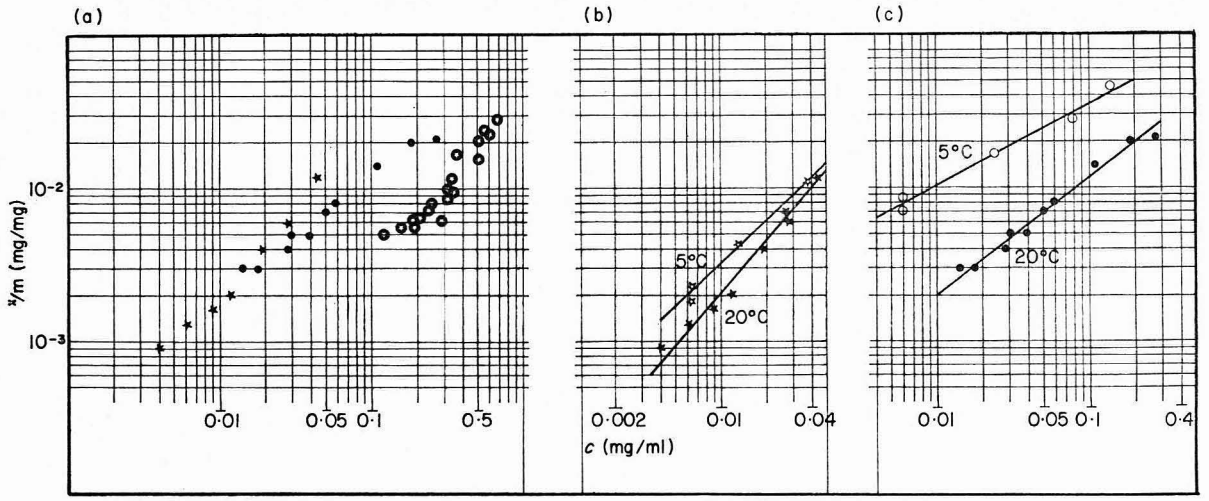

FIG. 2. Freundlich plot of adsorption data for Polyclar. (a) Isotherms at 20°C of solutions with different anthocyanin content. ● Solution (a); ★ solution (b); ✪ solution (c). (b) Isotherms of solution (b) at two temperatures. ★ 20°C; ☆ 5°C. (c) Isotherms of solution (a) at two temperatures. ● 20°C; ○ 5°C.

Solution (c). A two-fold concentration product obtained by evaporation under vacuum of solution (*a*) (anthocyanin content = about 80 mg/100 ml).

The isotherms obtained from the three solutions may be compared in Figs 1a and 2a. The isotherms obtained at different temperatures may be compared in Figs 1b and 1c for nylon and Figs 2b and 2c for Polyclar. The calculated values of K and n are shown in Table 2.

As a general comment to the graphs it may be observed that the Freundlich plot fits the adsorption data to a good approximation, with the only exception of points

TABLE 2. Values of the constants K and n of the Freundlich isotherm calculated from curves in Figs 1 and 2

Reference (Fig.)	Adsorbent	Solution	Temperature (°C)	K	n
1b	Nylon	(b)	20	$3{\cdot}1 \times 10^{-4}$	0·674
			5	$9{\cdot}0 \times 10^{-4}$	0·452
1c	Nylon	(a)	20	$1{\cdot}9 \times 10^{-4}$	0·690
			5	$2{\cdot}8 \times 10^{-4}$	0·604
2b	Polyclar	(b)	20	$1{\cdot}7 \times 10^{-4}$	1·155
			5	$3{\cdot}5 \times 10^{-4}$	0·936
2c	Polyclar	(a)	20	$3{\cdot}8 \times 10^{-4}$	0·770
			5	$3{\cdot}0 \times 10^{-3}$	0·526

obtained at high equilibrium concentrations of anthocyanin. For both resins K is higher and n lower at 5°C than at 20°C. The result of this trend is that adsorption capacity is generally higher at the lower temperature, particularly at the lower equilibrium concentrations.

Comparing the values obtained with the two resins it can be seen that Polyclar gives higher values of K and therefore has a greater adsorbing capacity than nylon. Comparing data points in Figs 1a and 2a one may observe that isotherms relative to solutions of different concentration are roughly parallel, with the value of K decreasing with increase in equilibrium concentration of anthocyanin. In other words the adsorption capacity for anthocyanins decreases with increasing anthocyanin content. In trying to explain this result we must consider that we deal with a complex solution, containing different kind of phenolic compounds and particularly tannins, which are selectively adsorbed by the resins (Peri & Cantarelli, 1963). Thus an increase in their concentration tends to saturate the adsorbing sites of the resin, reducing its ability to bind anthocyanins. In all cases the maximum adsorption capacity of the resins was between 2 and 4 g of anthocyanin per 100 g of resin. The kinetics of adsorption is quite different for the two resins as is clearly apparent from comparison of the curves in Fig. 3, in which the amount of adsorbed anthocyanins has been reported as a function of time.

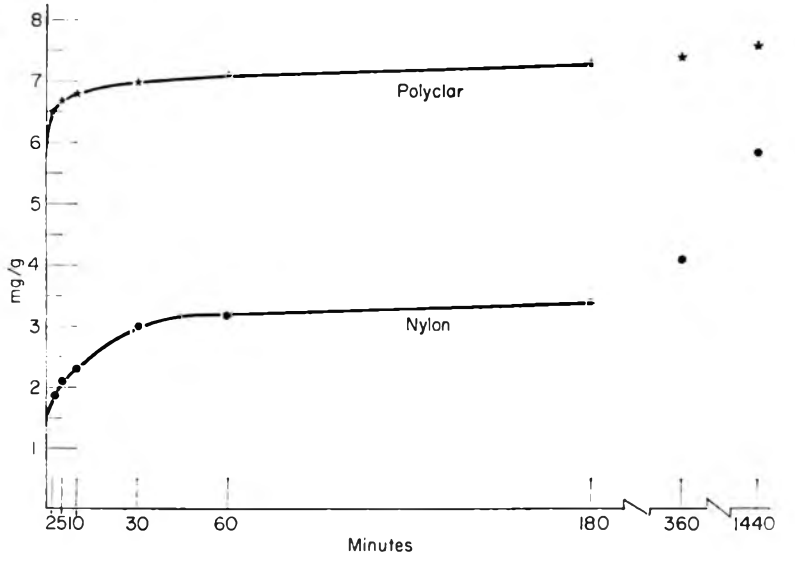

FIG. 3. Adsorption kinetics. Data represent mg of anthocyanin adsorbed per g of resin as function of time.

The data relative to Polyclar show that adsorption of most of the anthocyanin is practically immediate. In contrast, the quantity adsorbed by nylon tends to increase significantly with time. We presume that the reason for this behaviour can be found in

the different physical structure of the resins. Polyclar is a finely divided powder of uniform particles whose available surface area contains most of the adsorbing sites. Therefore adsorption takes place almost immediately as the resin comes into contact with the solution. Nylon, on the other hand, is a very porous powder in which adsorbing sites are not only present at the surface, but also distributed in the porous structure. Diffusion of anthocyanins and penetration of the solution into this structure may be the limiting phenomenon of the process.

Desorption

(*a*) *Simple extraction.* Figure 4 reports the results of the desorption process carried out by simple extraction, using different quantities of solvent. Comparison of the two curves shows that the quantity of anthocyanins which was desorbed from nylon was much greater than the quantity which was desorbed from Polyclar in spite of the fact that the latter had adsorbed a greater quantity. This result indirectly confirms that Polyclar is a stronger adsorbent than nylon. The quantity of anthocyanin desorbed increases with the quantity of solvent used, as was expected considering that a partition equilibrium of concentrations tends to be established between the resin and the surrounding solution. However, the increase in the desorbed quantity becomes negligible for quantities of solvent greater than 500 ml/g.

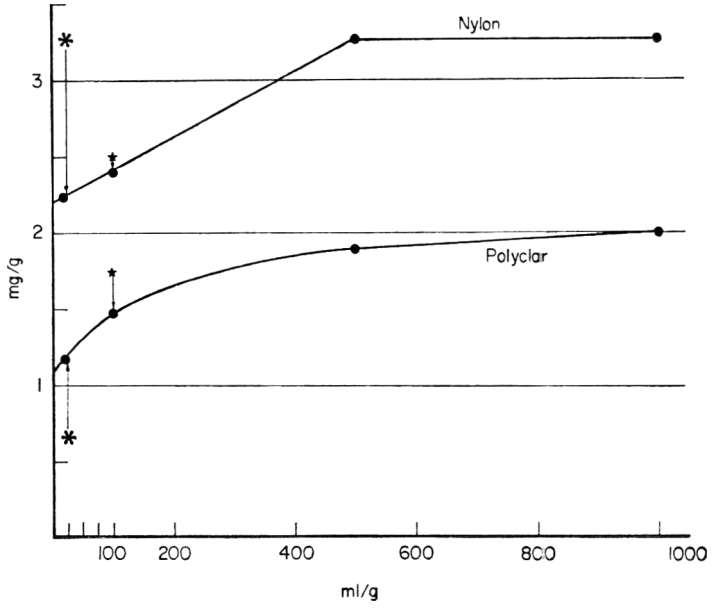

FIG. 4. Desorption yield in 'simple extraction'. Data represent mg of anthocyanins desorbed per g of resin as a function of ml of solvent used per g of resin. * Countercurrent extraction; ★ multiple extraction.

TABLE 3. Results of 'multiple extraction' experiments

Solvent fraction	mg of anthocyanin desorbed from 1 g of the resin-anthocyanin complex	
n	Nylon	Polyclar
1	1·82	0·94
2	0·43	0·41
3	0·11	0·15
4	0·09	0·14
5	0·05	0·11
Total	2·50	1·75

(*b*) *Multiple extraction.* The multiple extraction was carried out using 5 fractions of solvent of 20 ml each, in 5 successive extractions of 1 g of the resin-anthocyanin complex. Data obtained in these experiments are reported in Table 3.

In total 1·75 mg of anthocyanin was desorbed from Polyclar; this value should be compared with a value of 1·48 mg obtained by using 100 ml of solvent in one single extraction operation. For nylon the quantities were 2·50 and 2·40 for the multiple and simple extraction, respectively. It may therefore be concluded that multiple extraction slightly improves the yield of the process; the difference is more significant for Polyclar than for nylon. For the purpose of comparison, the results of the multiple extraction experiments are reported with stars in Fig. 4.

(*c*) *Countercurrent extraction.* Finally, in Fig. 5, typical results from the countercurrent extraction experiments are reported. After reaching the steady state of operation, several extraction cycles were repeated; the results of a single cycle are reported in Fig. 5 as representative of all. In fact concentration data obtained from the three extractors were very similar in all cycles, never differing by more than 10% as a result of experimental error. When applied to nylon the system worked as expected with the anthocyanin content increasing from Ex_3 to Ex_2 and finally reaching its maximum in Ex_1. Considering that we used batches of solution of 25 ml of solvent for each gram of the resin anthocyanin complex, the reported value of 3·27 mg of anthocyanin desorbed is very high compared to the quantities desorbed by simple or multiple extraction. This value is also reported for comparison with an asterisk in Fig. 4.

Unexpectedly, with Polyclar the behaviour was completely and consistently reversed. At all extraction cycles the concentration of anthocyanins decreased from the third to the second and finally to the first extract.

We are unable to explain this behaviour which indicates that re-adsorption takes place

at the second and first stages. The practical result is that, in the case of Polyclar, countercurrent extraction is less effective than multiple or even simple extraction, as is clearly apparent from a comparison of the data points in Fig. 4.

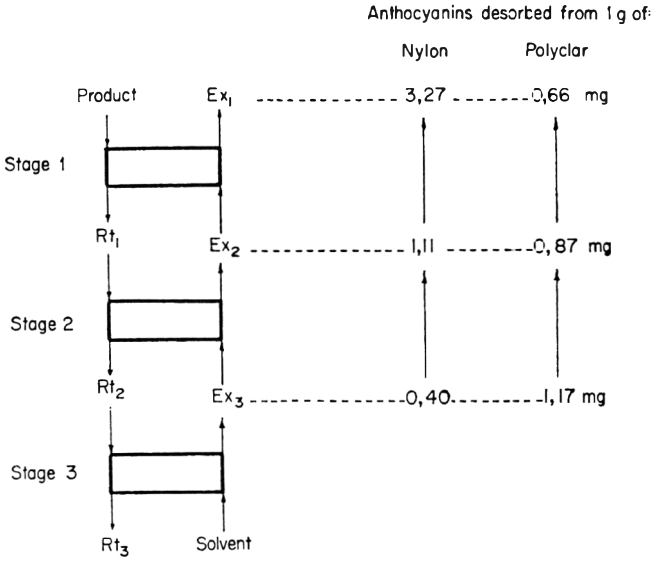

FIG. 5. Schematic representation and typical results of the countercurrent extraction experiment. Ex = extract stream; Rt = resin stream.

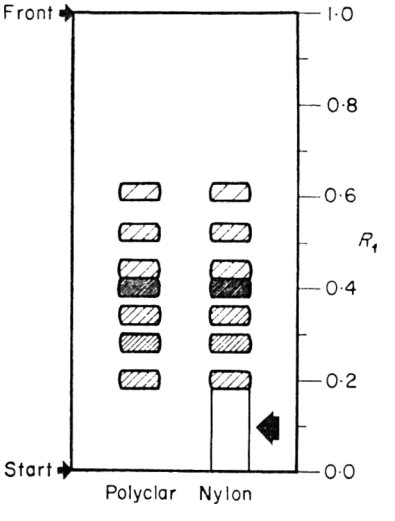

FIG. 6. TLC of Nylon and Polyclar extracts. Arrow indicates the band of tannic material in the nylon extract.

Composition of the extracts

Qualitative comparison of the solutions obtained by desorption from the two resins was carried out. Simple thin layer chromatography separation on Cellulose-F plates using PAW (*n*-pentanol-acetic acid-water, 2:1:1, v/v) as solvent is shown in Fig. 6.

TABLE 4. Phenolic fraction distribution in the nylon and Polyclar extracts

Phenolic fraction	Percent distribution in the extracts from	
	Polyclar	Nylon
Simple phenolics	82·9	46·3
Non-tannin flavonoids	17·1	17·4
Tannins	0·0	36·3
Total phenolics	100·0	100·0

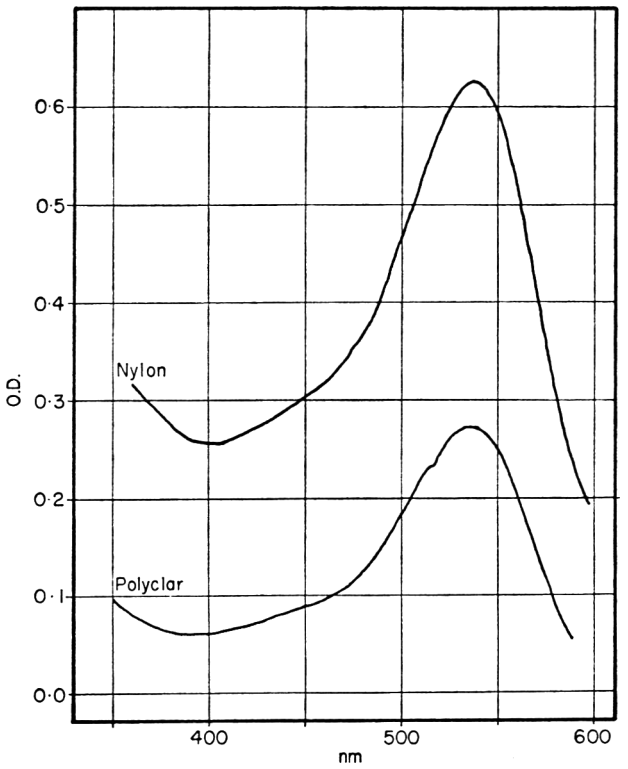

FIG. 7. Absorption spectra of Nylon and Polyclar extracts.

As pointed out in the experimental section the purpose of the TLC is simply one of comparison and not one of analytical identification.

Seven anthocyanin spots were clearly separated in the plates and were present in both the nylon and the Polyclar extracts. The main difference is the presence of a diffuse band of clear-brown material in the lower Rf range of the chromatogram of the nylon extract. This material is probably composed of tannins and tannin-associated anthocyanins. Justification of this hypothesis was obtained by analysis of the phenolic fractions from the two extracts and data are reported in Table 4 for their composition.

Finally, we determined the absorption spectra in the range of 400–600 nm in two extracts obtained from nylon and Polyclar under identical experimental condition. The spectra have been reproduced in Fig. 7.

If we calculate, as a measure of purity of the anthocyanins the ratio of maximum to minimum Absorbance values, which in both cases corresponds to 538 and 400 nm, respectively, the following values are obtained:

Resin	$A_{538\ nm}/A_{400\ nm}$
Polyclar	4·50
Nylon	2·35.

Thus, these data also confirm that a purer anthocyanin solution was obtained from Polyclar.

Conclusions

Adsorption by nylon and Polyclar may be used for the separation of anthocyanins from red-wine distillation wastes. Both resins adsorb all phenolic compounds from the solution, thus eliminating acids, salts and other contaminants. Carrying out the desorption process with methanol-0·1% HCl allows the recovery of the anthocyanins although complete purification cannot be achieved. In fact the nylon extracts contain minor amounts of tannin-associated anthocyanins and both the nylon and Polyclar extracts contain a high proportion of simple phenolics. It may be suggested that, if necessary, the simple phenolics can be removed from the solution before the adsorption process by extraction with ethylacetate, for example, but this procedure is hardly justified in practice.

Considering the technology for desorption, simple or multiple extraction can be applied to the Polyclar complexes, while countercurrent is the most efficient extraction mode for the nylon complexes.

It may be further considered that wine distillation wastes have a high polluting potential and are unsuitable for treatment in biological depuration plants because of the strong bacteriostatic action of phenolics, particularly tannins.

Thus, the suggested process allows the recovery of an interesting by-product and, at the same time, leaves a solution perfectly suitable for microbial degradation.

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Comparison of methods of freshness assessment of wet fish

IV. Assessment of commercial fish at port markets

J. J. CONNELL, P. F. HOWGATE, I. M. MACKIE, H. R. SANDERS AND G. L. SMITH

Summary

Two sensory methods (General Appearance and Raw Odour) and two instrumental methods (Torry Fish Freshness Meter and Intelectron Fish Tester V) were tested on Aberdeen and Hull Markets. The variability of each method, comparisons between the methods and the time required to carry them out under market conditions were obtained. The instrumental methods (particularly the averaging version of the Torry instrument) are the most economical to operate.

Introduction

Papers I, II and III of this series (Burt *et al.*, 1975, 1976a, b) have reported on comparisons between some sensory, chemical and instrumental methods of assessing the freshness of iced cod (*Gadus morhua*). In Paper I it was shown that the different sensory factors—general appearance, raw odour, cooked flavour and cooked odour—gave similar results when used in the laboratory to assess the freshness of boxed experimental fish. In Paper II the two instrumental methods (Torry Fish Freshness Meter and Intelectron Fish Tester V) and two chemical methods (hypoxanthine determination and trimethylamine determination) were used. In Paper III all the above tests were applied in the laboratory to bulked and boxed commercial fish. Regression coefficients against age-in-ice were estimated, and it was shown that the chemical methods were the most consistent at predicting time of storage in ice.

One of the most important stages at which assessment of freshness is required is at port markets where fish, which have been bulked or shelved on board, are laid out in market containers. It is at this stage that much of the fish landed in the United Kingdom is sold for the first time, and freshness is an important factor in determining price. It was therefore decided to investigate whether the methods of freshness assessment previously described could be applied to grading in this situation. The study was stimulated by the impending entry of the UK into the European Economic Community and the conse-

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quent requirement to introduce grading schemes for wet fish. As in Paper III no distinction was made between fish from the two types of stowage.

The following conditions under which a grading scheme might operate were assumed:

(1) The fish are cod, whole, gutted, well iced on board the vessel.

(2) The fish are laid out with little or no ice in market containers of about 50–70 kg (i.e. 1 kit) capacity.

(3) The fish are roughly graded for size.

(4) Some mixing of freshnesses exists within and between containers.

(5) The number of inspectors available for testing would be sufficient to examine all the fish in the market, but would not be so large as to be prohibitively expensive.

(6) Limited laboratory facilities are available if necessary.

(7) Destructive sampling is permitted.

(8) The time available to inspect the fish on the market would be limited by the unloading and auction practice; at the main UK markets this would normally be 3–6 hr.

The requirements for a grading scheme were as follows.

(1) The criterion for grading is defined in terms of the method specified. If, for example, raw odour were specified, grade boundaries would be at points on its scale. There would be no attempt to relate back to any other method of freshness assessment, or days-in-ice, before grading a batch.

(2) The inspector sets out to grade batches of fish on the market into one of four grades, using the method chosen as the criterion for grading.

(3) The inspector wishes to place batches of fish in grades so that, in the long run, not more than a certain percentage of batches is misgraded.

Materials and methods

Methods of assessment

All methods used in the laboratory in the earlier parts of this series are theoretically applicable to a market situation, but some were excluded from further consideration in this exercise because they require too much time, facilities or manpower. The sensory methods on cooked fish are not considered, because they would offer no advantage over general appearance or raw odour. They require larger sample numbers, are lengthier and more inconvenient to carry out and it is unlikely that appropriate facilities would be available. The chemical methods were also excluded on the grounds that the difficulties of organizing sampling and the associated operations within the time and space available would be too great. However, it was felt that if the rapid hypoxanthine screening method for use in the field (Burt, Stroud & Jones, 1969) had similar precision to that of the automated method, it might be feasible.

The experiment was carried out, therefore, using general appearance (GA), raw

odour (RO) (Shewan *et al.*, 1953), the Torry Fish Freshness Meter (TFM) (Jason & Richards, 1975) and the Intelectron Fish Tester V (IFT) (Hennings, 1963, 1965). Sensory assessments were carried out by a panel of two judges. In addition to the version of TFM used in Papers II and III, an averaging version was available (TAV), which could be set to display the arithmetic mean of 15 or 30 consecutive readings.

Choice of ports

It was decided that the investigation should be carried out on the fish markets at Aberdeen and Hull, the largest fishing ports (in terms of weight of fish landed) in Scotland and England, respectively. At Hull, all the wet fish landings are from distant water vessels fishing mainly Iceland, the Norwegian Coast, or Barents Sea, and it is also the base for most of the UK freezer fleet. In 1971 landings of wet and frozen fish at Hull from British vessels totalled 165 000 tonnes out of a UK catch of 976 000 tonnes (MAFF, 1972). Landings at Aberdeen, apart from those from a smaller number of distant water vessels, are predominantly from near or middle-water grounds, i.e. North Sea, West Coast of Scotland, Shetland and Faroes, and amounted to 106 000 tonnes in 1971 (DAFS, 1972). Thus between the two ports a large range of freshnesses, typical of UK wet fish, could be obtained, up to 10 days in ice for Aberdeen landings and between 5 and 18 days in ice for fish landed at Hull.

Market layout and unloading procedure

On Aberdeen market, bulked cod is sorted on landing into five size grades and set out on the market floor, all but the largest fish being laid in nominal 8 stone (51 kg) boxes. (At the time of the exercise, wooden boxes were in use, but they have since been largely replaced by plastic boxes.) At Hull, two size grades are used, the fish being placed in nominal 10 stone (64 kg) metal tub-shaped kits.

At Aberdeen, boxes are laid out in rows of 10 or 15. Landings from each boat are kept together, ordered by size and age, the most recently caught fish generally at the outside of the array. At Hull each bay of the market contains a rectangular array of 8 rows of 10 kits with a narrow passage between the two halves, or 'heaps' of 4 rows each. Fish of different size grades are separate and there is a rough ordering according to the age of the fish, but the pattern varies with boats.

The type of stowage on board the vessel can affect the distribution of freshnesses throughout the array on the market floor. On distant-water trawlers, it has been the practice for the earlier caught fish to be placed in bulk stowage, several layers high, while the later part of the catch is shelved in single layers. It is the intention to discharge the catch in reverse chronological order of catching, but some mixing of freshness is inevitable. The mixing is increased when unloading is carried out through two hatches at opposite ends of the deck, or when fish are unloaded by mechanical conveyor.

Unloading starts at Aberdeen at 04.00 hours with up to seven boats being unloaded simultaneously, the number of boats depending on their size. On one of the

mornings of the exercise, a large distant-water trawler was landing its catch, and only a further four boats could be unloaded at the same time. The first auction starts at 07.30 hours by which time only catches from the first unloading may be completely ready for sale. Unloading continues if necessary until approximately 10.00 hours, when a second sale takes place. At Hull unloading commences at 02.00 hours and is usually completed by 07.00 hours. The auction starts at 07.30 hours.

Batch size

The size of a batch for sampling purposes was chosen in accordance with the market layout and the expected size of a 'lot', or quantity of fish of the same species and size grade caught by one boat on one day.

Records of fish landings at Aberdeen and Hull from the corresponding period of the previous year were inspected, and it was calculated that the mean day-catch (of all sizes) of cod from a motor trawler was 27 boxes at Aberdeen and 132 kits at Hull. The day-catch at Aberdeen was lower than that at Hull because the Aberdeen figure is the average for distant, middle and near-water trawlers of a variety of sizes whereas landings at Hull are all from distant-water vessels which are larger. The expected number of day-catches on the market on one day was 70 at Aberdeen and 40 at Hull.

It was decided therefore that for Aberdeen a batch should be one row of 15 boxes, or two adjacent rows of 10 boxes, provided that they contained fish of the same size grade. From a first unloading of five boats, there were expected to be 40 batches out of a total landing for the day of between 50 and 180. A day-catch was expected to cover one to two batches. However, when a day-catch is small or includes several size grades, a batch may contain more than one lot. For Hull, it was decided that a batch should be a 'heap' of 40 kits, the number of batches expected ranging from 60 to 210, and each day-catch extending over about three batches.

Tolerances

A viable grading scheme would have to enable all batches of fish on the market to be graded, but it would be beyond the endeavour of a reasonable number of judges to assess every individual fish within the time available. A typical batch, as defined above, might contain as many as 400 fish. It is therefore necessary, if all batches are to be graded, that within each batch only a sample of the fish should be taken. However, since grade boundaries would be defined in terms of the freshnesses of all the fish in the batch (e.g. 'a batch shall be assigned to Grade 2 if the mean RO score is less than 8 but not less than 6), correct grading can only occur if all the fish in the batch are inspected.

A representative sample of the fish in the batch will give an estimate of the overall freshness of the batch, and the grade will be assigned on the basis of the sample. Sampling error would make itself evident as misgrading of batches, i.e. for some batches the grade

assigned on the basis of the sample would not be the same as that which would be assigned had all fish in the batch been inspected.

The larger the sample taken, the less will be the chance of misgrading, but beyond a certain point the level of misgrading is sufficiently low that further effort to reduce it would not be justified. It was decided to choose a sampling scheme such that, in the long run, 10% of batches would be misgraded.

It may be shown, given a uniform distribution of freshnesses over a reasonable range, that this tolerance of 10% is achieved when batch means are estimated, with 95% confidence, to within the equivalent of $\pm 1{\cdot}5$ days in ice.

Sample size

Sample sizes were calculated on the basis of the standard deviations observed in boxed fish (Burt *et al.*, 1975, 1976a) and an assumed pattern of mixing such that a batch contained 5, 10, 70, 10 and 5% respectively of fish caught on each of five successive days. One fish was to be taken from each container sampled.

If σ is the standard deviation, b the regression coefficient against days-in-ice for the method and z the standard normal deviate for the appropriate confidence limits, then the sample size n required to predict the batch mean to within $\pm L$ days is

$$n = \left(\frac{z\sigma}{Lb}\right)^2.$$

For the sensory methods the observed standard deviations were based on mean scores for a panel of five, and had to be adjusted to allow for the use of only two judges.

Under these conditions, the required sample sizes for the accepted degree of misgrading were 4 for GA, 3 for RO, 15 for TFM and 10 for IFT. It was assumed that in the sensory tests the same fish would be assessed by both judges, but after a preliminary run on Aberdeen market it was concluded from the relative values of between-fish and residual variances that better precision would be obtained for the same effort if judges assessed different fish.

Teams (personnel taking part)

For each of the methods of assessment, two teams were used. A team for TFM or IFT consisted of two members, an operator and a recorder, the roles being exchanged each half hour. The operator applied the instrument to each sampled fish and called out the score to the recorder who noted it on a form. The recorder summed the scores for the fish in a batch and assigned a grade to the batch on the basis of the total.

For TAV only one operator was required, who recorded the mean score and assigned the grade. As already stated, a sensory team (RO or GA) consisted of two judges who assessed different samples in each batch. Each judge recorded his scores on a pad attached to his arm, and when the batch had been completed the judges together assigned a

grade to the batch on the basis of their combined total. RO teams were instructed to break for 10 min after every sixth batch tested, to avoid sensory fatigue.

With each team, sensory or instrumental, there was an observer who directed the team to each batch they were to grade and recorded the time taken to assess each fish, grade the batch and move to the next batch. The observer took no part in assessment or grading.

Sampling plans

A systematic sampling plan was used for Aberdeen. Where a batch consisted of a single row, testers using instruments sampled one fish from each box, taking a second if necessary, until 15 (for TFM) or 10 (for IFT) fish had been sampled. Where a batch consisted of two rows of ten boxes, a starting point was chosen at random and one fish from every second box—going clockwise or anticlockwise—sampled from there. Sensory judges took a starting point at random and each judge chose a systematic sample of 3 (for RO) or 4 (for GA) boxes. Fish were taken from below the top layer of each box for all methods of testing, in case the top layer was not representative of the contents of the box. This requirement was not made for TAV, as it would have caused great difficulty for the single operator.

At Hull, a systematic sample of the sizes required would tend to favour certain parts of the heap, and a random sampling scheme would be practically impossible to operate in the time available in a busy market. Judges were therefore given two representative sampling schemes from which they could choose one at random for each batch. As at Aberdeen, one fish was taken from each of the required number of containers.

Results and discussion

The standard deviation for each team within each batch was calculated and the pooled

TABLE 1. Pooled within-batch standard deviations

Market	Method and team							
	GA		RO		TFM		IFT	
	1	2	1	2	1	2	1	2
Aberdeen	0·53	0·50	0·79	0·68	14·8	14·8	—	12·3
Hull	0·53	0·48	0·93	0·70	13·7	12·4	10·8	13·2
Pooled	0·51		0·78		14·0		12·1	
Prior estimates	0·27*		0·42*		8·6		10·2	
	0·33†		0·50†					

* Based on mean score from two judges.

† Assuming judges assess independent samples.

values on each market are shown in Table 1. The value for each method over the whole investigation is also given, along with the prior estimated values that had been used to calculate the sample sizes. For the sensory methods, estimates are shown not only for the originally intended situation of judges assessing the same fish, where they are based on panel means, but also recalculated to allow for judges assessing independent samples, where they are then based on individual scores.

Within each method, there were no significant differences between the standard deviations for the two teams on either market.

For all the methods the actual standard deviations are higher than the forecasts, which had allowed for an assumed pattern of mixing. The increase may be partly due to the degree of mixing being greater than expected, but some of it is probably caused by rougher handling; it was noted in Paper III that commercial fish had a larger standard deviation than experimentally caught and handled fish. Furthermore, it should be appreciated that the sensory assessments were being performed in quite different conditions from those in the laboratory, as described in the earlier parts of this series. Working conditions on a fish market are usually cold and somewhat smelly and there are continuous distractions of noise and bustle. Since fish have just come out of the vessel's hold, they will be at or near 0°C and with reduced volatility the more subtle and faint odours will be more difficult to detect on the market than in a heated laboratory. The requirements of a grading scheme also result in the judges having to assess more fish than in a laboratory experiment.

Since standard deviations were higher than anticipated, larger sample numbers would then be required to obtain the same confidence (in terms of the test used) in determining the sample mean.

Calibration

If the methods are to show agreement in their grading, it is necessary to determine the numerical relationships between them. It was deemed appropriate to use the linear functional relationship (Lindley, 1947) to calibrate one method of assessment against another. Sample sizes had been chosen so that standard errors of batch means would be equivalent whatever the method of assessment, and the functional relationship can be obtained in the situation where the ratio between errors on the two variables is known. The functional relation was chosen, rather than linear regression, since calibration of two methods was required, not prediction of one from the other.

The functional relationships, obtained over all days, are shown in Table 2. The number of batches is not the same throughout, because not all teams assessed every batch, and a relationship between two methods was estimated only on those batches which were assessed by both.

Figures 1–3 show batch means for TFM, IFT and GA respectively plotted against batch means for RO, with the functional relationship line drawn in for each figure. For the same sensory score, instrumental readings are lower than for the experimental

TABLE 2. Functional relationships

Methods		No. of batches	Correlation coefficient	Intercept	Slope
y	*x*			*a*	*b*
TFM	RO	60	0·86	−50·6	13·3
IFT	RO	60	0·87	−42·5	13·6
TFM	GA	60	0·86	−32·8	22·2
IFT	GA	61	0·79	−25·9	23·0
GA	RO	77	0·92	− 0·862	0·605

The relationship may be expressed as $y = a + bx$ where x and y are the appropriate pair of methods.

All correlations in the table are significant at the 0·1% level.

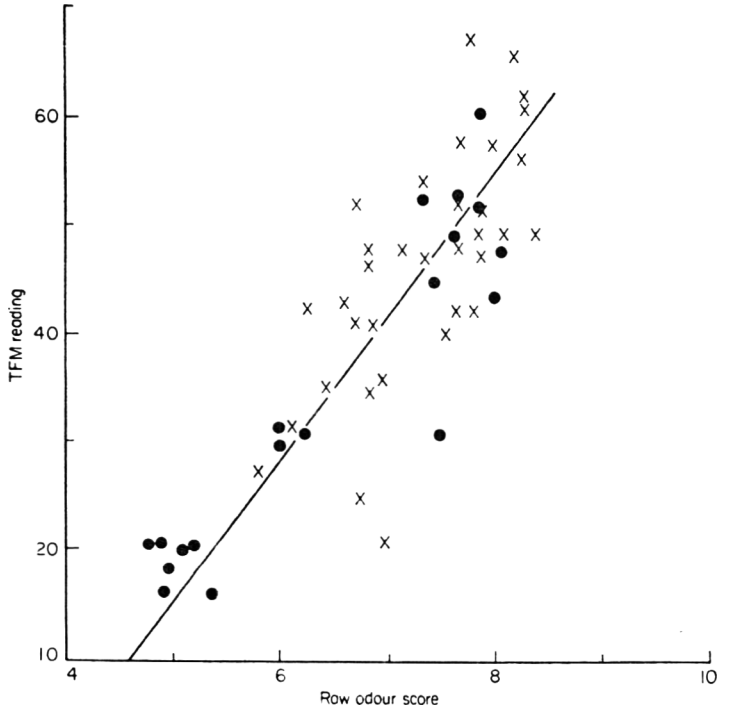

FIG. 1. Relationship between Torry Fish Freshness Meter reading and raw odour score. Batch means and fitted functional relationship line. × Aberdeen; ● Hull.

fish in Paper II. This affect has been noted in laboratory assessments of commercial fish (Burt *et al.*, 1976b) and was attributed to physical damage to the skin and the underlying tissues. Provided that the instruments are calibrated appropriately to the kind of fish to be tested, this effect does not invalidate their use as indicators of freshness.

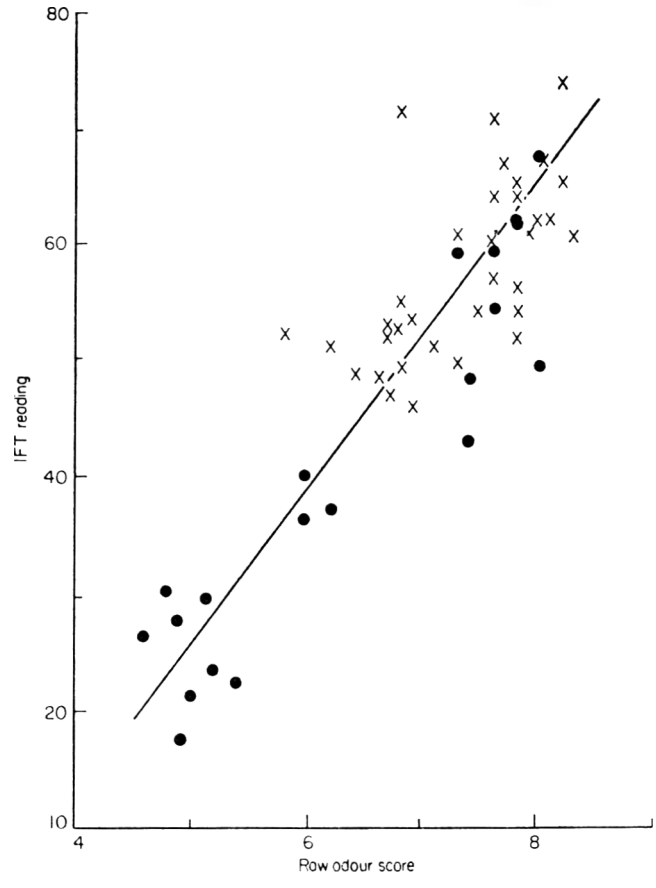

FIG. 2. Relationship between Intelectron Fish Tester reading and raw odour score. Batch means and fitted functional relationship line. × Aberdeen; ● Hull.

Speed and accuracy of operation

An essential part of an investigation into the applicability of the methods to grading under commercial conditions is an examination of the time required to grade a batch by each method. As the total time available for grading all fish on the market is limited to that between unloading and auction—at the largest UK ports this could require 120 batches to be graded inside 2 hr—for a method to be acceptable, the work must be completed with a sufficiently small number of inspectors, in the time available.

The mean rate of grading achieved throughout the exercise, at each port, is shown in Table 3. In all cases, grading was slower at Hull than Aberdeen, mainly because it was more awkward moving from one batch and gaining access to the next. With TAV, it was not necessary to interrupt testing to write anything until 15 fish had been tested. All methods except TAV required two people to test and record.

The estimated rate of grading which would be achieved, based on sample sizes

TABLE 3. Rate of grading

(a) No. of batches per team-hour in the exercise

Sample size	GA	RO	TFM	TAV	IFT
	4	3	15	15	10
Aberdeen	18	16	14	21	13
Hull	14	14	10	15	11

(b) Estimated no. of batches per team-hour, using sample sizes derived from obtained standard deviations and functional relationships

Sample size	GA	RO	TFM	TAV	IFT
	6	5	18	18	13
Aberdeen	14	11	13	19	11
Hull	11	10	8	13	9

RO, GA, two judges; TFM, IFT, one operator, one recorder; TAV, one operator.

derived from the observed standard deviations and between-method relationships, are also shown in Table 3. The rates show little change for the instrumental methods.

The differences between teams using the same sensory method were examined. One of the teams using RO gave a mean score which was on average 0·3 unit higher than that for the other team, while for the teams using GA the mean difference was 0·4 unit, both these effects being significant at the 0·1% level. This could be caused by a bias by one or more judges. Where a bias is consistent, it can be monitored and the panel mean subsequently adjusted (Shewan *et al.*, 1953) but if grading is the aim the effect could be serious if many batches are close to a grade boundary. It is important therefore when grading is carried out by sensory methods that steps be taken to reduce biases to the smallest possible extent.

Conclusions

All the methods can be used under market conditions. However, the number of sensory judges required, and the expense incurred in training them, probably rules out sensory methods in the forms that were used in the exercise. The use of the sensory methods under commercial conditions would necessitate some change in the requirements for the grading scheme; for example, the degree of misgrading could be set higher

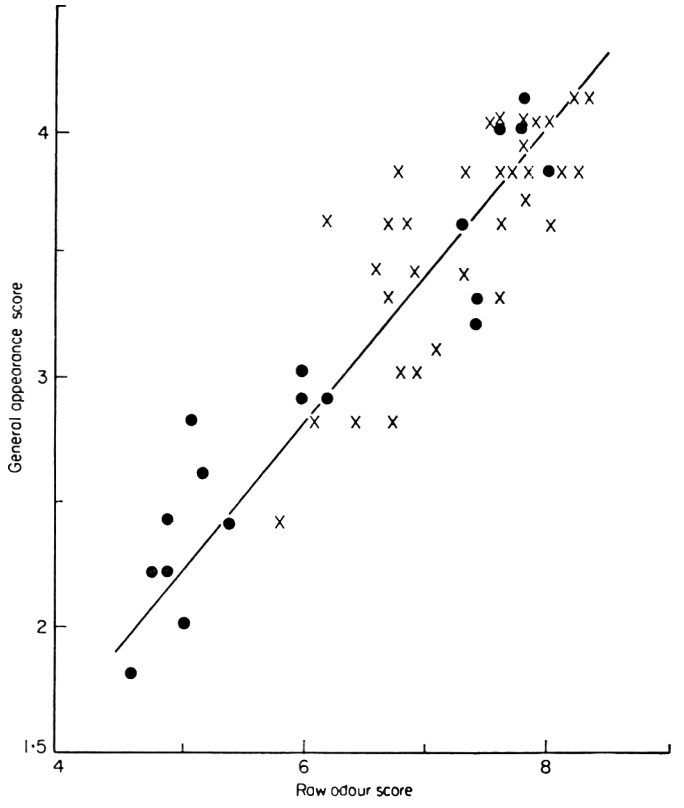

FIG. 3. Relationship between general appearance score and raw odour score. Batch means and fitted functional relationship line. × Aberdeen; ● Hull.

than 10% or some preliminary screening could be undertaken so that only batches near a grade boundary need be assessed in detail.

Since fish-to-fish variation was higher than expected, larger sample numbers than used in the exercise would be needed to estimate a batch mean with the desired precision.

The speed of operation depends on the type of market and the ease of access to each container. Thus, for all methods times were slower at Hull than at Aberdeen. For the required sample sizes the averaging version of the Torry Fish Freshness Meter was not only the fastest method, but, as it required only one operator, was by far the most economical method.

There was considerable variation in the number of completed batches available for testing at any particular time, depending on the number and size of the boats being unloaded. At Aberdeen unloading at one end of the market is generally still proceeding when auctioning has started at the other. For some batches, therefore, very little time is available between their completion and their sale. Difficulties could therefore some-

times arise if a grading scheme of the type outlined here were to be applied without changes in market practice.

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Technical note: An alkali degradation test and an alcoholic alkali bran-staining test for determining the approximate degree of milling of rice

K. R. BHATTACHARYA AND C. M. SOWBHAGYA

The degree of milling (d.m.) of rice indicates the per cent by weight of brown rice (i.e. shelled paddy) lost during its milling (or whitening) to produce milled rice: in other words the amount of 'bran' (i.e. 18 mesh powder) produced during milling expressed as a percentage of the brown rice. The d.m. is a measure of the extent to which the various outer layers, including the germ, have been removed from the rice kernel. As such it is a very important parameter, for it not only determines the aesthetic appearance of rice but also profoundly influences its nutritional, chemical and storage behaviours (Hogan & Deobald, 1965). Specification of the d.m. is therefore important not only in experimental work but also in grading for marketing and trade.

Rigorous laboratory tests for determining the d.m. of rice are available (Hogan & Deobald, 1965; Bhattacharya & Sowbhagya, 1972a). But these are generally too elaborate and complicated for routine testing for grading and marketing. Visual examination is still relied upon for the latter purposes (Hogan & Deobald, 1965). Clearly there is a need for simple tests for this purpose.

F.A.O. (1970), while formulating a model system of rice grading for international trade, tentatively proposed an iodine test for classifying rice according to its d.m. In this a few kernels of the rice are immersed in a 0·02% solution of iodine when the residual bran streaks on the kernels would stand out on a blue background. However, we found the test quite unsatisfactory. The residual bran streaks did not in fact stand out distinctly and it was difficult to distinguish between samples milled to different extents. Two alternative simple and more reliable tests have been developed and are described here.

A local *indica* variety of rice, Coimbatore Sanna, milled to several degrees of milling ranging from 0–10% by the method described earlier (Bhattacharya & Sowbhagya, 1972a) was mainly used in the studies. The last sample mentioned (10% d.m.) represented the limit of milling by the McGill miller No. 1 used.

(1) *Alkali degradation test*

Attempts to see whether the wellknown alkali degradation test for rice quality

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(Little, Hilder & Dawson, 1958; Bhattacharya & Sowbhagya, 1972b) possibly gave any difference in the rate or pattern of reaction when applied to rice with different degrees of milling generally gave negative results. But it was observed that there was a tendency for the residual bran tissue to get detatched from the rice endosperm when subjected to the test. It was then seen that if the rice kernels were immersed in a concentrated aqueous alkali solution (3–5% KOH or 2% NaOH), the endosperm in all the samples was rapidly attacked and gelatinized, leaving the residual bran layers intact as a separated skin.* The following test was developed on this principle.

A few (5–6) kernels of the rice to be tested are taken in a 5 cm diameter Corning Petri dish standing on a black sheet. The kernels are covered with 10 ml of 2% NaOH and the dish is covered and left undisturbed. The endosperms are rapidly attacked and completely gelatinized within 3–4 hr, when, preferably after overnight standing, the detached residual bran layers stand out very well against a nearly transparent background. This is shown in Plate 1(a). The approximate d.m. can then be determined from the nature and amount of the separated bran layers by means of a score card (Table 1).

This technique can also be perhaps utilized to isolate bran tissue for experimental

TABLE 1. Classification of rice according to degree of milling and their testing

D.m. (%)	Designation	Alkali degradation test	Alcoholic alkali bran-staining test
0–2	Husked (or unmilled) rice	Bran skin one whole; or an intact leaf-like skin (main), with some additional slightly disorganized skin (subsidiary); germ > 1 mm dia	Over 50% of kernel face covered with unbroken yellow-brown bran film
2–4	Undermilled rice	Main skin full but slightly damaged; subsidiary skin quite disorganized and usually disposed on either side of main skin; germ about 1 mm dia	Distinct 4 full-length yellow-brown streaks (on top face of kernel); some inter-streak space also filled (usually the dorsal rectangle completely filled)
4–6	Lightly milled rice	Main skin rather disorganized, splitting and thinning; subsidiary strands powdery-fibery; germ like a pin head	Distinct 3–4 hair-line streaks, some broken; inter-streak space clear
6–8	Reasonably well milled rice	No compact skin; all dispersed into small strands of fibre, a few whole, majority fragmented; germ like a needle point	At least 1 rather distinct and nearly full-length very thin streak, with 1 or 2 more very faint and fragmented ones
8–10	Well milled rice	A few faint strands of fibre or nil; germ like a needle point	Kernel face practically clear

* This observation was first made in mid-sixties by Dr P. V. Subba Rao, then in this laboratory.

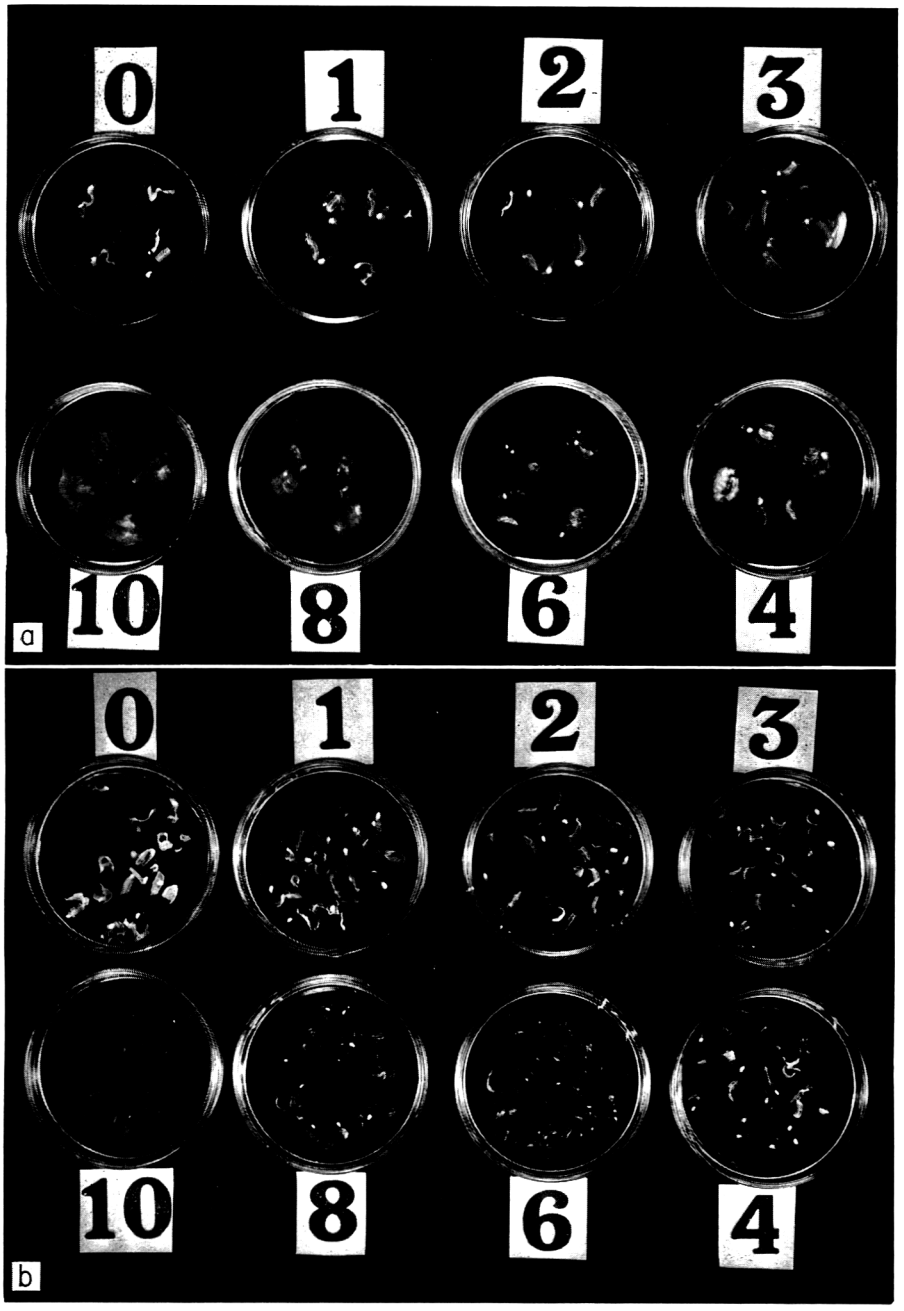

PLATE 1. (a) Alkali degradation test for approximate d.m. of rice. Photograph showing gelatinization of rice endosperm in 2% NaOH (18 hr), leaving the residual bran as a separated skin clearly visible against the degraded endosperm. The numerals identify the d.m. of the samples. (b) Isolation of bran tissue from rice of different d.m. (indicated) by alkali. Rice (0·25 g) was soaked with occasional shaking in 10 ml of 2% NaOH in a glass-stoppered test tube overnight. Next morning the slightly viscous medium containing the gelatinized endosperm was shaken and drawn off; the bran skins were washed twice with distilled water and then suspended in water in a Petri dish. The diffused bits of matter visible in all the dishes are a part of the gelatinized endosperm that remained.

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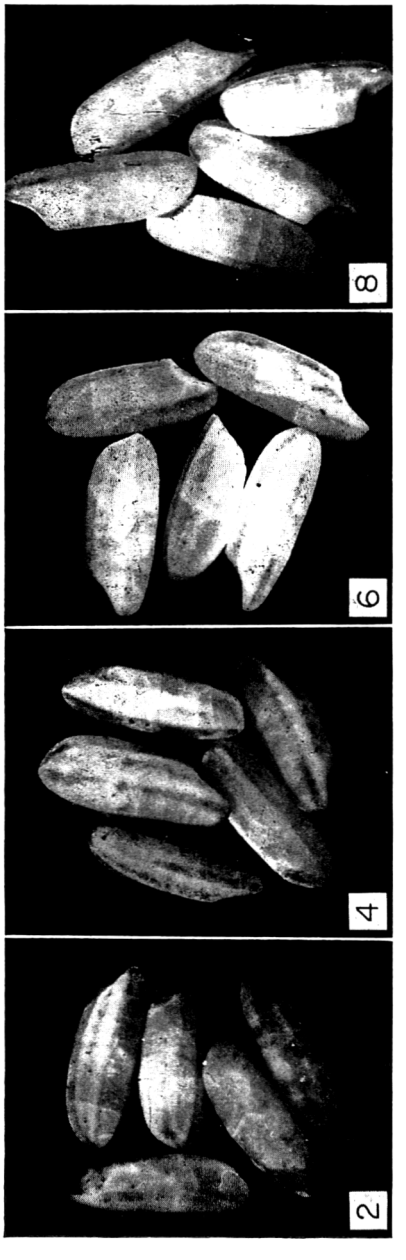

PLATE 2. Alcoholic alkali bran-staining test for approximate d.m. of rice. Yellow-brown patches or streaks of bran are clearly visible up to 6% d.m. rice (in decreasing order); they are faint in 8% d.m. rice. Photographed with blue filter to emphasize the yellow colour. Photographed here on a black background to eliminate the shadows of the kernels for better reproduction; but in actual testing, the streaks show up much better against a white background. D.m. of samples 2, 4, 6, 8%.

or other purpose by conducting the test with larger quantities of rice in a suitable container (Plate 1(b)).

(2) *Alcoholic alkali bran-staining test*

It was earlier shown (Bhattacharya & Sowbhagya, 1972a) that a 1:2 (v/v) mixture of 2% aqueous KOH and *n*-propanol extracted the residual bran pigments in rice as a yellow solution, which could be quantitatively measured as an index of its d.m.

In trying to adapt the above method as a rough test with whole rice kernels, it was observed that if the alcoholic alkali solvent was poured on the rice kernels taken in a Petri dish, the kernels immediately turned pale yellow while the residual bran soon turned dark brownish-yellow. The yellow-brown patches or streaks of bran thus stood out clearly on the kernel surface and hence gave a very good indication of the approximate d.m. of the rice. This is shown in Plate 2. The streaks became distinct after about 15 min and gradually faded in intensity after 1 hr.

Ethyl alcohol was substituted for *n*-propanol for the test, being equally satisfactory for the present purpose. Even denatured spirit can be used in place of alcohol; it gives a slightly turbid solution, but this does not interfere with the test overmuch.

For the test, the kernels to be tested (1 g) are taken in a 5-cm diameter Corning Petri dish, placed on a white background, and then covered with 10 ml of the 2% aqueous KOH:ethyl alcohol (or denatured spirit) (1:2, v/v) solvent. After 15–30 min, the kernels are observed carefully with a magnifying glass and the relative size and amount of the yellow-brown patches or streaks of residual bran are noted and then compared with a score card (Table 1).

Both these simple methods should be quite suitable for determining the approximate d.m. of rice for the purpose of rice trade. Method 1 is very objective because it actually separates out the bran that is still remaining on the kernel, but Method 2 has the advantage of being almost an instant test (15 min) and hence can also be used for quality control in the rice milling industry.

Preliminary testing with two other varieties showed that the methods were applicable to them as well and also to parboiled rice (one variety). Method 2, in fact, gave better results with parboiled rice because the yellow streaks stood out even better against the translucent kernels.

These methods, however, would not reveal the actual d.m. as understood in rice research, but only the approximate d.m. in terms of some verbal expressions. The F.A.O. (1970) in the study earlier mentioned classified rice on the basis of its approximate degree of milling into the following categories: 'husked', 'undermilled', 'reasonably well milled', 'well milled' and 'extra well milled'. These were defined in terms of further subjective expressions, such as 'paddy from which . . . the outer bran layers and the greater part of the inner bran layers have been removed. . . .' These expressions and definitions are somewhat confusing and arbitrary, and the designations and their definition in terms of per cent d.m. suggested in Table 1 seem to be preferable.

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Technical Note: Improvement of texture of frozen vegetables by stepwise blanching treatments

E. STEINBUCH

The quality of frozen vegetables is sometimes inferior to that of fresh prepared products. Especially, the texture of some frozen vegetables is seriously softened after cooking. For example, the cooked frozen green beans have lost the crispness, being still detectable in cooked fresh beans. Frozen carrots are somehow characterized by a softened rubber-like tough structure.

Texture changes in frozen vegetables are generally considered to be affected by blanching, freezing, storage and cooking conditions. However, high blanching temperatures are preferred, in order to obtain a rapid enzyme inactivation. Consistency and other quality aspects are protected by the application of high freezing rates and proper storage temperatures.

As reported by Van Buren, Moyer & Wilson (1960) and confirmed by Hoogzand & Doesburg (1961) there exists a relationship between the applied blanching conditions and the firmness of canned vegetables. The firming effect of a low temperature long time (LT-LT) treatment is considered to be related to activation of pectinesterase.

The effect of LT-LT blanching conditions is studied in regard to the maintenance of the desired texture of frozen vegetables. However, LT-LT blanching conditions are not suitable for inactivation of those enzymes, which are responsible for quality decrease during storage of the frozen product. Therefore, a second heat treatment at elevated temperatures is necessary for enzyme inactivation.

Results of first experiments (Steinbuch, 1973) indicate that a LT-LT heat treatment prior to normal blanching procedures at 98°C favourably affects the texture of frozen beans after cooking. Continued experiments confirm these findings. Figure 1 shows the relation of the blanching time at 70°C with the tenderometer value of the prepared green beans. The more the blanching time is prolonged at lower temperatures, the more the hardness of the cooked product is increased. Water cooling between the two blanching treatments does contribute to an increased hardness of the texture in some cases.

The effect of the temperature of the pre-blanching treatment is shown by Fig. 2. Obviously, the enzyme pectinesterase is inactivated at 80°C and higher temperatures, as illustrated by the decrease of the hardening effect on bean texture. There are some indications that a preheat treatment at 75°C activates pectinesterase optimally, resulting in a maximal increase of texture hardness.

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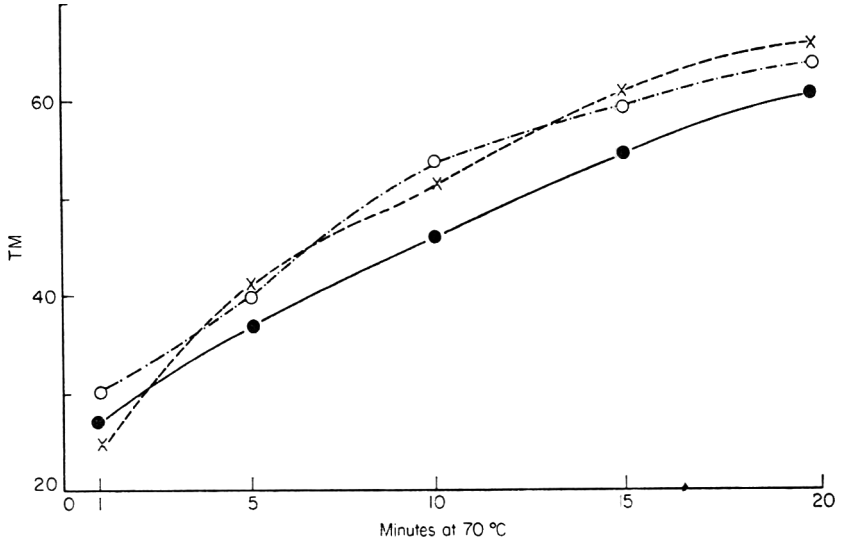

FIG. 1. Effect of a long low blanching process prior to a short high treatment on the texture (tenderometer value) of prepared frozen green beans. ●, Blanched 4 min at 98°C; ×, water cooled and blanched 4 min at 98°C; ○, water cooled and blanched 20 sec with steam and a holding time of 3 min.

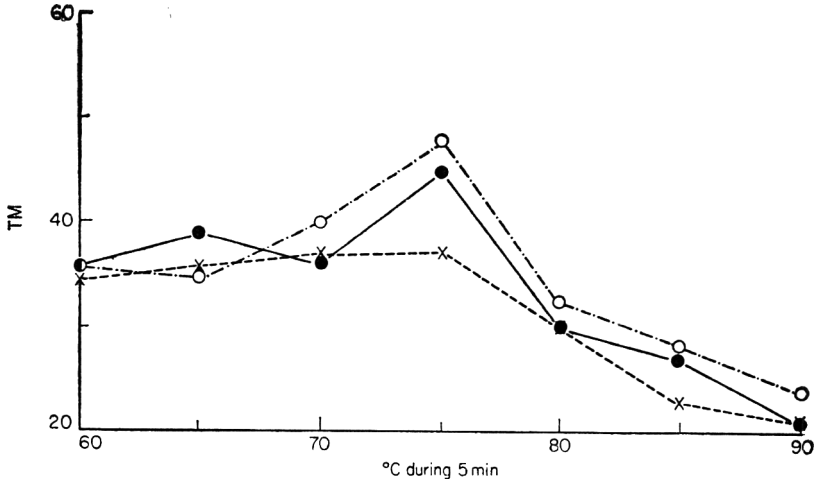

FIG. 2. Effect of the temperature during a heat pretreatment, prior to a short-high blanching process on the texture (tenderometer value) of prepared frozen green beans. Symbols as in Fig. 1.

Texture hardening by stepwise blanching treatments can be produced in broad beans to a slight degree, but not in peas. The leaching of valuable soluble material during the prolonged stay in heated water can be inhibited by the application of short-high steam treatments or saturated blanching water.

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(*Received* 20 *November* 1975)

J. Fd Technol. (1976) **11**, 317–318.

Book Reviews

Plastic Films and Packaging. By R. C. Oswin.
London: Applied Science Publishers, 1975. Pp. xi + 214. £8.00.

Dr Oswin's book, *Plastic Films and Packaging*, will be of interest to all those engaged in either the manufacture of plastic film or their use in packaging.

The subject matter is treated in logical sequence dealing firstly with the polyolefines and then progressively substituted olefines, polyesters, polyethers and polyamides. For each of the many individual films there is given a brief description of manufacture of the polymer, methods of film manufacture, film properties and applications. The main physical properties are tabulated for each film and this facilitates comparisons between different materials. The chemical structures are given and, where appropriate, diagrams are included to show spatial aspects of the chemical molecule. In this section of the book perhaps insufficient consideration is given to the films which have large commercial applications. For example, only four pages are devoted to LD polyethylene and eight to the fluoropolymers. Also, discussion on plastic film laminates is brief and possibly inadequate. Subsequent chapters outline the principles of packaging, machinery operations (heat sealing, cutting, etc.) and the practical and theoretical aspects of selecting films for packaging and non-packaging outlets as well as costs and benefits arising from the use of packaging films.

Overall the book is written with a style and content which should keep the reader's interest. For the specialist technologist there are over 200 well selected literature references.

E. K. Munday

Doughs and Baked Goods. Chemical, Air and Non-Leavened. By D. J. DeRonzo.
New Jersey: Noyes Data Corporation, 1975. Pp. xii + 435. US$36.

The Noyes Data Publications Food Technology Review No. 26 deals with patents granted since 1960 concerned with chemically leavened bakery products such as angel food cakes and with non-leavened products such as pies and tortillas. As usual the patent coverage is very extensive: 186 patents are reviewed. Each patent is concisely described and sufficient detail is given to make them entirely comprehensible.

Subjects covered include agglomeration, anti-staling and anti-mould additives, formation of refrigerated cake mixes, water proofing of baked products, dietetic products, shelf stable intermediate moisture products, improved baking powders and a variety of shortening compositions including powdered fats. Flavouring processes and fillings, toppings and coatings, etc. are also considered.

The book is thoroughly recommended for its comprehensive coverage of the subject.

Judith V. Russo

Books Received

Consumer Health and Products Hazards. Cosmetics and Drugs, Pesticides and Food Additives. Volume 2 of *The Legislation of Products Safety*. Ed. by S. S. Epstein and R. D. Grundy.
Cambridge, Mass.: M.I.T. Press, 1974. Pp. xi + 389. £7.50.

A collection of six review articles on consumer protection in the U.S. One of these the development and principles of the present legislation relating to food additives.

Feed Energy Sources for Livestock. Ed. by H. Swan and D. Lewis.
London: Butterworth, 1976. Pp. iii + 158. £4.00.

A collection of papers read at Nutrition Conference for Feed Manufacturers in 1975.

The Second Edition

Handbook of Flavour Ingredients

Edited by Thomas E. Furia and Nicolo Bellanca, *Dynapol, Palo Alto, California*

The new data incorporated into this well-known reference is so extensive that the work has been expanded into two volumes. The general format and contents of the first edition have been retained and the information provided is essential to anyone concerned in the use or development of flavour ingredients.

Volume 1, 1975. 526 pages. £24.50

Volume 2, 1975. 926 pages. £32.20

Storage, Processing and Nutritional Quality of Fruits and Vegetables

Edited by D. K. Salunkhe, *Utah State University*

1. Assessment of nutritive value, quality and stability of cruciferous vegetables during storage and subsequent processing
Nutrients and quality of cruciferous vegetables and their products
Needs for future research

2. Developments in technology and nutritive value of dehydrated fruits, vegetables and their products
Developments in technology
Storage stability
Nutritive value
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Utilization
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Yield: quantity and quality
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Stimulation of plant growth and yield
Increase in protein content by plants
Increase in carbohydrate content
Increase in vitamin content
Increase in mineral content
Increase in pigment content
Retention of nutrients and quality in plants produced by chemical treatments

4. Developments in technology of storage and handling of fresh fruits and vegetables
Chemicals that delay ripening and senescence
Chemicals that hasten ripening and senescence
Chemicals that may hasten or delay ripening and senescence
Chemicals that control postharvest microbial growth
Controlled atmosphere storage
Ionizing radiations
Other important and associated considerations to control ripening, senescence and microbial growth

1975. 176 pages, 32 illustrations. £13.30

CRC Press

Blackwell Scientific Publications
Oxford London Edinburgh Melbourne

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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.

Standard usage. The *Concise Oxford English Dictionary* is used as a reference for all spelling and hyphenation. Verbs which contain the suffix ize (ise) and their derivatives should be spelt with the z. Statistics and measurements should always be given in figures, i.e. 10 min, 20 hr, 5 ml, except where the number begins the sentence. When the number does *not* refer to a unit of measurement, it is spelt out except where the number is greater than one hundred.

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'.

Tables. There should be as few tables as possible and these should include only essential data; the data should not be crowded together. The main heading should be in capitals with an Arabic number, e.g. TABLE 2. Each table must have a caption in small letters. Vertical lines should not be used.

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