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The relevance of *Clostridium botulinum* type C in public health and food processing

T. A. ROBERTS AND ANGELA M. GIBSON

Summary

A survey of published information suggests that measures taken in the food industry to control *Clostridium botulinum* types A and B would also control type C should its numbers have increased locally as a consequence of outbreaks of botulism in wildfowl or broiler chickens.

Introduction

Recent outbreaks of type C botulism in the U.K. in wildfowl and broiler chickens have prompted food processors to question whether *Clostridium botulinum* type C has become more common in the environment, whether processes to control *Cl. botulinum* types A and B will also control type C, and raise the question of the possible public health significance of *Cl. botulinum* type C in man.

Outbreaks of type C avian botulism have been reported from many parts of the world, including North America, Canada, Australia, Sweden, Denmark, The Netherlands, Britain, Uruguay and S. Africa (Hariharan & Mitchell, 1977; Haagsma, 1974a). Botulism in poultry was frequently reported in the early literature (see Roberts & Collings, 1973) but had become rare until 1969 when two outbreaks, one in California (Sadler & Brownell, 1969) and one in the U.K. (Blandford & Roberts, 1970) occurred almost simultaneously. Since 1969, U.K. outbreaks of type C botulism in birds have been reported by Keymer *et al.* (1972); Blandford, Roberts & Ashton (1969) and Roberts *et al.* (1972) and outbreaks in broiler chickens by Blandford & Roberts (1970); Roberts & Collings (1973); Roberts, Thomas & Gilbert (1973); Smart & Roberts (1977).

Botulism is classically regarded as an intoxication – a preformed toxin being ingested and absorbed. The cause of death is usually muscular paralysis resulting in impaired breathing and terminal asphyxia (Lamanna, 1959). There are six

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Table 1. Relative toxicities of the types of *Cl. botulinum* toxin (subcutaneous injection)

Type	A	B	C	D	E	F	Reference
Mouse*	1	1	1	1	1	1	Prévot & Brygoo, 1953
Rat			6	320	40		Prévot & Brygoo, 1953
Guinea-pig			1	0.3	0.5		Prévot & Brygoo, 1953
						25	Dolman & Murakami, 1961
Rabbit			0.1	0.2	1		Prévot & Brygoo, 1953
						$>4\times10^{3}<2\times10^{4}$	Dolman & Murakami, 1961
Dog			1×10^{3}	$>1\times10^{5}$	100		Prévot & Brygoo, 1953
Cat			800	1.5×10^{4}	400		Prévot & Brygoo, 1953
Monkey			0.3	100	1		Prévot & Brygoo, 1953
						40	Dolman & Murakami, 1961
Fowl	10	20	2×10^{3}	$>1\times10^{5}$	25		Prévot & Brygoo, 1953
Pigeon	200	110	20	2×10^{3}	25		Prévot & Brygoo, 1953
Mink			18				Yndestad & Loftsgård, 1970
Fox			5×10^{5}		1×10^{4}		Yndestad, Helgebostad & Loftsgård, 1977

* = intraperitoneal injection

types of *Cl. botulinum* each producing antigenically distinguishable neurotoxins designated A, B, C, D, E, F. A seventh type, G, has been suggested (Gimenez & Ciccarelli, 1967) although no outbreak of type G botulism has yet been reported in man or animals. Animal species differ in their sensitivity to the various toxins of *Cl. botulinum* (Table 1). Man is generally only affected by types A, B, E and F, while type C commonly affects birds and animals, and type D animals, mainly cattle (Dolman, 1964). However suspected outbreaks of type C and D botulism in man have been reported (Meyer *et al.*, 1953; Prévot *et al.*, 1955; Demarchi *et al.* 1958).

The general ecology, distribution and host spectrum of *Cl. botulinum* type C has been fully discussed by Smith (1976) and Hariharan & Mitchell (1977) and the purpose of this short review is to bring together available information of *Cl. botulinum* types C and D, and discuss their public health and food processing implications.

Public health implications

Reported human botulism of types C and D

(1) In 1953 a case of 'human type C botulism' was reported in the U.S.A. (Meyer *et al.*, 1953). Stomach contents from the fatal case produced symptoms of botulism in mice and guinea-pigs, although the toxicity could not be neutralized by specific botulinal antisera. When stomach contents were cultured *Cl. botulinum* was isolated and guinea-pigs immunized with toxoid from this organism were resistant to type C toxin but not toxins of types A, B, D and E. The source of the intoxication was not stated, and no serological evidence was provided to confirm botulism as the cause of death.

(2) Another case of 'human type C botulism' was reported in France (Prévot *et al.*, 1955). *Clostridium botulinum* type C spores survived 'cooking' in a pâté de campagne which was stored for approximately 2 months prior to consumption. Type C toxin was detected in the pâté and two persons were poisoned, one recovering without treatment, and the other after treatment with polyvalent antitoxin. Again no serological tests were reported to confirm the presence of type C toxin in the blood of those affected.

(3) Matveev *et al.* (1967) reported two cases of human type C botulism in Russia between 1964 and 1966, but offered no details of these outbreaks.

(4) Demarchi *et al.* (1958) described an outbreak of type D botulism in Chad, involving six Europeans who had eaten uncooked salted ham prepared as an hors d'oeuvre. All six cases showed varying symptoms of botulism but recovered over a range of 1 day to 1 month. No confirmatory tests on serum from any of the cases were reported, but *Cl. botulinum* type D and its toxin were detected in the ham.

(5) In 1973 a further case of 'human type C botulism' apparently occurred in France (Fournaud, 1977, personal communication) when type C toxin was

detected in the blood of one fatal case. However, according to our information, samples of food were not tested for the organism or its toxin.

Although these reports are offered as 'outbreaks of types C or D botulism in man' and the symptoms observed corresponded to those of botulism, in no instance have adequate tests been made to confirm those claims. In addition to observing characteristic symptoms of botulism in affected cases, *Cl. botulinum* toxin should be demonstrated in the blood and/or faeces of those cases, and ideally, the source of the toxin identified. It is not uncommon however, in genuine cases of botulism for the quantity of toxin in a patient's serum or faeces to be close to the limit of detection using the mouse bioassay. Demonstration of *Cl. botulinum* by enrichment procedures should not be taken as adequate confirmation since it can also be demonstrated in foods not associated with outbreaks of botulism (Roberts & Smart, 1977). All the above 'outbreaks' were reported by workers experienced in *Cl. botulinum*.

Type C botulism in birds and animals

Since 1969 outbreaks of type C botulism in the U.K. have been reported frequently in wildfowl (Keymer *et al.*, 1972; Blandford *et al.*, 1969; Roberts *et al.*, 1972) and a comprehensive review of botulism in waterfowl was published by Smith (1976). In the summer of 1975 at least 4000 birds were known to have died from botulism in eighteen different outbreaks (Lloyd *et al.*, 1976) and small outbreaks are now confirmed each summer.

Similar outbreaks of botulism in waterfowl were first recognized in The Netherlands in 1970, with subsequent outbreaks in 1971, and 1972 (Haagsma, 1973, 1974a). The serious outbreak in 1970 prompted research into the etiology and epidemiology of botulism amongst waterfowl in The Netherlands. Thermal pollution of surface water may prolong outbreaks of botulism among waterfowl because the birds tend to concentrate in areas of warmer water throughout the autumn and winter (Haagsma, 1974a).

Since the report by Kalmbach & Gunderson (1934) that *Cl. botulinum* type C was able to grow and produce toxin in decomposing marsh vegetation rich in organic matter, it was long assumed that outbreaks of botulism in wildfowl arose in that manner. However Hunter (1970) showed that rotting vegetation failed to support the growth of *Cl. botulinum* type C, and experimental lakes did not become toxic to ducks even though the lakes simulated areas of natural outbreaks. Haagsma (1973) also demonstrated that *Cl. botulinum* type C failed to grow and produce toxin in samples of mud or water taken from areas of high botulism mortality, even though these samples were heavily contaminated with *Cl. botulinum* type C and were incubated at optimum temperature, unless they were enriched with animal material (liver and meat particles).

Natural outbreaks of type C botulism are now believed to be caused by either the ingestion of invertebrates which have died following flood or drought, a proportion of which will contain botulinum toxin as a result of growth of *Cl.*

botulinum in the alimentary tract after death, or the ingestion of maggots which often develop on the carcasses of ducks which have died of botulism or other causes and frequently contain *Cl. botulinum* toxin (Hunter, 1970).

Clostridium botulinum was demonstrated in 35% of samples taken from aquatic environments throughout the U.K. (Smith, Milligan & Moryson, 1978) type B being more common than types C or E and type D least common. Samples from the Norfolk Broads yielded almost uniform contamination with *Cl. botulinum* of one type or another and types C and E occurred as frequently as B (Borland, Moryson & Smith, 1977) but there was little difference between the Broads where bird mortality from botulism had occurred, and those where it had not. Similarly Haagsma (1974a) showed that 30% samples from Dutch inland waters, and approximately 75% of waterways themselves contained *Cl. botulinum*. Type E was more common than C or B, except in areas where botulism in waterfowl had occurred.

In a survey of 260 soil samples throughout the U.S.A., *Cl. botulinum* was detected in only 23.5% of the samples (Smith, 1978). Types A and B were the most common (approximately 10%) but type C was isolated from only three samples not associated with aquatic environments.

Of 4345 samples of soil and water from the U.S.S.R. examined by Kravchenko & Shishulina (1967), *Cl. botulinum* was demonstrated in 10% of the soil samples, and 4% of the water samples. Type E was the most common in soil samples, type C least common.

In the first outbreak of type C botulism in broiler chickens in the U.K. (Blandford & Roberts, 1979) intoxication was the result of birds cannibalizing decomposing carcasses in which *Cl. botulinum* type C was growing, and a relatively small number of birds were affected. The remaining outbreaks (Roberts & Collings, 1973; Roberts, Thomas & Gilbert, 1973; Smart & Roberts, 1977) involved from 400 to 3500 birds, but in no case was the source of toxin discovered, although *Cl. botulinum* type C was detected readily in the litter and sometimes in feed samples. It seemed possible that the organism could have grown in the gut of the birds and produced sufficient amounts of toxin to intoxicate them – a process termed 'toxico-infection' (Minervin, 1967). Low numbers of *Cl. botulinum* in the feed and numbers up to 10 000-fold higher in the gut and litter in the last outbreak (Smart & Roberts, 1977) appear to confirm this mode of intoxication. Miyazaki & Sakaguchi (1978) confirmed that growth of type C can occur in the live chicken, particularly in the caecum, and thus cause a toxico-infection.

Similarly, on a poultry farm in The Netherlands, 30 000 broiler chickens died from type C botulism over a 15-month period. The farm was widely contaminated with *Cl. botulinum* type C, but the source of the toxin was not located (Haagsma, 1974b).

In a survey in the U.K. of 1120 dressed chickens from six large packing stations which had obtained the birds from 185 broiler houses on ninety-four separate farms, plus 129 samples from ten other packing stations, by methods previously successful in detecting low numbers of *Cl. botulinum* in chicken,

only four samples (0.32%) carried *Cl. botulinum*, all type C (Willis *et al.*, 1971).

Botulism was almost certainly the cause of death of calves (Davies *et al.*, 1974) since *Cl. botulinum* type C was isolated from both soil and liver enrichments and type C toxin was also found in the bulked bowel contents of the two calves. However serum samples were not available and confirmatory tests for the presence of *Cl. botulinum* toxin could not be performed.

Botulism in cattle has also been reported in The Netherlands. The first outbreak was of type C botulism (Haagsma *et al.*, 1977) and was caused by the use of contaminated litter from a broiler farm as bedding for the cattle. The following two outbreaks were of type B botulism caused by the supplementary feeding of brewers grains contaminated with *Cl. botulinum* type B (Haagsma & Laak, 1978; Breukink *et al.*, 1978).

In an outbreak described by Blandford, Roberts & Ashton (1969) ferrets died after being fed carcases of mallard collected from an area where botulism was later demonstrated. Type C botulism is relatively common in mink which are fed uncooked offal (Gitter, 1959; Roberts *et al.*, 1972). Good protection is afforded by 'vaccination' but unvaccinated stock are susceptible to very small amounts of toxin in the offal.

One possible risk to the public would arise from a dead broiler chicken being accidentally included with birds for slaughter, or a bird suffering from the disease being slaughtered with healthy birds. The former should never occur and in the unlikely event of the latter, the level of *Cl. botulinum* toxin in a fresh carcase would be much lower than the 2000 mouse MLD/g of leg muscle detected in one sample of decomposing broiler chicken (Blandford & Roberts, 1970). In the outbreak described by Roberts & Collings (1973) birds from the affected flock that had remained healthy were processed normally. After processing and freezing they were still contaminated with *Cl. botulinum* type C. The minimum growth temperature of *Cl. botulinum* type C is 12.8°C (Segner, Schmidt & Boltz, 1971) but at that temperature about 23 days would be required for toxin production, by which time the chicken would be spoiled by other bacteria.

Botulinal toxins are simple globular proteins (Lamanna, 1959) and relatively heat sensitive. Type C toxin was destroyed by heating at 90°C for 2 min (Prévot & Brygoo, 1953). Thus normal cooking procedures would inactivate any preformed toxin.

Food processing implications

Physiological properties of Cl. botulinum

Metabolism in relation to pH. The minimum pH for germination, vegetative growth and toxin production of *Cl. botulinum* type C was similar or slightly higher than that for types A, B and E (Table 2) consequently the possibility of growth of *Cl. botulinum* type C is similar to that of other types.

Table 2. Metabolism of the types of *Cl. botulinum* in relation to pH

Type	Minimum pH	Reference
Germination		
A	5.3	Baird-Parker & Freame, 1967
B	5.0	Baird-Parker & Freame, 1967
C	5.9	McKee, Bell & Hoyer, 1958
C	6.0	Dozier, 1924
E	5.0	Baird-Parker & Freame, 1967
E	5.01	Segner, Schmidt & Boltz, 1966
Growth in vegetative form		
A	4.8	Townsend, Yee & Mercer, 1954
	5.2–5.3	Meyer & Kietzeman, 1957
	5.3	Baird-Parker & Freame, 1967
	5.5	Bonventre & Kempe, 1959
	5.3	Ingram & Robinson, 1951
	6.0	Ohye & Christian, 1967
	5.68	Huhtanen *et al.*, 1976
B	4.93	Townsend, Yee & Mercer, 1954
	5.2–5.3	Meyer & Kietzeman, 1957
	5.0	Baird-Parker & Freame, 1967
	5.24	Huhtanen *et al.*, 1976
	5.1	Ingram & Robinson, 1951
	6.0	Ohye & Christian, 1967
C	5.0	Dozier, 1924
	7.0	Bengston, 1924
	5.1	Segner, Schmidt & Boltz, 1971
E	5.25–5.3	Meyer & Kietzeman, 1957
	6.0	Ohye & Christian, 1967
	5.3	Baird-Parker & Freame, 1967
F	4.5	Holdeman, 1964
Toxin production		
A	5.45	Ito *et al.*, 1978
	5.5	Bonventre & Kempe, 1959
	6.0	Ohye & Christian, 1967
B	6.0	Ohye & Christian, 1967
	5.58	Ito *et al.*, 1978
C	5.7	McKee, Bell & Hoyer, 1958
	7.0	Bengston, 1924
E	6.0	Quortrup & Holt, 1940
	6.0	Ohye & Christian, 1967

Minimum growth temperatures. The minimum growth temperature for *Cl. botulinum* type C is 12.8°C (Segner, Schmidt & Boltz, 1971) hence it is unlikely to grow under good commerical chill storage (Table 3).

Heat resistance of spores. Clostridium botulinum type C spores are no more resistant to heat than those of the other types of *Cl. botulinum* (Table 4).

Chlorine resistance of spores. The sensitivity of *Cl. botulinum* type C spores to chlorine is not significantly different from that of types A, B or E (Table 5).

Table 3. Minimum growth temperatures of *Cl. botulinum*

Type	Minimum temperature (°C)	Reference
A (proteolytic)	10	Tanner & Oglesby, 1936
	12.5	Ohye & Scott, 1953
B (proteolytic)	10	Tanner & Oglesby, 1936
	12.5	Ohye & Scott, 1953
B (non-proteolytic)	3.3	Eklund, Wieler & Poysky, 1967
C (Terrestrial)	12.8	Segner, Schmidt & Boltz, 1971
C (Marine)	15.6	Segner, Schmidt & Boltz, 1971
C	12.5	Haagsma, 1974a
E	3.3	Schmidt, Lechowich & Folinazzo, 1961 Schmidt & Segner, 1964
F (proteolytic)	4.0	Walls, 1967
F (non-proteolytic	3.3	Eklund, Poysky & Wieler, 1967

Sodium chloride tolerance. Clostridium botulinum type C is more sensitive to NaCl than types A, B or E (Table 6).

Sodium nitrite tolerance. No data are available for types C and D: Table 7 lists the inhibitory concentration of sodium nitrite for *Cl. botulinum* types A, B, E and F.

Radiation resistance of spores. The radiation resistance of spores of *Cl. botulinum* type C in aqueous suspension does not differ significantly from that of types A, B, D, E or F (Table 8).

Physiological properties of Cl. botulinum *toxin*

Resistance of toxin diluted in water. Clostridium botulinum type C toxin decomposes more slowly than types A, B, D or E when diluted in water (Table 9).

The stability of botulinal toxins in simple buffers was lower than in buffered systems containing gelatin (Littauer, 1951). Consequently, toxin would not be expected to remain for any length of time in lake water, but the evidence is conflicting: Brygoo (1953) reported that 80% toxicity was lost in 1–7 days at room temperature. However when Haagsma (1973) transferred *Cl. botulinum* type C toxin, produced experimentally, to an area of severe botulism outbreaks among waterfowl, the toxin remained stable even after 9 months, although toxin was not found in mud and water samples collected from areas of high botulism mortality. Graham *et al.* (1978) reported that type C toxin retained 1% of its toxicity after 344 days in screw-capped bottles in lake water. Hence toxin may remain in lake water or mud for long periods and areas of botulism mortality may be a potential danger to foraging waterfowl for many months following an outbreak.

Effect of pH. There are no data available for *Cl. botulinum* type C toxin, but type A toxin remained moderately stable in alkaline solutions up to a pH of

Table 4. Relative heat resistance of the spores of *Cl. botulinum*

Type	Temperature (°C)	D(min)	Author's z value (°F)	Reference
A	121.1	0.20	18	Schmidt, 1964
	115.6	0.74		
	115.5	0.3–0.8		Tsuji & Perkins, 1962
	112.8	1.1–1.2		Ito *et al.*, 1967
B	112.8	0.2–1.3		Ito *et al.*, 1967
	100	3.0–6.0		Roberts unpublished
C terrestrial				
468	101	2.31–2.58	11.5	Segner & Schmidt, 1971
571		1.07–1.27	10.0	Segner & Schmidt, 1971
468	104	0.82–1.00		Segner & Schmidt, 1971
571		0.4		Segner & Schmidt, 1971
468	107	0.25–0.3		Segner & Schmidt, 1971
571		0.1–0.13		Segner & Schmidt, 1971
468	110	0.10–0.14		Segner & Schmidt, 1971
C marine				
6813	93	2.26–2.39	10.7	Segner & Schmidt, 1971
6812	96	2.29–2.85	10.8	Segner & Schmidt, 1971
6813		0.92–1.23		Segner & Schmidt, 1971
6812	102	0.2–0.28		Segner & Schmidt, 1971
6813		0.06–0.11		Segner & Schmidt, 1971
6812	104	0.1–0.14		Segner & Schmidt, 1971
E	70	29.3–37.5	13–15	Ito *et al.*, 1968
	75	5.1–8.2		Ito *et al.*, 1968
	80	1.4–1.8		Ito *et al.*, 1968
	80	0.33–1.25	7.4–10.7	Roberts, Ingram & Skulberg, 1965
	70	36, 7.8		Ohye & Scott, 1957
	75	10.0, 3.1	14.0, 17.0	Ohye & Scott, 1957
	80	3.3, 0.4		Ohye & Scott, 1957
F	80	0.6, 3.3		Schmidt, 1964
	100	0.003, 0.017		Schmidt, 1964
	95	15		Roberts, unpublished

Table 5. The effect of chlorine on the spores of *Cl. botulinum*. 4.5 ppm free available chlorine in phosphate buffer pH 6.5

Type	Time* (min)	Reference
A & B	7.8	Ito *et al.*, 1968
C	*c.* 3	Ito, 1975 (personal communication)
E	4.0	Ito *et al.*, 1968

*Time for 99.9% reduction in viability

Table 6. Sodium chloride tolerance of *Cl botulinum*

Type	Highest concentration of salt tested allowing growth (%)	pH	Incubation temperature (°C)	Reference
A	6.1	7.0	30	† Baird-Parker & Freame, 1967
	4.58	6.0	30	† Baird-Parker & Freame, 1967
	8.0	7.0	40	† Ohye & Christian, 1967
	6.0	6.0	35	* Roberts & Ingram, 1973
	6.0	6.0	25	* Baird-Parker & Baillie, 1973
B	6.1	7.0	30	† Baird-Parker & Freame, 1967
	9.4	7.0	40	† Ohye & Christian, 1967
	4.58	6.0	30	† Baird-Parker & Freame, 1967
	6.0	6.2	35	* Roberts & Ingram, 1973
	6.0	6.0	25	* Baird-Parker & Baillie, 1973
C	2.0–2.5	7.2	30	† Segner, Schmidt & Boltz, 1971
E	4.5	7.0	30	† Segner, Schmidt & Boltz, 1966
	4.58	7.0	30	† Baird-Parker & Freame, 1967
	5.1	7.0	35	† Ohye & Christian, 1967
	2.9	6.0	30	† Baird-Parker & Freame, 1967
	4.0	6.0	35	* Roberts & Ingram, 1973
	4.5	6.0	25	* Baird-Parker & Baillie, 1973
F	6.0	6.2	35	* Roberts & Ingram, 1973
	4.5	6.0	25	* Baird-Parker & Baillie, 1973

* Vegetative cell inoculum
† Spore inoculum

Table 7. Sodium nitrite tolerance of *Cl. botulinum*. Concentration (ppm) of sodium nitrite inhibiting growth of *Cl. botulinum*

	Unheated		Heated		
Type	pH 6.0	pH 7.0	pH 6.0	pH 7.0	Reference
A	240		20		† Roberts & Smart, 1974
	160	2560	5	40	* Perigo & Roberts, 1968
	300				* Roberts & Ingram, 1973
B	160	1280	20	80	* Perigo & Roberts, 1968
	300				* Roberts & Ingram, 1973
E	80	320	10	20	* Perigo & Roberts, 1968
	160		15		† Roberts & Smart, 1974
	50				* Roberts & Ingram, 1973
	200				* Baird-Parker & Baillie, 1973
F	120	2560	10	80	* Perigo & Roberts, 1968
	150				* Roberts & Ingram, 1973
	200				* Baird-Parker & Baillie, 1973
G	100				* Baird-Parker & Baillie, 1973

* Vegetative cell inoculum
† Spore inoculum

Table 8. Radiation resistance of *Cl. botulinum* spores in aqueous suspension or beef stew

Type	D* exponential	Dose†	Reference
A	0.10–0.14	1.01–1.41	Roberts & Ingram, 1965
	0.22–0.33		Anellis & Koch, 1962
	0.25–0.31		‡ Schmidt, 1964;
			‡ Schmidt, Nank & Lechowich, 1962
B	0.13–0.33		Anellis & Koch, 1962
	0.18–0.28		‡ Schmidt, Nank & Lechowich, 1962
			‡ Schmidt, 1964
	0.10–0.11	1.05–1.18	Roberts & Ingram, 1965
C	0.14	1.05	Roberts & Ingram, 1965
	0.153–0.172		‡ Schmidt, 1964
D	0.22	1.50	Roberts & Ingram, 1965
E	0.13–0.14		‡ Schmidt, Nank & Lechowich, 1962
			‡ Schmidt, 1964
	0.08–0.16	0.77–1.17	Roberts & Ingram, 1965
F	0.25	1.96	Roberts & Ingram, 1965

* Mrad.
† Dose to give 10^6 inactivation
‡ In beef stew

10.0, within a temperature range of 5–25°C but the rate of inactivation increased rapidly between pH 10–11, and at pH 11.2 the inactivation time for type A toxin (7×10^7 mouse LD_{50}/ml) at 15°C is 60 min (Spero, 1958). Woolford, Schantz & Woodburn (1978) found type A toxin to be more heat resistant at pH 4.1–4.7 than pH 6.1–6.8, while Scott & Stewart (1950) and Losikoff (1978) found the toxin of type A to be most resistant to heat in the pH range 5.0–5.5.

Thermal resistance. Thermal resistance of *Cl. botulinum* types C and D toxin is higher than that of types A, B or E (Prévot & Brygoo, 1953; Ohye & Scott, 1957; Abrahamsson, Gullmar & Molin, 1965; Cartwright & Lauffer, 1958) (Table 10). A temperature of 90°C for 2 min must be reached to destroy

Table 9. Stability of *Cl. botulinum* toxins diluted in water*

Toxin	Initial conc. mouse MLD/ml	Time (days)	Reduction in toxicity (%)	Reference
A	10	1–2	80	Brygoo, 1953
B	10	2–3	80	Brygoo, 1953
C	10	5–7	80	Brygoo, 1953
	1×10^4	97	10	Graham *et al.*, 1978
	1×10^4	344	99	Graham *et al.*, 1978
D	10	2–4	80	Brygoo, 1953
E	10	3–4	80	Brygoo, 1953

* Tap water (pH not stated) stored at 'room temperature' was used by Brygoo, 1953.
Lake water (pH not stated) in 'natural surroundings' was used by Graham *et al.*, 1978.

Table 10. Thermal resistance of *Cl. botulinum* toxins

Type	Initial mouse MLD/ml	Temperature (°C)	Time (min)	Reduction in toxicity	pH	Reference
A	4×10^4	70	15	Total	7.3	Scott & Stewart, 1950
	4×10^5	60	2	99.9%	NS	Prévot & Brygoo, 1953
		60	30	99.9%	6.9	Cartwright & Lauffer, 1958
	6×10^4	68	2	99%	5.9	Woolford, Schantz & Woodburn, 1978
		68	15	99.9%	5.9	Woolford, Schantz & Woodburn, 1978
B	2×10^3	60	32	99%	7.6	Scott & Stewart, 1950
	5×10^3–4×10^4	70	2	Total	NS	Prévot & Brygoo, 1953
C	4×10^4–1×10^5	70	2	90%	NS	Prévot & Brygoo, 1953
		80	2	99%	NS	Prévot & Brygoo, 1953
		90	2	Total	NS	Prévot & Brygoo, 1953
D	8×10^4	80	2	None	NS	Prévot & Brygoo, 1953
		90	2	Total	NS	Prévot & Brygoo, 1953
E	2×10^2–2×10^3	60	2	None	NS	Prévot & Brygoo, 1953
		70	2	Total	NS	Prévot & Brygoo, 1953
	1×10^3	60	5	Total	7.5	Ohye & Scott, 1957
	5×10^3	58	5	Total	7.2	Abrahamsson *et al.*, 1965
	2.1×10^4	60	10	99%	6.2	Licciardello *et al.*, 1967

NS = pH not stated

type C or D toxin; 70°C for 2 min is sufficient to destroy types B and E toxin (Prévot & Brygoo, 1953), and 99.9% toxicity of type A is lost after heating at 60°C for 2 min.

The substrate in which toxin is heated has a marked effect on the inactivation rate. Type A toxin (initial titre 2500 MLD) produced in cooked meat medium (pH 5.0) was destroyed by heating for 16 min at 75°C, whereas type A toxin produced in canned asparagus, cabbage or turnips, (initial titre 620–1000 MLD) was still detectable after heating in the vegetable liquor (pH 4.8–4.95) for 32 min at 75°C (Scott & Stewart, 1950). Ionized substances dissolved in the liquor of canned vegetables afforded the toxin increased protection against heat (Scott, 1950).

Type B toxin was less resistant to heat than type A (Scott & Stewart, 1950) 2000 MLD type B toxin produced in cooked meat medium lost 99% toxicity after 32 min at 60°C, the same concentration of toxin produced and heated in cabbage liquor was destroyed after 8 min at 75°C.

Effect of chlorine: 5 ppm chlorine neutralized 10 LD_{50} of type A toxin at 22°C (Kalember-Radosavljević & Ilić, 1971), and 35 ppm calcium hypochlorite neutralized 4 MLD of type C toxin. Calcium hypochlorite is less effective at alkaline pH, and at temperatures below 20°C (Kalember-Radosavljević & Ilić, 1970).

Conclusions

There is no evidence that *Cl. botulinum* type C is more resistant to physical stress than the other types of *Cl. botulinum*, although type C toxin may be more resistant to dilution in water (Prévot & Brygoo, 1953; Haagsma, 1973; Graham *et al.*, 1978), and more resistant than types A, B and E to heat (Prévot & Brygoo, 1953). However heating for 2 min at 90°C was sufficient to destroy type C toxin from an initial concentration of 4–10 $\times$ 10^4 MLD/ml (Prévot & Brygoo, 1953).

Hence *Cl. botulinum* type C appears to pose no additional problems to the food industry, and measures to inactivate or control the other types of *Cl. botulinum* should also control type C.

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Thermal process calculations using sterilizing ratios

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Summary

Commonly used thermal process calculations are based on temperature differences. An improved method of calculation based on sterilizing ratios is described. This method is independent of the temperature scales used, and reduces the number and size of associated tables. The integration limits were able to be selected so that computational errors are negligible.

Introduction

The commonly used method of process calculation described by Ball & Olson (1957) and the revision of this method by Griffin, Herndon & Ball (1971) are based on temperature differences.

This paper describes an improved method of process calculation based on the concept of sterilizing ratios. This ratio is defined as the temperature difference between the thermal centre of the can and the heating (or cooling) medium divided by the slope of the thermal death time curve for the micro-organism of concern. As this sterilizing ratio is a dimensionless number, the calculations described in this paper are independent of the temperature scales used.

The heating phase

Ball & Olson (1957) evaluated the lethality of the heating phase, F_h from:

$$F_h = \exp[-\mu \cdot (T_b - T_1)/z] \cdot \int_{t=0}^{t_g} \exp[\mu \cdot (T - T_1)/z] \cdot dt \qquad (1)$$

where μ is the constant $\ln(10) = 2.3026$, T_b is the base or reference temperature, T_1 is the temperature of the heating medium, T is the temperature at the thermal centre of the can, z is the slope of the thermal death time curve, t is the elapsed time of heating, and t_g is the total time of heating.

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Equation (1) may be expressed in terms of sterilizing ratio S:

$$F_h = \exp(\mu \cdot S_b) \int_{t=0}^{t_g} \exp(-\mu \cdot S)\, dt \tag{2}$$

where

$S_b = (T_1 - T_b)/z$

and

$S = (T_1 - T)/z.$

The equation describing the straight line portion of logarithmic heating in terms of sterilizing ratios is:

$$t = f_h \cdot \log(j_h \cdot S_0/S) \tag{3}$$

where f_h is the slope of the straight line portion of the logarithmic heating curve, j_h is the lag factor, and S_0 is the sterilizing ratio at $t = 0$.

Differentiating eqn (3) with respect to S gives:

$$dt = -[f_h/(\mu \cdot S)] \cdot dS \tag{4}$$

F_h may be evaluated from eqn (5) which is obtained by first substituting eqn (4) into eqn (2):

$$F_h = f_h \cdot \exp(\mu \cdot S_b) \cdot [E_1(\mu \cdot S_g) - E_1(\mu \cdot S_0)]/\mu \tag{5}$$

where S_g is the sterilizing ratio at $t = t_g$, $E_1(x)$ is the exponential integral as defined by Gautschi & Cahill (1964).

$E_1(\mu \cdot S_0)$ is in practice very small and may be ignored. Values of $E_1(\mu \cdot S_g)/\mu$, denoted by F_h', for S_g ranging from 0.001 to 0.99 are presented in Table 1. For values of S_g not in Table 1 the following approximations, from Gautschi & Cahill (1964) may be used.

When $x = \mu \cdot S_g \leqslant 1.0$

$$E_1(x) = \sum_{m=0}^{5} a_m \cdot x^m - \ln(x) \tag{6a}$$

and when $x > 1.0$

$$E_1(x) = \exp(-x) \cdot (x^2 + a_6 \cdot x + a_7)/(x^3 + a_8 \cdot x^2 + a_9 \cdot x) \tag{6b}$$

where $a_0 = 0.57721566$, $a_1 = 0.99999193$, $a_2 = -0.24991055$, $a_3 = 0.05519968$, $a_4 = -0.00976004$, $a_5 = 0.00107857$, $a_6 = 2.334733$, $a_7 = 0.250621$, $a_8 = 3.330657$, $a_9 = 1.681534$.

The cooling phase

Griffin *et al.* (1971) revised the method that Ball & Olson (1957) used for the cooling phase calculations and made the calculations applicable to various

Table 1. F'_h values for values of S_g from 0.001 to 0.990

S_g	0.0000	0.0001	0.0002	0.0003	0.0004	0.0005	0.0006	0.0007	0.0008	0.0009
0.001	2.3881	2.3468	2.3091	2.2745	2.2424	2.2125	2.1846	2.1584	2.1336	2.1102
0.002	2.0881	2.0670	2.0469	2.0277	2.0093	1.9917	1.9747	1.9584	1.9427	1.9276
0.003	1.9130	1.8988	1.8851	1.8719	1.8590	1.8465	1.8344	1.8226	1.8111	1.7999
0.004	1.7890	1.7784	1.7680	1.7579	1.7480	1.7384	1.7289	1.7197	1.7106	1.7018
0.005	1.6931	1.6846	1.6763	1.6681	1.6601	1.6522	1.6445	1.6369	1.6295	1.6221
0.006	1.6149	1.6079	1.6009	1.5940	1.5873	1.5807	1.5741	1.5677	1.5614	1.5551
0.007	1.5490	1.5429	1.5369	1.5310	1.5252	1.5195	1.5139	1.5083	1.5028	1.4973
0.008	1.4920	1.4867	1.4815	1.4763	1.4712	1.4661	1.4612	1.4562	1.4514	1.4466
0.009	1.4418	1.4371	1.4325	1.4279	1.4233	1.4188	1.4144	1.4100	1.4056	1.4013
S_g	0.000	0.001	0.002	0.003	0.004	0.005	0.006	0.007	0.008	0.009
0.010	1.3970	1.3566	1.3198	1.2861	1.2549	1.2259	1.1988	1.1735	1.1496	1.1271
0.020	1.1058	1.0856	1.0664	1.0481	1.0306	1.0138	0.9977	0.9823	0.9675	0.9532
0.030	0.9395	0.9262	0.9134	0.9010	0.8890	0.8773	0.8661	0.8551	0.8445	0.8342
0.040	0.8241	0.8144	0.8049	0.7956	0.7866	0.7778	0.7692	0.7608	0.7526	0.7446
0.050	0.7367	0.7291	0.7216	0.7143	0.7071	0.7000	0.6932	0.6864	0.6798	0.6733
0.060	0.6669	0.6607	0.6546	0.6485	0.6426	0.6368	0.6311	0.6255	0.6200	0.6146
0.070	0.6093	0.6040	0.5989	0.5938	0.5888	0.5839	0.5791	0.5743	0.5696	0.5650
0.080	0.5605	0.5560	0.5516	0.5472	0.5429	0.5387	0.5345	0.5304	0.5264	0.5224
0.090	0.5184	0.5145	0.5107	0.5069	0.5031	0.4994	0.4958	0.4922	0.4886	0.4851
S_g	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.100	0.4816	0.4491	0.4201	0.3940	0.3705	0.3490	0.3294	0.3114	0.2948	0.2794
0.200	0.2652	0.2520	0.2397	0.2282	0.2174	0.2073	0.1979	0.1890	0.1806	0.1727
0.300	0.1652	0.1581	0.1515	0.1451	0.1391	0.1335	0.1281	0.1229	0.1180	0.1134
0.400	0.1090	0.1047	0.1007	0.0969	0.0932	0.0897	0.0864	0.0831	0.0801	0.0772
0.500	0.0743	0.0717	0.0691	0.0666	0.0642	0.0620	0.0598	0.0577	0.0557	0.0538
0.600	0.0519	0.0501	0.0484	0.0468	0.0452	0.0437	0.0422	0.0408	0.0394	0.0381
0.700	0.0368	0.0356	0.0345	0.0333	0.0322	0.0312	0.0302	0.0292	0.0283	0.0274
0.800	0.0265	0.0256	0.0248	0.0240	0.0233	0.0225	0.0218	0.0211	0.0205	0.0198
0.900	0.0192	0.0186	0.0180	0.0175	0.0170	0.0164	0.0159	0.0154	0.0150	0.0145

values of the lag factor j_c whereas Ball & Olson had assumed that $j_c = 1.41$ for all cooling curves.

The following equations, based on the method outlined by Griffin *et al.* (1971) were used to evaluate the lethality of the cooling phase F_c in terms of sterilizing ratios:

$$F_c = f_c \cdot F'_c \cdot \exp(\mu \cdot S_{cb}) \tag{7}$$

where f_c is the slope of the straight line portion of the logarithmic cooling curve,

$$S_{cb} = (T_{c0} - T_b)/z$$

T_{c0} is the temperature at the thermal centre of the can at the beginning of the cooling phase,

$$F'_c = \int_{t'_c=0}^{t'_\ell} \exp[\mu \cdot (S_c - S_{c0})] \cdot dt'_c \tag{8}$$

where $S_c = (T_c - T_w)/z$, $S_{c0} = (T_{c0} - T_w)/z$, T_c is the temperature at the thermal centre of the can during cooling, T_w is the temperature of the cooling medium, t'_c is the elapsed time of cooling divided by f_c, and t'_ℓ is the total time of cooling divided by f_c.

S_c may be expressed in terms of j_c, S_{c0} and t'_c by the following:

when $j_c = 1$,

$$S_c = S_{c0} \cdot \exp(-\mu \cdot t'_c) \tag{9a}$$

when $j_c < 1$,

$$S_c = j_c \cdot S_{c0} \cdot \exp(-\mu \cdot t'_c) + (1 - j_c) \cdot S_{c0} \cdot \exp[-\mu \cdot t'_c/(1 - j_c)] \tag{9b}$$

when $j_c > 1$,

$$S_c = j_c \cdot S_{c0} \cdot \exp(-\mu \cdot t'_c) - (j_c - 1) \cdot S_{c0} \cdot \exp[-\mu \cdot t'_c \cdot j_c/(j_c - 1)] \tag{9c}$$

Values of F'_c were evaluated from eqn (8) using numerical integration techniques on a Cyber 76 computer. Computational efficiency requires that the interval be as large as possible whilst achieving the required accuracy. Pennington (1970) described a technique, Simpson's rule with error control, for numerical integration in which the interval is selected according to the required accuracy; this technique was used in the calculations for the cooling phase with an error level of 0.00001.

Equation (8) only describes the cooling phase accurately for the small values of the upper integration limit t'_ℓ applying in practice. As t'_ℓ approaches infinity F'_c also approaches infinity. This is equivalent to saying that if a can is cooled for long enough, irrespective of the lethality of the heating phase, the contents will become sterile. Clearly this is unrealistic and consequently eqn (8) requires a finite integration limit. Griffin *et al.* chose the upper time limit to correspond to a temperature 50F degrees below the initial temperature and the upper time limit was found by a reiterative technique. A similar technique was used in this work except that the upper limit was chosen to correspond to $(s_{c0} - 5)$. Equation (8) was used to derive:

$$dF'_c/dt'_c = \exp[\mu \cdot (S_c - S_{c0})]$$

$$= 1 \times 10^{-5} \quad \text{when } S_c = S_{c0} - 5 \tag{10}$$

The upper limit then corresponds to the slope of the F'_c vs. t'_c curve of 1×10^{-5}. Since dF'_c/dt'_c decreases with increasing t'_c, an increase in t'_c by one unit increases F'_c by less than 1×10^{-5} units.

An important aspect of eqn (10) is that the slope, and hence the inherent error, depends upon z which is incorporated into S_c and S_{c0}. In the calcu-

Table 2. F_c' values for values of S_{c0} from 6 to 19 and of j_c from 0.5 to 2.0

j_c	$S_{c0} = 6$	$S_{c0} = 7$	$S_{c0} = 8$	$S_{c0} = 9$	$S_{c0} = 10$	$S_{c0} = 11$	$S_{c0} = 12$
0.50	0.02299	0.01941	0.01680	0.01481	0.01325	0.01198	0.01094
0.60	0.02175	0.01833	0.01585	0.01396	0.01248	0.01128	0.01029
0.70	0.02082	0.01749	0.01509	0.01328	0.01185	0.01070	0.00976
0.80	0.02037	0.01702	0.01462	0.01282	0.01141	0.01029	0.00937
0.90	0.02132	0.01758	0.01494	0.01299	0.01149	0.01029	0.00933
1.00	0.03414	0.02888	0.02502	0.02208	0.01976	0.01788	0.01633
1.10	0.06183	0.05542	0.05056	0.04673	0.04361	0.04101	0.03881
1.20	0.07745	0.06996	0.06421	0.05963	0.05587	0.05272	0.05002
1.30	0.08851	0.08018	0.07376	0.06862	0.06439	0.06083	0.05778
1.40	0.09699	0.08800	0.08105	0.07547	0.07087	0.06700	0.06367
1.50	0.10380	0.09427	0.08688	0.08095	0.07605	0.07192	0.06837
1.60	0.10942	0.09944	0.09169	0.08546	0.08031	0.07597	0.07224
1.70	0.11417	0.10379	0.09574	0.08926	0.08390	0.07937	0.07549
1.80	0.11824	0.10753	0.09921	0.09251	0.08697	0.08229	0.07827
1.90	0.12178	0.11077	0.10221	0.09533	0.08963	0.08481	0.08068
2.00	0.12488	0.11361	0.10485	0.09780	0.09196	0.08703	0.08279

j_c	$S_{c0} = 13$	$S_{c0} = 14$	$S_{c0} = 15$	$S_{c0} = 16$	$S_{c0} = 17$	$S_{c0} = 18$	$S_{c0} = 19$
0.50	0.01006	0.00931	0.00867	0.00811	0.00762	0.00718	0.00679
0.60	0.00947	0.00876	0.00815	0.00763	0.00716	0.00675	0.00639
0.70	0.00897	0.00830	0.00772	0.00722	0.00678	0.00638	0.00604
0.80	0.00860	0.00794	0.00738	0.00689	0.00647	0.00609	0.00576
0.90	0.00852	0.00785	0.00727	0.00677	0.00634	0.00596	0.00562
1.00	0.01503	0.01392	0.01296	0.01213	0.01139	0.01074	0.01016
1.10	0.03691	0.03525	0.03379	0.03249	0.03132	0.03026	0.02930
1.20	0.04769	0.04564	0.04383	0.04221	0.04075	0.03943	0.03822
1.30	0.05514	0.05281	0.05075	0.04890	0.04724	0.04573	0.04435
1.40	0.06079	0.05825	0.05599	0.05397	0.05215	0.05050	0.04899
1.50	0.06529	0.06258	0.06017	0.05801	0.05606	0.05430	0.05268
1.60	0.06900	0.06614	0.06360	0.06133	0.05928	0.05741	0.05571
1.70	0.07211	0.06913	0.06649	0.06412	0.06198	0.06003	0.05825
1.80	0.07477	0.07169	0.06895	0.06650	0.06428	0.06227	0.06043
1.90	0.07708	0.07391	0.07109	0.06856	0.06628	0.06421	0.06231
2.00	0.07910	0.07585	0.07296	0.07037	0.06803	0.06591	0.06396

lations described by Ball & Olson (1957) and Griffin *et al.* (1971), z was not taken into account in determining integration limits.

Ball & Olson (1957) chose a limit of 80F degrees below the initial temperature for the cooling curve. This represents a lethal rate (if $T_b = T_g$) of about 4.6×10^{-13} for $z = 6$ which is very small and may be neglected. For $z = 26$ the lethal rate is about 8.4×10^{-4} which although small is nevertheless large enough to affect the tabulated values and should not be neglected. Similarly Griffin *et al.* (1971) chose the final integration limit to correspond to a lethal rate of 4.6×10^{-9} for $z = 6$ but for $z = 26$ the corresponding lethal rate is 1.2×10^{-2} which would also affect tabulated values and should not be neglected.

The tabulated values therefore become less accurate as z becomes large. Thermal process calculation based on sterilizing ratios automatically take into account the value of z, and avoids these computational errors. Errors arising from the measurement of the required parameters and errors arising from the failure of the equations to fit the temperature history of some cans during the cooling phase may be orders of magnitude greater than the computational errors. F'_c values for j_c from 0.5 to 2.0 and S_{c0} from 6 to 19 are presented in Table 2.

Calculating the lethality F of a thermal process

Data required are:

(1) The processing time, B.
(2) The lag factors for heating, j_h and cooling, j_c.
(3) Slopes of the straight line portion of the logarithmic heating, f_h and cooling, f_c curves.
(4) The initial temperature at the thermal centre of the can, T_0.
(5) The temperature of the heating medium, T_1.
(6) The reference or base temperature, T_b.
(7) The temperature of the cooling medium, T_w.
(8) The slope of the thermal death time curve, z.

When data for the cooling curve are not available we recommend that the contribution of the cooling curve be neglected so that F is underestimated and the safety margin of the process is increased.

Procedure

(A) Calculate S_g, the sterilizing ratio at the end of the heating from:

$$S_g = j_h \cdot S_0 \cdot \exp(-\mu \cdot B/f_h)$$

where

$$S_0 = (T_1 - T_0)/z$$

(B) Use Table 1 or eqns (6a) and (6b) to find the corresponding

$$F'_h = E_1 (\mu \cdot S_g)/\mu$$

(C) Calculate F_h, the lethality of the heating phase from:

$$F_h = f_h \cdot F'_h \cdot \exp(\mu \cdot S_b)$$

where

$$S_b = (T_1 - T_b)/z$$

(D) When cooling phase data are available and when cooling is started immediately heating is finished calculate S_{c0} from:

$$S_{c0} = S_w - S_g$$

where

$S_w = (T_1 - T_w)/z$

(E) Use Table 2 to find F'_c corresponding to j_c and S_{c0}. When a high degree of accuracy is not required the following approximations are suitable for programmable calculators. The errors in the estimates of F'_c using these approximations are less than 2% for $j_c \geqslant 1$ and less than 4% for $j_c < 0.8$. When $j_c < 1.0$,

$$F'_c = \exp\left[\sum_{m=0}^{5}\left(\sum_{n=0}^{3} a_{m,n} \cdot S_{c0}^n\right) \cdot j_c^m - \ln(j_c)\right]$$

when $j_c \geqslant 1.0$,

$$F'_c = \sum_{m=0}^{1}\left(\sum_{n=0}^{3} b_{m,n} \cdot y^n\right) \cdot x^m + \sum_{m=2}^{5}\left(\sum_{n=0}^{3} b_{m,n} \cdot S_{c0}^n\right) \cdot x^m$$

where

$x = \ln(j_c)/j_c$,

and

$y = 1/S_{c0}$

The coefficients for these approximations are presented in Tables 3 and 4.

(F) Calculate F_c from:

$F_c = f_c \cdot F'_c \cdot \exp(\mu \cdot S_{cb})$

where

$S_{cb} = (T_{c0} - T_b)/z = S_b - S_g$

(G) Add the value of F_c to F_h to calculate the lethality of the whole process.

Table 3. Values of $a_{m,n}$ for approximations to the cooling phase lethality for j_c less than one.*

m	n = 0	n = 1	n = 2	n = 3
0	32.901	−17.524	0.98817	−0.018275
1	−265.11	126.58	−7.1944	0.13347
2	763.49	−368.01	21.016	−0.39124
3	−1074.64	528.56	−30.342	0.56698
4	471.50	−375.00	21.652	−0.40623
5	−201.26	105.37	−6.1266	0.11549

*A fifth order least squares regression analysis of $\ln(F'_c \cdot j_c)$ against j_c yielded seventy-eight coefficients for the thirteen values of S_{c0}. These coefficients were regressed against S_{c0} using a third order least squares analysis to yield the twenty-four coefficients above.

Table 4. Values of $b_{m,n}$ for approximations to the cooling phase lethality for j_c greater than or equal to one.*

m	n = 0	n = 1	n = 2	n = 3
0	3.0072×10^{-4}	0.18637	0.077141	0.13935
1	0.21906	3.19845	−18.597	40.509
2	−0.86696	−0.22413	0.012891	-2.4561×10^{-4}
3	4.4589	1.01155	−0.053642	9.6439×10^{-4}
4	−15.1299	−1.91128	0.09480	-1.5849×10^{-3}
5	22.481	0.89434	−0.03811	4.6892×10^{-4}

*A fifth order least squares regression analysis of F_c' against $\ln(j_c)/j_c$ yielded seventy-eight coefficients for the thirteen values of S_{c0}. The coefficients for m = 0 and m = 1 were regressed against $1/S_{c0}$, the coefficients for m = 2 to m = 5 were regressed against S_{c0}, using third order least squares analysis to yield the twenty-four coefficients above.

Calculating the time of a thermal process

Data required are the same as for the lethality calculations except that the processing time is not known and the required lethality is known.

Procedure where the cooling phase data are not available:

(1A) Calculate F_h' from:

$$F_h' = F_h / [f_h \cdot \exp(\mu \cdot S_b)]$$

(1B) Use Table 1 to find S_g. When a high degree of accuracy is not required the following approximations are suitable for programmable calculators. The errors in the estimates of S_g using these approximations are less than 0.3% for F_h' greater than 0.5. When $0.5 < F_h' \leqslant 2.4$

$$S_g = \exp\left(\sum_{m=0}^{5} - b_m \cdot y^m\right)$$

where

$$y = \ln(F_h')$$

and $b_0 = 3.655$, $b_1 = 2.444$, $b_2 = 1.044$, $b_3 = 0.3916$, $b_4 = 0.1142$, and $b_5 = 0.02058$. The coefficients were evaluated using a fifth order least squares regression analysis of $\ln(S_g)$ against $\ln(F_h')$. When $F_h' > 2.4$

$$S_g = (1/\mu) \cdot \exp(-0.577216 - \mu \cdot F_h')$$

(1C) Calculate the processing time from:

$$B = (f_h/\mu) \cdot \ln(\mu \cdot j_h \cdot S_0/S_g)$$

Procedure when cooling phase data are available:

(2A) Take an initial guess of the cooling phase lethality ($F_c = 0.1 \cdot F$ is a satisfactory starting point).

(2B) Calculate F_h and use steps (1A) and (1C) to calculate the process time.
(2C) Calculate F_c from steps (D) to (F).
(2D) Use this estimate of F_c and repeat (2B) to (2D) until the estimates of the process time agree to the required accuracy (0.1 min is satisfactory).

Conclusions

Thermal process calculations based on sterilizing ratios offer four main advantages over the presently used methods. Firstly, the method may be used for any temperature scale as long as the same scale is used in determining the sterilizing ratios. Secondly, the number of associated tables is less than in other methods as z is incorporated in the sterilizing ratio and consequently the calculations are more generally applicable as the tables may be used for any value of z. In addition, the tables are more easily reduced to simple approximations for programmable calculators as one variable has been eliminated and finally the selection of integration limits is such that errors in the tabulated values are negligible.

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Histological measurements of ice in frozen beef

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Summary

A histological study of pieces of meat frozen under controlled conditions which simulate the operation of industrial freezers, showed differences in the ice morphology according to the registered thermal histories.

The diameter of the intracellular dendrites and extracellular ice crystals has been expressed mathematically in terms of a characteristic freezing time, while the percentage of the tissue water that was frozen and the variation of the ice-fibre interfacial area have been measured from the micrographs of transverse sections of frozen beef.

Introduction

The different quality obtained in frozen foods according to whether the freezing is rapid or slow, has been the subject of many studies (Fennema, Powrie & Marth, 1973; Fennema, 1966; Meryman, 1966; Penny, 1974).

These studies generally consider the different sizes of the crystals formed as one of the factors responsible for this changes in the quality obtained.

Histological studies of animal tissues frozen under different conditions have been performed by several authors (Partmann, 1973; Partmann & Schlaszus, 1973).

These histological examinations have generally been made on small samples, frozen under controlled conditions in the laboratory and have shown a relatively uniform ice morphology.

On the other hand, in the freezing of big pieces of meat, and therefore with important temperature gradients, zones of different ice morphology exist, (Tchigeov & Tsuranov, 1973; Menegalli & Calvelo, 1978).

In the present investigation pieces of meat with sizes similar to those used in industrial plate freezers have been examined histologically along the heat flux direction.

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The experimental work was carried out with the following objectives:

(a) To verify the appearance of the morphological zones of the ice in a piece of meat frozen under industrial conditions.
(b) To determine the percentage of frozen water in samples that have suffered different thermal histories but that reached the same final temperature.
(c) To establish a limit in the value of the characteristic time over which no more intracellular ice is produced.
(d) To provide an experimental correlation between the average diameter of the intracellular and extracellular ice crystals in terms of the characteristic parameters of the freezing velocity.
(e) To use these studies in the interpretation of the causes that originate the damage in a frozen tissue.

Materials and methods

Freezing Method

In each experiment samples of post rigor beef (Musculus Semitendinosus), were frozen in an acrylic cylindrical cell, 2.4 mm wall thickness, 5 cm in diameter and 10 cm in length.

This was insulated with 5 cm of expanded polystyrene on all sides, except on one of its bases that rested on a metallic heat exchanger through which alcohol from a cryostat, Lauda UK 50 D W, was circulated. In this way, conditions close to a one direction heat flow were obtained, similar to what happens when an industrial plate freezer is used.

Different surface heat transfer resistances were achieved by placing acrylic slabs of different thickness b and known thermal conductivity k_a, between the heat exchanger and the piece of meat to be frozen.

The approximate surface heat transfer coefficient was calculated from the equation $h = k_a/b$.

The thermal history of the different points of the piece of meat was registered by means of thermocouples disposed along it.

Two moving freezing boundaries of constant temperature were defined, one at $-1°C$ (start of freezing in the meat) and another at $-7°C$ (temperature at which 80% of the total water had frozen).

Curves were plotted for the position of each of the boundaries versus time (Fig. 1) and for the temperature as a function of the position from the border x.

Three parameters, characteristic of the freezing velocity, were defined:

$R(x)$: Advance velocity in mm/min of the boundary at $-1°C$, which was obtained from the slope of the $-1°C$ curve (Fig. 1).

$t_c(x)$: Characteristic freezing time (min) defined as the time necessary for a point to pass from $-1°C$ to $-7°C$ (Fig. 1).

$G(x)$: The temperature gradient (°C/mm) evaluated at $-1°C$, which was obtained from the curve of temperature as a function of position.

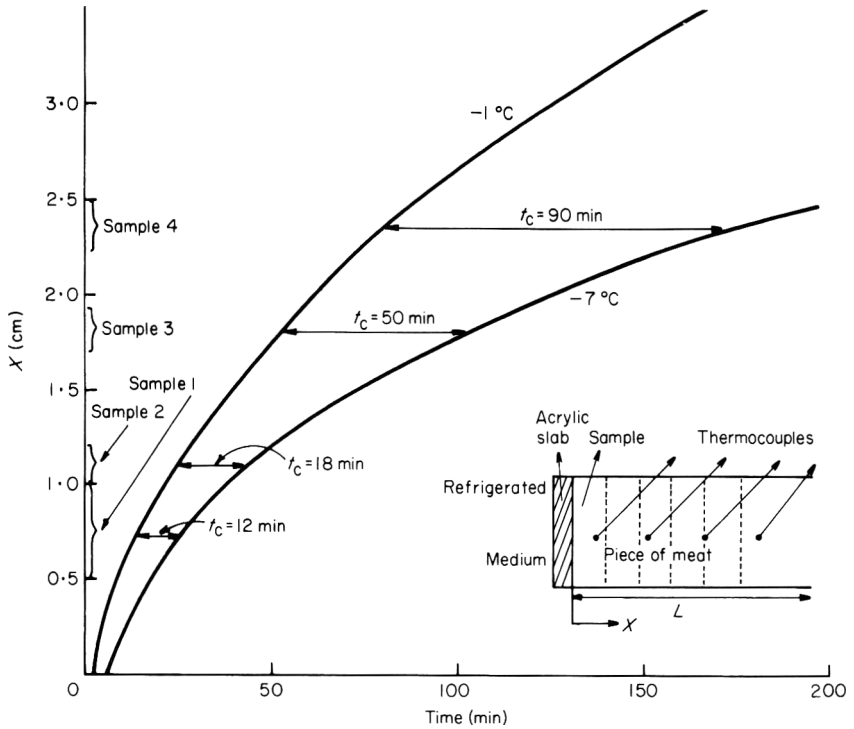

Figure 1. Experimental determination of the characteristic freezing time. Freezing conditions T_i = 15.3°C, T_f = −25.6°C, L = 6.6 cm, Bi = 23.9.

The piece of frozen meat was kept at the final freezing temperature for 12 hr to ensure that thermal equilibrium had been established. Subsequently, the piece was cut in slices of approximately 5 mm thickness from which small samples were cut for histological examination.

Histological method

For the microscopic analysis of the frozen tissue, an indirect technique was used based on the observations of the holes left by the ice in the tissue. The technique used was a modification of the classical freeze-substitution method (Cerrella & Zaritzky, 1975) in which the samples were fixed at the final freezing temperature with Carnoy fluid which has a low freezing point and diffuses rapidly through the tissue.

Sections of the samples were stained with hematoxylin-eosin for histological examination and in each experiment a sample of unfrozen meat was processed at the same time, to act as a control (Fig. 2a).

Frozen water fraction

The micrographs were also utilized to measure the proportion of water converted to ice for samples subjected to different thermal histories and the same final temperature.

This method was applied by Love (1966) in fish and consists in evaluating the areas occupied by fibre and ice, respectively.

From this information the ratio A_T = area of ice/total area, was obtained and used to calculate the fraction of frozen water (ω) that was:

$$\omega = A_T \rho_h / \gamma_0 \rho_c \quad (1)$$

where:

ρ_h is the density of ice (0.916 g/cm^3).

γ_0 is the water content of the meat (mass of water/mass of meat) and for which an average value of 0.73 was adopted.

ρ_c is the density of the partially frozen meat and was calculated as:

$$\rho_c = \rho_0 / 1 + \omega \rho_0 \gamma_0 (1/\rho_h - 1/\rho_a) \quad (2)$$

where:

ρ_0 is the density of the unfrozen meat (1.053 g/cm^3) and ρ_a is the density of water (1 g/cm^3).

Equally the proportion of water converted to intracellular ice is given by

$$\omega_i = \rho_h A_i / \gamma_0 \rho_c \quad (3)$$

where A_i = (area of intracellular ice)/(total area).

Crystal size determination

In order to measure the size of the crystalline ice formation in the tissue, the holes left by the ice were redrawn on the micrographs and the diameters of each one was measured along its minimum axis.

In this way the error that could arise from histological cuts not perpendicular to the direction of crystalline growth, was avoided.

For $t_c < 23$ min the crystalline size was obtained from the intracellular ice while for $t_c > 23$ min measurements were taken on extracellular ice crystals.

Ice-fibre interfacial area

The reabsorption process, which will take place during thawing, will be affected by the ice-fibre interfacial area.

Measurements of the ice-fibre interfacial area per unit ice volume A_v (1/μm) were made on projected images of the micrographs quantifying the ice-fibre perimeter per unit tissue surface. This information was transformed to A_v using the values of frozen water fraction ω previously obtained.

Results

Histological zones

The histological analysis of successive transverse sections of meat frozen in a direction parallel to the fibres reveals the following structures of ice formed in

the tissue:

(a) *Intracellular ice.* This was observed at short characteristic freezing times (high freezing rates), while for long values of this parameter no evidence of intracellular ice was detected.

At a characteristic freezing time of t_c = 0.5 min the ice crystals formed inside and outside the cells were uniformly distributed and had small diameters (Fig. 2b).

As we move toward the thermal centre of the piece of meat the characteristic freezing times increase, leading to fewer crystals per fibre but of bigger size (Fig. 2c).

For characteristic freezing times between 15 and 23 min the meat tissues presented an image where practically only one intracellular crystal occupied each fibre, and it was difficult to distinguish between intracellular and extracellular ice (Fig. 2d).

Beyond this zone, there was no ice in the intracellular space.

Pictures a and b of Fig. 3 were obtained from sections separated by 60 μm in the direction of the thermal gradient. As can be observed, a correspondence exists between the holes, showing that the ice crystals adopted the shape of approximately cylindrical needles in the intracellular zone.

(b) *Extracellular ice.* For values of $t_c > 23$ min the presence of ice was only registered in the extracellular spaces.

The extracellular ice grows at the expense of the water from the inside of the cell because the interfibre fluid has become concentrated through freezing. Therefore the fibres suffer a dehydration process, become distorted and contract adopting irregular shapes (Fig. 3c, t_c = 67 min).

The size of the ice crystals were even larger when moving toward the thermal centre.

At a characteristic freezing time of 380 min the fibres were grouped together and surrounded by columns of ice (Fig. 3d).

The morphological zones of ice described in this section, show a good coincidence with those reported by other authors (Tingakov, Pimenskaya & Kostenko, 1972).

Frozen water fraction

The fraction of water frozen at −14°C was found to be 0.72 and was independent of the freezing rate (Fig. 4).

These data contrast which those of Riedel (1957), who using a calorimetric technique, found that at −14°C, the fraction of water frozen was higher (ω = 0.84).

In the same figure, experimental data for the fraction of water that was frozen as intracellular ice, have also been included. As can be seen, the intracellular ice extends up the values of $t_c \approx 23$ min.

The low value of ω (0.72) obtained in the present work at −14°C could be attributed to the freeze substitution histological method employed.

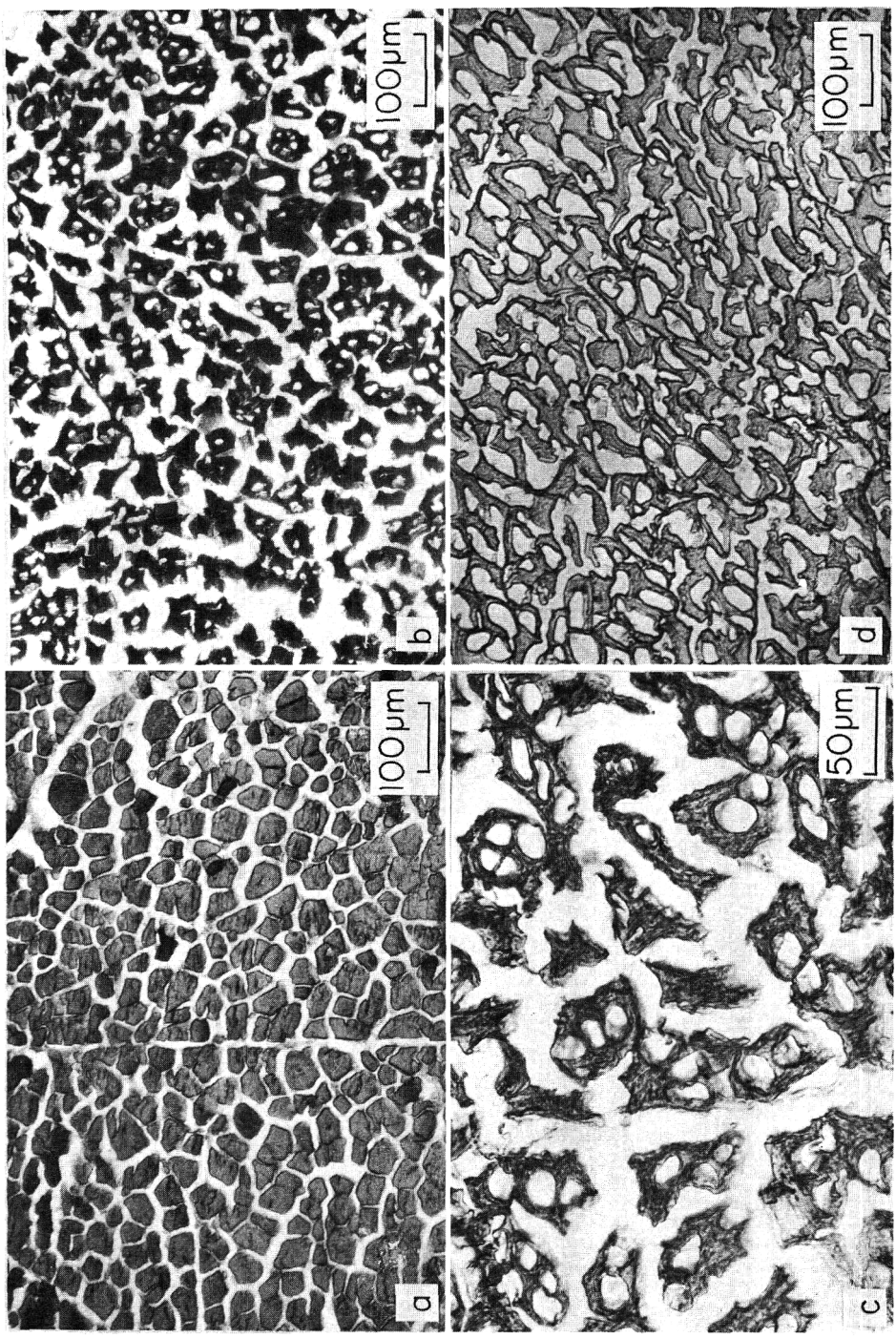

Figure 2. Histological cross-sections of beef: (a) unfrozen; (b), (c) frozen; t_c = 0.5 min; (d) frozen; t_c = 20 min ($T = -14°C$).

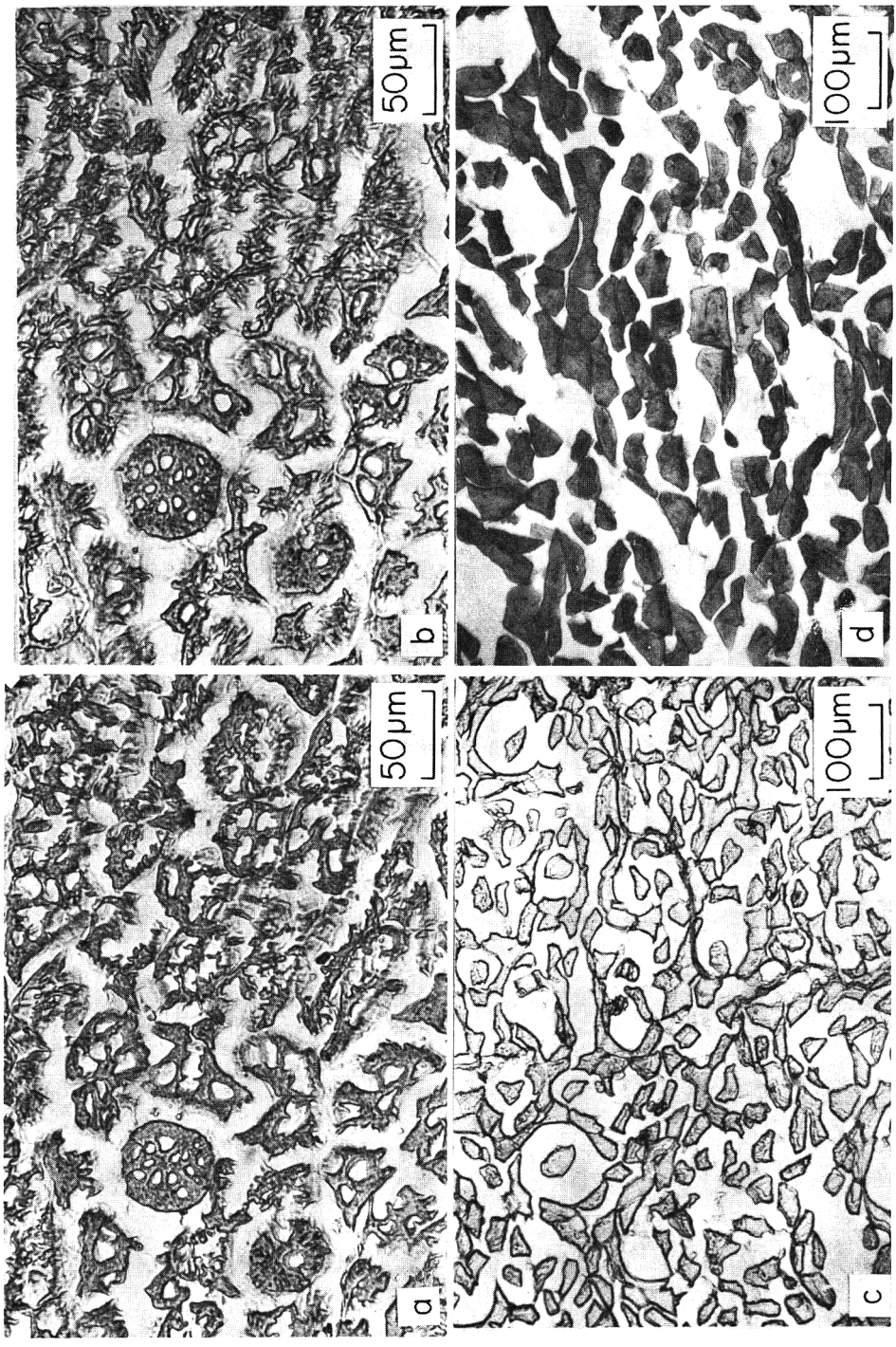

Figure 3. Histological cross-sections of frozen beef: (a), (b) samples separated by 60 μm, t_c = 0.3 min; (c) t_c = 67 min; (d) t_c = 380 min ($T = -14\,^{\circ}C$).

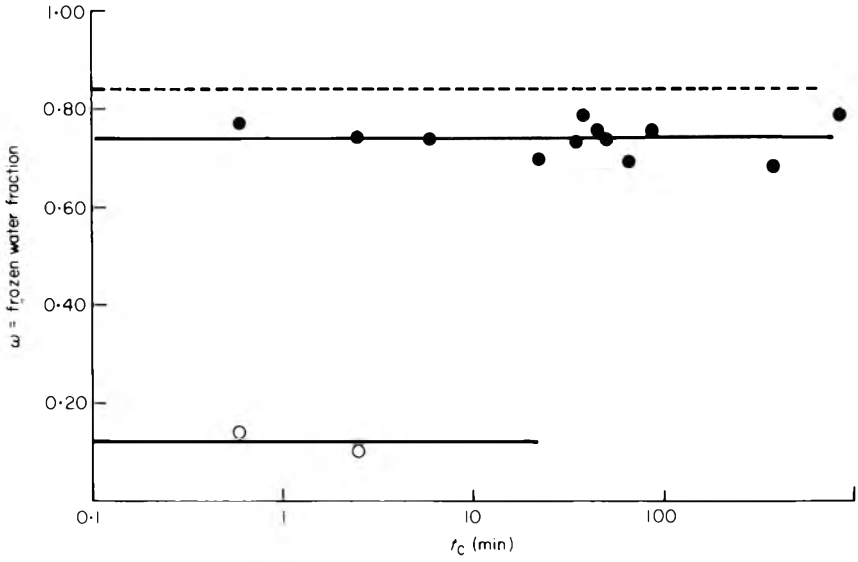

Figure 4. Histological measurement of the fraction of water that is frozen in post rigor beef muscle at −14°C (●). Fraction of water frozen intracellularly (○). (——), Average values; (– – –), Riedel's (1957) calorimetric data.

Even though Love (1966) applying a histological method, indicated that the relative amounts of water converted to ice in cod tissues cannot be comparable with data obtained by calorimetry, if equation 1 was applied to his A_T (area of ice/total area) results, the agreement in this case, is quite good.

Average diameter of intracellular and extracellular ice crystals

The average diameter D of the ice crystals in samples frozen over a range of conditions given in Table 1 was related to the characteristic freezing time t_c by the equation:

$$D = a + b \ln t_c \qquad (4)$$

where t_c was expressed in minutes and a, b are constants determined from the regression of the experimental data (Fig. 5) as a = 15.45 ± 1.9 μm and b = 4.38 ± 0.52 (with 95% confidence).

The correlation coefficient of the regression was $r = 0.96$.

Discussion and conclusions

In order to interpret the results obtained, it must be taken into account that meat is a multicomponent system that in a simplified way can be considered to be formed by insoluble components, soluble compounds, free water and bound water. The freezing of meat can then be considered in a similar way to what

Table 1. Average diameter of ice crystals in samples frozen under different freezing conditions

Freezing conditions	x/L	t_c (min)	D (μm)
$T_i = 17^\circ C$ $T_f = -20^\circ C$ $L = 6.5$ cm $B_i = 23.5$	0.04	35.0	31.4
	0.19	67.0	32.3
$T_i = 15.3^\circ C$ $T_f = -25.6^\circ C$ $L = 6.6$ cm $B_i = 23.9$	0.11	12.0	29.3
	0.16	18.0	29.3
	0.27	50.0	34.8
	0.35	90.0	37.2
$T_i = 18.7^\circ C$ $T_f = -21.5^\circ C$ $L = 7.4$ cm $B_i = 29.7$	0.02	3.0	18.2
	0.15	36.0	29.1
$T_i = 18.7^\circ C$ $T_f = -21.5^\circ C$ $L = 7.85$ cm $B_i > 100$	0.005	0.5	10.4
	0.17	22.0	33.4
$T_i = 7^\circ C$ $T_f = -21.6^\circ C$ $L = 6$ cm $B_i = 21.7$	0.016	22.0	28.3
	0.06	38.0	32.0
	0.24	116.0	33.0
	0.38	380.0	43.0
$T_i = 7^\circ C$ $T_f = -24.1^\circ C$ $L = 3.6$ cm $B_i = 13.05$	0.05	9.0	24.1
	0.20	27.5	28.4
	0.44	44.2	30.3
$T_i = 11.5^\circ C$ $T_f = -21^\circ C$ $L = 6$ cm $B_i > 100$	0.03	5.0	25.9
	0.15	14.0	28.9
	0.27	32.0	31.4
	0.46	97.0	35.2
	0.66	260.0	38.9
$T_i = 12^\circ C$ $T_f = -25.7^\circ C$ $L = 6.2$ cm $B_i > 100$	0.09	6.2	22.8
	0.26	22.5	28.1
	0.53	133.7	35.6

T_i = Initial temperature of the piece of meat.
T_f = Temperature of the refrigerant medium.
L = Total length of the piece.
x = Position of each histologically analysed sample measured from the refrigerated border.
B_i = Biot dimensionless number defined as $B_i = hL/k_c$ where h is the heat transfer coefficient in each experiment and k_c is the thermal conductivity of unfrozen meat (k_c = 0.50 W/m °C).

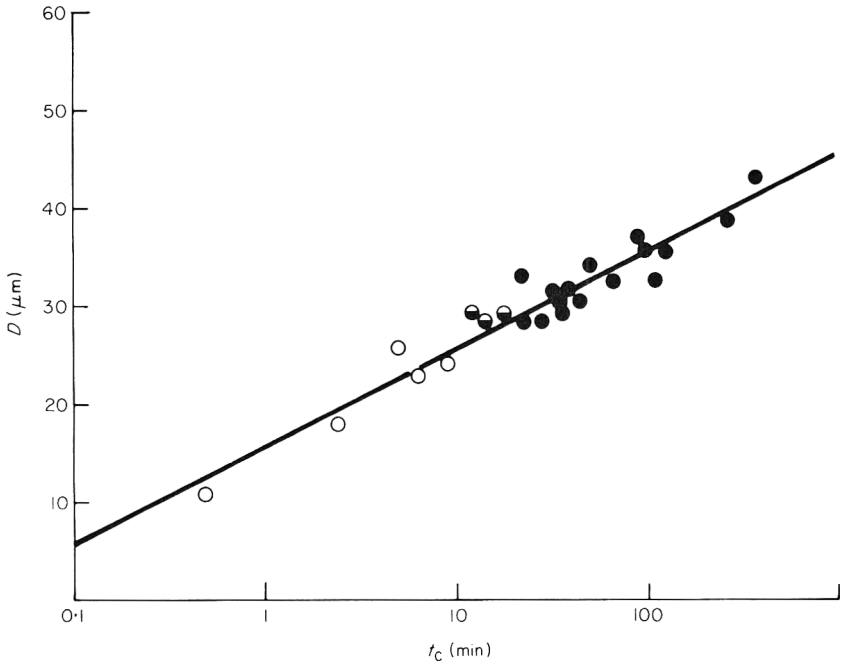

Figure 5. Average diameters of intracellular and extracellular ice crystals as a function of the characteristic freezing time. ○, Intracellular ice; ◒, Intracellular ice + extracellular ice; ●, Extracellular ice.

happens in freezing a multicomponent system. However, in the freezing of meat tissues the problem is complicated because the solutions are contained inside and outside the fibres. As a consequence, the ice crystals can be intracellular or extracellular giving rise to different configurations of the tissue.

When the multicomponent solution comes into contact with the refrigerating medium, a substantial supercooling is reached which generates a number of nuclei proportional to it. In the interphase of the crystals formed, the equilibrium temperature is soon reached due to the release of latent heat of crystallization. As crystal growth elevates the temperature, no further nucleation is allowed. The appearance of intracellular ice depends on the magnitude of the supercooling allowed by the refrigerated border.

It is generally accepted that the intracellular supercooling reached is always lower than in the interfibre space (Meryman, 1966). This effect, either due to a greater solute concentration in the intracellular fluid, or to the existence of a temperature difference between the interior and the exterior of the fibres, leads to the fact that the beginning of the nucleation is always produced in the extracellular space. However, if more heat is extracted from the system than that originated by the formation of extracellular nuclei, the temperature falls till the supercooling necessary for nucleation inside the cells is reached. According to these concepts, the appearance of intracellular ice should be expected at low

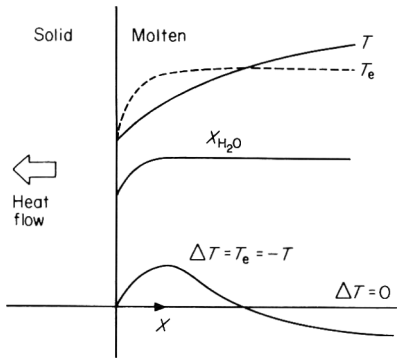

Figure 6. Temperature profile during constitutional supercooling.

low values of the characteristic freezing time as was histologically verified in the present investigation.

During ice crystal growth, the separation of practically pure solid increases the concentration of solutes in front of the interphase. This modifies the molten equilibrium temperature profile, which adopts the shape shown in Fig. 6 and leads to a supercooling in the interphase (constitutional supercooling).

This supercooling produces an irregular interphase (dendrites or columns) where crystalline protuberances grow from the interphase toward the liquid.

Studies performed in metallurgy for solidification of multicomponent systems (alloys), (Chalmers, 1967; Chadwick, 1972) confirm that nucleation takes place at the refrigerated border from which the sample is cooled.

The formed ice crystals grow toward the thermal centre of the system as protuberances accompanied by lateral diffusion of solutes; therefore the protuberances do not tend to expand on their sides and this can lead to a columnar structure.

These crystal columns grow in the direction of the heat flow and away from the refrigerated border. Only those crystals that have their fastest growth direction nearly parallel to the direction of heat flow will subsist. As a consequence, as they grow in length they increase their cross sectional area.

The existence of cylindrical ice crystals growing toward the centre of the piece of meat explains the needles which were detected histologically in the intracellular space (Pictures a and b of Fig. 3).

On the other hand, the growing of the average crystal diameter with the characteristic freezing time, already described, can be interpreted in terms of the above analysis.

Tiller & Rutter, (1956) found, for metallurgical systems, that the average diameter of the crystal columns correlated with $1/RG$ as a straight line, where R is the advance velocity of the solid-melt interphase (mm/min) and G is the temperature gradient (°C/mm) at this interphase.

The measured average diameters of the extracellular ice followed a straight line when plotted against $1/RG$. For ice crystals in meat, a temperature of

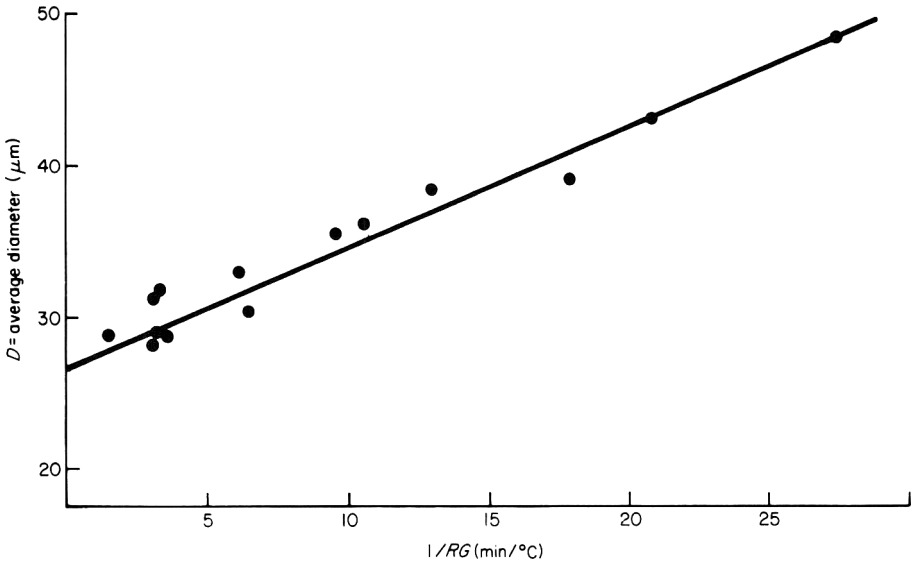

Figure 7. Average diameter of extracellular ice crystals as a function of $1/RG$ where R = advance velocity of the solid-molten interphase at $-1^{\circ}C$ (mm/min), G = temperature gradient at this interphase ($^{\circ}C$/mm).

$-1^{\circ}C$ was assumed in the solid-melt interphase. The correlation coefficient of the regression is 0.97 (Fig. 7).

Freezing injury

The damage caused by the freezing of a tissue can be quantified by measuring the volume of drip produced during thawing.

Añón & Calvelo, (1977) froze pieces of meat in a similar way to that used in the present work and found that the volume of drip was maximum at t_c = 18 min. A similar behaviour was reported by other authors (Love, 1955; Crigler & Dawson, 1968). At this value of the characteristic freezing time (t_c = 18 min), only one intracellular ice column would have existed in each fibre, and crystals of similar size would have occupied the extracellular space.

A first attempt to explain the different exudate productions during thawing might be based on the hypothesis that water reabsorption depends upon the ice fibre interfacial area. Other factors being equal, high values of this variable should generate, according to the described mechanism, low values of exudate.

Measurements of the ice fibre interfacial area per unit ice volume (A_v) expressed in $1/\mu m$, were performed for different t_c (Fig. 8). The highest values of interfacial area were registered in the zone of intracellular ice. When t_c increased A_v decreased, because the number of intracellular columns diminished. The interfacial area adopted its lowest value when the ice was formed only in the extracellular space. This continuous diminishing of the interfacial

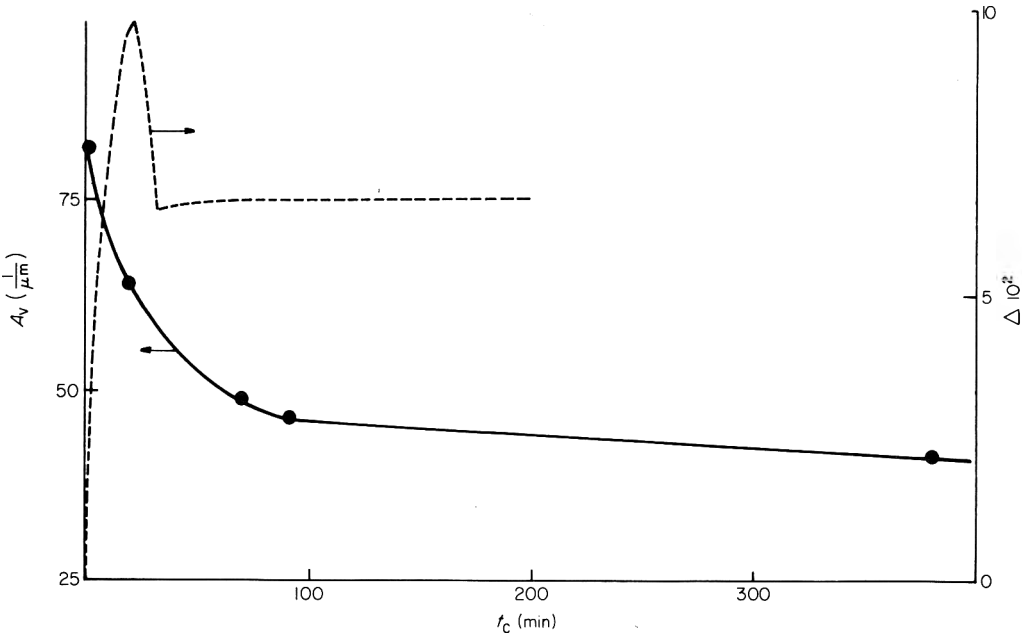

Figure 8. Relationship between the ice-fibre interfacial area per unit volume of ice and the characteristic freezing time of post rigor beef muscle (——). Weight of exudate Δ as a function of the characteristic freezing time (– – –), where

$$\Delta = \left|\frac{\text{weight of exudate}}{\text{weight of meat}}\right|_{\substack{\text{frozen}\\\text{meat}}} - \left|\frac{\text{weight of exudate}}{\text{weight of meat}}\right|_{\substack{\text{unfrozen}\\\text{meat}}}$$

area would have to generate, according to the proposed hypothesis, the highest values of exudate in the zone corresponding to extracellular ice.

If the amount of exudate on thawing is determined by the different interfacial areas originated in different ice morphologies then, the production of exudate must also depend on at least one other factor which produces high exudate in the intracellular zone.

One possible factor is the mechanical damage produced when the ice dendrites have diameters similar to those of the fibres. Therefore, for short values of t_c, intracellular and extracellular dendrites are produced in great number and small diameter, the fibres are not distorted very much and in the thawing the water is easily reabsorbed by the fibres giving low exudate volumes. For values of t_c around 23 min only one intracellular column is formed giving rise to mechanical damage which generates a greater production of exudate. In the zone where only extracellular ice is produced the above described type of damage would disappear. Consequently the exudate volume diminish although not below the values registered at very short values of t_c. In this zone the decreasing in the interfacial area effect remains producing a slight increment in the volume of exudate, as can be observed in Fig. 8.

From the above analysis it can be concluded that:

(1) The existence of intracellular columns of ice has been verified histologically in frozen beef muscle.

(2) A zone of formation of intracellular ice has been established in terms of the characteristic parameters of the thermal history. Thus, for freezing with a heat flow parallel to the fibres, the intracellular ice will be formed for values of t_c up to 23 min. Consequently, for meat pieces frozen under industrial conditions, the region of intracellular ice will extend deeper as the freezing rate increases.

(3) The diameter D of intracellular and of extracellular ice crystals was highly correlated with the characteristic freezing time t_c by the equation $D = a + b \ln t_c$.

(4) For $t_c > 23$ min (extracellular ice) the diameters of ice crystals showed a good correlation with $1/RG$ where R is the advance velocity of the solid melt interphase at $-1°C$ (mm/min) and G is the temperature gradient (°C/mm) at this interphase.

(5) The frozen water fraction was found to be independent of the freezing rate. The low value of ω obtained in this work could be attributed to the freeze substitution method employed.

In spite of this, in general, the histological method should give comparable results with those obtained by calorimetry if the relation:

$$\omega = A_T \rho_h / \rho_0 \gamma_0$$

was applied to obtain the amount of water converted to ice.

(6) The variation of the exudate volume with the characteristic time t_c, registered experimentally in other studies, cannot be solely explained on the basis of the different interfacial areas originated by the ice morphology.

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Studies on an alleged toxic hazard of heat-bleached palm oil

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Summary

It has recently been suggested that the high-temperature heat-bleaching procedure sometimes used to decolorize palm oil for use in margarine and similar products could lead to the oil becoming contaminated with (unspecified) toxic substances, these being derived by thermal degradation of the orange-red carotenoids originally present. Several of the non-glyceride constituents of such an oil have been identified (as squalene, hentriacontane, and various known sterols and triterpenes: all common food constituents) while degradation products of the type said to be present have been sought without success. In addition, the degree to which one of the known carotenoid thermal-degradation products is removed from the oil during the usual refining procedure has been measured and the oil's toxicology has been briefly examined. Neither the results obtained here, nor the allied data reported by others and noted herein, appear to provide any evidence in support of the above suggestion.

Introduction

Palm oil production has been increasing steadily over recent years and is currently in excess of 3 million t per annum (Anon, 1977), much of which is eventually incorporated in edible products such as margarine, cooking fat, shortenings and frying oils. For these end-uses the oil has to be bleached to remove the 0.05–0.1% carotenoids normally present. This is usually accomplished either by the thermal destruction of these compounds at ca. 240° *in vacuo* (Jasperson & Pritchard, 1965; Pritchard, 1975), which converts them into a mixture of essentially colourless products, or alternatively by a combination of (less vigorous) heat and absorbent-earth treatment. Precisely what happens to each of these compounds (mainly β- and α-carotenes with some γ- and ζ-carotenes, lycopene, and hydroxylated carotenes: Argoud, 1958; Loncin, Jacobsberg & Evrard, 1971) when pyrolysed in palm oil has never been firmly established.

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However, model experiments in which β-carotene alone, either as a solid or in an inert solvent, was pyrolysed *in vacuo* showed that many compounds are formed under such conditions. These range from volatile substances such as toluene and *m*-xylene through 2,6-dimethylnaphthalene and ionene (1,1,6-trimethyl-1,2,3,4,-tetrahydronaphthalene), all of which apparently arise from intramolecular cyclization reactions with concomitant cleavage of the polyene chain, to a mixture of non-volatile, and as yet unidentified, compounds (Becker *et al.*, 1966; Davis, 1968). A recent study which more accurately simulated the bleaching process by pyrolysing β-carotene dissolved in bleached palm oil showed that basically similar degradation products are produced in that medium (Hinnekens *et al.*, 1976). The proportion of volatile to non-volatile products formed in these experiments varied from 70% volatiles to less than 20%, depending on reaction conditions (Edmunds & Johnstone, 1965; Becker *et al.*, 1966).

During the commercial processing of palm oil, the volatile carotenoid-degradation products formed during heat-bleaching would be expected to evaporate from the oil at the subsequent 'deodorization' step; but Loncin (1975a, b) has pointed out that the less volatile degradation products may remain in the oil and so be incorporated in the eventual foodstuff. Further, he claimed that these substances could be toxicologically suspect and suggested that they are polycyclic compounds having around forty carbon atoms (as for β-carotene) but with only two or three double bonds per molecule. However no experimental evidence was cited in support of such structures, which apparently were inferred from Becker *et al.*'s (1966) brief study of the non-volatile fractions obtained from a vacuum pyrolysis of β-carotene, nor were any actual toxicological tests reported.

Although the above model experiments yielded interesting results, they do not fully equate the effect of heat-bleaching palm oil itself, with its complex mixture of carotenoids. In the present paper a typical batch of heat-bleached palm oil has been studied and attempts have been made to detect any compounds of the type Loncin claimed to be present. Since he implied that these compounds are hydrocarbons, this has been assumed to be the case and the hydrocarbon (petrol-soluble) fraction from a batch of saponified commercial heat-bleached palm oil was isolated; and its chemistry and, in a preliminary manner, its toxicology studied. Although Loncin did not specifically mention carcinogenic polycyclic aromatic hydrocarbons (mainly C_{20-22} and far more unsaturated than the structural types he suggested) as possible constituents of the carotene-pyrolysis mixture, this possibility has been borne in mind since others may read this interpretation into his comments (cf. Rost, 1976).

Materials and methods

Solvents

Throughout 'hexane' means BDH 'Hexane fraction b.pt. 67–70°' and '30/40 petrol' Hopkin and Williams GPR A-grade petroleum ether of b.pt. 30–40°.

Both were distilled from potassium hydroxide pellets through a 60 cm Vigreux column, rejecting a small forerun and residue.

Preparation of the test material

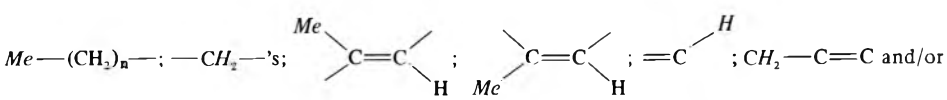
250 g of a commercial heat-bleached deodorized palm oil was saponified cold (to minimize rearrangement of labile constituents) by dissolving in a mixture of 2.2 litres analytical-grade 96% ethyl alcohol and 500 ml hexane, adding a freshly made solution of 250 g potassium hydroxide in 420 ml water, and leaving at room temperature for 24 hr. One volume of water was added with swirling and the solution subjected to rapid continuous liquid–liquid extraction with 30/40 petrol (ca 700 ml) for 72 hr, the extracting solvent being changed at the 24-hr point to minimize heating of the extracted material. The combined extracts were filtered, to remove insolubles washed over during the extraction, washed with water, dried with sodium sulphate, filtered and evaporated under reduced pressure. Several such experiments were carried out and yielded, on average, 450 mg (0.18%) of clear brownish oil. These oils were bulked and subjected to the tests below.

Results

Spectral tests on the crude test material

Infra-red (in carbon tetrachloride): showed CH_2/CH_3 bands at the usual positions and a (carbonyl) band at 1720 cm^{-1} which was unaffected by sodium borohydride treatment. No band attributable to triglyceride ester functions was detected (at 1740/1750 cm^{-1}).

Nuclear magnetic resonance (in deuteriochloroform at 60 MHz using Me_4Si as reference): showed it to be a mixture of components containing the following groupings:



Ultraviolet/visible region (in ethanol): steadily increasing absorption from 450 nm into the ultraviolet with no hint of distinct peaks at the positions expected for the major carcinogenic polycyclic aromatic hydrocarbons (3,4-benzpyrene and 1,2:5,6-dibenzanthracene have λ_{max} 385, 405 and 385, 395 nm respectively: Friedel & Orchin, 1951). On spiking a 250 g sample of bleached oil with 1.0 mg (equivalent to 4 p.p.m.) anthracene, processing as above, and measuring the u.v. spectrum of the resulting extract, peaks corresponding to anthracene's 376, 357, 254 nm bands superimposed on the absorption noted above were seen, with an intensity corresponding to a 65% recovery.

Separation of the test material into its constituents, and their characterization

Thin layer chromatography of the test material on silica gel using ether-hexane (40 : 60 v/v) as solvent and iodine vapour as detector gave nine main spots, from R_F 0.65 to R_F 0.1, with the following apparent (visual) intensities: A(5%), B(70%), C(tr.), D(tr.), E(5%), F(5%), G(1%), H(3%), I(10%) – of which 'A' and 'I' had similar R_F values to a paraffinic hydrocarbon and a typical monohydroxy steroid respectively. The major spot, 'B', was isolated by chromatographing a sample of the mixture on an equivalent column chromatogram (silica gel with hexane, hexane-ether, ethyl acetate as solvent: eluate monitored by t.l.c.) and was shown by g.l.c., as below, to be identical with g.l.c. component 'd'; i.e. squalene. An attempt to separate the other constituents similarly was only partially successful due to overlapping of zones.

Gas–liquid chromatography of the mixture using a Pye 104 instrument with flame ionization detector, 1.5 m × 4 mm i.d. glass-coil column packed with 3% SE30 on Chromosorb W silanized with hexamethyldisilazane *in situ*, and 40 ml/min N_2 as carrier, gave:

(i) *At 225°*: three close peaks ('a', 'b', 'c') with retention times 4.1, 5.1. and 5.8 min followed by a large peak ('d') at 47 min.

(ii) *At 295°*: peaks 'a'–'c' partially resolved at ca. 1.5 min followed by 'd' at 5.3 min and then a series of smaller peaks ('e' to 'k') at 6.6, 8.6, 9.7, 11.7, 12.3, 13.6, and 16.3 min respectively.

The peak areas, as measured by an electronic integrator, were as follows:

Peak:	a	b	c	d	e	f	g	h	i	j	k
%:	3	15	3	50	1	1	1.5	5	2	17	1.5

(iii) *At 315°* (using a 3% OV1 on Universal B column in place of the SE30): peak 'd' appeared after 1.3 min followed by peaks 'e' to 'k' at 1.5 to 3.3 min. No further peaks appeared up to a retention time of 30 min, even using conditions which would have clearly revealed a peak corresponding to 0.1% of the total. For comparison, the paraffinic hydrocarbon $C_{40}H_{82}$ appeared at 5.5 min under these conditions.

Peaks 'a' to 'k' were then subjected to g.l.c. – mass spectrometry using (for peaks 'a'–'f') a Perkin-Elmer Hitachi RMU-6 mass spectrometer and, for the less volatile constituents ('g'–'k': data provided by PCMU, Harwell), an AEI MS50 – after first silanizing the sample to convert any OH groups present to the corresponding trimethylsilyl (t.m.s.) ether derivatives.

Compound 'a': M^+ at 196 with significant fragment ions at m/e 178 (43%), 152, 149, 123, 95, 71, 69, 57 (100%), 43.

Compound 'b': M^+ at 272; fragments at m/e 203, 189, 135, 119, 93, 81, 69 (100%).

Compound 'c': M^+ at 272; fragments at m/e 187, 161, 147, 135, 119, 93, 69 (100%), 41.

Compound 'd': M^+ at 410; fragments at m/e 341 (M-69), 205, 203, 149, 137, 123, 109, 95, 81, 69 (100%), 55, 41. Comparison with authentic squalene on g.l.c. as above showed identical retention times and no separation on mixing.

Compound 'e': peak 'e' on the g.l.c. trace had an attached shoulder and the mass spectrum taken at the centre of the peak showed molecular ions at 440 and 430 (and smaller at 426) with fragments at m/e 422, 408 and 322; 100% peak at 69 with further low-mass peaks at 41, 55 and 81 – as for squalene, above.

Compound 'f': M^+ 436, corresponding to for example $C_{31}H_{64}$ or $C_{30}H_{60}O$. Comparison of the retention time of this compound with those of the n-alkanes $C_{28}H_{58}$ and $C_{32}H_{66}$ strongly suggested the $C_{31}H_{64}$ (hentriacontane) formulation and g.l.c. comparison with an authentic sample confirmed this.

Compound 'g': M^+ at 467.4644 with other peaks at m/e 458, 443, 368, 353, 329, 69 (100%: and other squalene-like peaks at 41 etc.).

Compound 'h': M^+ at 472.4092 with fragments at 457, 382, 367, 343 and 129 (100%).

Compound 'i': g.l.c. peak 'i' occurred on the tail of 'h' and its mass spectrum was therefore superimposed on that of 'h' (peaks at 472, etc.). However it could be inferred that 'i' has M^+ at 484.4096 with fragments occurring at 469, 400, 394, 255 and 129 (100%).

Compound 'j': M^+ at 486.4253 with fragments at 471, 396, 381, 357 (100%) and 129.

Compound 'k': M^+ at 495 with fragments corresponding to 'j' due to overlapping of g.l.c. peaks, and at 440, 425 and 393 due to 'k' itself.

Toxicological tests

The crude test material gave an LD_{50} value, as determined on mice by the intraperitoneal route, of $\geqslant 500$ mg/kg. 1.2 g of the same material was then incorporated at a concentration of 0.05% in a standard feed ('41B') which was pelleted with acacia solution and air-dried at 50°. A short-duration feeding trial with this material using rats showed negligible acute toxicity. In addition, an Ames (bacterial-mutant) test for mutagenicity (McCann *et al.*, 1975) was carried out using a *Salmonella* sp. and proved negative (test kindly carried out by Dr S. Vennitt of Pollards Wood Research Station, Chalfont St. Giles).

The fate of one of the known carotene-degradation products during processing

A sample of ionene (1,1,6-trimethyl-1,2,3,4-tetrahydronaphthalene) was prepared from β-ionone by iodine-catalysed dehydration/cyclization as described by Bogert & Fourman (1933); b.p. 238–240°. Examination on g.l.c. (Pye 105 with f.i.d., 1.5 m × 4 mm i.d. glass-coil column, 5% Apiezon L on Universal B, 40 ml/min N_2 as carrier; 180°) showed one main peak, ret. time 5.7 min. Structure checked by n.m.r.: τ 8.74 (6H: C_1–Me's), ca. 8.3 (4H m: C_2–/C_3–H's), 7.74 (3H: Ar–Me), ca. 7.3 (2H m: C_4–H's) and a 3H pattern at 3.17 (1H d, J = 1), 3.09 (1H double d, J's 1 and 7.5) and 2.80 (1H d, J = 7.5) (benzenoid – H's disposed 1,2,4).

To 20 g of commercial heat-bleached deodorized palm oil was added 0.04 g (0.2% w/w) of ionene and the mixture was subjected to high-temperature, *in vacuo* deodorization using conditions as close as possible to those used commercially (typically 240°/5 mm with steam stripping for ca. 1 hr: Pritchard, 1975) except that the steam was replaced by a rapid stream of dry nitrogen. The rate at which the ionene was removed from the oil under these conditions was monitored by cooling the reaction flask to 50°, shutting off the vacuum and allowing it to fill with nitrogen, removing a sample of the oil, weighing, adding a weighed amount of β-ionone as internal standard and subjecting the mixture to g.l.c. using the conditions above. The ionene appeared at 5.7 min and the β-ionone at 6.6 min, and from the areas of the two peaks the amount of ionene in the sample, and hence in the flask, was calculated. This showed that after 10 min treatment as above, the amount of ionene remaining in the palm oil had fallen to 4.5% and to <0.5% (two runs) of the amount present initially. In addition, the colourless liquid which collected in the outlet tube leading from the reaction flask was removed and subjected to g.l.c. This gave one main peak with a retention time identical with that of ionene run under the same conditions.

Duplicate tests on an oil of different origin

The above experiments were all based on a single batch of one company's palm oil. For comparison, a second company's heat-bleached/deodorized palm oil was therefore saponified etc. as before and the clear brown oil resulting subjected to t.l.c. and g.l.c. examination as before with the following results:

(a) t.l.c.: same series of nine spots seen, the only differences being that spots 'C' to 'I' appeared rather fainter as compared with 'B'; and 'G' was virtually absent.

(b) g.l.c.: a similar series of peaks was seen but with somewhat different intensities and with two small additional peaks (c′ and c″) immediately following peak 'c'; the squalene peak ('d') was rather bigger relative to the other peaks as compared with the previous oil, in agreement with the t.l.c. result.

Peak:	a	b	c	c′	c″	d	e	f	g	h	i	j	k
%:		18		4	1	68	1	tr.	1.5	0.5	tr.	3	2

Discussion

Chemical nature of the petrol-soluble unsaponifiable fraction of heat-bleached palm oil

Firstly, some idea of the general nature of this material's constituents was obtained spectrally. Thus, i.r. and n.m.r. spectroscopy indicated that it consisted mainly of compounds containing saturated $-CH_2-$ groups, of the kind

present in paraffinic hydrocarbons or in alicyclic ring systems, and/or isoprenoid ($-CH_2-C(Me)=CH-CH_2-$) units. (The cause of the 1720 cm^{-1} i.r. band remains unknown). The u.v. spectrum was uninformative; but in conjunction with an anthracene marker it was used to check that had a 'polycyclic hydrocarbon' (cf. Loncin's claim) actually been present in the oil, it would have been satisfactorily carried through the saponification-petrol extraction procedure used.

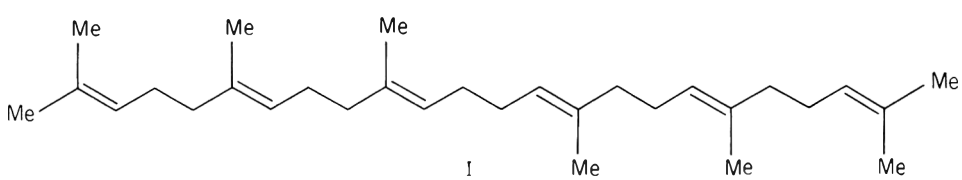
An attempt was then made to identify some of the major constituents and also to see if any compounds in Loncin's category of 'a polycyclic hydrocarbon with 2–3 double bonds and around 40 carbon atoms' could be detected. Thus thin layer chromatography gave nine spots ranging in polarity from 'A' with an R_F corresponding to a saturated hydrocarbon to 'I' with an R_F of a monohydroxy-steroid, the most intense of which ('B') being isolated on an equivalent column chromatogram which showed it to be the substance responsible for g.l.c. peak 'd' (i.e. squalene). G.l.c. examination gave a series of peaks which were then subjected to g.l.c.–m.s., with the following results.

The first three compounds seen on the g.l.c. trace were all comparatively volatile and so were only studied briefly. Compound 'a' gave a molecular ion at 196, corresponding to $C_{15}H_{16}$ or $C_{13}H_{24}O$, with a strong M-18 (H_2O) peak; thereby implying the latter formulation with the oxygen present as, probably, a secondary –OH (significant M^+ ion but no m/e 31). Compounds 'b' and 'c' both gave molecular ions at 272 corresponding to $C_{20}H_{32}$ as for the many known natural diterpene hydrocarbons but lacking the usual M-15 peak of the polycyclic members (Anon, 1974) and having instead squalene-like ions at 41 etc. (see below) indicative of $Me_2C=CH-CH_2-CH_2-C=$.

Compound 'd', the major g.l.c. constituent, had a mass spectral mol. wt and fragmentation pattern suggesting squalene (I, $C_{30}H_{50}$). Thus the intense peaks at 69 and 137, and weaker at 205, are characteristic of the cleavage of the doubly allylic CH_2-CH_2 bonds in such a molecule (Hemming, 1967; Weeks *et al.*, 1969):

$$Me_2C=CH.CH_2-CH_2.CMe=CH.CH_2-CH_2.CMe=CH.CH_2-\ldots\rightarrow$$
$$Me_2C=CH.CH_2^+ \text{ (m/e 69) etc.}$$

In addition, it seems likely (Hemming, 1967) that the peaks seen at 41, 55 and 81 are also due to the $Me_2C=CH.CH_2.CH_2-$ moiety, while we suspect the analogous $x + 68$ group of peaks seen at 109, 123 and 149 can similarly be ascribed to fragmentation of the $Me_2C=CH.CH_2.CH_2.CMe=CH.CH_2.CH_2-$ unit. Although comparison with published spectra (Tornabene *et al.*, 1969; Anon, 1974) did show slight discrepancies, these seemed likely to be due to instrumental differences or to squalene's inherent instability (Stedman, Swain



I

& Rusaniwskyj, 1960); and 'd' was finally confirmed as squalene by comparing it on g.l.c. with authentic material. Compound 'e' was apparently a mixture of three compounds with molecular weights 440, 430 and 426. Several known triterpenes have molecular weights in this region (McCrindle & Overton, 1969); and the 440 and 426 peaks were tentatively ascribed to, respectively, 24-methylenecycloartanol ($C_{31}H_{52}O$; 440) and to butyrospermol and/or cycloartenol (both $C_{30}H_{50}O$; 426) since these compounds have all previously been identified in crude palm oil (Fedeli *et al.*, 1966; Karleskind, 1967, 1968, 1969). The 422 and 408 peaks also seen would then be due to the corresponding $M-H_2O$ fragments from these constituents. In addition, the latter compounds both contain a $-CH_2.CH_2.CH=CMe_2$ side chain so explaining in part the strong m/e 69 etc. peaks also seen (the balance probably being due to contamination by the previous peak). The constituent with M^+ 430 remains unidentified but could be the tetrahydroderivative of butyrospermol. Compound 'f' was shown to be hentriacontane ($C_{31}H_{64}$) from its mass spectral molecular weight and by g.l.c. comparison with authentic material.

The mass spectra of compounds 'g' to 'k' were measured after conversion into the corresponding trimethylsilyl (t.m.s.) ethers and therefore included M-90, M-129 and m/e 129 peaks due to the loss of Me_3SiOH, loss of $Me_3SiO^+=CH.CH=CH_2$, and to the latter ion itself. The M^+ values and the common occurrence of x 15 peaks in the spectra suggested that these compounds were steroids; and the intensity of the M-129 and m/e 129 peaks suggested in turn these were of the 3-hydroxy-Δ^5 type (Budzikiewicz, 1972). Thus compound 'g' appeared to be a mixture of (i) an unknown with apparent M^+ of 467*; and (ii) a component having m/e 458 with associated M-15, M-90, M-90-15 and M-129 peaks at 443, 368, 353 and 329; and which was identified as the t.m.s. ether of cholesterol (or an isomer). Compound 'h' had accurate M^+ corresponding to $C_{31}H_{56}OSi$, equivalent to a parent sterol with formula $C_{28}H_{43}O$, and fragments at M-15, M-90, M-90-15, M-129 and m/e 129 in agreement with the data expected, and reported (Anon, 1974), for campesterol ($C_{28}H_{48}O$) t.m.s. ether. Compound 'i' was similarly identified as stigmasterol ($C_{29}H_{48}O$) t.m.s. ether and compound 'j' as β-sitosterol ($C_{29}H_{50}O$) t.m.s. ether. The observed g.l.c. elution sequence was as reported by others for these sterols on an SE30 column (Fedeli *et al.*, 1966; Karleskind, 1967, 1968, 1969). Compound 'k', a minor component of the mixture, gave an apparent M^+ value of 495*.

Summarizing, the g.l.c. volatile constituents consisted of:

A C_{13} alcohol ('a'; 3%), two C_{20} hydrocarbons ('b' and 'c'; total 18%), squalene ('d'; 50%), 24-methylenecycloartanol, butyrospermol and/or cycloartenol (triterpenes) ('e'; trace of each), hentriacontane ('f'; 1%), an unidentified sterol and possibly cholesterol ('g'; tr. of each), campesterol ('h'; 5%), stigmasterol ('i'; 2%), β-sitosterol ('j'; 17%), and an unidentified compound ('k'; 1.5%).

*The tendency of certain t.m.s. ethers to lose a methyl group from the Me_3SiO entity (Beynon, 1960) suggests that these components may have mol. wts of 482 and 510 respectively.

The unsaponifiable fraction derived from the second company's heat-bleached palm oil gave a generally similar g.l.c. pattern but had a larger peak 'd' (68%; presumed to be squalene) but significantly smaller peaks 'h' (0.5%) and 'j' (3%); and showed two additional, but small, peaks just after 'c' which were not identified. Although in either case there may have been further constituents before the C_{13} alcohol 'lost' in the solvent peak, there appeared to be none following the highest boiling constituent ('k'); in particular no peak following 'k' which could have corresponded to Loncin's C_{40} hydrocarbon with two-three double bonds and a molecular weight, by implication, of around 536. This point was checked by repeating the g.l.c. examination on an OV1 column at a sufficiently high temperature to elute all the above-mentioned components within only about 3 min and then searching the trace from then onwards for higher boiling constituents using the paraffinic hydrocarbon $C_{40}H_{82}$ as marker (in the absence of any precise idea as to the nature of Loncin's C_{40} compound). Even with a high load and high sensitivity, conditions which would have revealed peaks with an area of 0.1% of the total, no peak was seen in the vicinity of the C_{40} marker. This is equivalent to there being less than 2 p.p.m. of such a constituent in bleached palm oil itself.

Finally, although any constituents with molecular weight >ca. 700 could well have been missed by the g.l.c. investigations, the generally good correlation between the g.l.c. data on the one hand and the t.l.c. (and n.m.r.) data on the other suggests that most of the compounds present were in fact detected.

Toxicological aspects

The results obtained, considered in conjunction with allied work by others, provide no evidence of either acute or chronic toxicity. Thus our check for the presence of traces of the carcinogenic polycyclic aromatic hydrocarbons reached the stage of showing (by u.v. spectroscopy) the equivalent of less than 1 p.p.m. present in the bleached oil when a detailed survey by Rost (1976) was published which showed there to be less than 1 p.p.b. (10^9) of either 3,4-benzpyrene or 1,2:5,6-dibenzanthracene present in palm oil both before and after heat-bleaching. Similarly the negative response obtained from the Ames test, carried out on an isolate (the crude test material) which would have concentrated any hydrocarbon carcinogens 500-fold as compared with the oil itself, showed that the sample had no mutagenic tendencies in this test system; and hence, by implication (McCann *et al.*, 1975), was almost certainly not carcinogenic. In addition the brief tests (on the same isolate) for acute toxicity in mice and in rats were negative, and thereby substantiate the model experiments which others have conducted on the material obtained on subjecting β-carotene, the major palm oil carotenoid destroyed by heat-bleaching, to vacuum pyrolysis. For example, Lang *et al.* (1966) added large amounts (100 times more than would be produced from the carotene naturally present) of such a pyrolysate to a sample of bleached palm oil and fed it to rats over 2–3 years but neither acute nor carcinogenic effects were detected. Falk showed

that the non-volatile fraction of such a pyrolysate, prepared at 300° (Edmunds & Johnstone, 1965), was not carcinogenic on subcutaneous injection in mice. Jones & Sharpe (1948) subjected a similar pyrolysate (24 hr at 260°) to column chromatography in a search for polycyclic aromatic hydrocarbons of the above type but concluded that the pyrolysate contained less than 0.1% of any one of them (equivalent to <0.5 p.p.m. in bleached palm oil).

Fate of one of the carotene thermal-degradation products

The approach here was to ascertain to what extent the carotene-derived compounds introduced during the heat-bleaching step are removed during deodorization of the oil, the least volatile of the known degradation products, ionene, being used as the test substance. This was prepared from β-ionene and added to a batch of bleached palm oil which was then subjected to conditions simulating the deodorization procedure used commercially, the ionene concentration in the oil being monitored by gas chromatography using an internal standard. This showed that even using deodorization conditions rather less vigorous than those used commercially (a stream of nitrogen was used instead of steam), the ionene was removed from the oil very rapidly, virtually none of it remaining 10 min after treatment.

Conclusions

It is difficult to see any need for immediate concern over the compounds here identified (list, p. 8) in the petrol-soluble unsaponifiable fraction from two typical batches of heat-bleached palm oil. The major constituent found, squalene, occurs widely in nature, in common foodstuffs (including various vegetable oils) (Fedeli & Jacini, 1971; Gutfinger & Letan, 1974), and in parts of the human body (Goodman, 1964; Nicolaides, Fu & Rice, 1968). Of the others, hentriacontane is a common constituent of natural waxes (such as those in cabbage and spinach leaves: Deuel, 1951, 1957; Eglinton *et al.*, 1962) and the sterols and triterpenes identified are all common natural products. All these compounds have previously been identified in crude (unbleached) palm oil (Becker *et al.*, 1966; Fedeli *et al.*, 1966; Karleskind, 1967, 1968, 1969). In addition, no C_{40} polycyclic hydrocarbons of the kind Loncin suggested may be present were detected despite a search down to a level equivalent to 2 p.p.m. in the oil itself; and we have found no evidence of either carcinogenicity or overt toxicity. Finally it was shown that the carotenoid degradation products of b.pt. up to and including the ionene, which in the non-oxidizing conditions prevailing in a modern heat-bleaching plant should account for at least 70% of the small quantity (0.05% or so by weight) of such materials produced during bleaching, would be removed from the oil by subsequent processing. Taken in conjunction with the results reported by others and cited herein, there appears

to be no evidence of any particular toxic hazard associated with the consumption of heat-bleached palm oil. However until the experimental (chemical and/or toxicological) evidence on which the original claim was based is published in detail, it will not be possible to know what particular substance(s) or finding caused concern, to judge the likely practical effect, or to work towards eliminating it.

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Study of the water extractable components of the red seaweed *Eucheuma spinosum*

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Summary

A study of the components of the red seaweed *Eucheuma spinosum*, found growing abundantly in Singapore waters, was made with a view to improving its commercial value. Carbohydrates was found to be the chief component, accounting for over 70% of the dry weight. Proteins accounted for between 5–10% of the dry weight.

The nature of the carbohydrates present was determined and found to consist mainly of polysaccharide/s extractable with water under the mildest of conditions. Centrifuging a 1% homogenate of the dried *Eucheuma* plant and freeze drying the supernatant resulted in the isolation of over 80% of the polysaccharide present. By sun bleaching the plants before extraction, the red pigments present in the red alga was not extracted with the polysaccharide/s, resulting in a white fluffy product. The sugar units of the polysaccharide/s were galactose and 3,6-anhydrogalactose in the proportion of 3 : 2.

Introduction

Eucheuma spinosum is a red seaweed found growing abundantly in Singapore territorial waters. It is the major component of a variety of *Eucheuma* species which are exported under the trade name 'Singapore Weed'. The *Eucheuma* seaweeds serve as raw materials from which polysaccharides with gel forming properties of the carrageenan type are extracted for use as stabilizers or the formation of emulsions in food industries. The gel forming properties of the polysaccharides obtained are unfortunately rather weak, consequently, the commercial value of these seaweeds are relatively poor. Singapore being located in an area where labour is comparatively cheaper than in the countries to which the seaweeds are exported, it was felt that the economic value of the seaweed could perhaps be increased, if instead of exporting the seaweed plants the polysaccharide/s could be extracted and exported. An attempt was therefore made to develop a simple procedure of extracting the polysaccharide for

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export. The present paper describes the method and the carbohydrate content of the polysaccharide/s extracted from the seaweed under mild conditions. Some of the chemical composition of the polysaccharide/s is also described.

Materials and methods

Chemicals

General chemicals were obtained either from BDH (England) or E. Merck (Germany). Biochemicals were obtained mainly from Sigma Chemicals (U.S.A.). Whenever possible, chemicals of the highest purity available commercially were used.

Source of seaweed

The *Eucheuma spinosum* plants were gathered from the shores of Singapore, washed in running water, then dried and bleached in the sun. For most purposes, appropriate amounts of the seaweed were cut into smaller pieces and dried in an oven at 60 °C to constant weight before use.

Preparation of extract

Depending on the temperature at which the polysaccharide/s was extracted, care was taken to maintain that temperature throughout the procedures (e.g. when cold water extracts of the seaweed were made at 4 °C, the procedure was carried out in a cold room). The dried seaweed was soaked in cold distilled water (1 litre/4 g seaweed) for 30 min before homogenizing in a Warring blender for 10 min. The homogenate was centrifuged at 26 000 *g* at 4 °C for 30 min. The residue was then re-extracted by homogenizing with further amounts of cold distilled water, and the supernatant obtained combined with the first extract or stored separately at 4 °C depending on the nature of the experiments. This process was repeated until the carbohydrate content of the supernatant obtained, showed only a very faint reaction with the anthrone reagent. The extracts obtained were immediately freeze dried or dialysed before being freeze dried. The residue after the final extraction at 4 °C was usually stored frozen overnight for further extractions with hot water. It was found that freezing at this stage did not appear to affect the properties of the hot water extractable polysaccharides.

Hot water extracts at 100 °C were made by gently boiling the homogenate with water for 20 min with continuous stirring with a magnetic stirrer on a hot plate. Centrifugation was then carried out at 26 000 *g* at 10 °C for 30 min. Extraction with boiling water on the residue was repeated until the supernatant showed it contained little extractable carbohydrate on reaction with

the anthrone reagent. The extracts, either kept separately or pooled, were stored at 4 °C and freeze dried subsequently. Storage was never more than 3–4 hr.

Analytical methods

(1) *Elemental analysis.* (a) Carbon and hydrogen contents were determined by Pregl's method (Grant, 1951) using 5 mg samples. (b) Nitrogen was determined by the micro-Dumas gasometric and or the micro-Kjeldahl method. (c) Sulphur was determined as ionic sulphate from the ash obtained in the carbon determination. The ash was dissolved in 10 ml distilled water and the pH of the solution adjusted to 2.5 to 4.0 and ethanol added to a concentration of 80% ethanol. The ionic sulphate was then determined by titration against barium perchlorate (Fritz & Yamamura, 1955) using Wagner's thorin indicator (1957). The addition of 2 drops of 0.125% methylene blue solution enhanced the end point of titration.

(2) *Analysis and estimation of carbohydrates.* (a) *Paper chromatography* was done according to the method of Partridge (1949). 10 mg sample was hydrolysed with 1 ml M H_2SO_4 in 5 ml test-tubes, stoppered with marbles in a boiling water bath for 2 hr. After neutralization of the acid with solid barium carbonate and centrifugation at 2000 *g* for 10 min, 20 μl of the clear supernatant was spotted on Whatman No. 1 chromatographic paper. Sugar standards using 10 μl of a 50% alcoholic solution of concentration 10 mg per ml were also spotted and chromatographed together with the samples. (b) *Thin layer chromatography.* Microcrystalline cellulose thin layer plates of thickness 0.25 mm were prepared and air dried overnight. 2–5 μl of the polysaccharide hydrolysate and monosaccharide standards prepared for paper chromatography were used. The chromatograms were run in n-butanol : acetic acid : water (50 : 17 : 25) for 7 hr. After having been dried in the oven at 100°C for 10 min, they were sprayed with aniline-diphenylamine reagent (Buchan & Savage, 1952) and further dried in the oven for 10 min. (c) *Total carbohydrate and galactose* were estimated by the Anthrone reaction (Tong, Lee & Wong, 1973). *Glucose* was estimated by the glucose oxidase method of Huggett & Nixon (1957) after the polysaccharide/s samples were first hydrolysed with 2 N HCl in a boiling water bath for 3 hr and the hydrolysate neutralized with 1 N NaOH. *Pentoses* were estimated by the phloroglucinol method of Euler & Hahn (1946) as modified by Dische & Borenfreund (1957); *3,6-anhydrogalactose* by the modified resorcinol method of Yaphe (1960); and *protein* by the Lowry's procedure (1951).

Experimental results

Fresh *Eucheuma spinosum* plants were found to have an average moisture content of 92.4%. Drying at 60 °C was found to be as effective as drying at

Table 1. Protein and carbohydrate content as % dry weight*

Protein from dried plants			Protein and carbohydrate from 0.1% homogenate			
	N (%)	Protein (%)		Homogenate (%)	Centrifugate (%)	Residue (%)
Method			Carbohydrate (as galactose)	68.7 ± 0.9	57.2 ± 0.2	11.5 ± 0.6
Microkjeldahl	1.18 ± 0.09	7.37 ± 0.56	Protein (Lowry's method)	11.5 ± 0.4	10.0 ± 0.6	—
Dumas	1.16 ± 0.02	7.25 ± 0.12				

* All results are expressed as mean ± s.e. mean.

100 °C and was preferred if charring was to be avoided. The seaweed, however, is usually sun dried and bleached at the site of collection. This process was found to remove most of the moisture and probably prevented deterioration of the seaweed during harvesting and storage. The sun dried material was found to contain between 14.1% and 16.1% of moisture when it was further dried in the oven.

Eucheuma spinosum contains only a small amount of proteins as shown by the results of Table 1. A higher value was obtained by the Lowry method than from the estimation of nitrogen. With this method the homogenate had a higher value than the centrifugate. This could have been due to the presence of insoluble proteins in the homogenate. The dialysed centrifugate was found to contain a higher percentage of proteins as measured by Lowry's method. The exact cause for this was not known. The enhanced value could probably be due to the release of non-nitrogenous groups which could react positively with the reagent.

Carbohydrates appeared to be the main constituent of *Eucheuma spinosum* and amounted to 64–78% of its weight (with a mean of 68.7%) when expressed in terms of galactose (Table 1). A large percentage of the carbohydrates was water soluble since the centrifugate of the 0.1% homogenate contained about 80% of the total carbohydrates. A preliminary experiment showed that galactose was probably the main carbohydrate constituent (Fig. 1). This was confirmed by paper and thin layer chromatography of the acid hydrolysate of the polysaccharide/s (see below).

Table 2 shows the results of a typical experiment for the extraction of water soluble material from the seaweed. At 4 °C, one single extraction was sufficient to remove 90% of the water soluble fraction. Comparable results were obtained for extractions made at 28 °C (ambient temperature) and at 40 °C (results not shown). Fractions obtained at these temperatures appeared to have the same chemical composition as those extracted at 4 °C (Table 3).

Table 4 shows the loss of about 3–4% of the total weight of the extract on dialysis. Mono- and oligosaccharides accounted for very little of this and it was rather impossible to identify them. Enzymic methods for the estimation of glucose and galactose showed that there was hardly any of these present in the free state. The phloroglucinol method for the estimation of free pentoses also showed the absence of these sugars. An attempt was made to concentrate all the dialysates from a typical experiment for analysis. The concentrate was found to contain only negligible amounts of carbohydrates.

Acid hydrolysate of the crude polysaccharide preparation extracted from the seaweed with cold water showed that it contained mainly galactose with a small amount of xylose, detectable only on TLC plates. Similarly, small amounts of glucose were found as an additional component of the polysaccharide/s subsequently extracted at 100 °C after exhaustive extraction with cold water. Table 5 shows the xylose and glucose contents of the crude polysaccharide preparations from various water extracts. The amounts of both glucose and xylose were negligible. The amount of pentoses present

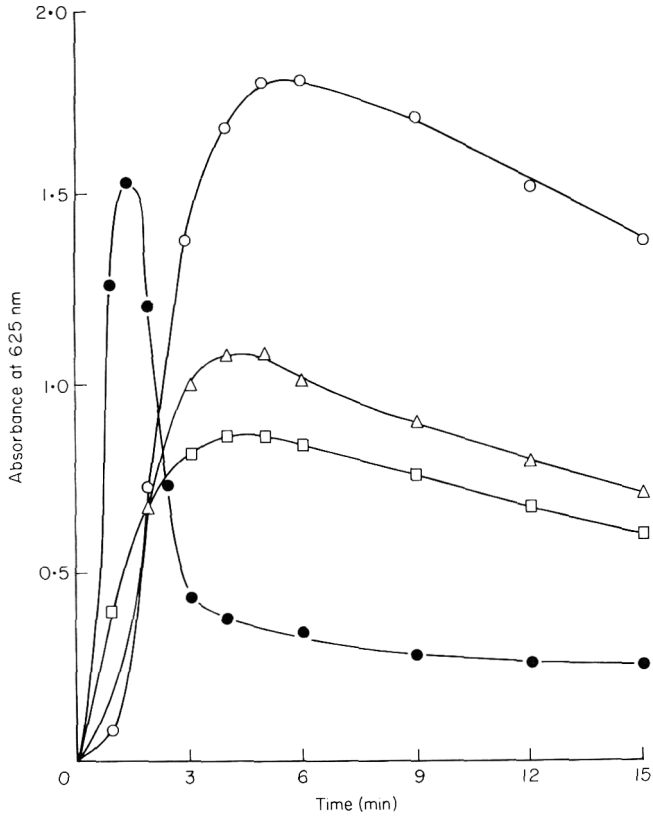

Figure 1. The anthrone-carbohydrate reaction rate curves for xylose, glucose, galactose and crude *Eucheuma* polysaccharide/s. 2 ml of 0.01% carbohydrate was reacted with 4 ml 0.2% anthrone-sulphuric acid reagent. ●, xylose; ○, glucose; △, galactose; □, *Eucheuma* polysaccharide/s.

in the extracts before and after dialysis was determined in order to confirm that there was little or no free pentoses present. There was no difference in the amount of pentoses in the seaweed homogenate before centrifugation and that of the polysaccharide/s in the centrifugate. The amount of glucose in the subsequent hot water extract and the residue though small, was, however greater than that in the initial extracts. It would appear therefore that the glucose present in the seaweed is largely in a less soluble bound form either as glucans or mixed type of polysaccharide. It could also be an indication that Floridean starch consisting of glucose units is present in *Eucheuma spinosum*. This form of starch is known to be present in red seaweeds of the Rhodophyta family of which *Eucheuma* is a member.

3,6-anhydrogalactose has been shown to be present in polysaccharide/s from *Eucheuma* (Black *et al.*, 1965) and known to be of the iota carrageenan type (Anderson *et al.*, 1973). As it is also believed to be responsible for the gelling properties of seaweed extracts (Tsuchiya & Hong, 1965) its content in the polysaccharide extract was determined. A comparison of the value

Table 2. Recovery experiment

Extractions	Weight (g) of solid in supernatant (freeze dried)	Weight of dried seaweed (%)
At 4 °C		
(1) 2.0008 g + 200 ml Homogenize 10 min	1.5206	76.03
(2) Residue + 300 ml H_2O 20 min stirring	0.0609	3.05
(3) Residue + 300 ml H_2O 10 min stirring	0.0232	1.16
(4) Residue + 200 ml H_2O 10 min stirring	0.0213	1.07
Four extractions in 1000 ml at 4 °C	1.6260	81.30
At 100 °C		
(1) Residue from 4 + 250 ml H_2O	0.0147	0.74
(2) Residue + 250 ml H_2O	0.0010	0.05
(3) Combined extractions from residue extracted with 250 ml, 150 ml and 50 ml H_2O	0.0070	0.35
Five extractions in 950 ml at 100 °C	0.0227	1.14
Final residue	0.2227	11.14
Total recovery	1.8714	93.57

obtained with that obtained by other workers would probably serve as an indication as to whether the gel strength of the polysaccharide would be affected by the method of extraction proposed by the present study.

Paper and thin layer chromatography was used to confirm that galactose

Table 3. Carbon, hydrogen and sulphur contents of polysaccharide extracted at different temperature and from different fractions

Temperature	C (μmol)	H (μmol)	C/H	S (μmol)	C/S
First two extracts					
4 °C	2.87	4.98	0.58	0.228	12.58
28 °C (room temperature)	2.93	5.62	0.54	0.191	15.34
40 °C	2.90	5.62	0.53	0.203	14.29
Later extracts					
4 °C	3.82	7.29	0.52	0.0698	55.52
28 °C (room temperature)	3.69	7.33	0.50	0.0743	49.66
40 °C	3.85	7.78	0.49	0.0684	56.28
100 °C	3.71	7.32	0.51	0.0650	57.08

Results are mean of at least three experiments.

Table 4. Weight of dialysable material

		Average	Average
Expt. A. Weight of seaweed 2.04082 g			
Homogenize with 500 ml H_2O			
3 x 50 ml	Homogenate freeze dried	0.19491 g	95.51 %
3 x 50 ml	Homogenate centrifuged and supernatant freeze dried	0.14870 g	76.29 %
3 x 50 ml	Homogenate dialysed and centrifuged. Supernatant freeze dried	0.14027 g	71.97
Loss after dialysis		8.43 mg	4.32 %
Results of triplicates were within ± 5 mg			
Expt. B. Weight of seaweed 3.50028 g			
Homogenize with 700 ml H_2O			
2 x 150 ml	Homogenate freeze dried	0.69865 g	93.13 %
2 x 150 ml	Homogenate dialysed and freeze dried	0.67320 g	89.80 %
Loss after dialysis		25.45 mg	3.33 %

Results of duplicates were within ± 10 mg
Therefore, weight of dialysable material is approx. 3–4%

was the only sugar present in the acid hydrolysate of the water extractable *Eucheuma* polysaccharide. Due to the instability of 3,6-anhydrogalactose in acid, hydrolysis of polysaccharide containing it would have resulted in its conversion to galactose. Hence it is not shown in either the paper or thin layer chromatograms of acid hydrolysates of polysaccharides. 3,6-anhydrogalactose content of the polysaccharide/s extract however could be determined without first hydrolysing it with acid. The values for galactose as shown in Table 6 were obtained by subtracting the amount of 3,6-anhydrogalactose from the total amount of carbohydrate determined as galactose by the Anthrone method. A small amount of variation was observed from sample to sample. A somewhat similar observation has been made by Black *et al.* (1965) on the polysaccharides from Gigartina and Chondrus. An average of the results obtained

Table 5. Xylose and glucose contents (average of three experiments)

Hydrolysed sample	Xylose (%)	Glucose (%)
Homogenate	1.68	0.17
Cold water extract	1.68	0.13
Cold water extract, dialysed	1.72	0.12
Hot water extract	—	6.84
Residue	—	7.38

Table 6. Carbohydrate content of *Eucheuma spinosum* polysaccharide as percentage of dry weight

Seaweed polysaccharide	Total carbohydrate (as galactose) (%)	3,6-anhydrogalactose (%)	Galactose (%)
Kappa carrageenan	62.20	26.80	35.40
Lambda carrageenan	59.50	2.00	57.50
Eucheuma spinosum	45.23	16.30	28.93
	47.73	18.20	29.53
	43.29	17.97	25.32
	56.76	18.23	38.53
	42.77	17.16	25.61
	49.45	18.21	31.24
	46.42	19.47	26.95
	43.66	17.76	25.90
Mean ± s.e. mean	46.91 ± 1.63	17.91 ± 0.32	29.00 ± 1.55

was compared with that obtained for a sample of kappa carrageenan and that of lambda carrageenan from Dr N. P. Stanley of Marine Colloids Inc. U.S.A. The molar ratio of the 3,6-anhydrogalactose to galactose content of *Eucheuma* polysaccharide was 0.63 ± 0.03 which is lower than the value of unity obtained by Anderson, Dolan & Rees (1973) though close to that of 0.71 obtained by Black *et al.* (1965). It is also much higher than that of the lambda carrageenan sample which was 0.04 but less than that of the kappa carrageenan sample with a molar ratio of 0.77. The total carbohydrate content of the *Eucheuma* extract was lower than that of either carrageenan samples. This could be an indication of the degree of purity of the crude *Eucheuma* extract. Alcohol precipitation of the polysaccharide/s from solution resulted in a colloidal compound which was difficult to dry or redissolve in water.

Discussion

The present study shows that a simple method could be used to obtain a crude preparation of the polysaccharide/s responsible for the commercial value of the red seaweed *Eucheuma spinosum*. The polysaccharide/s consisting mainly of galactose and 3,6-anhydrogalactose, was easily extractable with water under extremely mild conditions. Centrifuging a 1% homogenate of the sun bleached dried seaweed, and freeze drying the supernatant, resulted in the extraction of a white fluffy polysaccharide/s which accounted for about 70% of the dry weight of the seaweed plants (Table 2). One extraction with cold water at 4 °C was sufficient to extract 85% of the total extractable polysaccharide/s. At ambient temperatures (28 °C), the extraction was as efficient as at 4 °C if

not better. It was found that the polysaccharide/s extracted at either temperature was similar in chemical composition (Table 3) and possibly as well as gelling properties.

The water extract of the dried seaweed contained little free sugars and dialysable material. The protein content was between 5–10% (Table 1). The total carbohydrate content ranged from 43% to 56% and although it was lower than that of the kappa and lambda carrageenan samples supplied to us by Marine Colloids Inc. U.S.A. (Table 6), it probably indicated that the *Eucheuma* water extract consisted almost entirely of polysaccharide/s. Higher value comparable to the latter would probably have been obtained if some purification of the water extractable *Eucheuma* polysaccharide/s had been made.

Thus from the results of the present study, it was found that the commercial value of the seaweed *Eucheuma spinosum* could be easily enhanced. The cost involved in processing the water extractable polysaccharide/s and marketing it instead of the dried seaweed was economically feasible due to the simple procedure with which it could be extracted. In fact it is felt that the method we have used is simpler and superior to the usual method of boiling the seaweed for several hours and precipitating the polysaccharide from solution with alcohol. The relatively cheaper labour costs in this region than in consumer countries in the West, coupled with a reduction in freight costs should be additional factors favouring this.

Acknowledgments

The authors wish to thank Professor A. K. Tham of the Fisheries Biology Unit of the Univeristy of Singapore for the collection of the *Eucheuma spinosum* plants and Mr W. W. Leong and Miss K. L. Lim for technical assistance. They are also indebted to Dr N. P. Stanley of Marine Colloids Inc. U.S.A. for the samples of lambda and kappa carrageenan.

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Mechanism of gel formation by low methoxyl pectins

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Summary

For stable gel formation, low methoxyl pectin (LMP) should contain at least 50% of the galacturonic acid (GA) in the free carboxylic form. This is achieved by precipitating acid and sodium hydroxide deesterified LMP at pH 0.5, 1.5 or 3.0, and ammonia deesterified LMP at pH 0.5 or 1.5, but not at pH 4.5. With such LMP, depending on the concentration of calcium added and methoxyl content, the sol becomes gel. The changes are classified into five regions: (i) sol, (ii) soft gel, (iii) good gel, (iv) brittle gel and (v) coagulated gel.

The mechanism of gel formation by LMP involves the reaction of 40–50% of the GA present in the free carboxylic form with calcium which causes the precipitation of 75–90% of the total LMP including the entire galacturonic acid units present in the esterified form. The LMP so precipitated, holds 50–60 g of water per gram and forms a stable gel. Higher concentrations of calcium than the optimum increases the reactivity to 80–90% which results in strong cross linkages, incipient precipitation of LMP and loss of gel state.

Introduction

Pectins form two types of gels – conventional high methoxyl pectin (HMP) gels with 65% or more sugar and low methoxyl pectin (LMP) gels with or without sugar using calcium or other polyvalent ions. The former are formed predominantly through hydrogen bonds, while the latter, by covalent bonds (Kertesz, 1951; Doesburg, 1965). The low solids gels are made from LMP having 3 to 5% methoxyl, at 25 to 30 mg of calcium per g of LMP. The bivalent cations interlink adjacent pectin molecules to form a three dimensional network by covalent linkage with carboxyl groups (Hills *et al.*, 1949; Owens, McCready & Maclay, 1949; Hinton, 1950). The texture of LMP gels has been found to vary with pH and concentration of polyvalent cations; the gel strength depends upon methoxyl content and molecular weight of the LMP (Lopez & Li-Hsieng, 1968).

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Factors which influence the formation of LMP gels and the mechanism of gel formation are presented in this paper.

Experimental

Preparation of gels

LMP prepared by acid, NaOH and ammonia deesterification procedures (Padival, 1976) were used. Table 1 gives the analytical data of LMP. To prepare gels without sugar, 1.0 g of LMP was wetted with a few drops of alcohol in a tared 250 ml beaker, 100 ml of water was added and heated to boiling with stirring. Unless otherwise stated, the LMP solution was adjusted to pH 3.0 using sodium citrate, and the required quantity of calcium (30 to 60 mg per g of LMP) was added as calcium chloride, dropwise using a pipette and with constant stirring of pectin solution. The gels were cooked to a final weight of 101 g, poured into jelly glasses and allowed to set overnight. To prepare gels

Table 1. Acid, sodium hydroxide and ammonia deesterified LMP and their gelation characteristics

Particulars of deesterification					Particulars of LMP			Gelation characteristics[b]	
Agent and pH	Temp (°C)	Time (hr)	pH of pptn	Moisture (%)	Ash (%)	MeO AMFB[a] (%)	Optimum Ca (mg/g LMP)	Gel strength (ml H_2O)	Appearance[c]
HCl at	60	9.00	0.5	5.6	0.46	4.21	30	40	++++
0.5	70	5.00	0.5	5.4	0.50	4.61	30	38	++++
	80	1.00	0.5	4.3	0.51	5.20	60	30	+++
HCl at	60	9.00	4.5	4.8	9.40	4.10	—	10[d]	+
0.5	70	5.00	4.5	5.1	8.60	4.65	—	10	+
	80	1.00	4.5	4.3	7.50	5.21	—	10	+
HCl at	60	6.00	0.5	5.5	0.25	5.20	50	33	++++
0.5	60	8.00	0.5	3.7	0.33	4.36	40	40	++++
	60	15.00	0.5	4.0	0.41	3.00	30	50	++++
NaOH	5	0.15	0.5	6.5	0.56	5.00	40	43	++++
at	5	0.25	0.5	6.5	0.52	4.17	30	38	++++
11.7	5	0.50	0.5	4.8	0.50	3.00	25	33	++++
NH_3 at	25	1.50	0.5	6.8	0.66	5.20	60	38	++++
10.5	25	2.50	0.5	5.7	0.56	4.32	50	50	++++
	25	3.50	0.5	4.9	0.51	3.00	40	60	++++

a Ash and moisture free basis

b Gels were prepared using 1% LMP solution at pH 3.0 without sugar at the calcium levels given in the table

c ++++ Very good gel; +++ good gel; ++ soft gel; + coagulated gel

d Acid washing of this LMP resulted in the formation of satisfactory gels and the gel strength (38) matched with that of the LMP precipitated at pH 0.5

containing 35% total soluble solids (TSS), 54 g of sucrose (C.P.) was added to LMP solution in the above procedure, and the final weight of the gel adjusted to 155 g.

The gel strength was determined using the B.F.M.I.R.A. jelly tester with a water flow rate of 75 ml per min at 30° torque.

Acid washing of LMP precipitated at pH 4.5

To remove sodium ions from the LMP precipitated at pH 4.5, 500 ml of alcohol containing 30 ml of concentrated HCl were added to 5 g of the LMP, stirred for 30 min and strained. The LMP was washed repeatedly with alcohol of increasing concentration and dried at 60°C overnight.

Neutralization of free carboxylic groups of LMP

To study the effect of the extent of neutralization of the carboxylic groups on gel formation, calculated quantities of Na_2CO_3 were added to the solution of acid deesterified LMP precipitated at pH 0.5 to neutralize 25, 50, 75 and 100% of the carboxylic groups. The gels were prepared using these solutions at the optimum concentration of calcium (30 mg per g of LMP).

Reaction of calcium with carboxylic groups of LMP during gel formation

In the determination of methoxyl content of pectin, the initial titre value represents the galacturonic acid units with free carboxylic groups (hereafter referred to as GA-COOH), and the saponification titre value represents the galacturonic acid units esterified with methyl alcohol (hereafter referred to as GA-$COOCH_3$). Together, they represent the total galacturonic acid (total GA) content of the pectin. The GA naturally present as metallic salts is negligible and hence, not included.

To study the calcium reactivity with GA, the gels were mixed with equal quantities of water, and the LMP precipitated using two volumes of alcohol. The precipitate was washed repeatedly with increasing concentration of alcohol to remove sugar or excess calcium, and dried overnight at 60°C. One gram of the recovered pectin was divided into two equal portions. In one portion, initial and saponification titre values and in the other, calcium (AOAC, 1975) were determined. The GA-COOH was calculated on the assumption that 20 mg of calcium reacts with 176 mg of galacturonic acid.

Distribution of LMP in gelphase and solphase

Gels prepared with or without sugar using 10 to 140 mg of calcium per g of LMP were disturbed using a mechanical stirrer (at 100 r.p.m. for 1 min). The

disturbed gel, after allowing to stand for 1 hr was transferred to a closely knit terylene cloth and drained for 1 hr.

The pectin present mainly as calcium pectinate complex in the gelphase (i.e., the gel portion remaining on the cloth) was recovered by alcohol precipitation, weighed and then ashed. The LMP in the gelphase was calculated on ash and moisture free basis using the following expression:

$$\text{LMP precipitated in gelphase} = \frac{\text{Weight of LMP in gelphase} - \text{Weight of ash of LMP in gelphase}}{\text{Weight of LMP in gel (1 g)}} \times 100$$

The water-holding capacity of the LMP in the gelphase was calculated from the following expression:

$$\text{Water-holding capacity (g of water (per g of LMP)} = \frac{\text{Weight of gelphase} - \text{Dry weight of LMP recovered from gelphase}}{\text{Dry weight of LMP recovered from gelphase} - \text{Weight of ash}}$$

The pectin in the solphase (i.e., liquid portion draining through the cloth) was determined similarly.

Results and discussion

In the preparation of LMP, adjustment of the saponified extract to pH 4.5 prior to precipitation of the LMP has been invariably recommended to improve the stability during storage, to increase solubility and to overcome undue alteration of the pH in gels (Kertesz, 1951). Acid deesterified LMP precipitated at pH 4.5 and having 4.3% methoxyl did not form satisfactory gels at 30 to 60 mg of calcium per g of pectin (Table 1). Adjusting the pH of the LMP solution to 3.0 with citric acid did not improve the gel characteristics. In contrast, LMP prepared by adjusting the pH to 0.5 formed good gels (Table 1).

Gelation characteristics of the LMP prepared by adjusting the pH to 1.5 or 3.0 were the same as that of the LMP prepared similarly at pH 0.5.

In the determination of methoxyl content, the initial titre values of the above LMP precipitated at pH 4.5 and 0.5 were 0.12 and 3.08 m.eq. of NaOH per g of LMP respectively. These results showed that 90% of the GA-COOH in the former were bound with sodium ions and hence, failed to form satisfactory gels. After washing this LMP with acidified alcohol, the initial titre value was 2.72 m.eq. per g of LMP, and it formed good gels.

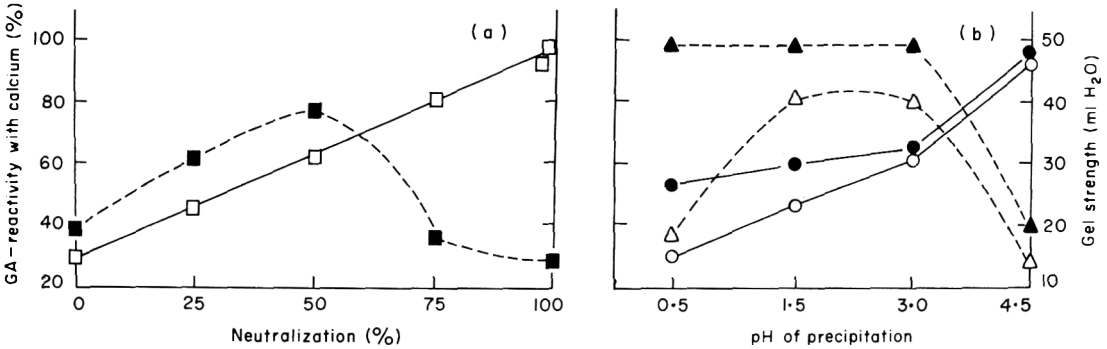

Figure 1. Effect of neutralization and pH of precipitation of acid deesterified LMP (5% methoxyl) on the gel strength and GA-reactivity with calcium. □, GA-reactivity with calcium in gels of LMP neutralized to varying extent using Na_2CO_3; ■, Gel strength of the gels prepared as in □; ○, GA-reactivity with calcium in gels of LMP prepared by precipitating at different pH values; ●, Same as in ○ but the gels were prepared after adjusting the pH of the pectin solution to 3.0 irrespective of the pH of precipitation; △, Gel strengths of gels of LMP precipitated at pH 0.5, 1.5, 3.0 and 4.5, in the absence of buffers; ▲, Same as △ but after adjusting the pectin solution to 3.0 with buffers (citric acid or sodium citrate).

When the GA-COOH in the LMP precipitated at pH 0.5 was neutralized to the extent of 25% and 50% using sodium carbonate, the gels were good; at 75% level, the gels showed a tendency to coagulate which became predominant at 100% level. This is obvious from the gel strength (Fig. 1).

In general, the LMP samples precipitated after adjusting the pH of the deesterified extract to 0.5 yielded good gels in the pH range of 2.9 to 3.6, and at 30–50 mg of calcium per g of LMP depending on the methoxyl content (3–5%) and the mode of deesterification, but not the LMP precipitated at pH 4.5 (Table 1).

Reaction of calcium with GA-COOH during gel formation

When calcium is added to an aqueous solution of LMP, a gelatinous precipitate is formed which, under controlled conditions, is transformed to a stable gel by salt formation (Owens, McCready & Maclay, 1949; Hinton, 1950) or by chelation (Hamm, 1963). Acid deesterified LMP having 4.3% methoxyl and precipitated at pH 0.5 used in this study had a pH of about 2.0 to 2.4 in 1% solution. One gram of this LMP contained 820.2 mg of total GA of which 28.4% (232.9 mg) was present as GA-$COOCH_3$ and 71.6% (587.3 mg) as GA-COOH (Table 2). In the LMP recovered from gel, only 40.5% (240.6 mg) of GA-COOH was bound with calcium, and the gelphase contained 27.0 mg of calcium (Table 2). Using the stoichiometric relation that 20 mg of calcium reacts with 176 mg of GA, the value of 240.6 mg tallied extremely well with the GA content calculated. In this experiment, the gel was prepared after

Table 2. Reactivity of acid deesterified LMP with calcium in sugar gel[a]

Particulars	LMP with 4.3% MeO			
	pptd at pH 0.5		pptd at pH 4.5	
	LMP as such	LMP recovered from gel	LMP as such	LMP recovered from gel
(1) GA* with free COOH[b] (mg/g)	587.3	346.7	21.1	20.6
(2) GA in the esterified form[c] (mg/g)	232.9	232.6	232.9	232.0
(3) GA present as sodium salt[d] (mg/g)	—	—	566.2	—
(4) GA present as calcium salt[e] (mg/g)	—	240.6	—	566.7
Total GA (mg/g)	820.2	819.9	820.2	819.3
(5) Calcium – Calcd[f] (mg/g)		27.3		
– found[g] (mg/g)		27.0		20.6
(6) Extent of GA bound by calcium[h] (%)		40.5		96.4[i]

*GA = galacturonic acid

a Sugar gel containing 35% TSS was prepared with 1% LMP solution at pH 3.0 using 30 mg and 20 mg of Ca for LMP precipitated at pH 0.5 and 4.5 respectively

b Calculated from (1) (10 ml of 0.1 N NaOH = 23 mg of Na = 176 mg GA)

c Calculated from saponification titre value as in 'b'

d Calculated from the difference in the 'b' of LMP pptd at pH 0.5 and 4.5

e Calculated as in 'b' from the difference in initial titre value of the original LMP and that recovered from gel

f Calculated from (4) (176 mg of GA = 20 mg of Ca)

g By ash analysis of LMP recovered from gel

h Calculated (e/b x 100)

i Calculated (d/b x 100)

adjusting the pH of the LMP solution to 3.0. In gels prepared without adjusting the pH to 3.0, only 30% of GA-COOH reacted with calcium giving a soft set.

Neutralization of 25 and 50% of GA-COOH with Na_2CO_3, or buffering to pH 1.5 and 3.0 in the above LMP had similar effect on GA reactivity with calcium. The reactivity increased to 46 and 60% respectively and the gels were good. When neutralized to the extent of 75 and 100%, 80 and 100% respectively of the GA-COOH reacted with calcium resulting in coagulated gels (Fig. 1).

When the methoxyl content of the LMP was 3.95%, 45.5 and 54.2% of the GA-COOH reacted with 30 and 40 mg of calcium per g of LMP resulting in good gels; the reactivity was more in gels prepared without sugar than in gels with sugar (Table 3).

In the LMP precipitated at pH 4.5, out of 587.3 mg of saponified GA, only 3.6% (21.1 mg) was present as GA-COOH and the remaining as sodium salt. The pH of a 1% solution of this pectin was about 4.0 to 4.3. On adding calcium, the GA reacted with it almost quantitatively forming coagulated gels. On buffering to pH 3.0 with citric acid and heating as in gel making, the LMP

Table 3. Reactivity of acid deesterified LMP with added calcium in sugar and sugar-free gels

Particulars	LMP with 3.95% MeO			
	Sugar gel[j]		Sugar-free gel[k]	
	LMP as such	LMP recovered from gel	LMP as such	LMP recovered from gel
(1) GA* with free COOH[b] (mg/g)	595.1	324.5	595.1	251.9
(2) GA in the esterified form[c] (mg/g)	224.3	221.8	224.3	223.0
(3) GA present as sodium salt[d] (mg/g)	—	—	—	—
(4) GA present as calcium salt[e] (mg/g)	—	270.9	—	343.3
Total GA (mg/g)	819.4	817.2	819.4	818.2
(5) Calcium – Calcd[f] (mg)		30.8		38.6
– Found[g] (mg)		30.1		39.0
(6) Extent of GA bound by calcium[h] (%)		45.5[l]		57.1

*GA = Galacturonic acid

b, c, d, e, f, g and h see footnote of Table 2

j Sugar gel was prepared with 1% LMP solution at pH 3.0 using 30 mg of calcium and 35% TSS

k Sugar-free gel was prepared with 1% LMP solution at pH 3.0 using 40 mg of calcium and without sugar

l Sugar gel prepared as in 'j' with 40 mg of calcium per g of LMP showed 54.2% of the GA reactivity with calcium

recovered by precipitation with alcohol contained only 19.2% GA-COOH which was much lower than the minimum of 40% required for the formation of good gels. On treating with alcoholic HCl, 88% of the carboxylic groups were set free, and the LMP formed good gels.

These results show that LMP should contain a minimum of 40 to 50% of GA-COOH to form good gels. This is achieved by precipitating the acid or NaOH deesterified LMP at pH 3.0 or lower. Ammonia deesterified LMP precipitated at pH 3.0 contained 10.5% ammonium salts on the carboxylic groups and 4.2% amides, and did not form satisfactory gels. The samples precipitated at pH 0.5 and 1.5 contained 0 and 5% ammonium salts respectively and formed stable gels although the amide content remained the same.

Besides the GA-reactivity with calcium, the other factors which influence the mechanism of gel formation are discussed below.

Acid deesterified LMP

Gel characteristics. Gel formation by LMP is the transformation of sol state to gel state caused by added calcium and its reactivity with GA-COOH. To a

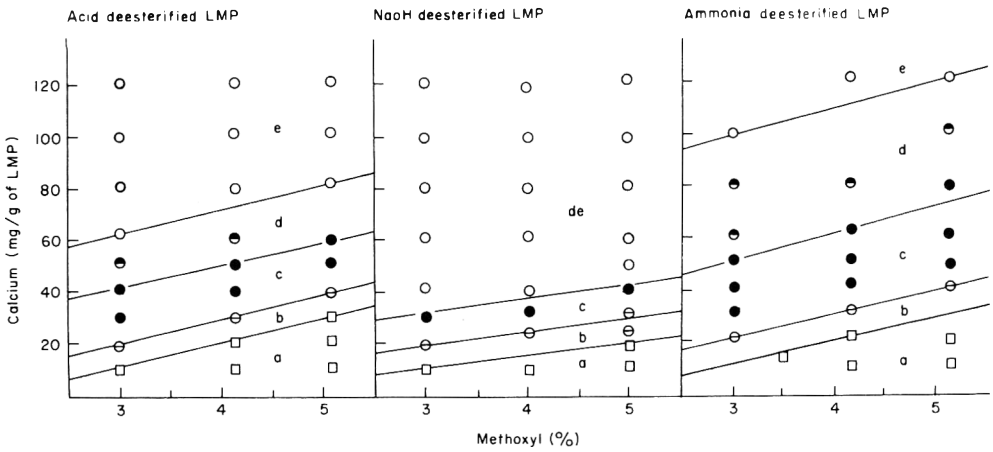

Figure 2. Schematic illustration of gelation characteristics of acid, NaOH and ammonia deesterified LMP preparations with increasing concentration of calcium. a, Sol region (□); b, Soft gel region (⊖); c, Good gel region (●); d, Brittle gel region (◓); e, Coagulated gel region (○).

LMP having 3% methoxyl (677.6 mg of GA-COOH), calcium added at 10–20 mg per g of pectin increased the viscosity but the LMP remained as a sol (region a in Fig. 2). Further increase in the concentration of calcium changed the sol to a gel; between the sol and the gel region, a soft gel region (b) existed which had a high consistency. Next to this, was a broad gel region which could be divided into three sub-regions: (i) region c having a good set (20–40 mg of calcium), (ii) region d in which gels were apparently good but were actually brittle and turbid (40–50 mg) and (iii) region e of coagulated gels in which the calcium concentration was high (60 mg) causing instantaneous precipitation of LMP.

The above observations were similar with LMP having 4 and 5% methoxyl (599.9 and 581.3 mg of GA-COOH) except that the calcium concentrations required to bring about these changes were higher as the methoxyl content increased from 3 to 5% (Fig. 2).

Gel strength. With increasing concentration of calcium, the gel strength increased which was maximum at the optimum, decreased rapidly thereafter, and levelled off at 100 mg or more per g of LMP (Fig. 3a). In gels containing 35% TSS, the optimum concentration of calcium required to exhibit maximum gel strength was lower than in gels containing no sugar (Fig. 3d).

Gelphase and solphase. When good gels were disturbed and allowed to stand for 1 hr, a distinct separation into a gelphase and a solphase occurred. This was not seen in soft gels, and they reset after disturbing and allowing to stand. The weight of gelphase in good gels was more than that in brittle gels which, in turn, was more than that incoagulated gels (Fig. 3b).

Extent of LMP precipitated in gelphase. The concentrations of calcium (40 mg) and LMP (1 g) being the same, the extent of pectin precipitated in the gelphase for good gels was 77, 79 and 88% for pectin having 5, 4 and 3%

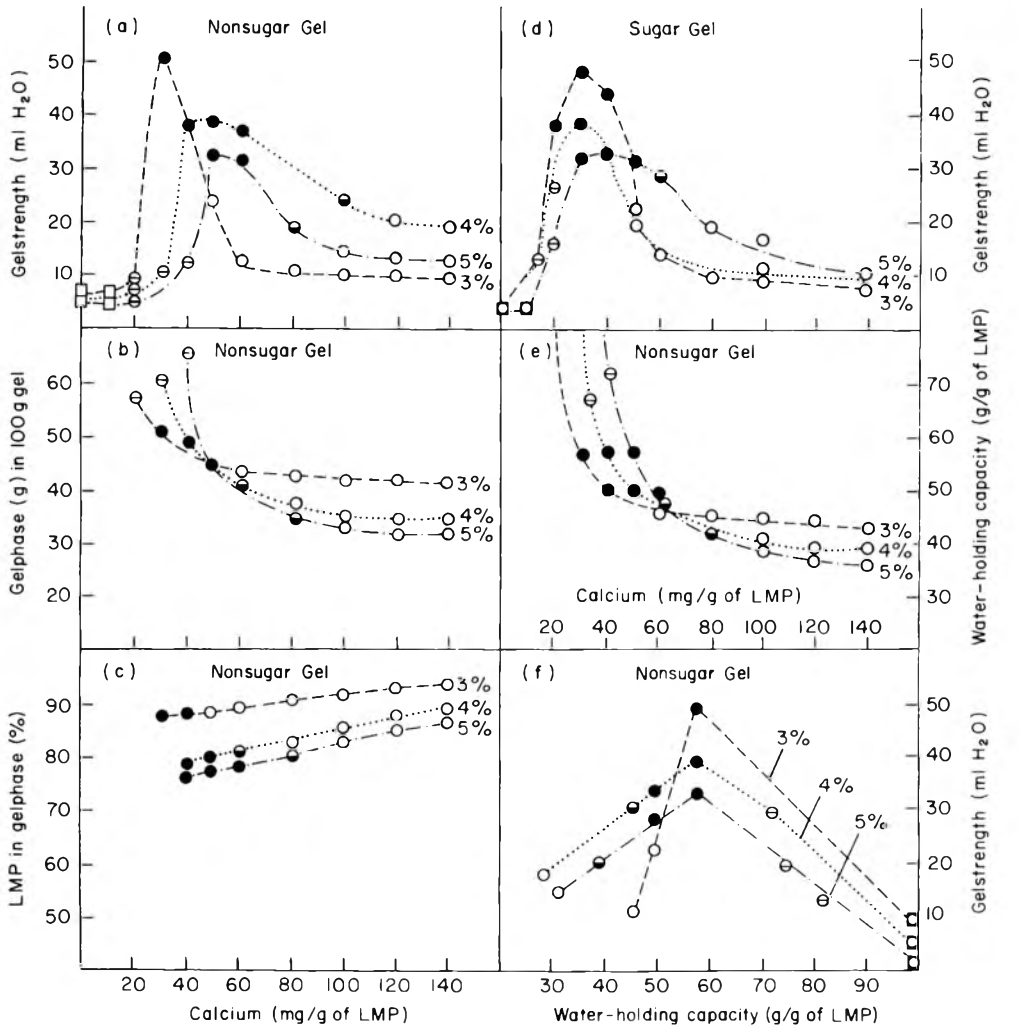

Figure 3. Effect of increasing concentration of calcium on gel strength, gelphase, pectin content in gelphase and water-holding capacity of pectin in gelphase of acid deesterified LMP preparations having 3, 4 and 5% methoxyl groups. □, sol; ⊖, soft gel; ●, good gel; ◒, brittle gel; ○, coagulated gel.

methoxyl groups respectively. The LMP present in the gelphase consisted of most of the GA-$COOCH_3$, the GA bound by calcium and the remaining GA-COOH (Table 4). With increasing concentration of calcium from 30 to 140 mg per g of LMP, pectin in the gelphase increased slightly (Fig. 3c).

Water-holding capacity. Irrespective of methoxyl content, at the optimum concentrations of calcium at which the gels exhibited maximum gel strength, the water-holding capacity of LMP in gelphase was the same (57 to 58 g of water per g of LMP) (Fig. 3e). In soft gels formed at sub-optimal concentrations of calcium, the water-holding capacity of LMP may be considered to be maximum, i.e., 100 g of water per g of LMP used in gel making, whereas at concen-

Table 4. Distribution of pectin between gelphase and solphase of the gel prepared from ammonia deesterified LMP having 3% methoxyl

Particulars	Original LMP (mg)	LMP recovered from gel[a]			
		Gelphase (mg)	(%)[b]	Solphase (mg)	(%)[b]
Weight of LMP	1000.00	883.5	88.4	75.9	7.6
GA* with free- COOH	674.7	303.2	44.9	70.9	10.5
GA present as Ca salt	—	293.2	43.5	0.0	—
GA in esterified form	166.8	161.2	97.0	4.0	2.4

*GA = Galacturonic acid

a The gel was prepared using 1.0 g of LMP and 30 mg of Ca without sugar and pH adjusted to 3.0

b Percent of the value shown under the column 'original LMP'

Table 5. Comparison of gel characteristics of non-sugar gels of acid, sodium hydroxide and ammonia deesterified LMP

Particulars	MeO (± 0.1%)	Acid deesterified LMP	NaOH deesterified LMP	NH_3 deesterified LMP
	(%)	(mg/g of LMP)		
(1) Optimum Ca concn for maximum gel strength	3	30	25	50
	4	40	30	60
	5	50	40	80
	(%)	(ml H_2O)		
(2) Gel strength at optimum Ca concn	3	50	33	57
	4	38	38	50
	5	32	43	38
	(%)	(g per 100 g gel)		
(3) Gelphase at optimum Ca concn	3	51	51	44
	4	48	45	41
	5	45	42	35
	(%)	(per cent)		
(4) LMP pptd in gelphase at optimum Ca concn	3	88	86	88
	4	79	76	79
	5	77	75	77
	(%)	(g of H_2O per g LMP)		
(5) Water-holding capacity at optimum Ca concn	3	58	55	51
	4	57	57	51
	5	57	56	49

trations of calcium above the optimum, the water-holding capacity decreased resulting in coagulated gels (Fig. 3f).

Sodium hydroxide and ammonia deesterified LMP

The transformations discussed above were similar in NaOH deesterified LMP except that the calcium range was narrower for good gels (Fig. 2); the brittle and coagulated gel regions (d and e) merged resulting in a broad coagulated gel region. In ammonia deesterified LMP, the calcium range for gel formation was broader and was divided almost equally into good gel, brittle gel and coagulated gel regions. The gel characteristics of acid, sodium hydroxide and ammonia deesterified LMP are given in Table 5. The calcium requirement for gels without sugar varied from 30–50 mg, 25–40 mg and 50–80 mg respectively per g of acid, sodium hydroxide and ammonia deesterified LMP having 3 to 5% methoxyl content. Addition of sugar in gels reduced sineresis and the calcium requirement to 25–30 mg per g of LMP irrespective of the type of LMP.

Acknowledgments

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Quality changes in frozen Brussels sprouts during storage.

I. Sensory characteristics and residual enzyme activities

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Summary

Sensory evaluation and quantitative analyses of catalase, peroxidase and lipoxygenase were performed in frozen Brussels sprouts during storage at three different temperatures. Within 6 weeks at −4°C all sensory attributes had reached the limit of acceptability, while peroxidase activity increased in the same period. At the end of this period catalase activity had increased strongly. Colour and flavour decreased slowly during 11 months storage at −9°C, while texture remained on a constant level. After 4 months at −9°C a slight catalase activity became detectable. At −18°C no detectable changes occurred during 11 months. Characterization of the preliminary blanching process in terms of residual activities of peroxidase and catalase remains possible after prolonged storage, provided microbial growth can be excluded.

Introduction

Discussions in both national and in international organizations concerning frozen vegetables, indicate a serious lack of objective assessments for the quality of the processed product. In answer to a request of the *Codex Alimentarius* Group of Experts for the Standardization of Quick Frozen Food Products, a research has been carried out into quality changes in frozen vegetables.

Several authors (Dietrich, 1957; Guadagni, 1961, 1968; Guadagni & Fenstel, 1961) indicate that frozen vegetables maintain their quality for at least one year, if stored at proper temperatures. From the authors' own experience, however, the quality of industrially processed vegetables shows striking variations within the first year of storage.

The purpose of the underlying work is to discover the extent of quality changes of frozen Brussels sprouts in practical conditions of storage time and

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temperature and the development of criteria for objective assessment. Sensory characteristics are inherently important indicators for quality. However, uncertainties, as related to the interpretation of the results of sensory tests, and their laborious nature enforce the development of feasible objective criteria. A non-sensory characterization of a frozen product with suitable methods for the determination of quality could certainly provide for a substantial need.

Therefore, a number of physical and chemical parameters was determined, including hardness, colour and ascorbic acid. In order to obtain measurable changes in a limited time, the extreme storage temperature of −4°C has been included. Microbiological tests have been carried out, mainly in view of the application of this high temperature. In order to characterize the applied degree of blanching, enzyme activities were determined before and after blanching. Enzyme residual activity during storage has been studied in view of the possibility of blanching characterization after prolonged storage. In part I of the investigation, the results of sensory evaluations and enzyme activity determinations are given in function of storage time and temperature.

Experimental

Materials

150 kg of Brussels sprouts, cultivar Parcival, were size graded to 2.5–3 cm and cleaned at the auction Mierlo (Netherlands) on 27 October 1975.

The sprouts were stored overnight at 3°C. Blanching was performed the next day for 3.5 min in water at a temperature of 98°C. The blanching time started after approximately 4 min, when the temperature of the blanching water had again increased to the desired level. The freezing of the sprouts at −38°C was completed within 2 hr in a tunnel freezer.

After mixing, the product was packed in cartons, each containing 250 g. The packed cartons were stored in three freeze containers at −4, −9 and −18°C, each containing 126 cartons.

Sampling

Every 3 weeks samples of 2 kg (eight cartons) were taken at random from each of the three freeze containers. The first sampling (zero time) was one week after freezing. After each sampling, the cartons were replaced by frozen dummies to avoid disturbance of temperature equilibrium in the freeze containers.

Samples from each storage temperature were mixed separately in a sterile room and divided into subsamples for analysis of hardness, colour – both before

and after cooking – bacterial count, ascorbic acid, sensory attributes and enzyme activity.

Sensory evaluation

Preparations of the frozen sprout samples were made by cooking 700 g of the product in 250 ml of tap water, with an original temperature of *c.* 15°C. The pans with this content were heated on electric plates. Within approximately 7–8 min the water started to boil and at this moment the cooking time of 5 min was considered to have commenced. During the whole heating treatment the pans were closed by fitting lids and the content was shaken up several times in order to stimulate regular heating of the individual sprouts. A trained panel of six to eight persons subsequently evaluated these samples for colour, texture and flavour. The applied evaluation system was based on the division of the product into three main quality groups: good, acceptable and inferior. Each main quality group is subdivided in three sections, according to the Karlsruhe scheme (Gutschmidt, 1951; Paulus, Gutschmidt & Fricker, 1969) for sensory quality evaluation of foods:

9, 8, 7 } good
6, 5, 4 } acceptable
3, 2, 1 } inferior

The frequency of judgements, evaluated by the panel members, divided over the three main categories, good, acceptable, and inferior, is given for each sample. In order to examine the significance of the effect of the storage time on the sensoric quality of frozen sprouts at different temperatures, the test of Jonckheere (Lehman, 1974) is used. This non-parametric test is appropriate for indicating increase or decrease of the mean ranks.

Enzyme extraction

A laboratory sample (200 g) was homogenized with 400 ml 0.05 M phosphate-buffer pH 7.0 containing 2% glucose and 2% sodium chloride (laboratory homogenizer). A convenient part of the homogenate was further comminuted in a high speed blender (type Edmund Bühler) under carefully controlled conditions (15 sec maximum speed – 15 sec stop – 15 sec maximum speed; chilling with ice-water).

Enzyme activity

Peroxidase activity. Part of the homogenate was cleared at 48 000 *g* for 20 min at 4°C (MSE high speed centrifuge). Peroxidase activity in the clear supernatant was measured according to Lück (1965) in such a way that the increase in optical density was recorded at 25°C (Beckmann DBGT spectrophotometer with a thermostated cellhouse). Because of an occasionally occurring lag-time the recording had to be followed over 10 min, especially at low activities. One arbitrary unit of peroxidase activity is defined as an increase in optical density equal to one in 1 min.

Catalase activity. Using a Gilson Oxygraph equipped with a Clark oxygen electrode the liberation of free O_2 from a sodiumperborate substrate was recorded against time, according to Goldstein (1968).

The Bühler homogenate from fresh material (high catalase activity) had to be diluted with buffer to 0.3 mg dry weight/ml or less, and 0.50 ml of the diluted suspension was taken for analysis. The homogenate from blanched material was used undiluted. One international unit (IU) of catalase liberated 0.5×10^{-6} Mol O_2 /min.

Lipoxygenase activity. To check linearity, a dilution series containing about 5, 10, 20 and 40 mg dry weight in 1 ml was made from the Bühler homogenate (fresh or blanched) and equilibrated to the air at 30°C 0.70 ml phosphate – buffer pH 7.0 (equilibrated to the air at 30°C) and 0.20 ml substrate solution (32 mM linoleic acid and 1% TWEEN-20 in 0.05 M Borate buffer pH 9.0 freshly prepared every week and stored under nitrogen) were brought together in the Clark electrode vessel. The reaction was started with 0.50 ml of the diluted or undiluted homogenate. The pH of the reaction mixture had to be 7.5. One international unit of lipoxygenase activity catalized the consumption of 10^{-6} Mol O_2 per min.

Results and discussion

Sensory evaluation

Table 1 shows the results of the sensory tests, which were performed fourteen times within a period of 11 months. The frequency distribution indicates a large variation at every evaluation session. The reasons for this rather serious variation might be the 'within sample' variation and the effect of the individual interpretations of the evaluation system by the members of the evaluation panel. Notwithstanding this large variation, the results of the evaluation tests indicate obvious changes in some quality aspects of frozen Brussels sprouts, over various storage times and at various temperatures.

The results of statistical tests, according to Lehman (1974) are given in Table 2. The decrease from 'good' to 'inferior' of the colour and flavour of sprouts stored at –4°C and at –9°C and the texture of sprouts stored at –4°C proved to be significant.

Sprouts stored at −4°C rapidly showed dull olive-like discolourations. When stored at −9°C, the 'colour' decreased more slowly; after 3 months a fairly good colour could still be observed, but gradual discolouration occurred during the next 8 months. No detectable changes occur in sprouts stored at −18°C.

The tests for flavour show similar results; sprouts stored at −4°C develop an off flavour obviously due to bacterial spoilage (Tijskens *et al.*, 1979). The more gradually developing off-flavours in sprouts, stored at −9°C coincide with a decrease in ascorbic acid content (Tijskens *et al.*, 1979). Sprouts stored at −18°C do not develop off-flavours at all. However, at the end of the storage period a slight decrease of flavour could be observed.

As it can be concluded from Table 2, frozen sprouts do not exhibit important changes in texture during a storage time of approximately 11 months at −9 or −18°C. However when the sprouts are stored at −4°C a rapid softening can be observed, which may be the result of spoilage.

Enzyme inactivation by blanching

The catalase and peroxidase activities in fresh Brussels sprouts appear to be sufficient for checking a normal blanching treatment by means of the residual enzyme activity. During blanching, catalase and peroxidase were inactivated to respectively $< 0.05\%$ and 0.5% of the original activity (Table 3). Lindquist *et al.* (1951) indicated a significant quality deterioration during 10 months of frozen storage after a blanching treatment resulting in a 2% catalase and 12% peroxidase residual activity. Samples with a respectively 'zero' and 0.04% residual activity were judged as 'good'.

Due to the low measurable lipoxygenase activity in the fresh product, this enzyme is less suitable as a blanching parameter for frozen Brussels sprouts. In how far the quality deterioration of frozen Brussels sprouts during storage at −9°C might depend on a remaining lipoxygenase-like activity is actually unknown.

Enzyme activity during storage

In Fig. 1 the values of the residual activities of the three enzymes are plotted against storage time. Lipoxygenase residual activity remained on a low and constant level for 12 weeks and was not investigated further. During storage at −9°C and at −18°C peroxidase activity did not change significantly, while catalase at −18°C remained below the sensitivity limit. Furthermore the variance in peroxidase and catalase activity between cultivar varieties seems to be low enough to derive a general value for the enzyme activity in fresh Brussels sprouts (see Table 4). So, blanching intensity may be estimated in a frozen product of unknown origin using the residual activity of these enzymes. The

Table 1. Frequency distribution of the sensoric evaluation of frozen Brussels sprouts

Date	Storage temperature	Colour									Flavour									Texture								
		Good			Acceptable			Inferior			Good			Acceptable			Inferior			Good			Acceptable			Inferior		
		9	8	7	6	5	4	3	2	1	9	8	7	6	5	4	3	2	1	9	8	7	6	5	4	3	2	1
5.11.75	−4°C			••••	•••			•				•••	•••	••							•••	•••	•		•			
25.11.75					•••	••••								••	••••		•				•		••	•••		•			
16.12.75					••••	••••							•	••	•	••	••						••	••••	••				
6.1.76				spoiled									spoiled									spoiled							
5.11.75	−9°C		•	••••	•••						•	••	••••		•						•	•••	•••		•			
25.11.75			•••	•••	•							••	•	••	••						••••		••	•				
16.12.75			•••	••	•••							•	•••	••	••						••	•••	••		•			
6.1.76			••		••••							•	•	••	••••						••	••••	••					
					••																							
27.1.76			•	•••	•	••	•						••••	•	••	•					•	••••	•••					
17.2.76				••	••••	•						•		••••		•	•				•••	•	•		•	•		
9.3.76				•	•••	••••							•	•	••••	•						••••	•	•••				
															•													
30.3.76					••••	•	•	•	•			•	•••	•	•	••					•	•	•••	•••				
20.4.76				•••	••	••	•						••	•	•••	••					•	••	••••	•				
11.5.76					••	•••	••		•				•	••	•••	•	•					••••	••	•				
																						•						
1.6.76					••		•	••••						•	••••	•	••				•	•	••••	•				
								•															•					
22.6.76				•	•	•	•	••••				•			••••	••					•	••••	••	•				
															•													
3.8.76				•	•	••	••	•	•						••••	••	••				••		•••	•••				
14.9.76						••	••	•	•					•	•	•	••	•				••••	••					

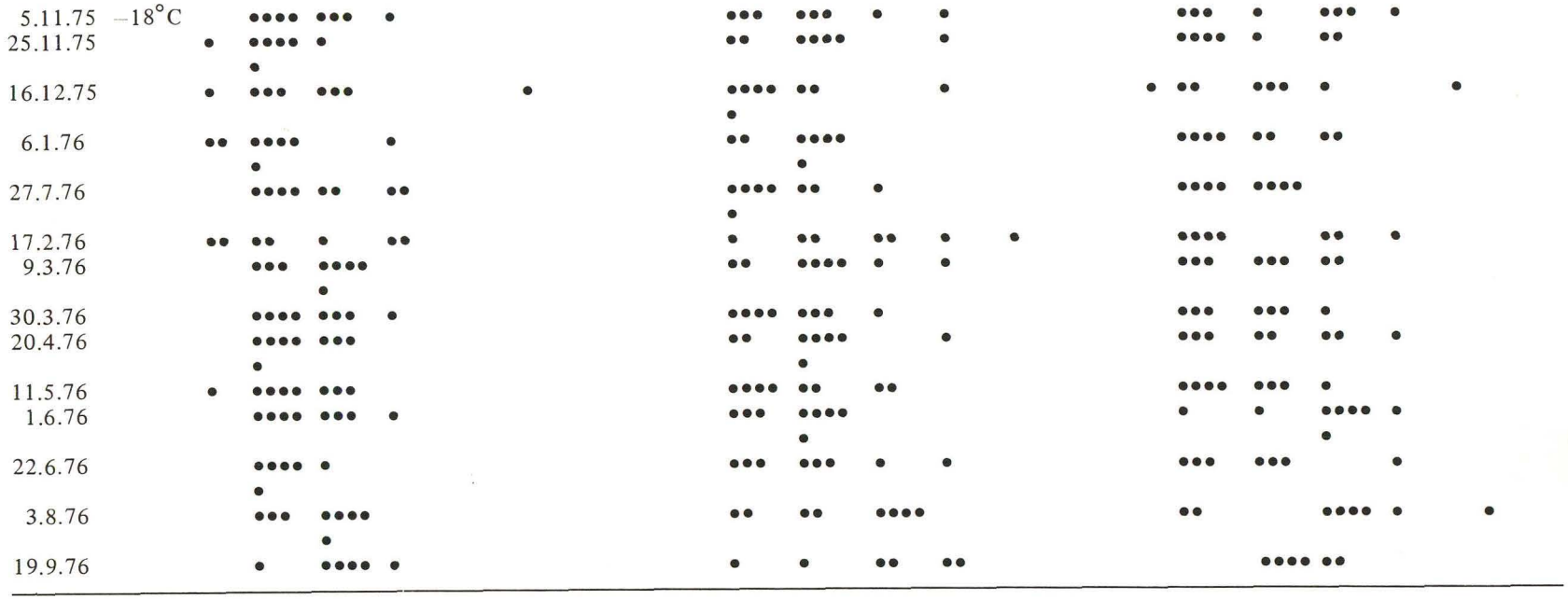
5.11.75 −18°C
25.11.75
16.12.75
6.1.76
27.7.76
17.2.76
9.3.76
30.3.76
20.4.76
11.5.76
1.6.76
22.6.76
3.8.76
19.9.76

Table 2. Frequency distribution of the sensory judges of frozen Brussels sprouts

Storage temp. (°C)	Storage time (weeks)	Colour			Flavour			Texture		
		Good	Acceptable	Inferior	Good	Acceptable	Inferior	Good	Acceptable	Inferior
−4°C	0	4	3	1	6	2		6	2	
	3		7			6	1	3	4	
	6		8		1	5	2	2	6	
	st. norm	u: −1.769*			st. norm	u: −2.730*		st. norm	u: −1.962*	
−9°C	0	5	3		7	1		4	4	
	3	6	1		3	4		4	3	
	6	5	3		4	4		5	3	
	9	2	6		2	6		6	2	
	12	4	4		4	4		5	3	
	15	2	5		1	5	1	4	3	
	18	1	7		1	7		4	4	
	21		6	2	4	4		2	6	
	24	3	5		2	6		3	5	
	27		7	1	1	6	1	5	3	
	30		3	5		6	2	2	6	
	33	1	3	4	1	7		5	3	
	39	1	5	2		6	2	2	6	
	45		4	2		3	3	4	1	
	st. norm	u: −5.280*			st. norm	u: −5.968†		st. norm	u: −1.182 n.s.	

−18°C	0	7	1	6	2	4	4
	3	7		6	1	5	2
	6	7	1	7	1	6	2
	9	7	1	8		6	2
	12	6	2	7	1	8	0
	15	5	2	3	4	4	3
	18	8		6	2	6	2
	21	7	1	7	1	7	1
	24	8		7	1	5	3
	27	8		6	2	7	1
	30	7	1	8		2	6
	33	8		6	2	7	1
	39	8		4	4	2	5
	45	5	1	2	4	4	2
	st. norm	u: +0.736 n.s.		st. norm	u: −1.880*	st. norm	u: −0.986 n.s.

n.s. not significant
* significant at a 5% level
† significant at a 1% level

Table 3. Enzyme inactivation during blanching of Brussels sprouts at 98°C for 3.5 min

	Activity (fresh product)	Activity after blanching	Sensitivity limit	Relative standard deviation of analysis[c] (%)
Catalase[a]	660 ± 210[d]	< 0.3	0.3	10.1
Lipoxygenase[a]	4 ± 2	1 ± 0.1	0.1	10.7
Peroxidase[b]	1300 ± 200	6.5 ± 0.5	0.5	4.3

a. International units per gram dry matter.
b. Arbitrary units per gram dry matter (see text).
c. Determined for activities ten times the sensitivity limit.
d. Confidence limits are given for $\alpha = 0.05$.

significance of such an estimation depends on the temperature history during storage in frozen condition. Thus the striking rise of catalase activity after 6 weeks at −4°C is not due to insufficient blanching but to newly formed bacterial catalase as it coincides with a strong increase in bacterial counts (Tijskens *et al.*, 1979). The apparent reactivation of peroxidase during the first 6 weeks at −4°C may be caused by mould growth. After 9 weeks at −4°C the samples were obviously moulded.

Enzyme activity as a quality parameter

Both sufficient blanching and storage at a sufficiently low temperature are essential for quality retention. Peroxidase and catalase residual activities

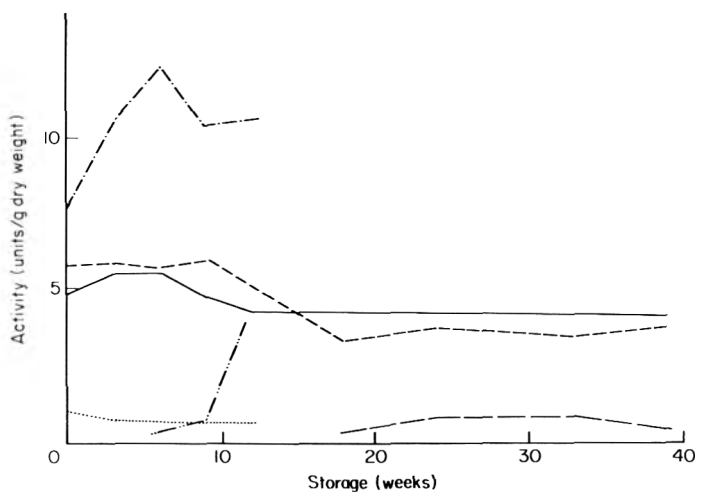

Figure 1. Enzyme residual activity of blanched and frozen Brussels sprouts during storage at three temperatures. – · –, Peroxidase −4°C; – – –, Peroxidase −9°C; ——, Peroxidase −18°C; – · · –, Catalase −4°C; — — —, Catalase −9°C; . . . . ., Lipoxygenase −4, −9 and −18°C.

Table 4. Enzyme activity[a] in fresh Brussels sprouts of different origin

		Catalase	Lipoxygenase	Peroxidase
Cultivar				
Parcival	1975	660	4.0	1300
Lunet	1976	310	2.3	1800
Perfect Line	1976	410	1.9	1010
Citadel	1977	640	0.9	1410
Leonor	1977	710	0.9	1010
Unknown	1977	370	2.0	1210
Mean		517	2.0	1290
Square root of variance		172	1.14	296

a. For definition see Table 3.

prove to be useful objective parameters for the blanching process and therefore for the quality retention during storage.

However, the interpretation of the measured residual activities depends upon the microbial integrity of the frozen product. Samples, showing a detectable catalase activity, should always be subjected to microbiological investigation.

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Quality changes in frozen Brussels sprouts during storage.

II. Objective quality parameters: texture, colour, ascorbic acid content and microbiological growth

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E. P. H. M. SCHIJVENS AND Y. DE WITTE

Summary

Brussels sprouts were stored at −18°C, −9°C and −4°C. During this storage, the colour of whole Brussels sprouts and Brussels sprouts slurry, measured as the greenness (Hunter − *a* value), decreased, probably can be described as a first order decay. The reflection at 680 nm of Brussels sprout slurry measures possibly the constant sum of chlorophyll and pheophytin. This can be a useful indication of the initial colour of the product. Also the ascorbic acid content can be a useful quality index, provided the initial level is known.

Although not a very sensitive one, the shear force seems to be the most reliable textural parameter.

From microbiological point of view, a storage temperature of −18°C as well as −9°C, is quite acceptable.

Introduction

In the first part of this publication (Steinbuch *et al.*, 1979), the sensory characteristics and the residual enzyme activities of frozen Brussels sprouts, stored at three different temperatures, were reported, and the importance of objective quality parameters was stressed. In this part, the behaviour of four objective quality parameters, measured during the same storage period, is reported.

It is long since known that conversion of chlorophyll to pheophytin takes place in vegetables during frozen storage (Dietrich *et al.*, 1957; Dietrich *et al.*, 1959; Dietrich, Huxsoll & Guadagni, 1970; Eheart & Odland, 1973; Walker, 1964; Hudson, Sharples & Gregory, 1974) and sterilization (Gold & Weckel, 1959). It is also known that this conversion can be measured as a change in sensorical appearance (Walker, 1964) and physical colour (Dietrich *et al.*, 1957; Dietrich *et al.*, 1959; Gold & Weckel, 1959). As apples during ripening show a substantial increase in light reflection at 680 nm (Adriaanse, 1968), it was expected that the Hunter parameters as well as the reflection at 680 nm could serve as valuable quality indexes.

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The ascorbic acid content also has been investigated by several authors for its usefulness as a quality parameter (Dietrich, Huxsoll & Guadagni, 1970; Eheart & Odland, 1973; Abrams, 1975; Blegen & Rognerud, 1972; Hudson *et al.*, 1974; Pimm, 1973; Woyke & Makowski, 1968).

Although texture is commonly considered as a property of vegetables important to the consumer, in recent literature there is little, if any research reported, devoted to the measurement of texture of Brussels sprouts. The instrumental methods chosen were the Kramer shear press and texture profile analysis. The first method was chosen because of its universal applicability, the second one because of its more fundamental aspects.

The microbiological tests have mainly been carried out in view of the application of rather high temperatures. Nevertheless, they can provide useful information about the efficacy of the blanching and freezing treatments.

Materials and methods

Materials and sampling techniques are described in the first part of this publication (Steinbuch *et al.*, 1979).

Colour measurements on whole Brussels sprouts

Colour measurements were carried out with a HunterLAB, model D25D2P. Because of the small size of the product (25–30 mm), the diameter of the reflection exit port was reduced with a black sheet from 28 mm to 20 mm. The measured reflection values for Y, X and Z were corrected for this small diameter exit port with the following formula:

$$R_{cor} = (R - R_{bs}) \frac{R_{wn}}{R_{ws} - R_{bs}}$$

where R = reflection (Y, X, Z respectively)
index b = black standard tile
w = white standard tile
s = small exit port
n = normal exit port

From the corrected Y, X and Z values, the Hunter L, a and b were calculated and from L, a, b other derived parameters.

From each sample of Brussels sprouts, one part was measured after 15 min thawing in water of 15°C, the other part after 5 min cooking in boiling water. Each Brussels sprout was measured on five different spots: four equatorial and one on the top. The first measurement on each was made on the spot that appeared the most green.

Colour measurements on mixed sprouts

In an AEG mixer, 100 g of Brussels sprouts were mixed with 100 g distilled water for 5 min at maximum speed (in duplicate). The slurry of product and water was poured into a glass cell (10 mm light path) and debubbled. The reflection at 680 nm was measured at both sides of the cell with a Bausch & Lomb spectrophotometer (model 505), equipped with a reflection sphere, With the Hunter colour meter, the *L*, *a* and *b* values were also measured at both sides of the cell.

Ascorbic acid content

For ascorbic acid determination, 100 g of Brussels sprouts were mixed in a Waring blendor with 200 g of 2% metaphosphoric acid. Ascorbic acid (AA) and AA + dehydroascorbic acid (DAA) were determined titrimetrically according to Zonneveld (1963).

Texture

Of each sample of Brussels sprouts, one part was measured after 15 min thawing in water at 15°C, the other part after 5 min cooking in boiling water. Both parts were measured with the following methods:

Shear press compression. The Brussels sprouts were cut longitudinally in two pieces, and divided into parcels of five halves of nearly equal weight. These parcels were compressed in the Shear press with a crosshead speed of 10 cm/min. The shear force was defined as the highest registrated force, and the mean shear force was calculated for each sample.

Texture profile analysis (TPA). The stalks were cut off at 15 mm from the top, in order to get a uniform height (very important in this method) and a stable positioning of the product during measurement. One by one, the Brussels sprouts were compressed two times between parallel plates to 20% of their original height, with a crosshead speed of 2 cm/min (Bourne, 1968; Friedman, Whitney & Szczesniak, 1963). According to the definitions of Bourne (1968) and Friedman, Whitney & Szczesniak (1963), the typical TPA parameters were determined and the mean for each parameter was calculated for each sample.

Microbiological tests

For each microbiological test, 200 g of Brussels sprouts were weighed out and put into a sterile polyethylene bag, together with 200 ml of physiological saline (0.85% NaCl in sterilized, distilled water).

The product was allowed to thaw in the bag at room temperature for about 1 hr. Subsequently, the Brussels sprouts were homogenized by the Colworth Stomacher 400. 1 ml of the homogenized product was pipetted (in duplicate) into a Petri dish. The agar medium was added afterwards.

Total viable count was determined according to two methods: (i) by incubation on Plate Count Agar (Oxoid CM 325) for 7 days at 17°C for detection of psychotrophic bacteria, and (ii) by incubation on Plate Count Agar for 3 days at 30°C for detection of mesophylic bacteria.

Finally the bacteria of the coliform group were determined by incubation on Violet Red Bile Agar (Oxoid CM 107) at 30°C for 2 days (Speck, 1976).

Results and discussion

Colour

Colour of whole Brussels sprouts. Comparing the $-a/b$ values with the $-a$ values, the first ones show much more variability within the sample than the last ones. Moreover, the b values do not change very much during storage. As a consequence, the decrease in $-a/b$ is mainly due to a decrease in $-a$ value.

Comparison of the first measurement on each Brussels sprout, which was at the spot judged as the most green, with the other four measurements, shows that the first spot always has the lowest L value, whereas the $-a$ value is about the same. So, the 'most green spot' is darker, but not greener.

The $-a$ value is decreasing with storage time and the slope depends on the storage temperature (see Fig. 1). The rate of decrease of the $-a$ value, measured on cooked product is almost the same as that of the uncooked Brussels sprouts at corresponding storage temperatures. Through the cooking process however, an additional variability is introduced. It is difficult to conclude from the data of Fig. 1, whether the degradation of the green colour, expressed as $-a$ value, is linear in time or not. A first order model, however, was found by Gupte, El-Bisi & Francis (1964) for the thermal degradation of chlorophyll in spinach pureé, and also by Dietrich *et al.* (1959) for the retention of chlorophyll in green snap beans during frozen storage. As the green colour of vegetables is largely determined by the chlorophyll content, the degradation of the green colour is likely to decay according to the same exponential model.

In Fig. 2 a semi-logarithmic plot is shown of the $-a$ value against storage time, together with the calculated regression coefficients. Figure 3 shows the temperature dependence of the regression coefficients, according to the Arrhenius equation. As three points are too few to be sure this relationship is correct, further experiments should include more temperature levels.

Colour of Brussels sprout slurry. The reflection of Brussels sprout slurry at 680 nm did not change with storage time nor temperature. Obviously, the constant sum of chlorophyll and pheophytin was measured. Perhaps this method can be used as an indication of the initial appearance of the frozen product.

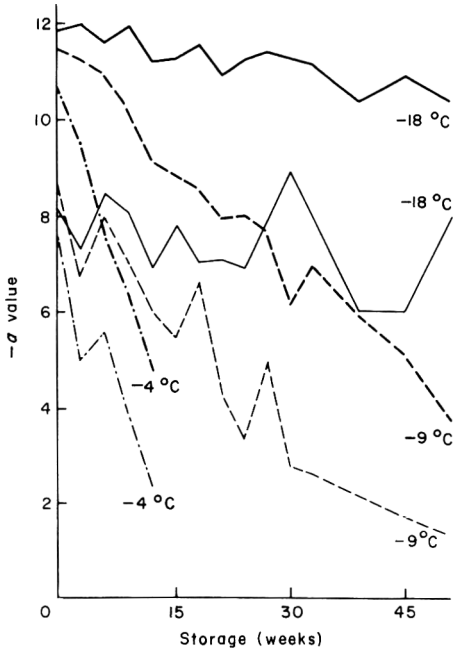

Figure 1. The effect of storage temperature on the green colour of Brussels sprouts, expressed as $-a$ value. ——, Thawed Brussels sprouts; ——, cooked in boiling water.

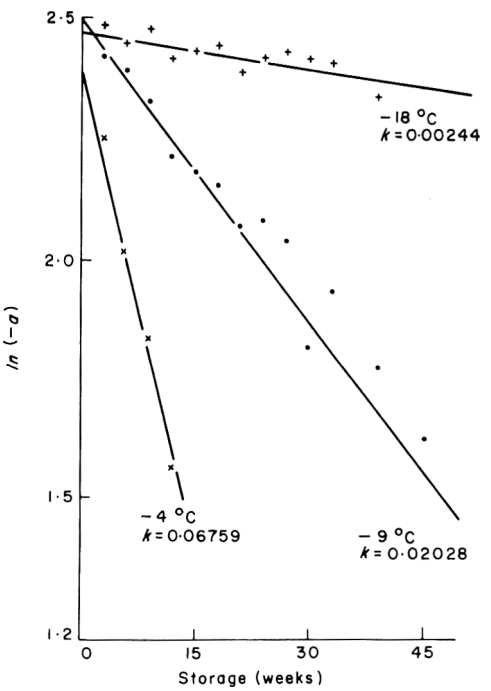

Figure 2. Semi-logarithmic plot of the green colour ($-a$ value) of thawed Brussels sprouts versus time ($\ln(-a) = \ln(-a_0) - kt$).

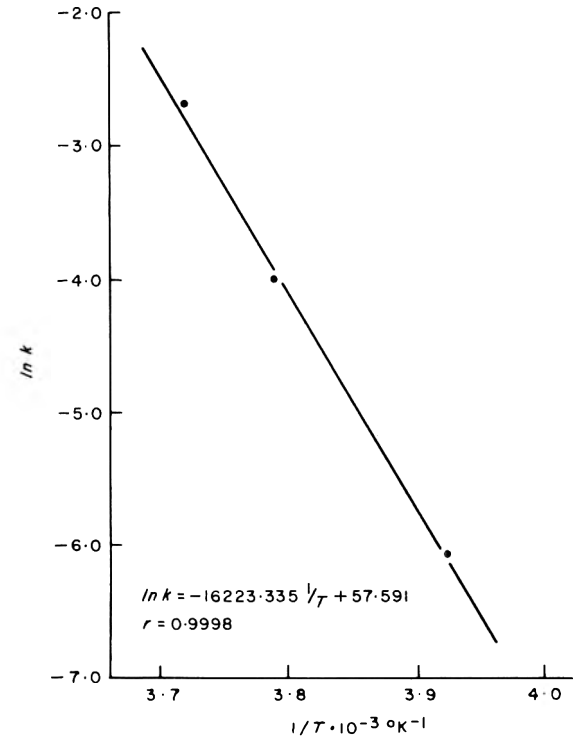

Figure 3. Effect of the storage temperature on the rate of degradation of the green colour (Arrhenius plot).

The results of colour measurements with the HunterLAB are shown in Table 1. There is a marked decrease in greenness ($-a$) with time and temperature, which is fully synchronous with the results of the measurements on whole product and also with the colour assessed sensorically (Steinbuch *et al.*, 1979). Below $-a = 8$, the colour was no longer acceptable to half the test panel. This is in good agreement with Dietrich *et al.* (1970), as they found a greater conversion of chlorophyll to pheophytin at −7°C (2 months) than at −18°C (6 months). They also mentioned that Brussels sprouts with $-a$ below 7, are quite brownish olive green.

Whereas Eheart & Odland (1973) found a substantial decrease in L value and a constant ratio of a/b, our results show only a very small decrease in L and b values and therefore a substantial increase in a/b.

Ascorbic acid content

Table 2 shows the AA content and the AA + DAA content of the Brussels sprouts. As the dry matter content, determined by drying 15 g of Brussels sprout slurry, was quite stable during storage at all temperatures, as could be expected for good sealed packages, the AA and AA + DAA content can be expressed on

Table 1. The colour of the slurry of Brussels sprouts measured with the Hunter colour difference meter

Storage time (weeks)	Stored at −18°C			Stored at −9°C			Stored at −4°C		
	L	*a*	*b*	*L*	*a*	*b*	*L*	*a*	*b*
9	47.1	−12.9	22.9	46.0	−11.7	22.4	46.2	−8.3	22.0
12	46.7	−12.4	22.3	45.6	−10.6	21.7	44.2	−5.9	20.4
15	46.8	−12.4	22.6	45.7	−10.4	21.9	44.0	−4.0	19.3
18	47.0	−12.0	22.4	46.1	−10.1	21.9			
21	47.1	−12.1	22.5	44.6	−9.2	21.3			
24	48.0	−11.3	22.4	45.4	−8.5	21.4			
27	47.5	−11.7	22.6	45.0	−8.3	21.1			
30	47.6	−12.1	22.6	45.7	−7.5	21.5			
33	46.6	−11.9	22.6	45.3	−7.6	21.4			
39	46.8	−11.4	22.0	43.9	−6.9	20.3			
45	46.6	−11.8	22.2	44.2	−4.7	19.9			
51	46.6	−11.9	22.1	44.0	−4.4	19.8			
s.d.	0.6	0.3	0.2	1.0	0.4	0.3	0.4	0.3	0.5

s.d. = standard deviation between duplicates

a fresh weight basis. The irregular results after 18, 21 and 45 weeks at −9°C and −18°C are probably due to sampling and/or analysis error. At −4°C storage, there is a rapid decrease of AA to 33% of the initial content after 12 weeks, but no consistent increase of DAA. The −9°C storage shows a gradual decrease of AA to 75% after 12 weeks, and to 54% after 39 weeks. These results are in fairly good agreement with Pimm (1973) and Dietrich *et al.* (1970).

Table 2. The content of ascorbic acid (AA) and of ascorbic acid + dehydroascorbic acid (DAA) in mg/100 g of frozen Brussels sprouts

Storage time (weeks)	Stored at −18°C		Stored at −9°C		Stored at −4°C	
	AA	AA + DAA	AA	AA + DAA	AA	AA + DAA
0	110	112	111	112	110	112
3	113	114	112	114	108	111
6	120	125	111	119	75	87
9	107	118	94	97	58	65
12	97	101	83	87	37	41
18	68	73	60	63		
21	78	82	72	74		
24	104	114	77	83		
27	110	122	66	70		
30	106	119	74	82		
33	97	108	63	72		
39	85	93	60	72		
45	66	67	34	35		

The −18°C storage shows fluctuating results: after 39 weeks, the loss of AA and AA + DAA will be about 20%. Here the references are not unanimous: Hudson *et al.* (1974) found no loss of AA, whereas Eheart & Odland (1973) and Woyke & Makowski (1968) reported greater losses. Ascorbic acid in frozen Brussels sprouts turns out to be susceptible to high temperature during storage, and may be an indicator for inferior handling or storage (Blegen & Rognerud, 1972). The main problem remains that for any stored sample (e.g. from a shop), the AA content directly after blanching is unknown. Moreover, there is a wide divergence in the AA content of fresh Brussels sprouts: Abrams (1975) mentions a range of 82–140 mg/100 g for nine varieties, and Woyke & Makowski (1968) 84–265 mg/100 g for twenty varieties.

Texture

Shear press compression. For the thawed product (Fig. 4) there clearly is, in spite of the fairly great fluctuations in the data, a decrease in shear force value with increasing storage time. Also the rate of decrease in shear force is clearly dependent on the storage temperature.

For the cooked product (Fig. 5) there is no longer any evidence of a change in shear force. The effect of storage time and temperature on the textural properties of the Brussels sprouts gets apparently lost in the rather violent cooking treatment.

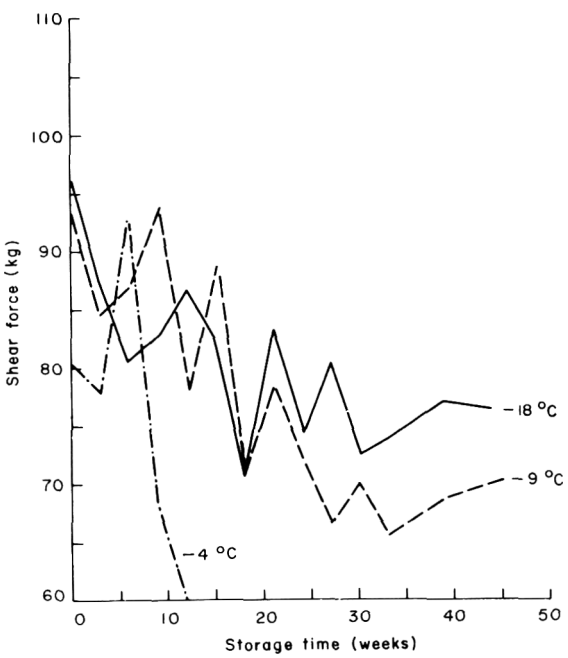

Figure 4. Shear force *v.* storage time for thawed Brussels sprouts.

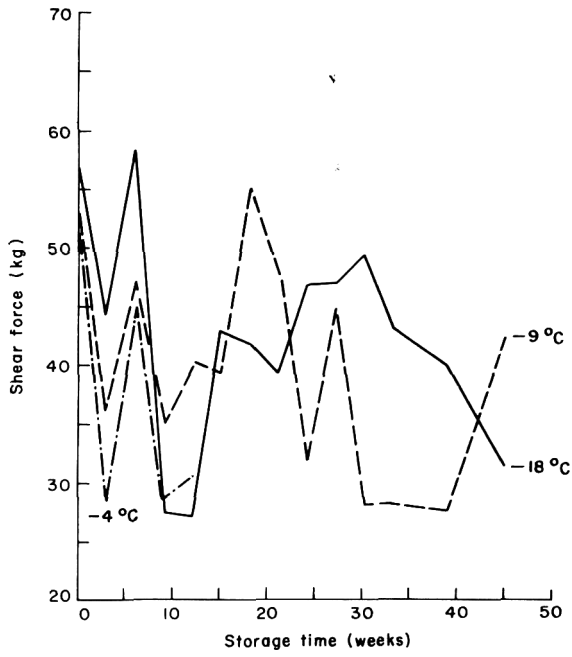

Figure 5. Shear force *v.* storage time for cooked Brussels sprouts.

Texture profile analysis. One outstanding feature in this method is that the product does not exhibit a brittleness peak and that the hardness peak always occurs at maximum compression. The process can best be described as a 'continuous failure'.

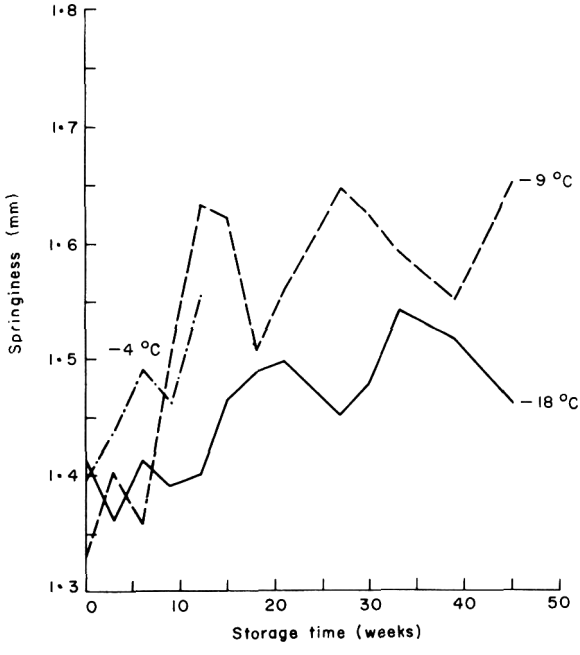

Figure 6. TPA springiness *v.* storage time for thawed Brussels sprouts.

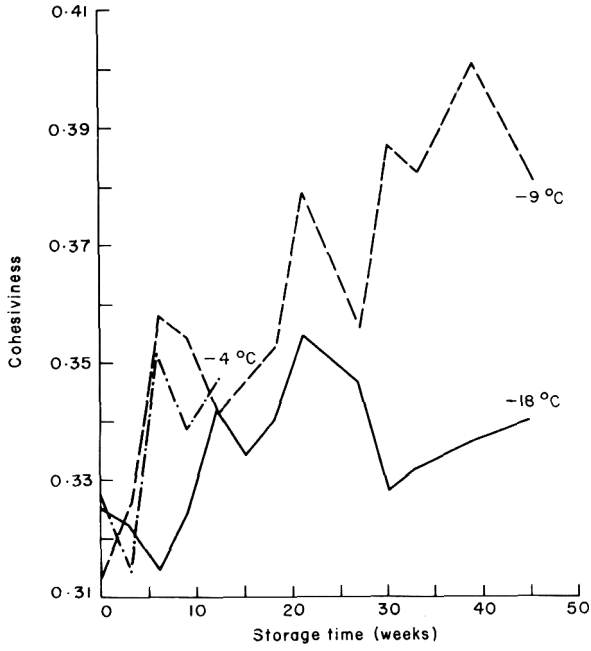

Figure 7. TPA cohesiveness *v.* storage time for thawed Brussels sprouts.

Of the typical TPA parameters, only 'springiness' and 'cohesiveness' are promising (Figs 6, 7). They both show an increase in value with increasing time and temperature, probably towards an asymptotic end value.

Because of the greater fluctuations, both TPA parameters seem to be less reliable than the shear force. All the other TPA parameters do not change during the storage, or exhibit such great fluctuations that no longer any correlation with neither time nor temperature can be detected. The great fluctuations in the different TPA parameters, including 'springiness' and 'cohesiveness', can to some extent be explained by the differences in size and dimensions of the Brussels sprouts: the contact area for large Brussels sprouts, especially at higher compressions, is greater than for smaller ones. It has been observed that large Brussels sprouts need more force and energy to be compressed over the same distance than the smaller ones, notwithstanding the fact that they all have been cut at 15 mm from the top.

For the cooked product one can make the same remarks for the same reasons as for the shear press compression.

Microbiological tests

In Table 3, the results are given for the fresh, the blanched and the deep frozen Brussels sprouts. The number of bacteria is decreased considerably by the blanching treatment.

Table 3. Results of microbiological tests before storage

Product	Total viable count		Viable count of the coliform group at 30°C (ct/g)
	At 30°C (ct/g)	At 17°C (ct/g)	
Fresh	3.4×10^6 4.0×10^6	2.4×10^6 2.4×10^6	3.4×10^5 3.2×10^5
Blanched	4.0×10^1 8.0×10^1	2.0×10^1 6.0×10^1	2 2
Deep frozen	1.3×10^3 1.4×10^3	7.8×10^2 6.8×10^2	2 0

Immediately after freezing the sprouts, the total viable count again increased considerably, due to infection by handling. This amount of bacteria seems to be inherent to the freezing and handling process (Schmidt-Lorenz, 1976). The average of the duplicates of the total count at 17 and 30°C in function of the storage time is shown in Figs 8 and 9. As can be seen, the total count at both 17 and 30°C increased enormously after 3 weeks of storage at −4°C to about 10^7/g after 9 weeks. By that time, a fungus had grown on the Brussels sprouts. Moreover, the product was no longer acceptable because of its high total count of bacteria.

The fungus was isolated and identified as *Cladosporium herbarum* (Pers.) Link. The minimum growth temperature of this fungus varies from −6°C down to −10°C. The bacteria, isolated from these Brussels sprouts, were identified with the Enterotube system (La Roche Diagnostics) and twice *Proteus rettgeri* and once *Pseudomonas* sp. was found.

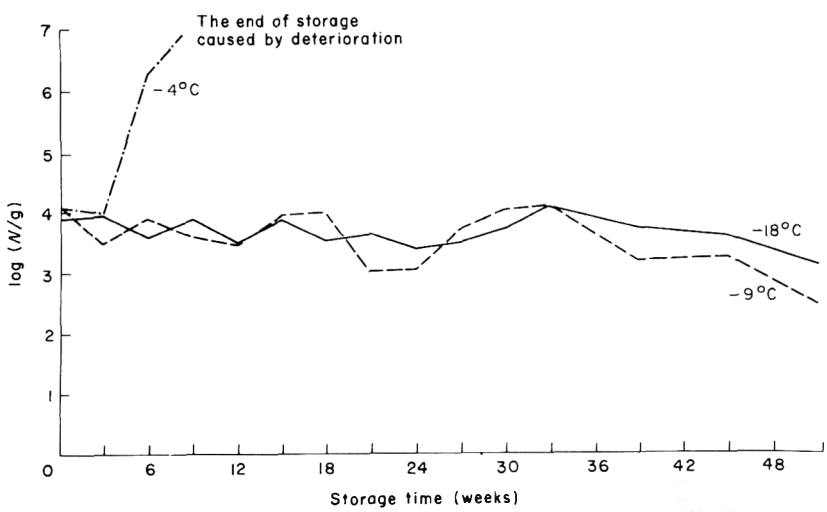

Figure 8. Semi-logarithmic plot of the total viable count (30°C) of Brussels sprouts *v.* storage time.

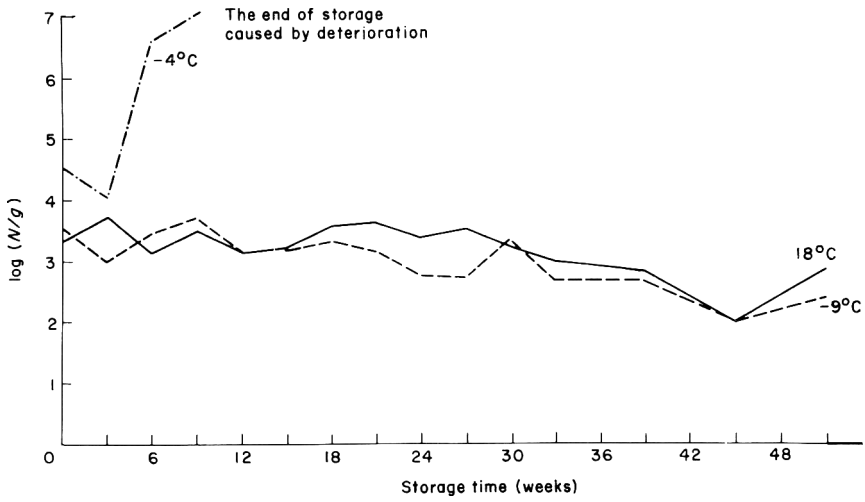

Figure 9. Semi-logarithmic plot of the total viable count (17°C) of Brussels sprouts *v.* storage time.

From a bacteriological point of view, the experiment has shown (Figs 8, 9) that deep frozen Brussels sprouts can be stored at −18°C for a period of 51 weeks without any problem for the product or the consumer. The product stored at −9°C for the same period of time seems to be safe too. But as the graphs show in Fig. 8, the product might be unbalanced and not under microbiological control any more if the storage conditions should become worse (Schmidt-Lorenz, 1976). As mentioned before the Brussels sprouts stored at −4°C are spoilt after 9 weeks of storage. At the moment the count of the coliform group was 1.3×10^7/g, which is a dangerous amount for this type of bacteria. The product stored at −9°C and −18°C gives no problem concerning the coliform group; the average of the group was < 2/g during the whole period.

Conclusions

Greenness, measured as the Hunter −*a* value on both Brussels sprouts and slurry, is found to be a very useful quality parameter, as it is fairly sensitive and shows a consequent temperature dependency. The reflection of Brussels sprout slurry at 680 nm seems to be useless as a quality parameter during storage, but may be important in determining the initial quality of the product.

The ascorbic acid content of the product is also a very useful quality index, provided the initial level is known.

Of the textural parameters tested, the shear force, measured with the Kramer shear cell, seems to be the most reliable, as it clearly indicates a decreasing texture with increasing storage time and temperature. The texture profile parameters are too much influenced by the size and weight of the individual Brussels sprouts. From a microbiological point of view, the experiments have

shown that a storage temperature of −9°C is quite acceptable, as long as the temperature does not increase any further, and the thawing period is not too long.

Acknowledgment

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Sensory analysis of bitterness in apple wine

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Summary

A dilution test determining the occurrence of basic tastes in apple wine and hard cider showed sourness, sweetness and bitterness to be present. For selection of assessors a bitterness standard was defined as a taste difference between two apple wines. Ranking and paired comparison tests were used for analysis of bitterness levels. Additions to the fermentations improving the growing conditions for the yeast decreased the bitterness of the apple wine.

Introduction

Bitterness is an important factor in the evaluation of hard cider and apple wine, which is made from juice ameliorated with sucrose to 20–22° Brix (Van Buren *et al.*, 1978). Quality improvement is often related to the lowering of the bitter taste. For this reason we have explored methods of bitter taste analysis with which treatments of the juice and/or the wine can be evaluated.

Materials and methods

The apple juices, some of which were ameliorated with sucrose (Table 1), were fermented with Montrachet 522 yeast to dryness (except for NY 260). The hard ciders and wines were bottled and kept in cold storage until use. For some experiments 130 ml air per min were bubbled through 300 ml wine in 500 ml Erlenmeyer flasks during 4 hr at room temperature.

The dilution-threshold testing was carried out with a hard cider and two wines. They were diluted with distilled water giving the series 10, 12.5, 25,

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Table 1. Description of the origin of the apple wines used

Code	Cultivar and preparation conditions
A	double strength apple juice (Seneca Food Co. 1974 concentrate diluted with water to 21° Brix)
B	Juice A plus an equal volume of 21° Brix sucrose solution
C	Juice A plus 3 volumes of 21° Brix sucrose solution
184	Cortland juice 1974 + sucrose added to give 22° Brix
174	Cortland juice 1974 + 0.1% (w/v) $(NH_4)_2 HPO_4$ + sucrose added to 22° Brix
164	Cortland juice 1974, oxidized + sucrose added to 22° Brix
154	Cortland juice 1974, oxidized, + 0.1% $(NH_4)_2$ HPO_4 + sucrose added to 22° Brix
NY 260	New York Agric. Exper. Sta. selected-seedling juice 1975 + sucrose added to 22° Brix
HC	(= hard cider) from Cortland juice 1975
FORT	Conical Rome juice 1975 → hard cider, fortified with ethanol to 12% alcohol content
CR	Cowin Rome juice 1974 + sucrose added to 22° Brix

50 and 100% wine or cider. Twelve panelists neither selected nor trained, were asked to indicate the dilution in which they could detect a sour, sweet, bitter or salty taste. The dilutions were tested from lower to higher concentrations. Sampling was done through a straw. The panelists used 7-Up for rinsing their mouths and restoring their taste ability. Colour differences between the samples were neutralized by placing sheets of orange paper under plastic cups filled with the dilutions. The panel tasted in the mornings at 11.00 and in the afternoon at 14.30 hours.

Scores were transferred to proportion values (ASTM, 1968), and the corresponding Z-scores (deviations from the mean in units of standard deviation) were found in a unit normal distribution table (Torgerson, 1967). According to Guilford (1954) one can assume that the regression of Z-scores on log (conc. %) is a straight line.

An analytical panel was selected for further evaluation of bitterness. The selection criterium for assessors was a taste difference between a low and a high bitter wine. Because of differences in bitterness perception (Amerine, Pangborn & Roessler, 1965) we avoided pure chemicals such as quinine or caffeine as standards for bitter materials in apple wine. The classification low and high bitter wine was determined by a small experts panel ranking five wines per session with a total of twenty-three wines. The low and high bitter wine of a previous session were always included in the next one.

All the people working in the Food Research Laboratory were invited for a selection test with the wines classified as low bitter: FORT, and high bitter: CR. The test consisted of a paired comparison test (which one is more bitter?) and a twofold five sample difference test (analogous to the triangle test with two and three identicals). A training in sensory methodology and taste charac-

teristics of apple wine was given to sixteen persons selected, from whom eight passed a second selection (six triangles). The assessors obtained were able to distinguish bitterness from other taste characteristics of apple wine, and had fairly equal sensitivity towards bitterness. Ranking and paired comparison test (ASTM, 1968) sessions of the analytical panel were held regularly in individual booths at 2.30 p.m. The samples were presented at room temperature in plastic cups standing on orange paper. Straws, crackers, 7-Up drink and water were freely available.

Results and discussion

The taste of apple wine/cider was analysed by a dilution-threshold test for basic tastes (Fig. 1). No salty taste was detected and sweet taste only in the NY 260 wine. The fermentation of the latter wine ended of itself while fermentable sugars were still present. The correlation coefficient of the regression line of

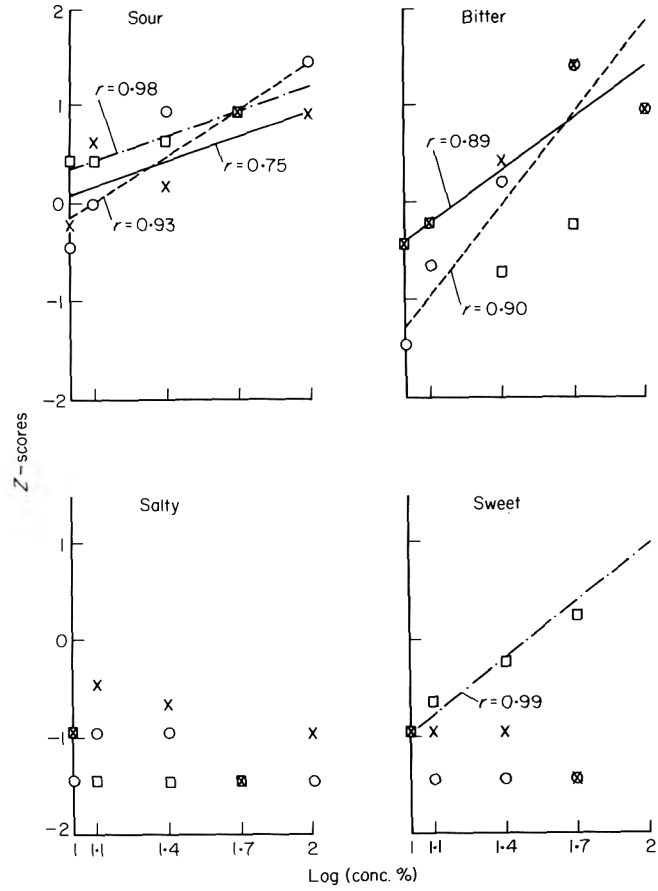

Figure 1. Plots of dilution tests for determining the occurrence of basic tastes in: Cortland apple wine (Code 184), x —x; Cortland hard cider, o– – –o; NY 260 apple wine, □ —·—□.

Z-scores on log concentration obtained for sweetness justifies the assumption of a cumulative normal distribution for sensitivity. The regressions for sourness indicate that this taste was present in every wine and that the wines (and hard cider) were not diluted far enough for exact determination of the thresholds. Bitterness was detected in both Cortland fermentations. The data scatter because of the wide distribution of bitter sensitivity of the assessors (Amerine *et al.*, 1965). For further application of the dilution method we should have used a much larger panel.

Apple wines A, B and C were made with different amounts of apple constituents. Differences in the bitterness of the wine were studied with a ranking test using the analytical panel (Table 2). Set I shows a higher bitterness with lower concentration of apple constituents, and this was confirmed with samples equalized in colour (Set II). Compounds from the apple juice seem to act on the yeast metabolism in a way that higher concentrations decrease the formation of bitter molecules.

Suomalainen & Nykänen (1968) conclude from their gas chromatographic analysis of aroma compounds in alcoholic beverages that, at least qualitatively, the same compounds were produced in both fermented sugar solutions and in beverages. So differences in bitter taste of A, B and C wines should be carried back to relative concentrations of compounds in the wines. Bubbling air through the wines, as we did for Set III, didn't change these relative concentrations enough to alter the results of the ranking test.

The addition of yeast growth factors to honey fermentations considerably improved the taste of these wines (Steinkraus & Morse, 1966). We modified our juices by oxidation with air and addition of $(NH_4)_2HPO_4$ to the fermentation solution. The resulting wines 154, 164, 174 and 184 were analysed for

Table 2. Relative bitterness of apple wines measured by ranking tests (increasing bitterness sequence)

Set	Assessments	Samples[1]	Rank totals	Significantly different[2]
I	14	A	19	yes
		B	29	no
		C	36	yes
II	16	A	23	yes
		B†	30	no
		C†	43	yes
III	14	A*	16	yes
		B*	29	no
		C*	39	yes

* Samples, bubbled air through

† Samples were brown coloured with acid soluble caramel to get the same colour as sample A

[1] Codes are explained in Table 1

[2] According to Kramer's tables for $P = 0.05$ (Amerine *et al.*, 1965)

bitterness with a paired comparison test (Table 3A). The statistical overall test of equality was carried out according to David (1969):

$\chi^2 = 4[S - \frac{1}{4} nm^2 (n-1)^2] / m \cdot n = 14.1$
d.f. $= n - 1 = 3$ (degrees of freedom)
S = sum of squares (Table 3)
n = number of samples (= 4)
m = members of panel (times 2 = 16)

Table 7 of the ASTM (1968) manual shows that our results are significant ($P < 0.5\%$). So the Cortland wine with both treatments (154) is significantly less bitter than the one without treatments (184). The same result was obtained with a ranking test (Table 3B). Reazin, Scales & Andreasen (1970) found that addition of ammonium salts to a fermenting solution resulted generally in a decrease of higher alcohol production. Their explanation is that excess ammonium ions promote an accumulation of amino acids, decreasing the formation of acetolactic acids and of fusel oil. Some fusel oils, tyrosol and tryptophol for example, were considered as bitter substances by Ribereau-Gayon & Sapsis (1965). The bitterness of many apple wines may be the result of fermentation conditions, particularly yeast nutrients.

Table 3. The influence of juice treatment on the bitterness of Cortland apple wine

A. *Paired comparison test*
Selections of the more bitter tasting sample

Pair*	154	164	174	184
154–164	5	11		
174–184			5	11
174–154	6		10	
184–154	3			13
174–164		9	7	
164–184		5		11
Totals	14	25	22	35
Sum squares (S) =	196 +	625 +	484 +	1225 = 2530

B. *Ranking test (increasing bitterness sequence)*

Assessments	Samples*	Rank totals	Significantly different†
16	154	29	yes
	164	29	yes
	174	51	yes
	184	51	yes

*Codes are explained in Table 1.
† According to Kramer's tables for $P = 0.05$ (Amerine *et al.*, 1965)

Summarizing the value of the tests used in measuring bitterness we conclude that the analytical panel should be composed of about ten selected assessors. The sensory method should be a discriminative one for a specific difference.

Acknowledgments

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Technical note: Quality retention of unblanched frozen vegetables by vacuum packing.

I. Mushrooms

E. STEINBUCH

In contrast to canning and drying, the freezing preservation method has been used on a limited scale for mushrooms. The flavour, texture and juiciness appear to depend highly on the freezing time (Åström & Löndahl, 1969). Tressler & Evers (1957) recommend preparation and freezing the same day on which the mushrooms are picked as well as steam blanching in order to maintain quality. Gormley (1972) mentions favourable effects of freon freezing, however, the industrial application of this method meets technical problems. Moreover, in comparison with other frozen vegetables, the quality of frozen mushrooms does not meet general standards for consumer acceptance. The blanching treatment will generally cause a sufficient inactivation of enzymes, resulting in a product without serious discolorations and off-flavours. However, the texture of blanched and normally frozen mushrooms indicates unfavourable toughness (Steinbuch, 1979).

Experiments with the processing of unblanched mushrooms confirm enzyme-induced defects with regard to colour and flavour after storage for 6–10 weeks at −20°C. Therefore, the retention of texture of these unblanched frozen mushrooms has indicated the necessity of investigation of alternative technological treatments, which might avoid the harmful action of enzymes in the unblanched frozen product. Since the oxygen-dependence of most enzymes is generally known, some experiments have been carried out in regard to the removal of oxygen from the fresh product. The removal of air has been achieved by vacuum packing of the sliced, unblanched mushrooms. The results of the freezing experiments with mushrooms, which have been pretreated (by various methods), are summarized in Table 1.

The sensory evaluation of the product a short time after freezing does not deliver relevant results, because quality deterioration cannot be correctly detected. On the other hand, the gravity of storage-time-depending quality aspects may be shown after prolonged storage (Table 2).

The comparison of the data of Table 1 and 2, indicates the obvious colour and flavour alterations of unblanched mushrooms during frozen storage, however, the quality of unblanched vacuum packed mushrooms shows

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Table 1. Sensory evaluation of the quality of frozen mushrooms, as affected by blanching and packaging, after 1 month's storage at $-20^{\circ}C$

Quality aspects being evaluated	Pretreatment		
	blanching ↓ cooling ↓ slicing ↓ freezing ↓ packing	slicing ↓ freezing (freeze flowing) ↓ packing	slicing ↓ vacuum packing ↓ freezing
Colour	yellowish	brownish	yellow
Flavour	flat	acceptable	natural
Texture	tough	good	good

promising prospects. Both colour, flavour and texture of this product obtain high scores of sensory evaluation, indicating only slight differences with fresh mushrooms. The maintenance of colour and flavour, however, appear to be highly dependent on the gas and oxygen permeability of the used packing materials.

In regard to shrinkage losses, the vacuum pack process also shows promising results. The weight decrease during cooking of the unblanched vacuum packed

Table 2. Sensory evaluation of the quality of frozen mushrooms as affected by blanching and packaging after 3 months' storage at $-20^{\circ}C$

Quality aspects being evaluated	Pretreatment		
	blanching ↓ cooling ↓ slicing ↓ freezing ↓ packing	slicing ↓ freezing (free flowing) ↓ packing	slicing ↓ vacuum packing ↓ freezing
Colour	a little dark yellowish	brown	yellowish
Flavour	flat	obvious off-flavour	natural
Texture	tough	good	good

frozen mushrooms was considerably less than the shrinking, which occurs during the blanching process, obviously caused by an unexplained retention or maintenance of the tissue, when the product is not blanched before freezing. Both the high quality and the limited weight losses of unblanched frozen mushrooms show favourable indications for the eventual application of this processing method.

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J. Fd Technol. (1979) **14**, 325–329

Technical note: Selected functional properties of a whey protein isolate

K. J. BURGESS AND J. KELLY

Cheese whey contains approximately 20% of the proteins present in milk and the recovery of these proteins is therefore an important feature of many whey utilization processes. The high nutritional value of whey protein is well documented (Wingerd, 1971; Delaney, 1976) and a denatured form of whey protein (known commercially as 'lactalbumin') has been manufactured for many years as a protein supplement. However, protein ingredients are not often added to foods for their nutritional value alone. Most protein additives are used for a specific physical function, e.g. foam formation, gelation, water and fat absorption, and in this respect 'lactalbumin' has limited applications because of poor solubility.

New processes for whey protein concentrate production are therefore designed to recover the proteins in a soluble form in order to fully exploit their functional properties. Such processes include ultrafiltration, gel filtration, complex formation and precipitation procedures. The composition and properties of a range of these whey protein concentrates have been reviewed by Morr, Swenson & Richter (1973) and Delaney (1976).

A disadvantage of most of these methods for whey protein recovery is that the lipid fraction of the whey is to a varying extent concentrated with the protein. Apart from causing off-flavours during storage (De Boer, De Wit & Hiddink, 1977), the presence of fat in whey protein concentrates is also known to be detrimental to their foaming (McDonough *et al.*, 1974) and gelling (Sternberg, Chiang & Eberts, 1976) properties. Several attempts have therefore been made to reduce the fat content of whey protein concentrates. These include centrifugal separation (Breslan, Gonlet & Cross, 1975), filtration (Burgess, 1977 unpublished) and a combination of demineralization and pH adjustment (De Wit, Klarenbeek & De Boer, 1978). However, recent developments in the use of ion exchange celluloses for protein recovery have made possible the isolation of whey protein of very high purity (Palmer, 1977).

It is claimed by the manufacturers that whey protein isolates produced in this way possess unique functional properties (Palmer, 1977) but to-date no independent assessment has been reported. Selected functional properties of

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the whey protein isolate were therefore examined and compared with those of fresh egg white and a whey protein concentrate prepared by ultrafiltration.

Materials and methods

Materials

Whey protein isolate (WPI) was produced by the Viscose Group Ltd. in a 700 litre pilot plant using regenerated ion exchange cellulose. Details of the recovery process have already been published (Palmer, 1977). Analysis (dry weight): protein, 95%; ash, 3%; fat, 0.5%.

Ultrafiltration whey protein concentrate (WPC) was prepared by batch ultrafiltration (D.D.S. Laboratory Module, type 600 membranes) of separated cheese whey to a total solids content of 22%. The concentrate was dried in a pilot scale spray drier (Anhydro Lab 3) with an outlet temperature of 85°C. Analysis: protein, 58%; ash, 4%; fat, 5%. Raw egg-white was obtained from fresh hen eggs.

Protein solubility

Solutions of 1% protein were made in distilled water and adjusted to pH 3, 4.5 and 8 with 1 N HCl or 1 N NaOH. After mixing for 30 min, solutions were centrifuged at 3000 *g* for 10 min. Total nitrogen was determined in the supernatant by the micro-Kjeldahl method (AOAC, 1970) and the percentage soluble protein calculated from this. No correction was made for non-protein nitrogen.

Gelation properties

Gelation temperature, gel water holding capacity and gel hardness and springiness were determined as described by Sternberg *et al.* (1976) using 11% protein solutions. This protein level was chosen because it compares with the protein concentration in egg-white (Shrimpton, 1969).

Whipping properties

Whipping tests were carried out on 11% protein solutions at their reconstituted pH.

Protein solutions (100 g) were whipped in a Kenwood Chef food mixer using the 'K' beater at full speed for 5 min. The specific foam volume was

measured by weighing a known volume of foam (180 ml) and defined as the reciprocal of the foam density.

The complete foam was then allowed to drip through a 1 mm nylon mesh into a measuring cylinder. Foam stability was defined as the volume of liquid drained in 30 min.

Results and discussion

Protein solubility

Protein solubility data for the whey protein isolate, the whey protein concentrate and egg-white are given in Table 1. The protein solubility of ultrafiltration whey protein concentrate always exceeded 90% with only a small drop in solubility around the isoelectric point. This is in agreement with previous work (Delaney, 1976). Similarly the protein solubility of egg white always exceeded 98%. However, while the protein solubility of the whey protein isolate exceeded 98% at high (8.0) and low (3.0) pH, the solubility fell to only 35% at pH 4.5.

Solubility at pH 4.5 is often used as a measure of denaturation in whey protein systems (Delaney, Donnelly & O'Sullivan, 1973) and the low protein solubility of the whey protein isolate at this pH therefore suggests that the protein recovery process in some way altered the native structure of the whey protein. This may be explained by the alkaline (pH 9) conditions used in the recovery process. At this high pH, the repulsive forces within the protein molecules are large and cause the molecules to open up and unfold. Processing temperature (50°C for the isolate studied) also affects this molecular unfolding since it has been shown that the protein solubility at pH 4.5 of whey protein isolate recovered at 20°C is over 75% (Phillips, 1977, personal communication).

In spite of its low solubility at pH 4.5, the whey protein isolate was almost completely soluble at pH 3 and is therefore suitable for the protein supplementation of carbonated beverages where a pH of 3.5 is usual (De Boer *et al.*, 1977).

Table 1. Protein solubility of egg and whey proteins

	Protein solubility		
Protein	pH 3.0	pH 4.5	pH 8.0
Whey protein isolate (WPI)	98.4	35.0	99.2
Whey protein concentrate (WPC)	94.6	90.2	95.9
Egg-white	98.6	99.3	99.0

Gelling properties

Some of the gelling properties of the WPI are given in Table 2, with the relevant properties of egg white and WPC included for comparison. The gelation temperature of egg white (60°C) is generally considered low compared to other protein sources but the gelation temperature of WPI was shown to be lower still, an indication of its excellent gelling properties. The gel temperature of WPI was not particularly pH sensitive, falling from 58°C at pH 7 to 56°C at pH 9, compared to 74 and 67°C respectively for WPC.

Table 2. Gelling properties of egg and whey proteins (11% protein)

Protein	pH	Gelation temperature (°C)	Water holding capacity (%)	Hardness	Springiness
Egg white	8.5	60	97.8	7.2	0.59
Whey protein isolate	7.0	58	98.4	8.4	0.65
	8.0	56	99.0	5.2	0.60
	9.0	56	98.2	5.2	0.58
Ultrafiltration WPC	7.0	74	90.6	7.8	0.42
	8.0	69	a	a	a
	9.0	67	a	a	a

a. Not measured

Ferry (1948) has described gelation as a 2-stage process: initial denaturation of native protein into unfolded polypeptides, then gradual association to form the gel matrix. The unfolded molecular nature of the WPI therefore accelerated gelation since the first stage of the process was at least partially complete. In the case of the WPC, the presence of lipid may have reduced the ability of the protein to gel through the competition of lipid components for the hydrogen bonding sites involved in association.

The water holding capacity of WPI gels was excellent and was approximately the same as that of egg white at 98% retention. WPC gels were slightly inferior with a value of only 90% retention.

WPI gels were harder than egg-white gels at pH 7 but softer at pH 9 and 8, though they were still firm under these conditions. WPI gels were also quite springy like egg white gels in contrast to the relatively inelastic WPC gels.

Whipping properties

Table 3 compares the whipping properties of some whey protein concentrates with those of egg white. The WPI solution (11% protein) gave a foam with approximately the same specific volume as egg white foam (10 ml/g), while the WPC gave a foam with a specific volume of only 2.5 ml/g.

Table 3. Whipping properties of egg and whey proteins (11% protein)

Protein	Specific foam value ml/g	Drainage at 30 min ml
Egg white	10.0	12
Whey protein isolate	10.2	8
Whey protein concentrate	2.5	100
*Whey protein isolate	3.1	4
*Egg white	3.5	2

*100 g protein solution + 100 g sucrose

The stability of the WPI foam was excellent with only 8 ml drainage after 30 min. This compared very favourably with the egg white foam where 12 ml drainage had occurred in the same time. In contrast, the foam of the WPC had completely collapsed after 30 min (100 ml drainage). The poor specific volume and stability of the WPC prepared by ultrafiltration was attributed to the relatively high fat content of this concentrate (5%).

In food systems, sugar is often present in protein foams. The effect of added sugar on the whipping properties of WPI and egg white is shown in Table 3. The specific volume of the foams was considerably reduced by the addition of sugar. The specific volume of the egg white foam (3.5 ml/g) was slightly higher than that of the WPI (3.1 ml/g) after sugar addition (100 g). However, the stability of each foam was improved by sugar addition with only 2 and 4 ml drainage from egg white and WPI foams respectively.

These limited data indicate that the whey protein isolate should find a number of uses in the food industry as an egg white replacer.

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Book reviews

The Analysis of Nutrients in Food. By D. R. Osborne and P. Voogt. London: Academic Press, 1978. Pp. vii + 251. £10.50.

This book is the latest in the series of Food Science and Technology monographs and sets out to provide the analyst with a set of reliable procedures for the determination of significant dietary nutrients. It is divided into two parts; Part I contains some useful but fairly elementary background information on the chemistry and biochemistry of micro and macro nutrients (Chapters 1 and 2) recommended intakes of nutrients (Chapter 4), food composition tables (Chapter 5), a reasonably comprehensive appendix of references to both books and published papers for further reading, and a short chapter on analysis of nutrients (Chapter 3) which would have been better placed as an introduction to Part II. Analytical methods are described in Part II for moisture and total solids, proteins and nitrogenous compounds, carbohydrates (Where, unfortunately, the methods for 'crude' and 'acid detergent' fibre are inadequate for the measurement of dietary fibre), lipids, inorganic constituents, and fat and water-soluble vitamins with a brief section on the calculation of calorific value. On the whole the methods are detailed, well laid out and easy to follow although the more sophisticated techniques such as gas and high performance liquid chromatography are introduced in a very matter-of-fact manner with no real indication of the problems likely to be encountered, and those wishing to analyse Vitamin D in margarine by gas chromatography may be put off by the suggested method when they learn that a mass spectrometer is required to overcome the effects of interfering substances. The book is well presented although Part I does seem overlong (97 pages compared to 141 pages in Part II); the typescript is clear and there are very few typographical errors. It should prove a useful addition to the library shelf although analysts requiring methods for one or two specific components might be better advised to try a literature survey first.

A. Hobson-Frohock

Books Received

Food Poisoning and Food Hygiene (4th edn). By B. C. Hobbs and R. J. Gilbert. London: Edward Arnold, 1978. Pp. VIII + 366. Paperback, £4.50.

The new edition of this well established book has been revised and brought up to date.

The Life of Yeasts (2nd edn). By H. J. Pfaff, M. W. Miller and E. M. Mrak.
Cambridge, Mass: Harvard University Press, 1978. Pp. IX + 341. £10.50.

A comprehensive text which has been written for non-specialists. The new edition has been revised to incorporate knowledge gained since 1968.

Advances in Food Research, Volume 24. Ed. by C. O. Chichester.
New York: Academic Press, 1978. Pp. VII + 378. US$32.00.

The present volume contains monographs on the following topics: sulphhydryl and disulphide groups in meat, histamine(?) toxicity from fish products, food irradiation, tea, honey.

Glutamic Acid. Advances in Biochemistry and Physiology. Ed. by L. J. Filer Jr, S. Garattini, M. R. Kare, W. A. Reynolds and R. J. Wurtman.
New York: Raven Press, 1979. Pp. XIV + 400. US$36.40.

A collection of twenty-six papers dealing with sensory and dietary aspects of glutamate, its metabolism in mammals, glutamate in the central nervous system, safety evaluation and clinical aspects of glutamate utilization.

Food and People (3rd edn). By M. E. Lowenberg, J. R. Savage, E. N. Todhunter, J. L. Lubawski and E. D. Wilson.
New York: John Wiley & Sons, 1979. Pp. VIII + 382. £8.50.

This book is intended for undergraduate students in food technology and home economics. It develops in a general context the importance of food and nutrition in the life and affairs of communities.

Animal Feeds and Pet Foods. Recent Developments
Food Technology Review No. 50. By C. S. Sedano.
New Jersey. Noyes Data Corporation, 1979. Pp. XI + 257. US$36.00.

Course in Rural Food Technology

The Netherlands Universities Foundation For International Cooperation (NUFFIC) is organizing an International Post Graduate Course in Food Science and Nutrition with emphasis on the theme, Rural Food Technology: its impact on food availability, food storage, processing and nutrition.

The course intends to provide further technical background and practical experiences on the choice of technology in food processing, appropriate food technology at different levels and their socioeconomic consequences in rural development.

Course content includes food science, food engineering, nutrition, economics, extension and project evaluation. Case studies, coming up during lectures and/or from literature, on the range of existing technologies and the choice of technology will serve to expand the topics.

Participants will undertake practical research work in small groups on developing a feasibility study for a certain production process. Attention will be paid to the process of development and extension of improved technologies starting from a survey of existing traditional technologies and the needs for improvement.

Requirements for Admission

Participants should meet the following requirements:

1. Academic degree (BSc as minimum) in food technology, food science, nutrition or a related field of study, and 3–5 years practical experience.
2. Professional position with tasks related to the theme of the course and through which dissemination of the acquired knowledge is possible and can be expected.
3. Drawn from the following types of organizations:
   - 3.1 Organization responsible for Agro Industrial Development.
   - 3.2 Institute for Food Technology.
   - 3.3 University or higher college in technical or agricultural disciplines.
   - 3.4 Private enterprises in the agrobased industries.
   - 3.5 National or Regional Development agencies.
4. Interested in the problem of choice of technology in food processing at various levels, related to rural development.

Course Period:	January–June 1980
Place:	Wageningen, The Netherlands
Fellowships:	The Netherlands Government has fellowship programmes. The diplomatic representative of The Netherlands in your country can give more information.
Application:	For further information about the course programme and for application forms, contact the Netherlands Embassy in your country or write to the course secretary. The closing date for application is 15 September 1979.
Address:	International Course in Food Science and Nutrition, Lawickse Allee 11, 6700 AB Wageningen, The Netherlands. Tel: 08370-19040.

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Selected contents

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

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

SI UNITS

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

NON SI UNITS

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

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

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 bold 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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